“ It matters not how strait the gate,

How charged with punishments the scroll,

I am the master of my fate,

I am the captain of my soul.”

William Ernest Henley

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Abbreviations index

AOC : Antioxidant Capacity

A_P : Aphron phase

a_W : Water Activity

CA : Cinnamic Acids

CAE : Caffeic Acid Equivalents

CGAs: Colloidal Gas Aphrons

CMC : Critical Micelle Concentration

CSE : Conventional Solvent Extraction

CyN : Cyanidin-3-glucoside

dm: Dry Matter

DMGS : Dried Milled Grape Skins

EtOH : Ethanol

GAE : Gallic Acid Equivalents

L_P : Liquid phase

MD: Maltodextrins

rpm : rounds per minutes

$T^{1/2}$: Half-life time

TT : Total Tannins

TAC : Total Anthocyanins Content

TFA : Total Flavonols

TPC_{280} : Total Phenolic Compounds measured at 280 nm

TPC_{Folin} : Total Phenolic Compounds measured with Folin method

Tw20: Tween20

WAE : Wine Anthocyaninis Equivalents

Wp : Wet Powder

WSI : Water Solubility Index

WSI : Water Solubility Index

ε : Gas Hold Up

1. Introduction

1.1 The Valorvitis project

The present PhD thesis work was carried out under the framework of the Valorvitis project coordinated by the Institute of Oenology and Agro-Food Engineering of the Università Cattolica del Sacro Cuore of Piacenza. The, overall project objective is the development of complete recovery strategies for wine-making wastes in order to reduce their environmental impact. The conceived strategies are meant to recover high added-value compounds in order to valorize the wine-making by-products that could, then, be sold at a profitable price. The Valorvitis project scheme in Figure 1.1 reports a possible approach for the complete utilization and valorization of wine-making wastes (stalks, skins and seed before and after distillation) .

The project started in June 2011 and is going to end in February 2015.

Research activities have been carried out according to the work packages sub-division shown in Figure 1.2.

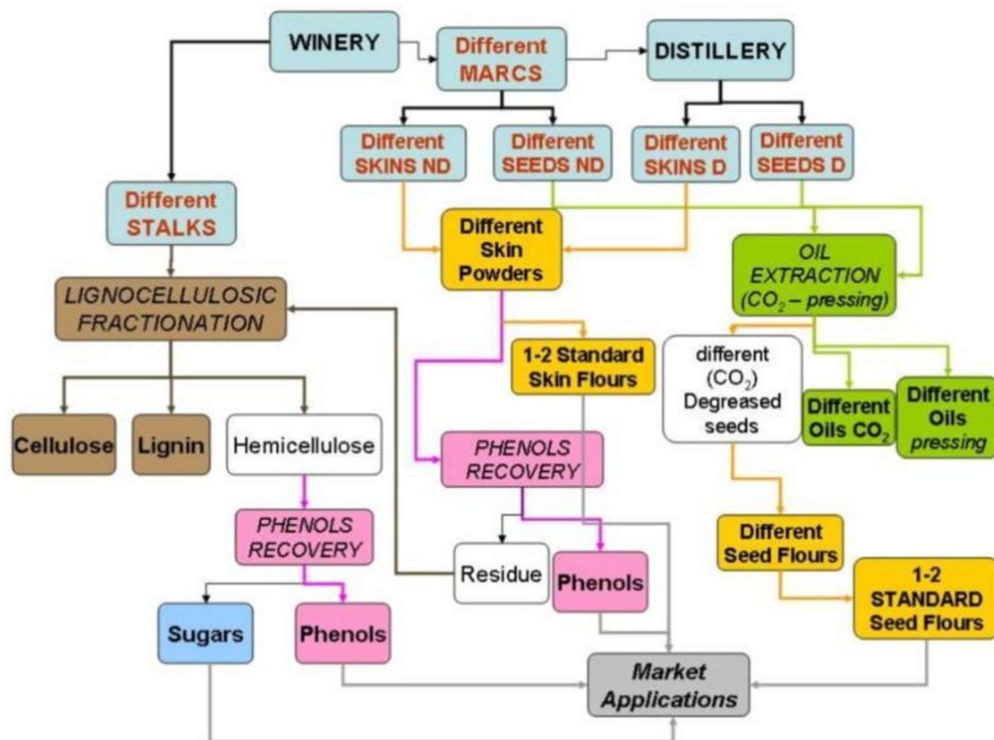


Figure 1.1. Scheme of the project approach for a complete recovery of wine-making wastes (ND: not distilled, D: distilled) (www.valorvitis.com)

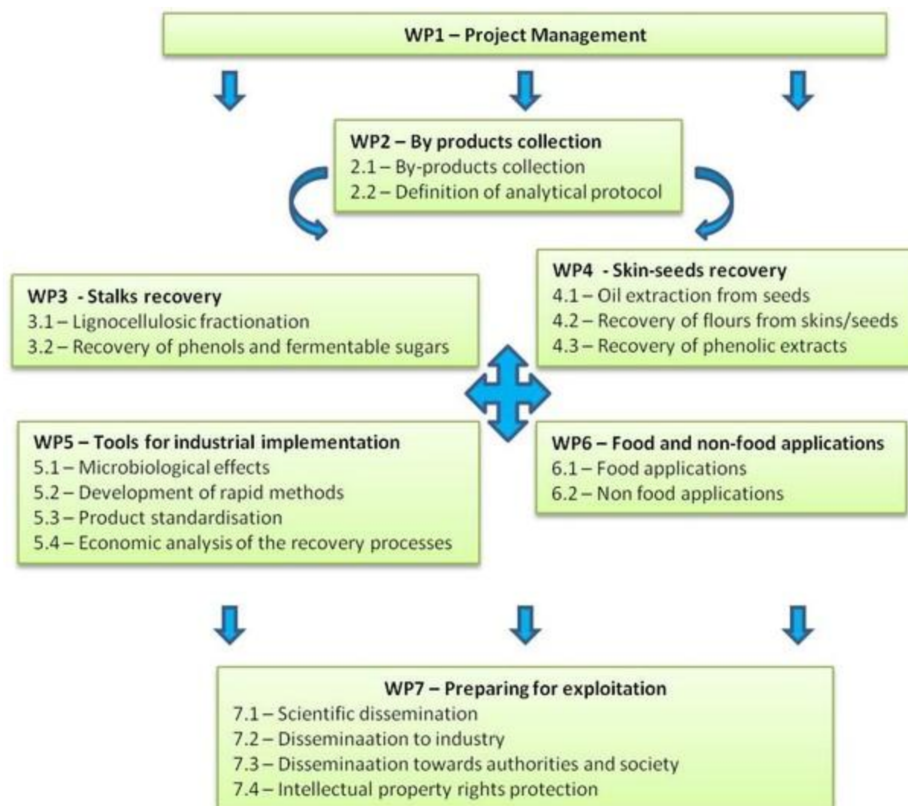


Figure 1.2 - Project structure (www.valorvitis.com)

In order to valorize the typical production of the Italian regions involved in the project (Emilia Romagna, Piemonte, Lombardia and Trentino Alto Adige), the most important cultivars of these areas have been selected for the by-products collection. The selection of the cultivars (3 red grapes Barbera, Nebbiolo, Pinot nero, and 3 white grapes Moscato, Chardonnay and Muller Thurgau) has been done considering several characteristics the phenolic and aromatic profile, their diffusion in other European countries (Chardonnay, Muller Thurgau, Pinot Noir), their representativeness of white and red varieties, of different vinification process (for example Pinot nero is often used for a white vinification process which produces not-fermented red marcs particularly rich in polyphenolic compounds), and their actual distillation in "purity" (this would allow to evaluate the influence of distillation on the aromatic profile and phenolic composition of recovered products). The utilisation of different cultivars is necessary to evaluate cultivar influence on the chemical-physical-functional properties of the recovered products (skin and seed flours and phenols extracts). On the other hand, heterogeneity of by-products batches could impair their industrial applications.

The activity of this research project was related to task 4.3 and contributed to task 7.1.
In particular, all the processes developed in Valorvitis need to be economically and environmentally-friendly since they are aimed to reduce the wine-industry pollution load.

- The project aims to produce functional ingredients, additives and high-added value products such as Phenolic extract recovered from skins

Their antioxidant properties are well known and at present exploited in many pharmaceutical and cosmetic products and a few food applications.

- Phenolic additives/ingredients with improved solubility in both hydrophilic and hydrophobic media by means of suitable modifications.

Based on previous results obtained by the coordinator's group, phenolic extracts have a low solubility in the solvent used for their recovery. Solubility improvement is therefore necessary to guarantee market exploitation. Addition of maltodextrins and surfactants have been investigated to produce modified phenolic extracts.

The reform CE/479/08 abolished the compulsory distillation of grape marc and lees, together with the economic helps to distilleries the minimum price given to wine-makers for the acquisition of wine-making residues and the EC acquisition of alcohol produced in excess. Even though for the moment Italy has decided to leave compulsory the delivery of marc and lees to recognized distilleries, as well as contribution to distilleries but only for the production of alcohol meant for energy or industrial application, it is clear how management of grape marc is going to become more expensive for wine industry. In fact, following the General EU Directive on wastes 2006/12/EC, Member States must take the necessary measures to ensure that waste is recovered or disposed off without endangering human health and without using processes or methods which could damage the environment. Such a directive is applicable to any substance the holder discards or intends or is required for discard.
The Valorvitis project wants to offer wine-makers and distilleries feasible technical solutions to solve their problems related to waste management. The proposed

solution will hopefully allow wine-makers to make a profit of their by-products, increasing their production margins which could be invested in further research and promotion necessary to gain competitiveness toward other European and world wine producers.

1.2 Valorization of grape marc

Wine production is one of the most important agricultural activities in the world with 7,528,000 hectares of vine. The world wine production in 2013 was around 281 million of hectolitres (Mhl). Europe is the main producer (Figure 1.3) and the leading countries are Italy and France with a production respectively around 45 and 44 Mhl, followed by Spain with 40 Mhl in 2013 (OIV, 2013).

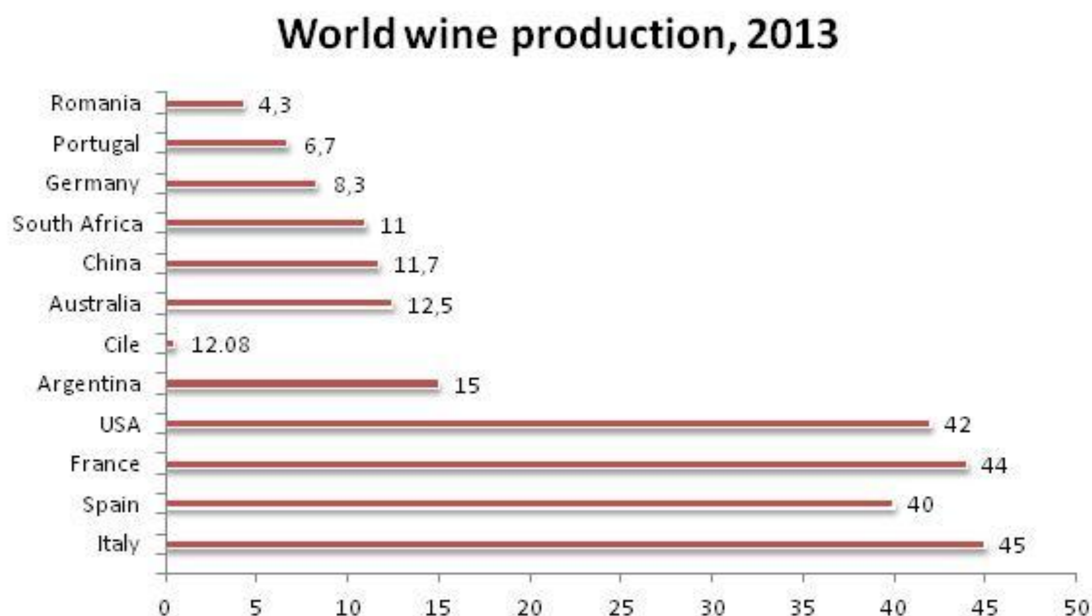


Figure 1.3. World wine production for the year 2013. The results are expressed as million of hectolitres (Mhl) (from www.oiv.int).

The yield in wine from full grape is around 65-70 %. This means that wine-making process produces more than 30 % of by- products as stalks, seeds and grape skin. In countries like Spain, Italy and France the annual production of this lignocellulosic waste is around 240million kg (Bustamante et al., 2008). At the end of the productive process the by-products still contain different bioactive compounds and could be exploited for further applications. Thanks to different

extraction methods, phenols and other bioactive compounds can be recovered, purified and applied in different food matrixes to produce added value foods. For example grape stalks are a source of dietary fibres that promote beneficial effect on human health as reduction in LDL cholesterol and sugars absorption. Grape seeds contain linoleic acid which is one of the most important omega six series fatty acids and is able to reduce the LDL cholesterol adsorption.

The largest amount of by-products is represented by grape skins that are commonly used to produce spirits and alcohol. Gallander and Peng (1980) have extracted different compounds as palmitic acid, stearic acid, arachidonic acid, linoleic and linolenic acid from grape peel although in small quantity. Otherwise, grape skins are rich in polyphenols. Grape polyphenols are monomeric and polymeric molecules located in the skin as anthocyanins, flavan–3–ols, flavonols, dihydro–flavonols, hydroxybenzoic acids and hydroxystilbenes (Ivanova et al., 2011). Polyphenols have shown many beneficial effects on human health such as anti–inflammatory effects, antimicrobial, anti–aging effects and they play a fundamental role preventing cardiovascular disease. Epidemiological studies have demonstrated that five to seven servings of fresh fruit and vegetables and two glasses of red wine per day can lead to a prolonged healthy life. This capacity has been attributed to wine because of its content of polyphenols, which are well-known antioxidant compounds (Alonso et al., 2002).

New technologies have been proposed to reuse the by–products not only in the agricultural sector. Today they can potentially be exploited for different purposes such as animal feeding (Klopfenstein et al.,2008), production of food and nutritional supplement (Fernández-López et al.,2004), pigment extraction (Vasso and Constantina, 2007), improvement of colour, aroma and phenolic profile of wine (Pedroza et al., 2013).

The polyphenolic compounds can be classified into two groups (Figure 1.4) based on of their primary chemical structures of hydroxybenzenes: flavonoids and nonflavonoids. The majority of the flavonoids found in grapes include anthocyanins, flavanols and flavonols, whereas non-flavonoids are mainly hydroxycinnamic and hydroxybenzoic acids (Liang et al., 2011).

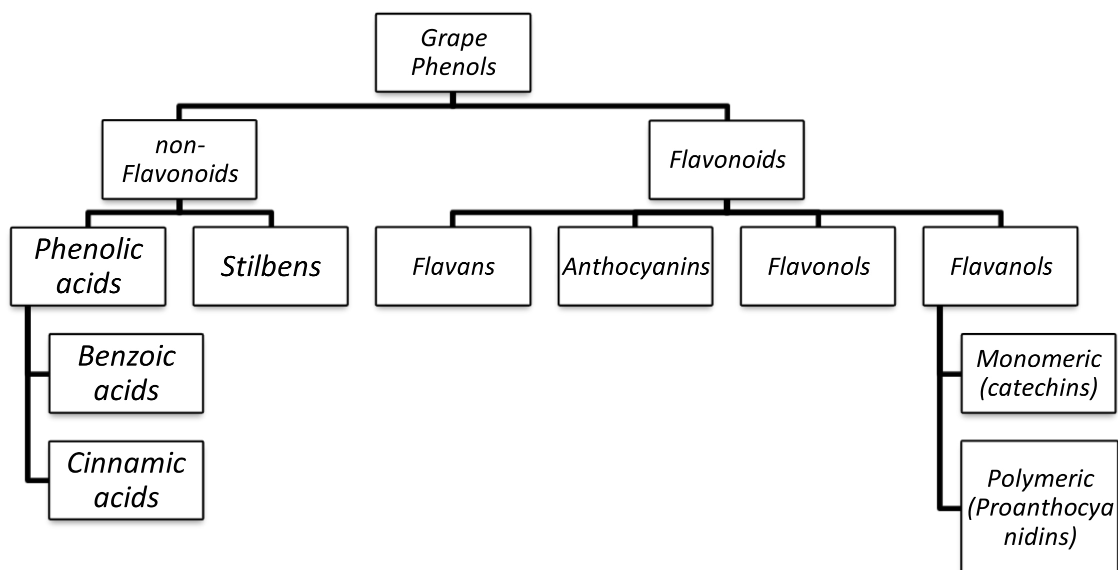


Figure 1.4 Main phenol classes present in grape skins and seeds.

1.2.1 Grape stalks

Grape stalks (GS) are obtained by the grape destemming operation and represent 2-8% of the processed material (Spigno et al., 2013). GS were investigated as bio-sorbent material for toxic compounds removal (Miralles et al.,2008), as a source of composting (Bertran et al.,2004; Bustamante et.,2009) and as a biomass for energy production (Fiori et al.,2010), also, they are actually considered an important source of phenols and fiber. The woody structure of the GS is composed of three polymeric fractions : lignin, cellulose and hemicelluloses (Figure 1.5).

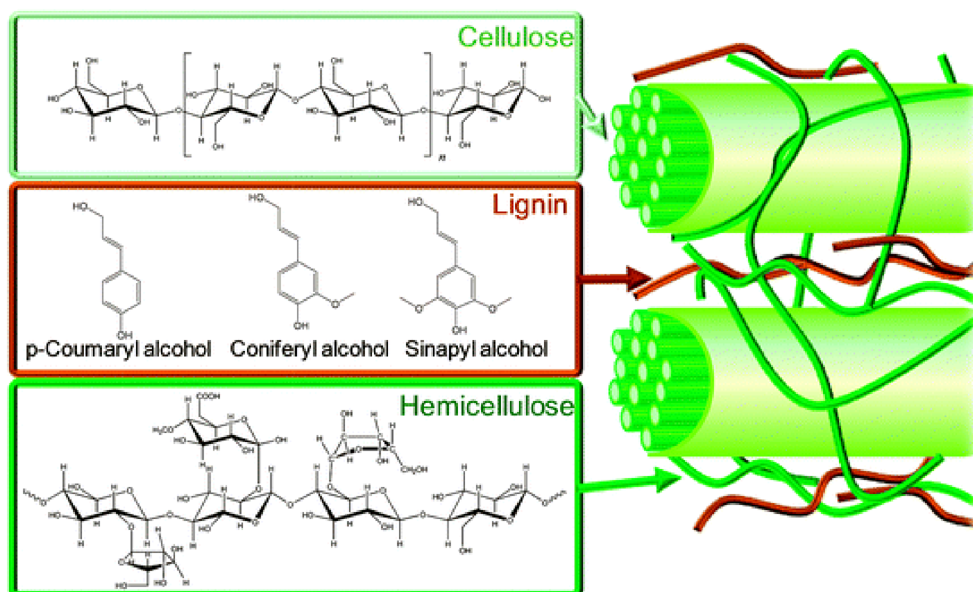


Figure 1.5. General lignocellulosic structure composed by lignin, hemicelluloses and cellulose(Alonso et al.,2012)

Lignin is a complex polymer of aromatic alcohols, it is a fibrous, tasteless material, insoluble in water and alcohol but soluble in weak alkaline solutions, and which can be precipitated from solution using acid. Lignin fills the spaces in the cell wall between cellulose, hemicellulose, and pectin components. Due to its chemical structure, lignin is classified as polyphenilpropanoid (Korkina, 2007). Lignin can be recovered by many delignification process and it is a versatile molecule that possesses manifold properties such as antioxidant (strong radical scavenger),anti-fungal and antibiotic activity (Mai et al., 2000; Lu et al., 1998; Barclay et al., 1997). Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands of β(1→4) linked D-glucose units (Crawford, 1981; Updegraff, 1969). It can be recovered and used for fiber production (Frederick et al.,2008) or sugar release (Moxley et al.,2008).

Hemicellulose is the second polysaccharides in nature, representing about 20-35 % of lignocellulosic biomass (Saha, 2003). Hemicellulases are classified according to their action on distinct substrates and Xylan is the major carbohydrate found in hemicellulose structures and its complete biodegradation leads to the formation of monomeric sugar (Perez et al., 2002). Hemicellulose is widely used in food industries as clarification and stabilization agent for fruit juice and beer (Viikari et al.,1993) and for bioethanol production (Spigno et al., 2014). Moreover grape

stems are rich in phenolic compounds such as stilbenic metabolites that are not frequently present in other by-products. This means that grape stems could be a source of particular phenolic molecules (Makris et al.,2007).

1.2.2 Seeds

Grape seed extracts (GSE) derived from whole grape seeds are a rich source of bioactive flavonoids such as resveratrol, oligomeric procyanidins, and linoleic acid. The microencapsulation of GSE allows a reduction in astringency and bitterness and as a consequence, the application of the extract can be use as a functional food (Davidov-Pardo et al., 2013). The grape seeds extract has shown some interesting results on the inhibition of growth and/or killing of some pathogenic microorganism. GSE encapsulated into a pea starch films with pork loins infected with *Brochothrix thermofacta* reduced the bacterial growth by 1.3 log CFU/mL after 4 days of incubation at 4 °C (Corrales et al., 2009). Water-soluble muscadine GSE exhibited strong antimicrobial activity against a cocktail of three strains of *Escherichia. coli O157:H7* and this activity increased after heat treatment, possibly because of increased acidity, tartaric acid, and individual and total phenolic content (Kim et al., 2008). Moreover the GSE inhibited antitoxin properties of the Shiga toxins Stx1 and Stx2 produced by *E. coli* O157:H7 bacteria (Quiñones et al., 2009).

1.2.3 Skins

The quantity of phenolic compounds in grape berries depends on the variety of grapevine and it is greatly influenced by viticultural and environmental factors, for example light, temperature, altitude, soil type, water, nutritional status, pathogenesis, and various developmental processes (Downey et al., 2006). Anthocyanins are the major phenolics in red grape skin. They are monoglucosides of five anthocyanidins, called delphinidin, cyanidin, petunidin, peonidin and malvidin (Guerrero et al, 2009) (Figure 1. 6).

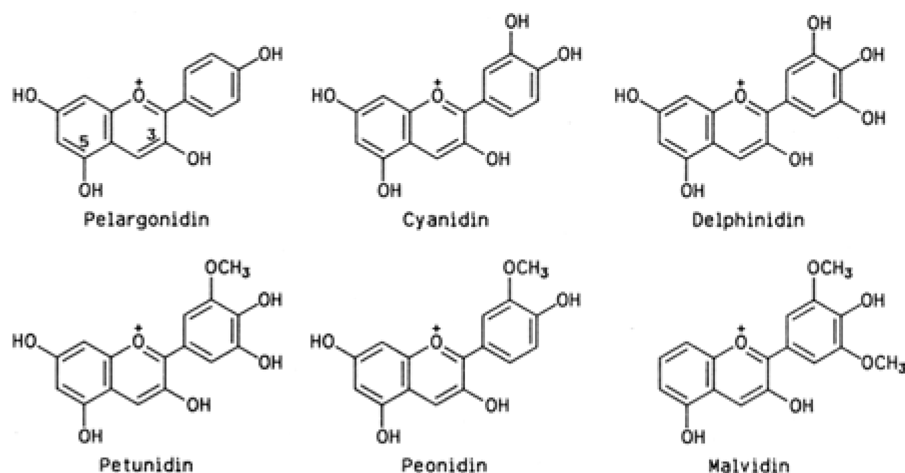


Figure 1.6. Chemical structure of the main anthocyanins present in the grape skin (from www.eplantscience.com)

Another important family of polyphenolic compounds in the grape skin are the Flavan-3-ols (monomeric catechins and polymeric proanthocyanidins). This class of phenolic compounds includes catechin, epicatechin, gallocatechin, epigallocatechin and their corresponding polymers knows as tannins (Figure 1.7). Hydroxycinnamic acids are the third most profuse group of phenolic compounds in grapes, this cluster includes p-coumaric acid, ferulic acid and caffeic acid (Figure 1.8)(Rodriguez et al.,2006).

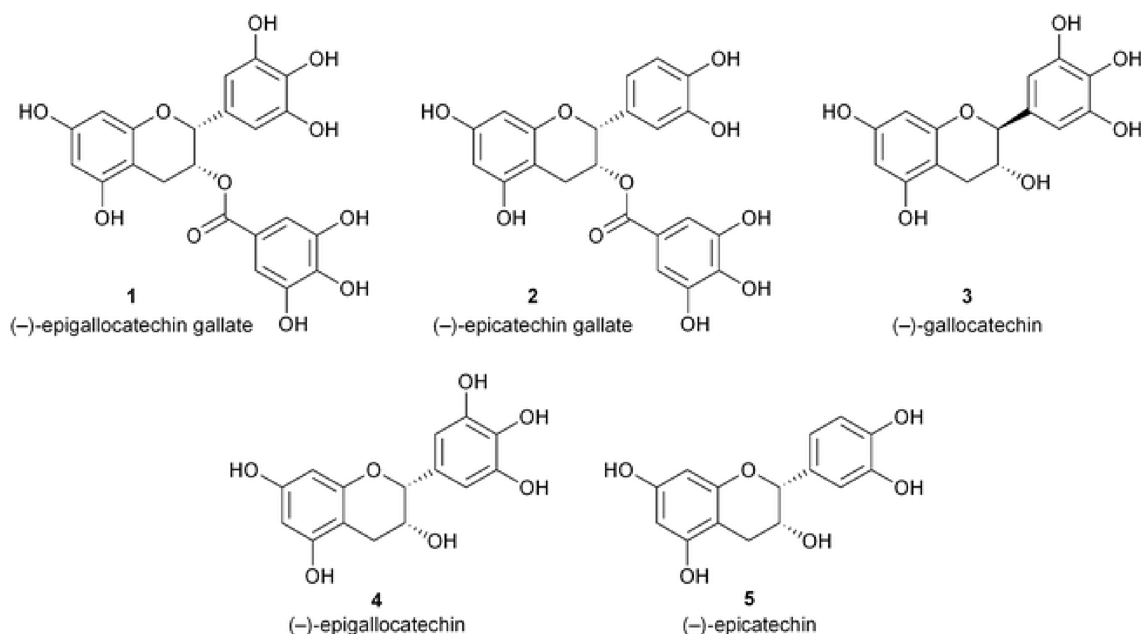


Figure 1.7. Chemical structure of the main flavanols in grape skin (from Drynan et al.,2010)

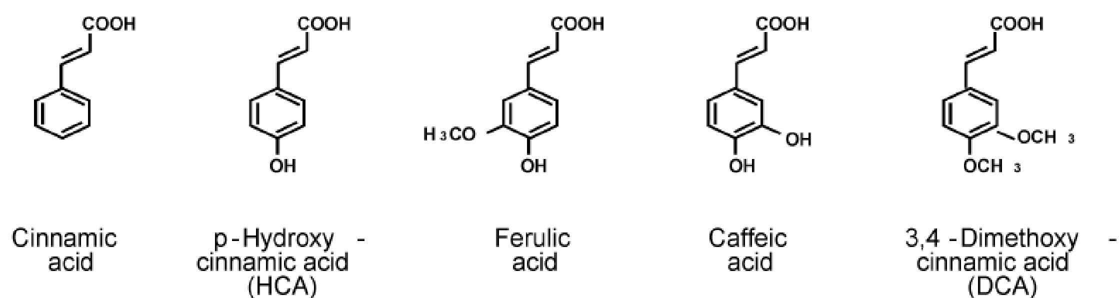


Figure 1.8. Chemical structure of the main hydroxycinnamic acids in grape skin (from Yamaguchi, 2012)

1.3 Conventional and alternative extraction technologies for antioxidants recovery

Presently several methods have been proposed and developed to enhance the extraction and isolation of bioactive compounds from different matrixes. In the following section a brief overview on the main extraction technologies adopted in the literature, is reported.

1.3.1 Solvent extraction

Solvent extraction (SE) is applied to recover some compounds from different materials like soil, polymers, microorganism and more frequently from plants residues (Hattab et al., 2007;Plaza et al., 2010). In the SE process the raw material is commonly pre-treated (such as drying and size reduction) and, after that, it is exposed to one or more extraction steps with one or different solvents to take up different interesting compounds. The obtained samples are generally submitted to centrifugation and/or filtration step to remove the solid residue. The final extract can be used as additives, food supplement or can be encapsulatefor the preparation of functional food or suitable for pharmaceutical purpose (Starmans & Nijhuis,1996). Purification of the extract can be required depending on the final destination.

The SE represents the most used technology for the recovery of bioactive compounds, with the main solvents adopted: methanol, ethanol, acetone, ether, chloroform, acetonitrile, benzene, hexane. Some applications are reported in Table 1.1. The solvents can be used pure or at different ratios with water (Vatai et al., 2009; Tokuoka et al., 2010; Pedroza et al., 2011). One of the main advantages of using certain organic solvents is their ability to recover both polar and non-polar molecules as alkaloids, phenols, fatty acids and more (Li et al., 2006). In opposite, the main disadvantage is the high toxicity for the human health and dangerousness for the environment. Furthermore the solvent has to be removed from the extract by evaporation or concentration, increasing overall process costs.

Table.1.1 Some literature studies on the use of organic solvent extraction for the recovery of bioactive compounds

Solvents /conditions	***Raw material***	***Compounds of interest***	***Bioactivity/Possible applications***	***Reference***
Methanol	Eggplant	Phenols and flavonoids	Positive health effect and possible use as food colorant	Akinitapichat et al., 2010
Ethanol, ethyl acetate and acetone	Edelberry and red grape mark	Phenols and anthocyanins	Positive health effect and possible use as food colorant	Vatai et al., 2009
Acetone: water 50% v/v	Refosk grape mark	Phenols and anthocyanins	Positive health effect and possible use as food colorants	Vatai et al., 2008
Hexane,ethanol, petroleum ether, chloroform	Wheat straw, germ, bran	Policosanols	Lowering LDL and increase HDL/ nutraceutilcal ingredients	Dunford et al., 2010
Hexane, ethanol, water	Algae	Volatiles, fatty acids and carotenoids	Antioxidant and antimicrobial/ functional food ingredients	Plaza et al., 2010
Water/ethanol, acidified with either acetic or citric acid	*Ceratonia siliqua* L.	Phenols	Antioxidant and antimicrobial/ functional food ingredients	Cavdarova & Makris, 2014

1.3.2 Microwave-assisted extraction

The microwaves are defined as an electromagnetic radiation with a wavelength from 0.001 m to 1 m that could be transferred in a wave form. The microwaves pass across the medium and their energy can be adsorbed and converted into thermal energy causing the water inside the cell to transform into vapor . This phenomenom increases the pressure on the cell wall with a modification of the physical properties of the biological tissues, improving the porosity of the biological matrix and resulting in a better penetration of the extracting solvent and improved yield of the bioactive compounds (Zhang et al., 2011). The microwave-assisted extraction (MAE) has been widely used for the recovery of bioactive compounds, in particular phenolic compounds (Li et al., 2011; Moreira et al., 2012; Spigno & De Faveri, 2009) and essential oils (Libran et al.,2013) from different matrixes (Table 1.2), due to some positive characteristics. In general, it allows a reduction of the solvent consumption and, above all, of the extraction time, with possible less energy utilization (Zhang et al., 2011).

1.3.3 Ultrasound-assisted extraction

Ultrasound –assisted extraction (UAE) has been proposed as mild technology alternative to the organic solvent extraction. The UAE method is based on the acoustic cavitations phenomenon: at certain intensity the expansion cycle could generate cavities or micro bubbles in the liquid. These bubbles are able to adsorb the energy from the sound waves and grow during the expansion cycles and recompress during the compression cycle. Therefore the implosion of the cavitations bubbles can hit the surface of the solid matrix and damage the cells causing the release of the bioactive compounds (Leighton 2007; Escaplez et al., 2011; Dahmoune et al., 2014). Many studies have shown that the recovery and the antioxidant capacity of the extract are strongly influenced by the extraction time, temperature and the frequency adopted (Hossain et al.,2011; Ghafoor et al.,2009). In addition, the stability of the UAE phenolic extract is higher than the extracts obtained with other extraction approach (Dobias et al., 2010).

Table 1.3.Some literature studies on the use of Microwaves and Ultrasounds-assisted extraction for the recovery of bioactive compounds.

Method /conditions	***Raw material***	***Bioactive compounds***	***Applications***	***Reference***
UAE-Ethanol	*Prunella vulgaris*	Flavonoids	Medical application, reducing fever	Zhang et al., 2011
UAE-Water	Litchi seeds by products	Polysaccharides	Food and biomedical applications	Chen et al., 2011
MAE-Ethanol 80 %	Pigeonpea leaves Plant	Cajanistilbene acid and pinostrobin	Medical treatment	Kong et al., 2010
MAE-Ethanol 30 %	Peanut skins by products	Phenolic compounds	Pharmaceutical application	Ballard et al., 2010
MAE-Ethanol 47.2 %	Grape seeds of different cultivars	Phenolic compounds	Pharmaceutical and food industries	Li et al., 2011

1.3.4 Pulsed Electric Fields

Recently, pulsed electric fields (PEF) and high-voltage electrical discharges (HVED) have been tested for polyphenols extraction from various by-products.

PEF induce the membrane electroporation phenomenon. When subjected to an external electric field, the difference in electrical potential across the cell membrane increases. If the induced electrical potential exceeds some threshold value (1–2 V for most plant tissues), the cell membrane loses its semi-permeability leading to pore creation: this process is called electroporation and allow an enhanced extraction of biomolecules from the cell. This permeabilization of cell membranes can be achieved at moderate electric fields of 0.5–1 kV/cm and treatment times in the range of 100 and 10,000 μs as tested by Vorobiev & Lebovka (2006).

Comparable effects have been obtained at higher electric field strengths (1–10 kV/cm) and shorter treatment times (5–100 µs) by Corrales et al. (2008), López et al. (2009), Schilling et al. (2008).

1.4 Surfactant and phenolic compounds interaction

Surfactants are usually classified as amphiphilic organic compounds, they contain both hydrophobic groups and hydrophilic groups. The hydrophobic part is generally related to the tail group and hydrophilic group is related to the head of the surfactant structure (Figure 1.9) (Rosen & Kunjappu, 2012). The word surfactant or surfactants derives from the expression *''surface active agent''* and it refers to the ability of a material , when present at a low concentrations, to adsorb onto the interface/surface of the system, altering in this manner the interfacial free energies of the interface (Rosen, 2004). One of the surfactants property is the capability to form micelles above their Critical Micelles Concentration (CMC). The micelles are composed by hydrophilic external surface and hydrophobic internal core. This particular structure allows the micelles to establish chemical and physical interaction with both hydrophilic and hydrophobic molecules (Hosseinzadeh et al., 2014).

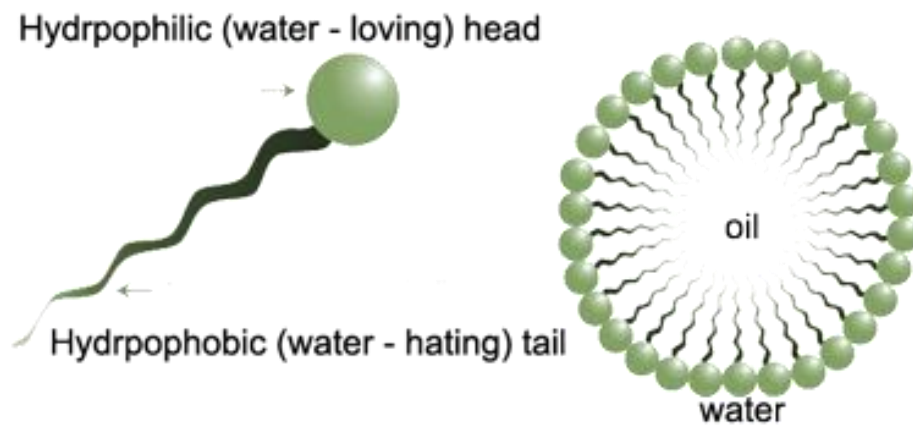


Figure 1.9. Surfactant (on the left) and micelle(on the right) structures (from www.hydrosoil.com.au)

In natural phenolic extracts, the phenolic fraction is actually a cluster composed of molecules with different structure and different polarity and the presence of surfactant, with the hydrophobic and hydrophilic part, during the extraction could be enhance their recovery and fractionation. Due to the aromatic structure, the phenolic compound adsorbs initially to the micelle interface and then enters deeper into the hydrophobic core (Liu et al., 2010). However, the interaction between surfactant and phenols depends by the kind of surfactant adopted and by the phenols ionization state.

Actually there are three main classes of surfactants:

- Ionic surfactants
- Non-ionic surfactants
- Zwitterionic surfactants

Ionic surfactants have a net charge on the hydrophilic head and they are classified as anionic surfactants if the charge is negative (the head contains groups as sulfate, sulfonate, phosphate or carboxylates)(Figure 1.10d) and cationic surfactant if the charge is positive (for example primary and secondary amines at pH lower than10)

(Figure 1.10b). The interaction between phenols and these kinds of surfactants are supposed to be mainly driven by electrostatic interactions.

Non-ionic surfactants (Figure 1.10a) are long chain molecules without net charge on the polar head. The cluster consists of fatty alcohols, cetyl alcohol, stearyl alcohol, and cetostearyl alcohol. In this case, the phenols-surfactant interaction are supposed to be mainly driven by hydrophobic interactions.

Zwitterionic surfactants (Figure 1.10c), called also amphoteric surfactants, have both cationic and anionic functions in the same molecule. The cationic part is based on primary, secondary, or tertiary amines or quaternary ammonium cations. The anionic part can be more variable and includes sulfonates.

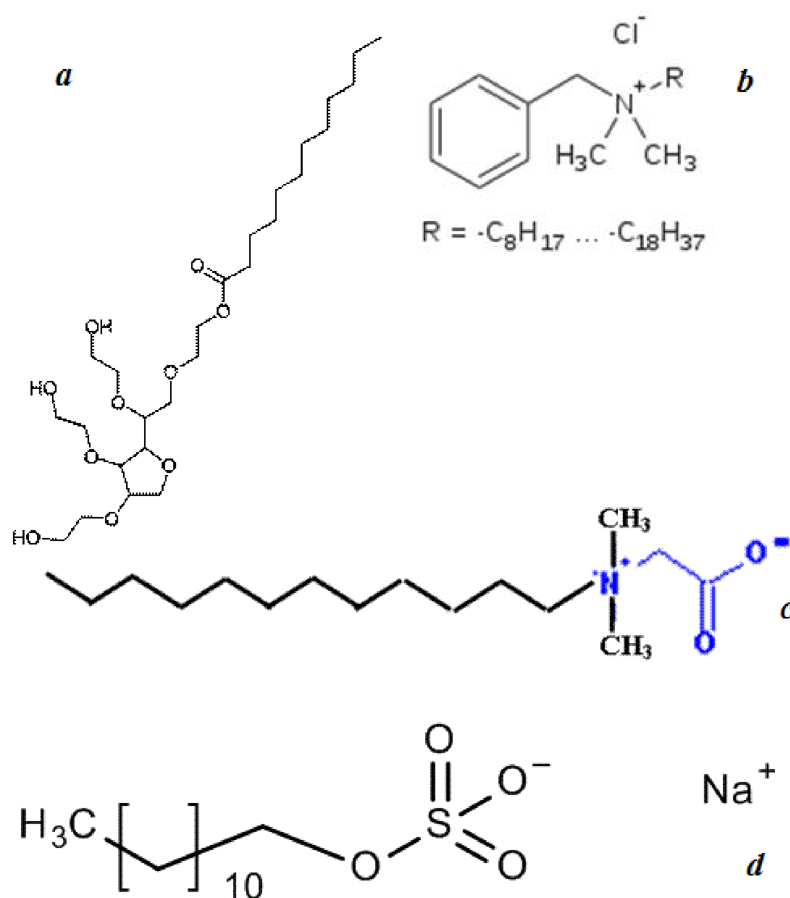


Figure 1.10. Structure of different surfactants: (a) Tween 20, non ionic (b) Benzalkonium Chloride, cationic (c) Alkilbetaine, Zwitterionic (d) Sodium Dodecyl Sulfate, anionic.

It is necessary to underline that the only group which includes non toxic surfactants, and for this reason allowed in a food formulation, is the non-ionic ones.

1.5 Colloidal gas aphrons for the recovery of bioactive compounds

Colloidal Gas Aphrons (CGAs) were first reported by Sebba (1971) as micro bubbles, with dimension among 10–100 μm, created by intense stirring of a surfactant solution above its CMC, composed of a gaseous inner core surrounded by a thin surfactant film (Figure 1.11) and for this reason they have different dispersion characteristics compared to conventional foams. The water near the two surfaces (viscous water) is different from the bulk water (external to the bubble), since it has more hydrogen bonds. This water structure involves, as a consequence, the orientation of surfactant molecules with the hydrophilic hydrocarbon head pointing inside and the hydrophobic hydrocarbon tail pointing outwards. This kind of orientation also prevents the CGAs coalescence. As previously reported by Spigno et al*.* (2010), surfactants are able to catch phenols thanks to both electrostatic and hydrophobic interactions depending on the surfactant (cationic, anionic or non-ionic) and on the phenolic compound. Depending on the surfactant used to produce CGAs, the outer surface of the micro bubble may be positively, negatively or non-charged, to which oppositely or non-charged molecules will adsorb resulting in their effective separation from the bulk liquid (Spigno et al., 2005), therefore the selectivity of adsorption can be adjusted (Fuda et al., 2006). The separation of the molecules coincides with the separation of the CGAs in two different phases due to the progressive collapse of the foam: the one on the top that contains the recovered molecules is called Aphron phase while the one on the bottom is called Liquid phase.

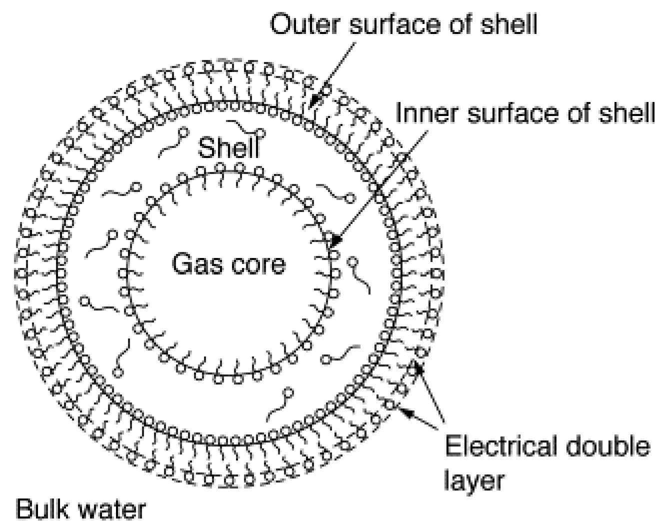


Figure 1.11. Proposed structure of colloidal gas aphron (Jauregi & Varley, 1999)

Despite of poor information about the structure and stability of the CGAs, some characteristics have been clearly defined (Jauregi, et al.,1997, 1999).:

- a large interfacial area per unit volume due to the small size bubble;
- high Gas Hold Up;
- relatively high stability;
- flow property very similar to water flow property, which allows an easy pumping from a site to another avoiding the foam collapse

Thanks to their properties, low cost and related high efficiency, CGAs have been found large application on the recovery of different bioactive compounds mainly from liquid samples, such as microbial cells (Save & Pangankar, 1995) and proteins (Jauregi & Varley, 1998; Fuda et al., 2005).

Hashim & Gupta (1998) applied CGAs also to recover fine cellulose from paper mill waste water with satisfactory results. Moreover, CGAs were frequently applied on waste water to remove some toxic compounds such as organic dyes (Roy et al.,1992; Basu & Malpani, 2001) and some useful molecules such as phenolic compounds (Gotzi et al., 2008). Spigno et al. (2011) and Dahmoune et al. (2013) proposed an interesting method for phenols purification and fractionation from grape skins extract based on CGAs.

Although a significant number of research works on CGAs application on liquid sample have been published, their application on solid sample is less studied. The

only relevant works are those by Zad Zidehsaraei et al. (2009) that have proposed the simultaneous extraction and recovery of glucoamylase from solid biomass, and by Roy et al. (1994a, 1994b) that removed oily waste from soil matrix with the CGAs.

1.6 Previous results on application of CGAs to the recovery of polyphenols from wine making wastes

CGA could also be applied for the purification of phenolic extracts in order to separate phenolic from non-phenolic compounds (such as sugars and minerals), and to fractionate different classes of phenolic compounds.

In a typical CGAs based separation process, CGAs are generated and placed in contact (e.g. in a flotation column) with the solution containing the target molecules to be removed or recovered. After contacting, a certain separation time is waited to allow the separation two phases: a top aphron phase ideally containing the desired molecules, and a bottom liquid phase. The two phases are then separately collected and, eventually, the surfactant is to be removed from the aphron phase by an additional processing step (e.g. membranes separation or solvent extraction). In case of using biodegradable and non-toxic surfactants, this methodology could result in an environmentally friendly process, while final products could also be safe for human consumption without the need of surfactant removal.

Spigno et al. (2010) used CGAs generated from a cationic surfactant (CTAB) to recover gallic acid from aqueous solutions, obtaining maximum recovery (close to 80 %) at conditions which promote electrostatic interactions between the cationic surfactant and the anionic form of gallic acid, that is at pH > pKa = 3.4. However, antioxidant capacity of the phenolic acid was greatly reduced at pH above 6. An increased ionic strength, that could be the case of real extracts containing natural salts, hindered electrostatic interactions with a consequent decrease in recovery efficiency.

CGAs have also been applied to real crude phenolic extracts obtained with aqueous ethanol solvent extraction from waste red grape pomace and skins (Dahmoune et

al., 2013; Spigno et al., 2014). In these trials, carried out in a batch flotation column, the influence of surfactant type (CTAB and the food-grad non-ionic Tween 20) and feed concentration was investigated. The crude extract was pre-concentrated to recover the solvent (ethanol), and then diluted with water to reach a pre-defined total phenols concentration before mixing with CGAs.

When CTAB was used, the natural extract showed a stabilising effect on CGAs (reduced drainage rate), leading to higher recovery. Anthocyanins revealed more affinity for the CGAs than other phenolics, probably due to the fact that they are polarized because of their high electron density in the aromatic ring. When the separation was carried out at pH 2, only a slight reduction in anthocyanins recovery (but not in that of other phenolics) was observed. This confirmed that separation was driven by electrostatic and hydrophobic interactions.

When Tween 20 was used, recovery decreased for both anthocyanins and other phenolics, confirming that electrostatic interactions enhanced the partitioning. Maximum recovery (78 % total phenols and 76 % total anthocyanins) was obtained at the highest volumetric ratio (22) and highest feed concentration (5 g/L of gallic acid equivalents). However, high concentrated feed led to the formation of insoluble aggregates in the aphron phase. Tween 20, interestingly, led to a lower loss of antioxidant capacity than CTAB.

In any case it was not possible to selectively separate non-phenolic compounds (reducing sugars and potassium), while a limited higher affinity for the aphron phase was shown by cinnamic acids and flavonols, which are smaller molecules compared to anthocyanins.

1.7 Encapsulation techniques

Microencapsulation is defined as a technology of wrapping gaseous, solid or liquid materials in small size, sealed off capsules that can release their contents at controlled rate under specific environmental conditions (Desai & Park, 2005; Vilstrup, 2001). The internal phase of the capsules can be pure or composed by a mixture of different substances and it can be called in different way such as core material, coated material. The external phase of the capsules, called wall material

or coating material, can be made on its turn of different compounds such as sugar, gums (Boiero et al., 2014), proteins (Costa et al., 2015; Flores et al., 2014), natural and modified polysaccharides (Souza et al., 2014), lipids (Mozafari et al., 2008) and synthetic polymers (Al-Qadi et al., 2014). The morphology of the capsules is strictly related to the encapsulation process and by the core and wall materials adopted. The most spread shapes are : the first one is mononuclear capsules, which have a single core enveloped by a shell; the second one is aggregates, which have many cores embedded in a matrix (Schrooyen et al., 2001) (Figure 1.12).

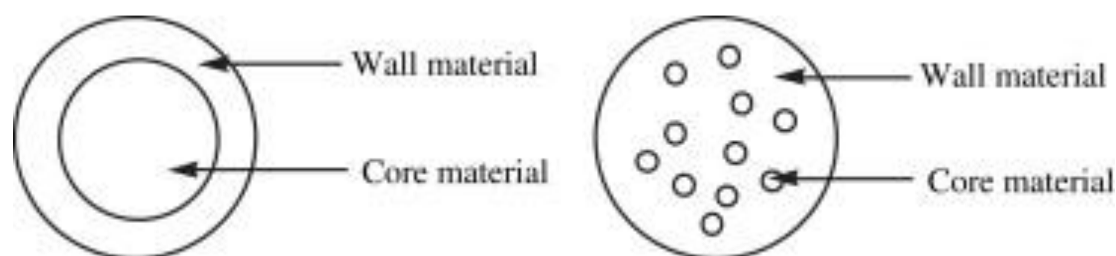


Figure 1.12. The two main encapsulation structures: mononuclear capsule on the left and aggregate on the right(Fang & Bhandari, 2010)

Currently, several encapsulation techniques have been developed such as spray drying, spray cooling or chilling, fluidized bed coating, coacervation, liposome entrapment, inclusion complexation, lyophilization, cocrystallization and emulsion (Augustin & Hemar, 2009; Desai & Park, 2005; Gibbs et al., 1999).
Independently of the used technique, the following aspects have to be guaranteed (Mozafari et al., 2008):

1. Formation of the wall around the packed compounds
2. Prevention of undesired release of the packed compounds
3. Ensuring that undesired materials are kept out

For encapsulation of bioactive compounds, the primary aim of the encapsulation process is to protect the core material from adverse environmental conditions such as light, moisture and oxygen and, at the same time, increase its shelf life and improve its controlled release (Shaidi & Han, 1993).
The encapsulation of phenolic compounds should offer the potential to overcome the liability of their instability and improve their bioavailability in the final application system (food or human body).

The main capsule structures obtained from the different encapsulation methods are reported in Figure 1.13.

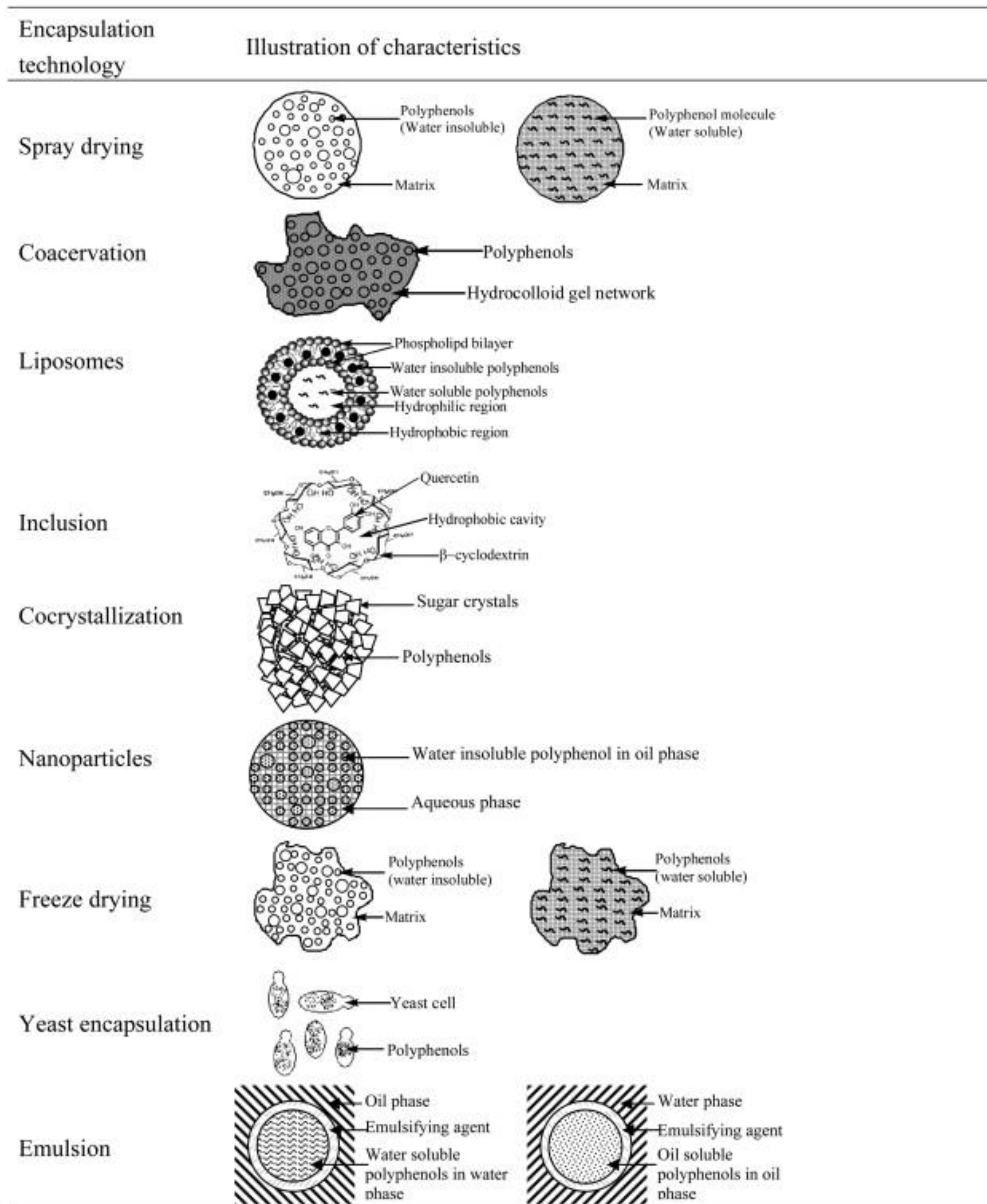


Figure 1.13. Structure of capsules obtained from several encapsulation method (Fang & Bhandari, 2010)

1.7.1 Spray drying

Spray drying techniques have been applied in the food industrysince the 1950s. This widely used encapsulation technique in the food industry is due to some positive characteristics such as cost-effective, flexibility, continuous operation and, moreover, production of final good quality particles (Desai & Park, 2005).
The spray drying process requires the presence of wall material that has to be hydrated and mixed with the core material. The final solution is fed into the spray dryer and atomized in small droplets. After water evaporation, the most commonly shape of the obtained dried droplets is spherical with a size from 10 to 100 μm (Fang & Bhandari, 2010).
The correct selection of the wall material is one of the most important parameters to ensure the stability and the good quality of the final powder. Also the inlet temperature has shown to play an important role on the phenols content in the final products (Georgetti et al., 2008).

1.7.2 Freeze drying

The freeze drying or lyophilization process is commonly used on heat-sensitive compounds. Freeze drying is a multi-stage operation stabilizing materials throughout the four main stages: freezing, sublimation (primary drying), desorption stage (secondary drying), and, finally, storage. Freeze drying results in superior-quality products, which are easily reconstituted, with a longer shelf-life. On the other hand, high energy consumption, long processing time (usually from 24 to 48 hours) and the final product withopen porous structures are the main drawbacks of freeze drying (Singh & Heldman 2009; Oetjen & Haseley, 2004). Freeze drying allows a very simple encapsulation of water soluble compounds and it does not require the presence of encapsulation material.

1.7.3 Coacervation

The coacervation technique involves the phase separation of a single or a mixture of hydrocolloids from a solution and the subsequent deposition of the newly set up coacervate phase on the active ingredient suspended or emulsified in the same reaction media (Fang & Bhandari, 2010). Also, a hydrocolloid hull can be cross-linked exploiting a suitable chemical or enzymatic cross-linker such as glutaraldehyde or transglutaminase, mainly to increase the robustness of the coacervate. The coacervation process is influenced by different factors including the biopolymer type (molar mass, flexibility, and charge), pH, ionic strength and concentration. All these factors affect the strange of the interaction between the used biopolymers, as well as the nature of the complex formed (Ezhilarasi et al., 2013). Electrostatic interactions between biopolymers of opposite charges, hydrophobic interactions and hydrogen bonding can also contribute significantly to the complex formation. The encapsulation by coacervation can be achieved in two ways through the application of only one colloidal solute or through a more complex process. The complex coacervation process frequently leads to particles withoutwell defined shape and it is considered an expensive method for encapsulating food ingredients, even though it could be a good solution for the encapsulation of high value, labile functional ingredients like phenols (Gouin, 2004).

1.7.4 Liposomes

Liposomes are defined as colloidal particles composed of a membranous system formed by lipid bilayers encapsulating aqueous space. The lipid and the aqueous phases of the liposome structure allow the entrapment, delivery, and release of water soluble, lipid soluble and amphiphilic compounds. The liposome mechanism formation is essentially a hydrophilic-hydrophobic interaction between phospholipids and water molecules (Fang & Bhandari, 2010). Liposome encapsulation have shown some interesting advantages as controlled release rate of the encapsulated compounds and delivering of the agent in the right place at the right time (Schäfer et al.,1992). Takahashi et al. (2007) have shown that the

bioactive compounds encapsulated into liposomes are protected by the digestion process in the stomach and show significant levels of adsorption in the gastrointestinal tract with an enhanced bioavailability and bioactivity.

Recent studies have demonstrated that liposomes are particularly suitable to incorporate phenolic compounds of grape seed extract with an encapsulation efficiency of 87% (Gibis et al., 2013; Gibis et al., 2012).

1.7.5 Co-crystallization

Co-crystallization is an encapsulation process that consists in the modification of sucrose structure from a perfect to an irregular agglomerated crystal in order to create a porous matrix suitable for the incorporation of active ingredients (Chen et al., 1988). The modification of the sucrose structure can be achieved at high temperature (around 120 °C) and low moisture (around 95° Brix). The addition of the ingredients to be encapsulated during sucrose crystallization results in their incorporation in the empty space present inside the agglomerates of the microsized crystals (less than 30 μm) (Bhandari et al., 1998). The advantages of the co-crystallization process can be summarized in improved solubility, wettability, homogeneity, dispersability, hydratation, anticaking, stability and flowability of the encapsulated materials (Beristain et al., 1996).

1.7.6 Nanoencapsulation

Nanoencapsulation process involves the formation of active particles with a diameter ranging from 1 to 1000 nm (Fang & Bhandari, 2010). The nanoparticles can be divided in two different categories:

- Nanospheres, where the active molecules can be adsorbed at the sphere surface or encapsulated within the particles;
- Nanocapsules, vescicular systems in which the active molecules are cramped to a cavity composed of an inner liquid core surrounded by a polymeric membrane (Couvreur et al., 1995).

The nanoparticles offer a greater surface area when compared with the micro-size particle and moreover they have shown enhanced solubility, bioavailability, release rate and,better precision targeting respect to the encapsulated compounds (Mozafari et al., 2008). Furthermore, several works have been proposed on nanoencapsulation of phenolic compounds (Spigno et al., 2013; Kaur & Saraf, 2012) and others bioactive molecules such as essential oils (Bilia et al., 2014) and bioactive peptide fraction (Mosquera et al., 2014).

2. Aims

In the framework of the Valorvitis project (described in chapter 1), this thesis work was focused on the valorisation of residual grape skins for the recovery of antioxidant compounds through both conventional solvent extraction and innovative strategies based on the use of surfactants for process intensification.

2.1 Conventional solvent extraction

The conventional solvent extraction process applied in this thesis was selected from the results of previous research works, therefore it was not further optimised with the exception of the number of extraction steps. In particular it was investigated the influence of a double extraction on the phenolic extraction yields.
The main objective of this part of the research was to evaluate the influence of grape variety, wine-making process and year on the extraction yield, the phenolic composition of the extracts and the antioxidant capacity of the extracts. All these aspects, in fact, are required to evaluate the possibility of obtaining waste grape skins extracts with standard characteristics independently of the original grape variety and year. This, on its turn, would allow to scale-up the process and implement it at commercial scale..
The trials were then carried out on waste skins obtained from six different grape varieties:

1. Barbera: a red grape extensively cultivated and processed in Italy and particularly rich in anthocyanins. Skins were recovered after fermentation.
2. Pinot noir: a red grape extensively cultivated and processed in Italy but also in the rest of the world. Skins were recovered before fermentation because this grape is often used to produce white wines. In this case, the residual skins are potentially richer in phenolic compounds than fermented ones. Furthermore, off-skins fermentation, the vintage is carried out early to limit the wine tannins content.
3. Nebbiolo: a red grape typically cultivated in Piemonte (a Northern particularly wine-vocated region of Italy) and with a low content in anthocyanins. Skins were recovered after fermentation. Compared to Pinot noir, vintage takes place late to maximise the wine tannins content.

4. Moscato: a white grape extensively cultivated in Piemonte for the production of aromatic and sparkling wines. Skins were recovered before fermentation.
5. Müller Thurgau: a white grape extensively cultivated in Trentino Alto Adige (a Northern particularly wine-vocated region of Italy) for the production of aromatic wines. Skins were recovered before fermentation.
6. Chardonnay: a white grape extensively cultivated and processed in Italy but also in the rest of the world. Skins were recovered before fermentation.

Skins were collected for three consecutive vintages (2011-2014) and the relative extracts obtained and characterized.

2.2 Process intensification: investigation of different strategies based on surfactant application

The conventional solvent extraction uses 60 % aqueous ethanol as a solvent, therefore its large scale implementation could be problematic in terms of processing costs and environmental impact. Furthermore, down processing purification steps may be required depending on the final application of the phenolic extracts. Different methods for phenols purification have been studied in literature, with the application of adsorption resins the most used one, even though it generally requires large volumes of different solvents for the elution and regeneration steps.

Investigation of alternative strategies is therefore necessary to make the overall production of phenolic compounds from waste skins more feasible from both, economic and environmental point of view.

In this work enforcement of a food grade surfactant solution to develop low cost and environmentally friendly extraction and/or purification methods was investigated. The overall idea comes from the results of previous researches showing the interactions between surfactants and phenolic compounds and between phenolic compounds and surfactants in the form of colloidal gas aphrons.

These interactions have been exploited for different purposes: solubilise or precipitate specific phenolic compounds; alter the phenolics partitioning in oil-in-water emulsions; enable phenolic compounds analytical determination; protect phenolic compounds from oxidation; improve phenolic compounds efficiency in topical formulations; recover phenolic compounds for pollution remediation.

The surfactant application was then investigated with the following different aims:

- Evaluate the possibility of replacing in part or completely the organic solvent (ethanol) with the food grade surfactant Tween® 20.
- Evaluate the possible integration of the extraction and purification steps with the application of CGAs immediately after the solvent extraction. This goal had the dual purpose of reducing the number of process steps and reducing the cost and environmental impact of a reference purification process with resins. .
- Obtain additional advantages, like reduction of the amount of encapsulation material and / or improved phenolic compounds stability / solubility in a spray drying encapsulation process for the production of powder phenolics formulations .

This part of the project was developed also in collaboration with the School of Chemistry, Food & Pharmacy of the University of Reading (UK).

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

The following reagents have been used: Gallic acid and Caffeic acid from Fluka; Sodium carbonate, Sodium phosphate monobasic, Ferric Chloride, Sodium Acetate, Potassium Chloride, Hydrochloric acid, Sodium Hydroxide, Sulfuric acid, Nitric acid, Potassium dihydrogen phosphate, Sodium chloride from Carlo Erba Reagents; Sodium phosphate dibasic, Folin reagent, Ethanol from VWR Chemicals; TPTZ (2,4,6-Tris(2-pyridyl)-*s*-triazine), Tween20, Quercetin, Metionin, AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride), Fluorescin sodium salt, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), Trolox, NBT from Sigma-Aldrich; Potassium sulfate from Merck.

3.1.2 Instruments

The following instruments have been used: Perkin Elmer Lambda BIO-40 UV/VIS Spectrophotometer; Shimatzu UV-1601 Spectrophotometer; Silverson L5M mixer; Silverson SL2T mixer; Flame Atomic Emission Spectroscopy (AES) Perkin Elmer AAnalyst 300; Buchi K-424 digestion unit; Thermo Scientific SL 16 R centrifuge; Varifuge 20 RS Hereus Sepatech centrifuge; Biotek Synergy HT Multidetection Microplate Reader; Peristaltic pump Watson–Marlon 502 S; glass flotation supplied by DISA Milano; hydrostatic balance Gibertini Idromatic;, Vibromix Pbi vortex; Buchi B-290 mini Spry Dryer; VELP Scientifica UDK 127 Distiller Unit; AquaLab Dew Point Water Activity Meter 4TE; Buchi Rotavapor R-114; Buchi Waterbath B-480

3.2 Antioxidant recovery from waste grape skins

3.2.1 Sample collection and characterization

Grape marcs of six varieties were kindly provided by different wineries in Northern regions of Italy (Piemonte and Trentino Alto Adige) during the vintages 2011, 2012, 2013. Table 3.1 resumes the characteristics and sampling of the investigated varieties.

Table 3.1 – Sampling and characteristics of the six grape marcs investigated in the project.

Cultivar	*Characteristics*	*Diffusion*	*Sampling*
Barbera	Red grape, rich in anthocyanins	Italian	Piemonte, after fermentation, 2011-12-13
Nebbiolo	Red grape	Italian	Piemonte, after fermentation, 2011-12-13
Pinot Noir	Red grape	International	Piemonte, after pressing, 2011-12-13
Chardonnay	*White grape*	International	Piemonte, after pressing, 2011-12
Moscato	*Aromatic white grape*	Italian	Trentino, after pressing, 2011-12
Müller Thurgau	*Aromatic white grape*	International	Trentino, after pressing, 2011-12

Cultivar selection was made under the Valorvitis Ager project based on their diffusion in the Italian regions (involved in the project), in Europe, their phenolic content and representativeness of wine-making process.

Three varieties are typical Italian cultivars and two of them, Barbera and Nebbiolo are red berries grapes cultivars, with Barbera well known for its high anthocyanins content and Nebbiolo for its high total phenols content and low anthocyianins content. The third, Moscato, is a white aromatic grape berry cultivar used for the production of aromatic sparkling sweet wine.

The other varieties, Pinot Noir (red grape berries), Chardonnay and Müller-Thurgau (white grape berries) are also internationally cultivated and Pinot Noir is a red grape processed as a white one.

Barbera and Nebbiolo grape marcs were collected after the alcoholic fermentation and maceration process, while Pinot Noir and the three white berries varieties were collected after the pressing operation and before maceration process. Since maceration is the main step to extract polyphenols (mainly anthocyanins) from grape skins, off-skins fermented grape marcs are potentially richer in phenolics.
Samples were dried at 53-54 °C for 48-72 h in a ventilated oven. After drying, skins were manually separated from the seeds, milled at a final particle size ≤ 2 mm and kept in air tight bags and darkness until use.
The separated dried milled grape skins (DMGS) were characterized for moisture, total fat, ash, nitrogen and minerals content.

3.2.1.1 Moisture content

The residual moisture was evaluated on DMGS by constant dry weight oven method at 105 °C.

3.2.1.2 Total Ash content

For total ash content analysis, 5 g of DMGS were previously dried at 105°C for 48 h and then placed into a muffle at 650 °C for 6 h or until complete mineralization (white ashes). The total ash content was expressed as percentage on dry matter (dm) content ($g_{ash}/100_{d.m.}$).

3.2.1.3 Total fat content

The total fat content was evaluated by solid – liquid extraction with a Soxhlet extractor.
Extraction was carried out on 10 g of DMGS with 300 mL of hexane for 5 h. After the extraction, the solvent was evaporated under vacuum to dryness and the residue weighted to obtain the total fat content which was expressed as percentage on dry matter ($g_{fat}/100_{dm}$).

3.2.1.4 Total nitrogen content

The total nitrogen content was evaluated according to the Kjeldahl method.
DMGS (3g) were placed into a oven at 103 ± 2 °C until constant weight. After that the sample was transferred into a glass tube and 22 mL of concentrated sulfuric acid (72%) was added. Potassium sulfate (in tablet form, purchased by Merck Germany) was added to increase the boiling point of the medium (around 400 °C). The tube was placed in a digestion unit (Buchi K-424) until the initially very dark-colored medium become clear and colorless.
The digested was then distilled in an automatic distiller (VELP Scientifica UDK 127 Distiller Unit) with 90 mL of sodium hydroxide (32%), in the presence of boric acid solution (4%) which converts the ammonium salt to ammonia. The amount of ammonia present is determined by back titration with 0.1 N sulfuric acid with phenolphthalein as indicator.
The total nitrogen expressed as percentage on dry matter was obtained by Equation 3.1:

$$\frac{g_N}{100\ g_{d.m.}} = \frac{mL_{H_2SO_4} * N_{H_2SO_4} * 14}{10 * (g_{DMGS} * (1 - \frac{U_R}{100})} * 100 \text{ (Equation 3.1)}$$

Where:

- mL H_2SO_4 are the mL of 0.1 N sulfuric acid used for the titration
- N H_2SO_4 is the normality of sulfuric acid
- 14 is nitrogen molecular weight
- g_{DMGS} are the grams of DMGS skins used for the analysis
- U_R is the percentage moisture content of the DMGS
- The total nitrogen value was multiplied for 6.25 to obtain the total protein content.

3.2.1.5- Minerals content

Minerals were quantified with the flame Atomic Emission Spectroscopy (AES) Perkin Elmer AAnalyst 300 on the previously obtained ash which were dissolved with 15 mL of 1 N nitric acid. After dilution with milliQ water (MilliQ Water Purification System) the solutions were filtered and analyzed for K, Na, Mg, Fe, Cu and Zn. Only for Ca determination, the sample was diluted with a lanthanum solution. The results were expressed as g of mineral in 100 g of dry matter. For the quantification of each minerals a calibration curve with standard solutions was performed.

3.2.2 Conventional solvent extraction process

The schematic diagram of conventional solvent extraction process (CSE) is presented in Figure 3.1.

In 2011, two extraction steps were carried out for each variety applying the method reported by Amendola et al. (2010) with some modifications.

DMGS were extracted with aqueous ethanol as a solvent (ethanol:water, 60:40 v/v) at 8:1 (v/w) solvent-to-solid ratio. Many studies have shown as a mixture of ethanol and water is more efficient than the corresponding mono-component solvent system in extracting phenolic constituents from winery by–products (Alonso et al., 1991; Yilmaz & Toledo, 2006; Pinelo et al., 2005).

In particular, 125 g of DMGS were mixed with 1000 mL of solvent using a semi – industrial mixer (Silverson L5M mixer) at 3500 rpm for 2 hours at 60 °C (the temperature was controlled by means of an electrically heated plate(Heidolph MR 2002). After this first extraction, solids were separated from the liquid by centrifugation (Thermo Scientific SL 16 R) at 5000 g for 15 min at room temperature and used for a second extraction at the same previous reported conditions.

The two extracts were stored separately in the refrigerator and analyzed within 24 h for total phenols content, anthocyanins, tannins, cinnamic acid, flavonols, and antioxidant capacity (ABTS assay).

The preliminary results obtained on grape skins collected in 2011 indicated that total phenols recovered on the second extract were, on average, four times lower than the first extract and, for this reason, the process was carried out with only one extraction step for the grape skins collected in the 2012 and 2013. Furthermore, for the year 2013, only the red grape varieties were extracted since the amount of total phenols in the white extract was quite low and other trials under the Valorvitis project (not shown in this thesis) revealed great difficulties in spray-drying these extract. Antioxidant capacity of the extracts was evaluated by three different methods: ABTS assay, Ferric reducing antioxidant power (FRAP) method, superoxide radical scavenging activity (only for 2012 DMGS) and, only for 2013 DMGS, also Oxygen radical absorbance capacity (ORAC).

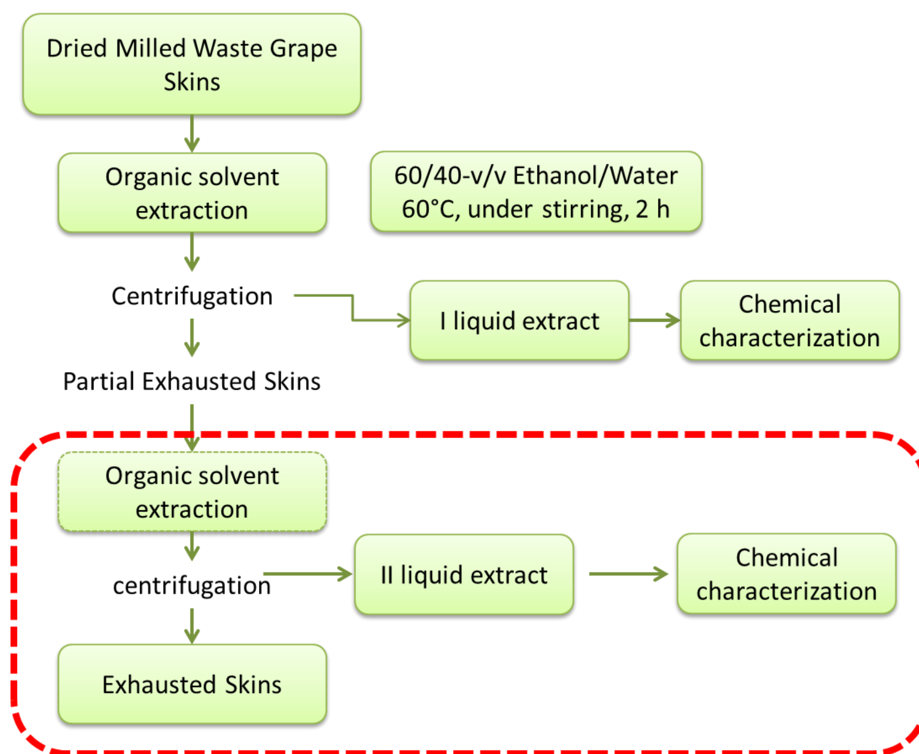


Figure 3.1. Schematic diagram of conventional solvent extraction process for 2011 grape skins. For the 2012 and 2013 DMGS the second extraction step was removed.

3.2.3 Characterization of grape skins extract

For each of the analysed phenolic classes (total phenolic compounds, cinnamic acids, flavonols, tannins and total anthocyanins), the recovery (RE_y) was calculated according to Equation 3.2:

$$RE_y = \frac{mg_y}{L_{extract}} * \frac{L_{extract}}{g_{DMGS}(1 - U_R/100)} = \frac{mg_{phenols}}{g_{d.m.}} \text{ (Equation 3.2)}$$

3.2.3.1 Total phenols

Polyphenols content from grape skins extract was evaluated by two different methods (Ribereau-Gayon et al., 2000). The first one is the Folin – Ciocalteau assay (Folin Index) and the second one is the direct measurement of the absorbance at 280 nm (total phenol index).
The two methods are based on two different principles described below.
In both cases, total phenolics were expressed as gallic acid equivalents (GAE) by means of calibration curves with a gallic acid standard.

3.2.3.1.1 Folin – Ciocalteau assay

The Folin – Ciocalteau assay is based on phenolic compounds oxidation by the Folin – Ciocalteau reagent composed by a mixture of phosphomolybdic acid and phosphotungstic acid.
The phenolic compounds in the extract react with the Folin reagent and cause the reduction of phosphomolybdic acid and phosphotungstic acid with a consequently production of molibdic oxide and tungstic oxide with a typical blue color and maximum absorbance at 750 nm. Since the method is based on the reducing capacities of phenolic compounds, the results can be influenced by the presence of other reducing substances (such as sugars, proteins and vitamins) and by the oxidative status of the molecules.
For the analysis, 25 mL of water, 0.5 mL of extract sample, 2.5 mL of the Folin reagent and 5 mL of 20 % sodium carbonate were mixed and brought to 50 mL. The absorbance was measured at 750 nm after 30 min of heating at 40 °C .
A mixture of water and reagents was used as a blank.

For each sample, different dilutions were analysed in order to cover the absorbance range 0.1 – 0.9 and average the results.

3.2.3.1.2 Total phenols index at 280nm

Total phenols index is based on the phenol's electronic spectra absorbance capacity around 280 nm. Phenols structure is composed by benzene ring bonded with hydroxyl group. The functional groups influence the conjugated system, giving a maximum absorption at 280 nm. This method is less sensitive but simpler and faster than the Folin Index. It is less influenced by the oxidative status of the phenolics but it also suffer some interference by other substances absorbing at 280 nm (such as proteins).

For the total phenolic index at 280 nm, the sample was diluted with distilled water, to obtain an absorbance range 0.1-0.9, and the absorption at 280 nm measured against water. For each sample, different dilutions were analysed in order to cover the absorbance range 0.1 – 0.9 and average the results.

3.2.3.2 Total anthocyanins content

Total anthocyanins were evaluated by diluting the extract in acid–ethanol (ethanol:water:HCl, 70:30:1, v:v:v) and reading the absorbance at 538 nm against the same acid-ethanol.

In this case, a calibration curve was not performed but the value was multiplied by the dilution factor and by 26.6, which is a literature conversion coefficient for a mixture of the five main grape anthocyanins (Di Stefano and Cravero, 2001). Results were then expressed as wine anthocyanins equivalents (WAE). For each sample, different dilutions were analysed in order to cover the absorbance range 0.1 – 0.9 and average the results.

3.2.3.3 Total tannins content

Tannins were estimated as described by Ribereau-Gayon et al.(2000).

For this analysis two samples were prepared, each containing 4 mL of extract diluted 1/50 with water, 2 mL of water and 6 mL of HCl (12 N). One sample was heated at 100 °C for 30 min and 1 mL of 96 % ethanol was added to solubilise the red colour that appears. The second sample was not heated but mixed with 1 mL of ethanol 96 %. Both samples were read at 550 nm against water.

Total tannins concentration was calculated by comparison with a standardized oligomeric procyanidin solution, and the concentration was calculated using the literature calibration equation (Equation 3.4) Ribereau-Gayon et al.(2000).:

$$Total\ tannins, TT\left(\frac{g}{L}\right) = 19.33 * (Abs_1 - Abs_o) \text{ (Equation 3.4)}$$

Where

- Abs_1 is the absorbance of heated sample
- Abs_0 is the absorbance of not heated sample

3.2.3.4 Cinnamic acids and Flavonols

Cinnamic acids were determined by direct absorbance reading of the sample at 320 nm, and expressed as caffeic acid equivalents (CAE) through a calibration curve (Spigno et al., 2007).

Flavonols were determined by direct absorbance reading of the sample at 370 nm, and expressed as quercetin equivalents (QE) through a calibration curve (Spigno et al., 2007).

For both the analyses, the sample was diluted with hydroalcholic solution (60/40, ethanol/water, v/v) to obtain absorbance values in the range 0.1-0.9. For each sample, different dilutions were analysed in order to cover the absorbance range 0.1 – 0.9 and average the results.

3.2.3.5 Antioxidant capacity of the extract

The antioxidant capacity of the extracts was evaluated with three different methods: ABTS assay, FRAP method and superoxide radical scavenging activity. Only the 2013 extracts were evaluated also with the ORAC assay.

3.2.3.5.1 ABTS assay

The ABTS assay (Re et al., 1999) is based on the ability of antioxidants to interact with the radical ABTS, reducing it and decreasing its absorbance at 734 nm (Figure 3.2)

A radical solution was prepared with 7 mM ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and 2.45 mM potassium persulfate and kept in the dark at room-temperature for 16 h before using. The radical was then diluted with aqueous ethanol 50 % to an absorbance of 0.70 (±0.02) at 734 nm (against 50 % ethanol). For the analysis, 2 mL of the diluted radical solution were mixed with 20 µL of the sample opportunely diluted with water and after 6 min the absorbance at 734 nm was read against ethanol 50 %. A blank sample (2 mL of diluted ABTS mixed with 20 µL of water) and a control sample (2 mL of diluted ABTS) were included. Antioxidant capacities (AOC) were calculated as percentage decrease of absorbance at 734 nm (Equation 3.5):

$$AOC\% = \frac{Abs_{Blank} - Abs_{sample}}{Abs_{control}} * 100 \text{ (Equation 3.5)}$$

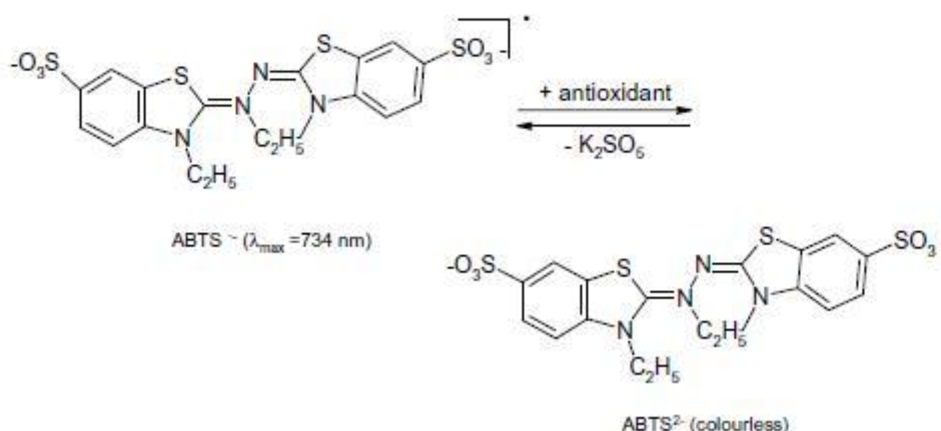


Figure3.2. ABTS Radical interaction with antioxidants during the ABTS test (Zulueta et al.,2009)

3.2.3.5.2 FRAP assay

The FRAP test is a method to measure the reducing capacity of antioxidant compounds on ferric (Fe) ions. It is based on electron transfer with a consequently switching from Fe^{+3} to Fe^{+2}. In presence of TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) and acid environment, the Fe ions form complex structure with different characteristics. The Fe^{+2} – TPTZ complex is blue colored and it shows the maximum absorbance at 593 nm.

The ferric reducing/antioxidant power (FRAP) was carried out as described by Pulido et al. (2000). The FRAP reagent contained 2.5 mL of 20 mM TPTZ solution (2, 4, 6-Tris (2-pyridyl)-s-triazine) in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \bullet 6H_2O$ and 25 mL of 0.3 M acetate buffer (pH 3.6). FRAP reagent (3.7 mL), prepared freshly and incubated at 37 °C, was mixed with 360 µl of distilled water and 120 µl of test sample or solvent (blank reagent). The test samples and blank reagent were incubated at 37 °C for 30 min in a water bath. At the end of incubation the absorbance readings were taken at 593 nm.

A reference calibration curve was obtained with the same procedure but with 120 µl of $FeSO_4 \bullet 7H_2O$ (200 – 2000 µM) instead of the sample.

The results are expressed as the amount of Fe(II) ($mM_{Fe(II)}$) correlated to the total phenols present in the sample (Equation 3.6):

$$\frac{mmol_{Fe(II)}}{g_{GAE-Folin}} = \frac{mM_{Fe(II)}}{\left(\frac{mg_{GAE}}{L}\right)_{sample}} * \frac{1000}{Sample\ dilution} \text{ (Equation 3.6)}$$

3.2.3.5.3 Superoxide radical scavenging activity

The method is based on the ability of phenolic compounds to react with superoxide anion (O^{-2}), hydroxyl radical (•OH) and others radical species. The presence of antioxidant compounds may reduce the radical oxidative activity.

The superoxide radical scavenging activity of extracts was measured by following the method described by Zhishen et al. (1999) with modifications. The reaction mixture was prepared using 3 µM riboflavin, 10 mM methionine, 100 µM NBT

(Nitroblue tetrozolium chloride) and 0.1 mM EDTA in phosphate buffer (pH 7.4). The phosphate buffer was prepared dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 1 L of distilled water. The final pH was checked and if necessary adjusted to 7.4. For the analysis, 3.0 mL of the reaction mixture were mixed with 100 μl of the sample (for the test) or solvent (for the control) and illuminated for 40 min under a fluorescent lamp (18 W).

The absorbance was then read at 560 nm against the blank (un-illuminated reaction mixture).

Results were expressed as % SRSA (superoxide radical scavenging activity) (Equation 3.7):

$$\%SRSA = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} * 100 \text{ (Equation 3.7)}$$

3.2.3.5.4 Oxygen Radical Absorbance Capacity (ORAC)

The accurate measurement of antioxidant capacity requires both inhibition degree and inhibition time to be taken into account. The oxygen radical absorbance capacity (ORAC) is the only method so far that combines both inhibition time and degree of inhibition into a single quantity (Cao & Prior, 1999).

The ORAC assay was performed by following the method described by Huang et al. (2002) using a micro plate fluorescence reader in a 96-well format with some modifications.

The assay measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride). AAPH is considered to produce the peroxyl radical by heating, which damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are considered to protect the fluorescent molecule from the oxidative degeneration. The degree of protection is quantified using a fluorimeter.

Fluorescein is currently used most as a fluorescent agent.

The degeneration (or decomposition) of fluorescein is measured as the presence of the antioxidant slows the fluorescence decay. Decay curves (fluorescence intensity vs. time) are recorded and the area between the two decay curves (with or without antioxidant) is calculated. Subsequently, the degree of antioxidant-mediated protection is quantified using the antioxidant Trolox (a vitamin E analogue) as a standard. Different concentrations of Trolox are used to make a standard curve (from 6.25 μM/L to 100 μM/L), and test samples are compared to this.

Results are expressed as Trolox equivalents or TEAC (Garret et al.,2010; Huang et al., 2005).

For the analysis, the samples, Trolox, AAPH (0.135 M) and fluorescein (40 nM) were diluted in a 0.075 M phosphate buffer at pH 7.4 composed by sodium phosphate monobasic and sodium phosphate dibasic.

The micro plate 96-well format was filled with: 0.33 μL of Trolox (for the standard curve) or with the diluted sample at different μM GAE/L (based on total phenols in the extract by Folin Index) and 200 μL of fluorescein.

The plate was incubated for 30 min at 37 °C, after that 0.33 µL of AAPH were added. The fluorescence was measured every minute for 1 h. The blank was made with 0.33 µL of phosphate buffer instead of sample.
The results are expressed as TEAC (Equation 3.8):

$$TEAC = \frac{AUC_{sample} - AUC_{Blank}}{AUC_{Trolox} - AUC_{Blank}} * \frac{Molarity_{Trolox}}{Molarity_{Sample}} \text{ (Equation 3.8)}$$

Where AUC indicates the Area Under the Curve of the sample, blank or Trolox.

3.2.3.6 Reducing Sugars content

The reducing sugars content in the extracts was evaluated with the enzymatic kit D-Fructose/D-Glucose Assay Kit (K-FRUGL) provided by Megazyme International Ireland, according to the Kit instructions.

3.3 Process intensification

Phenols extraction using an organic solvent is certainly the most widely used technology. Despite of a good yield in phenols, this process shows some disadvantages related to the use of organic solvent such as high toxicity (depending on the selected solvent), negative environmental impact and high processing costs.

For these reasons the implementation on large scale of this process could be difficult.

Starting from the organic solvent extraction process explained above (point 3.2.2), the objective is to develop an environmentally friendly and low cost process to recover polyphenols from wine by–products.

To achieve the aim, the applicability of Colloidal Gas Aphron (CGAs) and surfactant solution has been tested on grape skins for phenolic compounds extraction and/or purification.

Barbera grape skins and the food grade surfactant Tween® 20 (Tw20) were selected to carry out this part of the research.

3.3.1 Experimental plan

Figure 3.3 represents the potential application/integration of CGAs and surfactant solution into the process of recovering phenolic compounds from grape by-products.

The **APPLICATION A** consists in the partial and/or complete replacement of the extraction organic solvent with different solvents composed by surfactant dissolved in water or hydroalcholic solution.

The idea behind the **APPLICATION B** is the direct enforcement of the CGAs on ethanol suspension immediately after the solvent extraction step.

The ethanol suspension is composed by a liquid part, where the phenols are dissolved, and by a solid part which may still contain phenols. The ability of the CGAs to bind phenols could be exploited to further extract them from the solids. This application could be a solution to improve the conventional solvent extraction process in terms of yield in phenols and costs process reduction and, at the same

time, the presence of the CGAs could increase the purity of the final extract and provide an advantage in the antioxidant formulation for spray drying process.

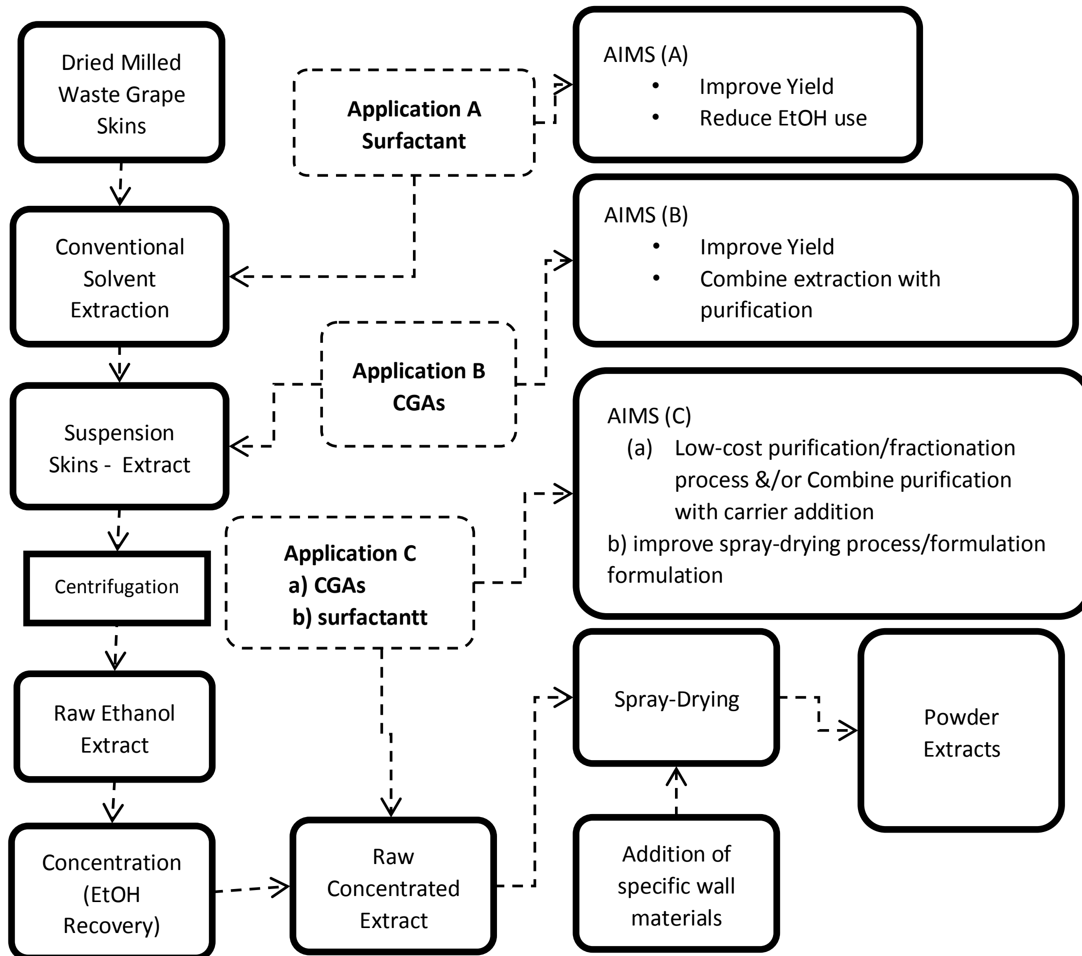


Figure 3.3 Schematic presentation of the three different applications of surfactant and CGAs (in orange) and the conventional solvent extraction (in green).

The **APPLICATION C (a)** uses CGAs to purify and/or fractionate the extract obtained with conventional solvent extraction (Spigno et al., 2010, 2014; Dahmoune et al., 2013).

Under this application some interesting and promising results have been obtained but research is still needed to improve process recovery and selectivity and to investigate the possible role of surfactant addition and CGAs in the preparation of feeding antioxidant formulations for the spray drying process. It should be verified if this could improve the stability and, above all, the solubility in specific media of

the final powdery extract. No additional experiments for this application have been carried out during this thesis project.

The **APPLICATION C (b)** consists in adding surfactant to the extract formulation before spray-drying in order to improve the spray-drying process &/or the final powder properties. Some trials have been carried out using this application.

3.3.2 Generation and characterization of CGAs

CGAs stability is a very important parameter to achieve the maximum phenols recovery. Foam which collapses very fast does not permit bond formation and phenols remain in the liquid phase. For this reason, the foam stability was studied at different Tw20 concentrations: 1 mM, 10 mM and 20 mM. The critical micelle concentration (CMC) is defined as the concentration of surfactant above which micelles form and all additional surfactants added to the system go to micelles (Mc Naught & Wilkinson, 1997). The Tw20 CMC in water was reported to be 0.08 mM (Kim & Hsieh, 2001).

CGAs were generated by intense stirring at 8000 rpm with Silverson SL2T mixer for 5 min of 1 L of aqueous surfactant solution. Tw20 at different concentrations (1 mM, 10 mM, 20 mM) was used and the corresponding CGAs were characterized for the stability in terms of half–life ($T^{1/2}$) and gas hold up (ε)The CGAs stability was also tested in the presence of different Ethanol concentrations (3, 12 and 30%)

For stability characterization CGAs were obtained from 200 mL of surfactant solution and transferred into a 500 mL volumetric cylinder and the volume of drained liquid was registered every minute until the15 minute, every 5 minutes until the 30 minute and the last volume of drained liquid was registered at the 60 minute (corresponding to complete foam collapse). The $T^{1/2}$ was then calculated as the time required for the first 100 mL to drain (corresponding to half volume of the initial surfactant solution). The ε is defined as the amount of air incorporated into the dispersion and was calculated based on Equation 3.9:

$$Gas\ Hold\ Up\ (\varepsilon) = \frac{V_{CGAs} - V_{drainedliquid}}{V_{CGAs}} \quad \text{(Equation 3.9)}$$

Where:

- V_{CGA} is the CGAs volume fed into the cylinder
- $V_{drained\ liquid}$ is the volume of the drained liquid after complete foam collapse.

Tests were carried out in duplicate for each concentration.

3.3.3 Application A

Direct extraction with different solvents in the presence of surfactant was conducted to evaluate direct extraction of phenolic compounds from DMGS. In particular, 125 g of grape skins were directly mixed with 1000 mL of solvent (1:8, w/v ratio) of different composition (Table 3.2). The dried skins and the solvent (pre-heated at 60 °C) were mixed for 1 h at 3500 rpm with Silverson L5M lab mixer at 60 °C(using an electric heating plate). After the extraction, solids were separated from the liquid by centrifugation at 5000 g for 15 min at room temperature. The supernatant was collected, its volume measured and analyzed for total phenols content, for total anthocyanins content and the antioxidant capacity was evaluated according to the methods reported in sections 3.2.2.1.1, 3.2.2.1.2, 3.2.2.3 and 3.2.2.5.1.

The application A experimental plan is reported in Figure 3.4.

The extraction yield was calculated according to the Equation 3.2.

Table 3.2. Composition of the different solutions tested for direct extraction of phenolic compounds from Barbera dried skins..

Trial	*Solvents*	*Tween20 Molarity (mM)*	*Ethanol amount (%)*
A	*Tween20 20mM*	20	0
B	*Tw20 20mM/EtOH 60% - 1:1 (v/v)*	10	30
C	*EtOH 60%*	0	60
D	*Tw20 20mM in EtOH60%*	20	60
E	*Tw20 20mM in EtOH30%*	20	30
F	*Tw20 10mM in EtOH60%*	10	60
G	*Tw20 10mM in EtOH30%*	10	30

Figure 3.4.Schematic presentation of alternative solvent extraction (Application A of Figure 3.3)

3.3.4 Application B

In the application B the potential of the CGAs in terms of recovery in the Aphron and Liquid phases and in terms of phenols additional recovery from grape skins after organic solvent extraction step, were evaluated.

In the CGAs process B, after the first extraction step of the conventional solvent extraction (CSE) process, the ethanol suspension was transferred into a glass flotation column (internal diameter of 0.25 m, total height 0.4 m) (Spigno et al., 2010) for CGAs application.

The CSE was made on small scale (Table 3.3) to ensure the complete transfer of the suspension (composed by solid and liquid part) into the flotation column and then, the column, was filled up to 780 mL (maximum volume admitted) with CGAs (the

CGAs were generated by intense stirring at 8000 rpm with Silverson L5M mixer for 5 min).

The solid – liquid ratio adopted was 1:8 (w/v) and the ethanol suspension was obtained mixing Barbera grape skins using pre–heated at 60 °C hydroalcholic solvent (60/40, ethanol/water, v/v) under magnetic stirring at 1300 rpm (with Heidolph MR 2002 heating and stirring unit) for 1 h. The solvent extraction was performed at constant temperature of 60 °C.

The amount of DMGS used and the corresponding solvent volume adopted are reported in Table 3.3.

The final ratios volume of ethanol suspension to volume of CGAs obtained in the flotation column, corresponded to 1:9, 1:12, 1:24 v/v.

The experimental plan is reported in Figure 3.6.

Table 3.3. Ethanol suspension – CGAs ratios adopted for the Application B trials.

Ethanol suspension/CGAs ratio (v/v)	***Barbera grape skins (g)***	***Volume of solvent adopted (mL)***	***Volume of CGAs adopted (mL)***
1:9	10	80	700
1:12	7.50	60	720
1:24	3.75	30	750

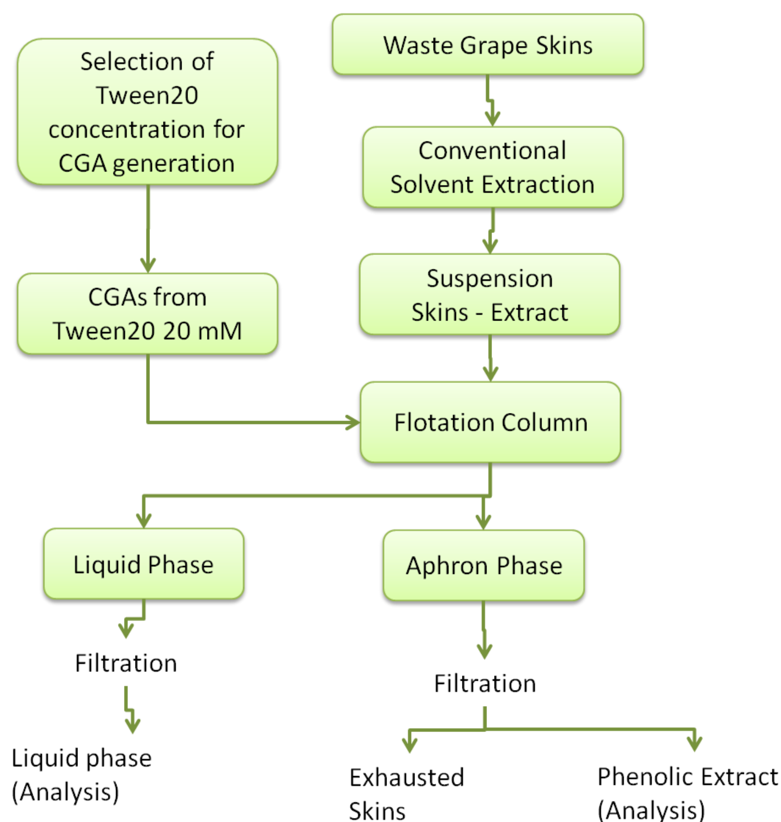


Figure 3.6. Schematic presentation of the experimental plan for Application B of Figure 3.4.

The CGAs were pumped into the column from the bottom at the minimum flow rates allowed by the peristaltic pump. The ethanol suspension was manually transferred into the column from the top. The flotation column loading process is reported in Figure 3.7.

The mixture of suspension and CGAs was left in the column for 5 min and then the separated liquid phase and aphron phase were pumped out from the bottom of the column. The volume of the liquid phase (V_{LP}) and the volume of the aphron phase (V_{AP}) after complete collapse were measured. The aphron phase was filtered and analysed for the total phenolic compounds (Folin index and total phenols 280nm index) and anthocyanins content and for the antioxidant capacity.

For the quantification of phenols, anthocyanins and antioxidant activity see sections 3.2.2.1.1, 3.2.2.1.2, 3.2.2.3 and 3.2.2.5.1.

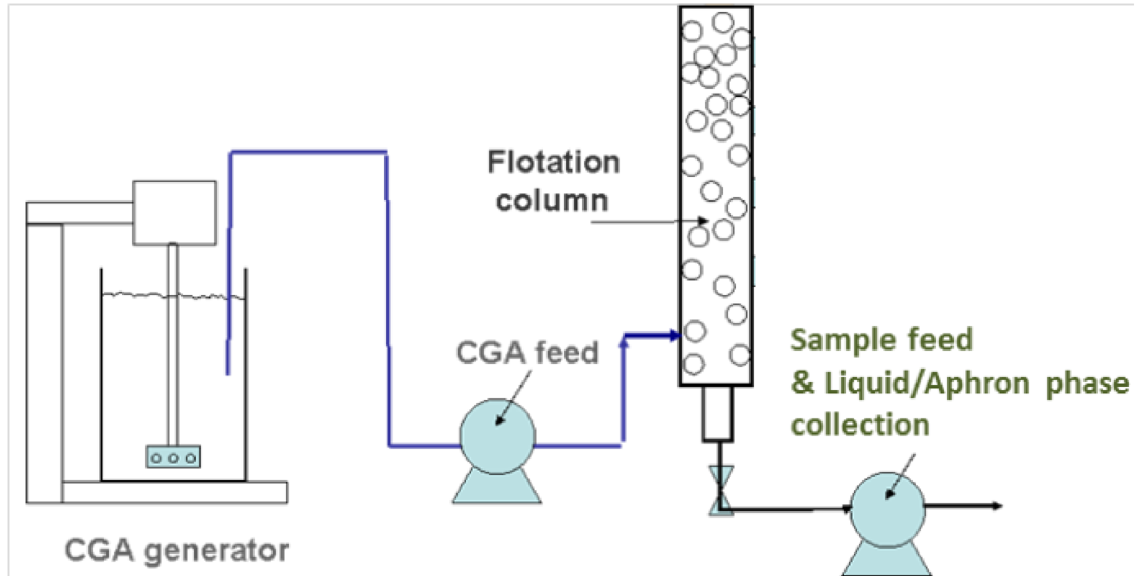


Figure 3.7. Flotation column loading process (from Dahmoune et al.,2013). The CGAs were pumped with a peristaltic pump from the bottom. In the figure the sample is loaded from the bottom too but, due to the presence of solids, it was manually transferred into the column from the top.

Each trial was carried out in duplicate and the results compared, in terms of phenols recovery, with the ethanol suspension obtained in the same operative conditions but without CGAs application.

The phenols additional recovery due to the CGAs process (RE_{CGA-B}) was then estimated, according to Equation 3.10:

$$Re_{CGAs-B} = \frac{mg_{phenolsAp} + mg_{phenolsLP} - mg_{phenolsFeed}}{g_{d.m.}} \text{ (Equation 3.10)}$$

Where :

- $mg_{phenolsFeed}$ indicates the total phenols contained in the suspension fed into the column
- $mg_{phenolsAp}$ indicates the total phenols contained in the aphron phase
- $mg_{phenolsLp}$ indicates the total phenols contained in the liquid phase

The phenols yield of the process, based on the phenols that remained in the aphron phase, was calculated in two different ways, according to Equation 3.11 and Equation 3.12:

$$RE_{Aphron(1)} = \frac{mg_{phenolsAp}}{mg_{phenolsAp} + mg_{phenolsLp}} * 100 \text{ (Equation 3.11)}$$

$$RE_{Aphron(2)} = \frac{mg_{phenolsAp}}{mg_{phenolsFeed}} * 100 \text{ (Equation 3.12)}$$

Equation 3.12, however, is based on the assumption that no additional compounds are extracted from the solids during the process.

In order to avoid any surfactant interference with the analytical methods, the concentration of the initial extract was evaluated diluting it with Tw20 20 mM.

3.4 Evaluation of surfactant influence on spray drying process

3.4.1 Experimental plan

A convenient way to increase the shelf-life and improve the organoleptic characteristics (with the application of particular encapsulation material)of a plant derivative is to transform them into a stable dry powder form by spray-drying with appropriate polymers (Laine et al., 2008; Kha et al., 2010; Ersus & Yurdagel, 2007). Spray drying is one of the most widely used encapsulation techniques to produce powder starting from liquids with the addition of appropriate encapsulation material. A schematic diagram of a spray dryer is reported in Figure 3.8.

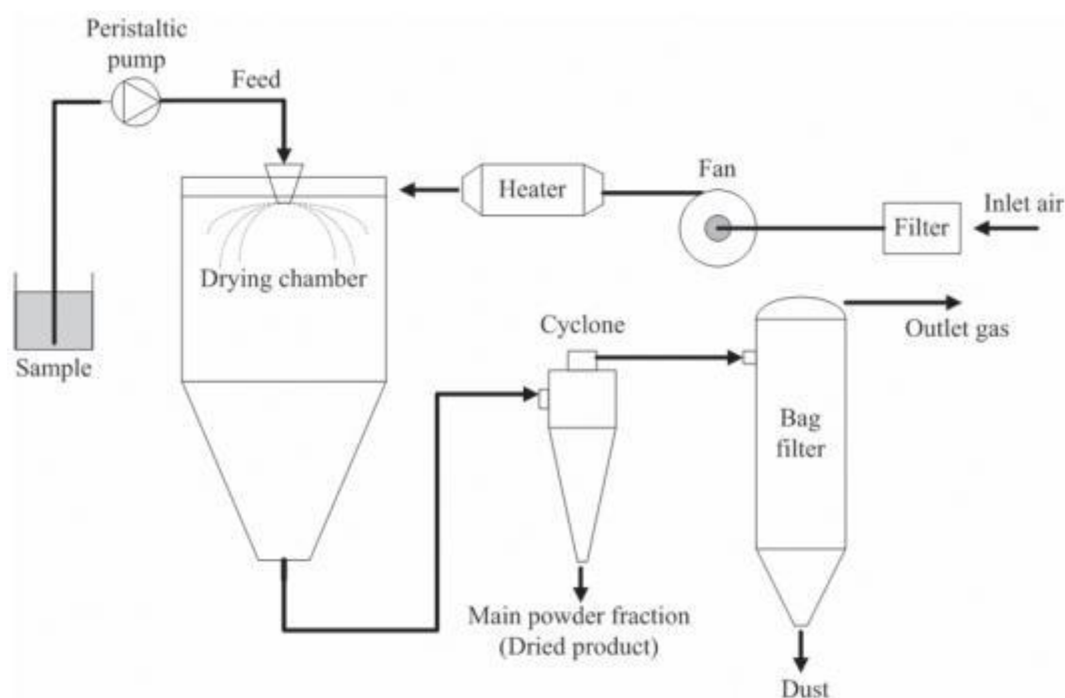


Figure 3.8. Schematic diagram of spray dryer (Jittanit et al.,2010)

On the market there are many encapsulation material but the most widely used are maltodexstrins (MD), which have a good solubility in water (are therefore used to improve water solubility of encapsulated material, but high cost. To reduce the amount of MD for the spray drying process a surfactant solution of Tw20 20mM was added to the ethanol extract.

The beneficial effects of Tw20 addiction were evaluated in terms of total phenols (Folin Index and 280nm Index, total anthocyanins, antioxidant activity (ABTS

assay) recovery and content in the final powder, powders solubility and water activity (a_W). Moreover, since a precipitate formed after surfactant addition, density and total solids content of the ethanol extract were evaluated before and after surfactant solution addition

The experimental plan is reported in Figure 3.9.

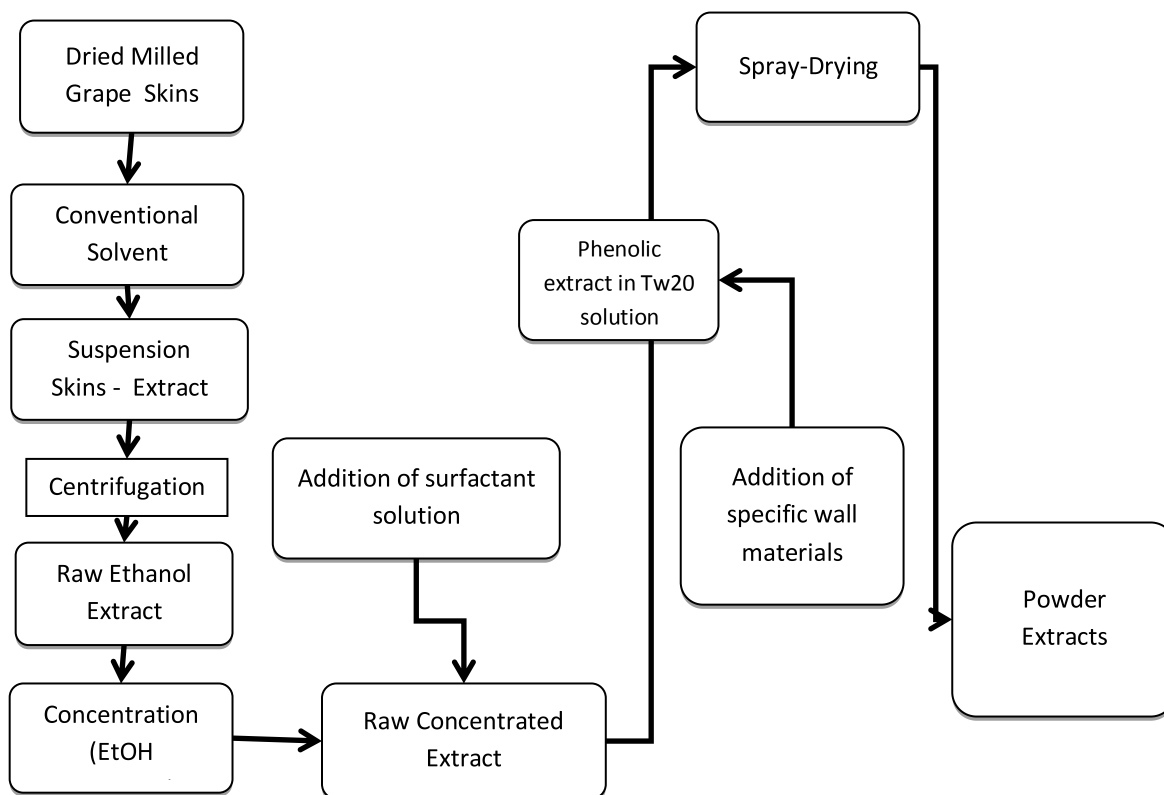


Figure 3.9. Experimental plan for the trials aimed to evaluate the influence of surfactant addition to the ethanol extract on the spray drying process.

Conventional solvent extraction was carried out on Barbera skins, as previously described in section 3.2.2 but using only one extraction step. The extract was collected, its volume measured and analyzed for total phenols content (see chapters 3.2.2.1.1 and 3.2.2.1.2), total anthocyanins content, and antioxidant capacity (see ABTS assay at point 3.2.2.5.1). The extract was concentrated three times under vacuum and then diluted back to initial volume with aqueous Tw20 20 mM. Since after dilution with Tw20 solution precipitation occurred, the extract was left in darkness for one night and then transferred to another flask to remove

the precipitate. Density and solids content of the extract were evaluated before and after Tw20 20 mM solution addition.

Total anthocyanins were quantified with a different method than that described at point 3.2.2.3.

In this case, the adopted method was proposed by Lee et al. (2005). The total monomeric anthocyanin concentration was quantified by the pH differential method, which is a rapid and simple spectrophotometric method based on the anthocyanin structural transformation that occurs with a change in pH value (colored at pH 1.0 and colorless at pH 4.5). In previous works (Duserm Garrido, 2012), it was observed that this method was more appropriate with the presence of maltodextrins, than that based on dilution with acid ethanol.

Two buffer solutions were prepared: the first one is pH 1.0 buffer (potassium chloride, 0.025M*)*; the second one is pH 4.5 buffer (sodium acetate, 0.4 M). The absorbance of the samples were measured after dilution with both buffers pH 1 and pH 4.5 at 520 nm and 700 nm. The samples were read against water.

The results are expressed as Cyanidin – 3 – glucoside equivalents (mg/L) applying Equation 3.13:

$$CyN\ \left(\frac{mg}{L}\right) = \frac{A*MW*DF*10^3}{\varepsilon*l} \text{ (Equation 3.13)}$$

Where

- $A = (Abs_{520} - Abs_{700})_{pH\ 1} - (Abs_{520} - Abs_{700})_{pH\ 4.5}$
- MW is the molecular weight of cyanidin – 3- glucoside (449.2 gmol)
- *l* is the path length in cm
- ε = is the molar extinction coefficient for cyanidin – 3 – glucoside (26900 $L*mol^{-1}*cm^{-1}$)
- DF is the dilution factor
- 10^3 is the conversion factor from g to mg

The density of the extract was measured with an hydrostatic balance (Gibertini Idromatic). The measurement was performed at 25 °C.

To evaluate the total solids content, 10 mL of the extract were placed in a crucible and left in an oven at 105 °C for 48 h. The crucible was then weighed and residual total solids quantified.

3.4.2 Spray drying process and powder characterization

The encapsulation process was performed with a lab-scale plant spray-dryer (Buchi B-290 mini Spray Dryer). The amount of MD added to the extract was related to the total phenols in the extract based on the Folin – Ciocalteau assay. The different powders were obtained starting from 50 g of extract, weighed in a beaker, and the related amount of MD added. The solution composed of extract and MD, was mixed under magnetic stirring for 1 h to allow the complete MD solubilization. After that the solution was pumped into the spray dryer.

Four different molar ratios MD (as moles of equivalent dextrose) / phenols (as moles of GAE) were tested and the powders obtained were analyzed for total phenols content (Folin index and 280 nm index), total anthocyanins and antioxidant capacity as reported for the extract before encapsulation. For analyse, powders were dissolved in water at a 20 g/L concentration.

Furthermore. the total wet weight powder recovery, and the total phenols and anthocyanins recovery were calculated comparing the content of the spray-dried extract with the content of the initial extract.

Solubility in water and a_W values were also evaluated.

The results were compared with the results of trials at the same working conditions (molar ratio, inlet and outlet temperature, pump speed) but without surfactant addition.

The operative parameters adopted are reported in Table 3.4.

Table 3.4. Operative parameters adopted for the spray drying trials.

Trials number	Feed Flow Rate (ml/min)	Inlet Temperature (°C)	Outlet Temperature (°C)	Molar ratio MD/GAE	Extract used (g)
1	3.6	150	83	2.44	50
2	3.6	150	83	1.28	50
3	3.6	150	83	0.64	50
4	3.6	150	83	3.85	50

3.4.2.1 Water solubility index (WSI)

The WSI of the powders was determined using the method described by Kha et al. (2010). The powder (2.5 g) and 30 mL of distilled water were vigorously mixed with Vibromix Pbi vortex in a 50 mL centrifuge tube, incubated at 37 °C in A water bath for 30 min and then centrifuged for 20 min at 10000 rpm (11,410 g). The supernatant was collected in a pre-weighed crucible and dried in a oven at a temperature of 105 °C until constant weight was obtained.
The WSI (%) was calculated as the percentage of solubilized powder (the residual solids at 105 °C) with respect to the initial 2.5 g.

3.4.2.2 Water activity (a_W)

Water activity is a fundamental parameter for food product design and food safety. Food designers use water activity to formulate shelf-stable food since under a certain water activity mould growth is inhibited. Higher a_w products tend to support microorganisms growth. Bacteria usually require at least a 0.91 a_w, and fungi at least 0.7 (Rockland et al.,1987).
The a_W measurements were performed with AquaLab Dew Point Water Activity Meter 4TE. Around 1 g of powder was placed into a plastic crucible and insert into the Water Activity Meter. After 5 min the a_W values recorded.

3.5 Statistical analysis

The results were all reported as mean ± SD and all the trials (except where specified) were made in triplicate. The results were statistically evaluated by ANOVA test to evaluate the influence of specific process variables on measured parameters. In case of significant influence (at a 99 % confidence level, p-value < 0.01), variance homogeneity was checked and the Tukey's post-hoc test was applied for means discrimination (p-value < 0.01). For comparison of less than three groups,, the t Test was applied always at p-value < 0.01. The statistical analysis was performed with the IBM SPSS®Statistics software v.19 (SPSS Inc, Chicago, IL, USA).

4. Results and discussion

4.1. Antioxidants recovery from grape skins

4.1.1 Raw material characterization

The grape skins of six different considered varieties were characterized for moisture, ash, lipids, proteins and minerals content (Table 4.1).

Table 4.1. Chemical characterization of dried milled grape skins from the six varieties tested in the project. Results are reported as mean ± s.d. Same letter under each parameter indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test ($p< 0.01$).

Year/variety	*Moisture (g/100 g)*	*Ash ($g/100\ g_{dm}$)*	*Proteins ($g/100\ g_{dm}$)*	*Lipids ($g/100\ g_{dm}$)*
2011				
Chardonnay	$5.66{\pm}0.11^{bcde}$	$6.43{\pm}0.06^{de}$	$10.46\ {\pm}0.28^{c}$	$4.81\ {\pm}0.45^{cde}$
Moscato	$8.37{\pm}0.19^{b}$	$5.47{\pm}0.01^{ef}$	$10.64{\pm}0.62^{bc}$	$5.23{\pm}0.25^{bcde}$
M. Thurgau	$4.83{\pm}0.02^{cdef}$	$7.14{\pm}0.03^{d}$	$13.06{\pm}0.25^{a}$	$4.84{\pm}0.30^{cde}$
Barbera	$3.41{\pm}0.06^{def}$	$10.91{\pm}0.38^{ab}$	$8.52{\pm}0.16^{d}$	$5.07{\pm}0.47^{cde}$
Nebbiolo	$2.70{\pm}0.11^{ef}$	$11.13{\pm}0.32^{a}$	$10.44{\pm}0.06^{c}$	$5.58{\pm}0.56^{bcd}$
Pinot Noir	$5.66{\pm}0.11^{def}$	$4.17{\pm}0.22^{gh}$	$10.53{\pm}0.39^{c}$	$7.44{\pm}0.31^{a}$
2012				
Chardonnay	$6.79{\pm}0.12^{bc}$	$5.19{\pm}0.52^{f}$	$9.03{\pm}0.20^{d}$	$4.56{\pm}0.04^{cdef}$
Moscato	$4.04{\pm}0.17^{cdef}$	$5.03{\pm}0.12^{fg}$	$7.35{\pm}0.07^{e}$	$1.71{\pm}0.38^{g}$
M. Thurgau	$5.96{\pm}0.22^{bcde}$	$5.40{\pm}0.04^{f}$	$10.31{\pm}0.06^{c}$	$3.20{\pm}0.02^{fg}$
Barbera	$2.92{\pm}0.00^{ef}$	$9.57{\pm}0.40^{b}$	$9.57{\pm}0.40^{d}$	$4.09{\pm}0.55^{def}$
Nebbiolo	$15.95{\pm}3.01^{a}$	$8.72{\pm}0.62^{c}$	$10.50{\pm}0.15^{c}$	$3.87{\pm}0.12^{ef}$
Pinot noir	$13.98{\pm}0.04^{a}$	$4.53{\pm}0.05^{fgh}$	$11.21{\pm}1.33^{b}$	$6.71{\pm}0.88^{ab}$
2013				
Barbera	$2.44{\pm}0.12^{f}$	$8.52{\pm}0.02^{c}$	$10.76{\pm}0.10^{bc}$	$5.72{\pm}0.58^{bc}$
Nebbiolo	$2.27{\pm}0.05^{f}$	$9.65{\pm}0.04^{b}$	$10.76{\pm}0.12^{bc}$	$4.66{\pm}0.37^{cdef}$
Pinot Noir	$5.46{\pm}0.10^{cde}$	$3.80{\pm}0.07^{h}$	$10.49{\pm}1.33^{c}$	$7.53{\pm}0.22^{a}$

The residual moisture content after drying was generally below 7 %. On average, not fermented skins (white varieties and Pinot noir) had the highest moisture contents. Only the 2012 Pinot noir samples had a very high moisture content which caused most of them to be spoiled by moulds during storage. Similar values have been reported in literature (Sousa et al., 2014; Bustamante et al., 2008; González-Centeno et al., 2004).

The samples did not substantially differentiate for the total crude protein content (generally around 10 % on dry matter), neither among varieties, nor among different years. Similar results were observed by Bravo & Saura-Calixto (1998) and Llobera & Canellas (2007).

Lipids are mainly concentrated in the grape seeds, which justifies the highest values observed for Pinot Noir for all the three years, since in these samples a higher residual presence of seeds was always visually observed compared to the other varieties.

Contrary to the lipids content, the ash content was the lowest in Pinot noir skins, and higher in the fermented skins (Barbera and Nebbiolo). The grape skins were then analyzed also for potassium, calcium, sodium, copper, iron and zinc (Table 4.2). The minerals amount in the grape skins depends on soil composition, agrochemical treatment and grape health condition. The values found are in agreement with the typical grape mineral profile (Ribereau-Gayon et al., 2000), with calcium and potassium being the two main compounds. Potassium revealed the same trend as that of total ash (with Barbera and Nebbiolo skins the richest ones), while the samples did not greatly differ for the Ca content. Similar K content was observed by Bustamante et al. (2008). Sodium content showed a great variability, ranging from 14.60 mg/kg_{dm} to 1468.27 mg/kg_{dm}. Chardonnay and Müller Thurgau skins generally showed higher contents. The values found for the micronutrients Cu, Fe and Zn were similar to those obtained by Bustamante et al. (2008).

Table 4.2. Minerals content of dried milled grape skins from the six varieties tested in the project. Results are reported as mean ± s.d. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test ($p < 0.01$).

Year/ variety	***K (g/kg_{dm})***	***Ca (g/kg_{dm})***	***Na (mg/kg_{dm})***	***Cu (mg/kg_{dm})***	***Fe (mg/kg_{dm})***	***Zn (mg/kg_{dm})***
2011						
Chardonnay	27.84±1.10^{de}	2.72±0.53^{ef}	33.96 ±3.86^{d}	61.32±2.06^{d}	93.40±16.47^{bcde}	11.67±0.71^{efg}
Moscato	23.10±0.59^{ef}	3.64±0.40^{bcd}	151.41±13.34^{d}	61.07±3.69^{d}	107±1.07^{bcd}	8.47±0.49^{g}
M. Thurgau	37.35±10.92^{bcd}	4.49±0.10^{b}	59.91±1..93^{d}	133.02±1.21^{c}	60.13±5.99^{def}	11.08±0.36^{fg}
Barbera	44.55±0.94^{b}	6.14±0.48^{a}	24.92±1.27^{d}	56.01±2.42^{bc}	74.14±2.32^{def}	8.60±0.44^{g}
Nebbiolo	45.17±1.20^{b}	4.01±0.17^{bc}	62.42±5.02^{d}	152.80±6.44^{e}	131.58±4.17^{b}	14.54±1.05^{cde}
Pinot Noir	13.49±0.63^{f}	3.94±0.05^{bcd}	14.60±0.36^{d}	21.81±0.49^{e}	53.96±2.97^{ef}	9.15±0.23^{fg}
2012						
Chardonnay	29.30±0.93^{cde}	1.44±0.09^{gh}	25.28±13.96^{d}	22.29±1.17^{d}	96.10±11.85^{bcde}	31.50±0.87^{a}
Moscato	31±1.42^{cde}	1.27±0.18^{gh}	682.13±9.30^{bc}	58±0.40^{ab}	69.71±7.93^{cdef}	12.22±0.49^{def}
M. Thurgau	28.5±1.61^{de}	1.09±0.12^{h}	814.1±106.4^{b}	167.39±3.42^{d}	55.63±3.96^{ef}	12.08±0.65^{def}
Barbera	77.25±0.14^{a}	2.1±0.29^{fg}	51.6±7.43^{d}	70.57±0.76^{b}	76.94±5.61^{cdef}	11.06±1.27^{fg}
Nebbiolo	41.06±6.81^{bc}	1.51±0.1^{gh}	92.34±23.8^{d}	164.95±26.37^{ab}	192.65±45.32^{a}	17.85±2.91^{c}
Pinot noir	23.44±2.43^{ef}	1.65±0.15^{gh}	567.2±115.09^{c}	21.56±1.59^{e}	110.47±6.27^{bc}	23.01±0.18^{b}
2013						
Barbera	44.12±0.61^{b}	4.03±0.04^{bc}	540.63±130.67^{c}	71.66±0.69^{d}	106.56±1.92^{bcd}	10.01±0.02^{fg}
Nebbiolo	65.84±0.92^{a}	3.46±0.00^{cde}	1468.27±0.00^{a}	178.66±1.41^{a}	138.76±5.23^{b}	15.27±0.07^{cd}
Pinot Noir	15.26±0.02^{f}	3.11±0.25^{de}	563.21±34.40^{c}	15.76±0.53^{e}	41.12±3.83^{f}	18.77±0.58^{c}

4.1.2 Phenolic extract characterization

4.1.2.1 Phenolic extracts from 2011 grape skins

4.1.2.1.1 Phenolic profile

The phenolic recovery with conventional solvent extraction from the six 2011 grape skins are reported in Table 4.3.

Table 4.3. Phenolics recoveries from 2011 grape skins by conventional solvent extraction. Results are reported as mean ± s.d. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test (p< 0.01). *indicates means not statistically different between first and second extraction for the same variety.

Variety	*TPC_{280} $g_{GAE}/100g_{dm}$*	*TPC_{Folin} $g_{GAE}/100g_{dm}$*	*TAC $mg_{WAE}/100g_{dm}$*	*CA $g_{CAE}/100g_{dm}$*	*TFA $mg_{QE}/100g_{dm}$*	*TT $g_{TT}/100g_{dm}$*
			First extraction			
Barbera	1.78±0.36^{b}	4.54±1.01^{b}	862.26±180.53^{a}	0.12±0.01^{bc}	244.95±35.50^{a}	3.02±0.57^{bc}
Nebbiolo	1.23±0.13^{c}	2.19±0.36^{c}	106.20±3.78^{c}	0.12±0.01^{bc}	103.83±24.08^{b}	1.90±0.13^{d}
Pinot Noir	2.88±0.31^{a}	7.07±1.15^{a}	171.06±3.94^{b}	0.16±0.00^{a}	107.82±10.88^{b}	8.34±0.33^{a}
Chardonnay	0.88±0.05^{de}	2.10±0.41^{c}	-	0.08±0.00^{d}	68.09±5.03^{c}	2.96±0.02^{c}
Moscato	0.83±0.06^{e}	1.92±0.39^{c}	-	0.09±0.00^{cd}	70.18±1.87^{c}	2.97±0.04^{c}
M. Thurgau	1.11±0.16^{cd}	2.46±0.16^{c}	-	0.10±0.01^{c}	69.70±1.28^{c}	3.57±0.17^{b}
			Second extraction			
Barbera	0.67±0.16^{a}	1.59±0.41^{ab}	176.07±20.40^{a}	0.13±0.02^{a}	94.69±12.54^{a}	1.03±0.11^{a}
Nebbiolo	0.58±0.10^{a}	1.08±0.27^{bc}	49.97±5.13^{b}	0.06±0.01^{b}	44.81±4.30^{b}	1.06±0.36^{a}
Pinot Noir	0.55±0.12^{a}	1.62±0.15^{a}	48.10±1.15^{b}	0.05±0.00^{c}	36.51±4.79^{c}	1.15±0.13^{a}
Chardonnay	0.29±0.07^{b}	0.57±0.12^{e}	-	0.02±0.00^{e}	20.84±2.82^{e}	0.74±0.02^{a}
Moscato	0.33±0.01^{b}	0.84±0.12^{cd}	-	0.03±0.00^{d}	26.54±1.92^{d}	1.13±0.07^{a}
M. Thurgau	0.40±0.05^{a}	0.75±0.19^{de}	-	0.04±0.00^{c}	33.72±6.51^{cd}	1.03±0.28^{a}

CA: Cinnamic Acids; CAE: caffeic acid equivalents; GAE: gallic acid equivalents; QE: quercetin equivalents;.TAC: Total Anthocyanins Content; TT: Total Tannins; TFA: Total Flavonols Content; TPC_{280}: Total Phenolic Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; WAE: wine anthocyanins equivalents.

A significant difference in terms of total phenols content, for both the analytical methods (Total phenols index and Folin Index), was found between the extracts obtained in the two extraction steps. The two methods gave different values of GAE due to the distinctive principles on which they are based, as explained in materials and methods section.

Also for all the other analyzed phenolic groups, significant differences were found between the yields of the two extraction steps, with the only exception being the cinnamic acids from Barbera skins. This was probably due to the higher concentration of cinnamic acids in the skins of this variety. Ferrandino et al. (2012), evaluated the presence of these compounds in 34 different grape genotypes, reporting a concentration of cinnamic acids in Barbera skins three times higher than in Nebbiolo and Pinot Noir.

On average, the first step led to a phenols yield about 3 times higher than the second step. Therefore, considering the total phenols extracted as the sum of the two steps, the 67-80-% was obtained during the first extraction. The low recoveries in the second step may not justify its application in terms of energy and solvent costs. It was then decided to apply only one extraction step for the next trials.

Within the six varieties, the highest total phenols and tannins RE were observed for Pinot Noir skins. This was an expected result due to the sample nature: unfermented red grape skins with the presence of residual seeds. Our result was similar to the value of 7.36 g/100 g reported by Rockenbach et al. (2011) for total phenols content of Pinot noir grape pomace. Nebbiolo skins extracts were the poorest in tannins.. Regarding this parameter, Lorrain et al. (2011) used the same analytical method and reporting 5.7 and 6.3 g of tannins on 100 g_{dm} for two red grape skins, values close to those we found considering the sum of the two extraction steps.

Even though the skins were collected after fermentation, Barbera confirmed its typical high anthocyanins content, with a WAE recovery up to 8 folds higher than Nebbiolo and Pinot Noir extracts. The values found for these last varieties were in agreement with literature results from other varieties (Katalinić et al., 2010; Pinelo et al., 2006; Lorain et al., 2011; Rockenbach et al., 2011). Higher amounts of anthocyanins were reported only by Ky et al. (2014) in six French grape varieties.

Barbera skins gave also the highest yields in flavonols.

As partly expected, even though white grape skins were not fermented, the related phenols recovery was on average lower than red varieties, except for M. Thurgau.

4.1.2.1.2. Antioxidant capacity

The antioxidant capacity (ABTS assay) of the extracts was correlated with the total phenols concentration as Folin Index (Figure 4.1 and 4.2). As observed by other authors (Rockenbach et al., 2011; Spigno et al., 2007) antioxidant capacity linearly increased with total phenols concentration up to a certain value, above which it reaches a plateau.

Looking at the first step extracts (Figure 4.1), all the samples showed similar activity, comparable or even higher than the standard Trolox. Nebbiolo extract appeared as the less powerful one.

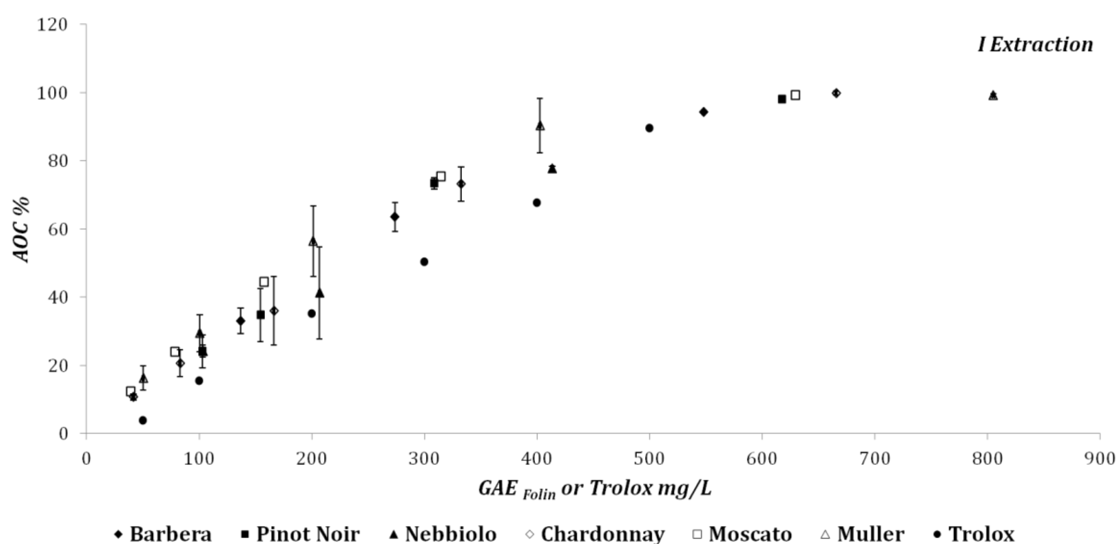


Figure 4.1. Antioxidant capacity (AOC% according to the ABTS assay) of first step extracts from 2011 skins, as a function of total phenols concentration (GAE_{Folin}). Error bars represent ± SD.

Considering the second step extracts (Figure 4.2), the activity was lower probably due to thermal degradation occurring during prolonged contact of the skins with the solvent at 60 °C. Nebbiolo was confirmed as the less powerful extract.

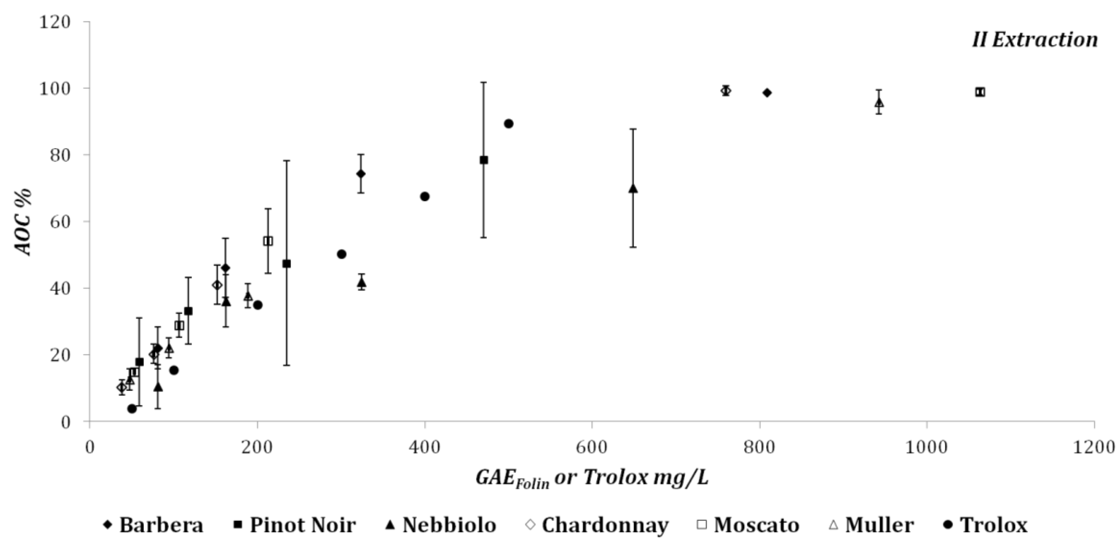


Figure 4.2. Antioxidant capacity (AOC% according to the ABTS assay) of second step extracts from 2011 skins, as a function of total phenols concentration (GAE_{Folin}). Error bars represent ± SD.

4.1.2.2 Phenolic extracts from 2012 grape skins

4.1.2.2.1 Phenolic profile

Table 4.4 reports the yields in term of total phenols contents and their subgroups from the 2012 samples. On average, the results confirmed what previously commented on 2011 extracts. Only the M. Thurgau extract was less rich in tannins.

Table 4.4. Phenolics recoveries from 2012 grape skins by conventional solvent extraction. Results are reported as mean ± s.d. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test ($p<0.01$).

Variety	TPC_{280} $g_{GAE}/100g_{dm}$	TPC_{Folin} $g_{GAE}/100g_{dm}$	*TAC* $mg_{WAE}/100g_{dm}$	*CA* $g_{CAE}/100g_{dm}$	*TFA* $mg_{QE}/100g_{dm}$	*TT* $g_{TT}/100g_{dm}$
Barbera	2.07 ± 0.17^{b}	3.55 ± 0.33^{b}	1200.66 ± 185.51^{a}	0.31 ± 0.01^{a}	230.40 ± 14.55^{a}	3.18 ± 0.25^{b}
Nebbiolo	0.88 ± 0.19^{cd}	1.51 ± 0.26^{d}	54.41 ± 4.22^{c}	0.09 ± 0.00^{c}	73.19 ± 11.05^{bc}	1.73 ± 0.22^{d}
Pinot Noir	2.95 ± 0.53^{a}	6.45 ± 0.67^{a}	99.72 ± 4.39^{d}	0.18 ± 0.00^{b}	87.06 ± 6.28^{b}	5.86 ± 1.15^{a}
Chardonnay	1.80 ± 0.40^{b}	3.18 ± 0.35^{b}		0.09 ± 0.00^{c}	56.77 ± 4.38^{d}	1.66 ± 0.03^{de}
Moscato	0.82 ± 0.07^{d}	1.80 ± 0.24^{cd}		0.08 ± 0.00^{c}	63.26 ± 5.19^{cd}	2.57 ± 0.35^{c}
M. Thurgau	1.06 ± 0.07^{c}	2.33 ± 0.33^{c}		0.08 ± 0.00^{c}	67.57 ± 2.05^{c}	1.25 ± 0.06^{e}

CA: Cinnamic Acids; CAE: caffeic acid equivalents; GAE: gallic acid equivalents; QE: quercetin equivalents;.TAC: Total Anthocyanins Content; TT: Total Tannins; TFA: Total Flavonols Content; TPC_{280}: Total Phenolic Compounds at based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; WAE: wine anthocyanins equivalents.

4.1.2.2.2 Antioxidant capacity

For the 2012 extracts, the antioxidant capacity was evaluated with different methods: ABTS test, FRAP and superoxide anion radical scavenging activity.

The highest ferric reducing-antioxidant power (Figure 4.3) was found for Nebbiolo extract, followed by Pinot Noir, Barbera (actually Barbera extract showed an activity comparable to both Pinot and white grapes) and the white varieties. Also Kataliníc et al. (2010) observed a higher activity for red grape skins extracts than white ones in the evaluation of 14 varieties. However, these authors attributed the difference to the presence of anthocyanins, which cannot be our case considering the higher anthocyanins content of Barbera extract.

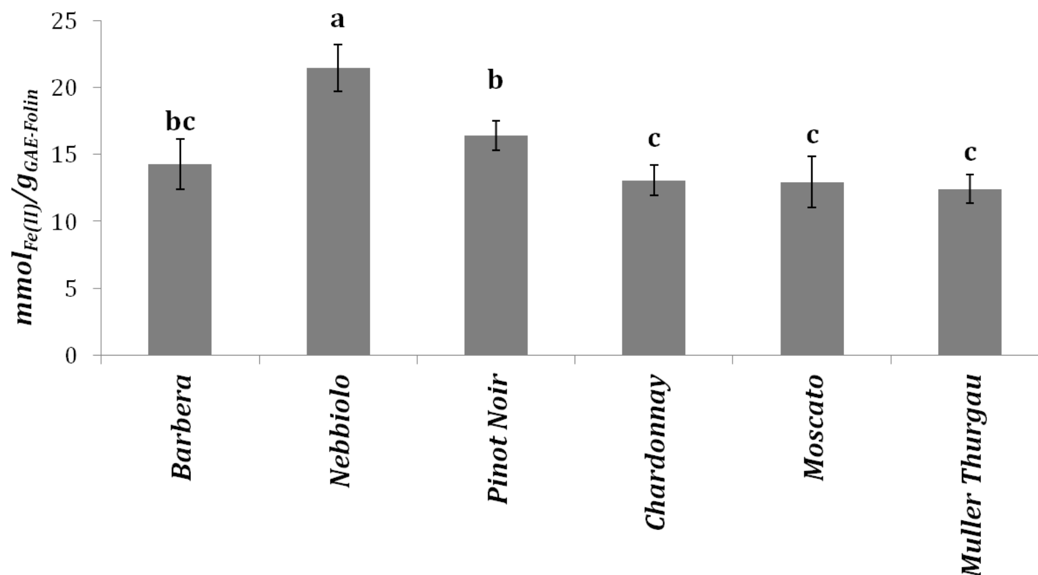


Figure 4.3. Reducing power (FRAP test) of 2012 skin extracts expressed as $mmol_{Fe(II)}/g_{GAE\text{-}Folin}$. Error bars indicate ± SD. Superscripts with different letters on the bars show significant differences according to ANOVA and Tukey's post-hoc test. ($p < 0.01$).

The degree of superoxide anion radical scavenging activity of the different extracts (% SRSA) versus total phenols concentration (GAE_{Folin}) is reported in Figure 4.4. For all the samples, % SRSA linearly increased up to a certain concentration (around 100 mg/L GAE_{Folin}), after which remained constant. All extracts presented similar behavior, with an apparent higher activity for Nebbiolo extract.

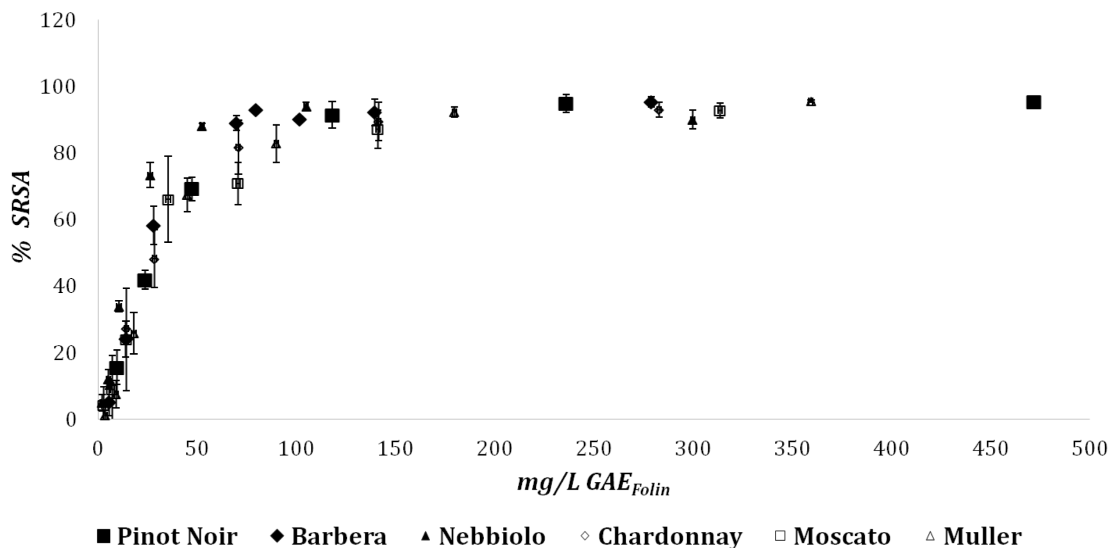


Figure 4.4. Superoxide anion radical scavenging activity of 2012 skin extracts. Error bars represent ± SD.

The antioxidant activity evaluated with ABTS assay is reported in Figure 4.5. The activity appeared independent of the grape variety and, compared to the 2011 results, Nebbiolo was not confirmed as the less powerful sample.

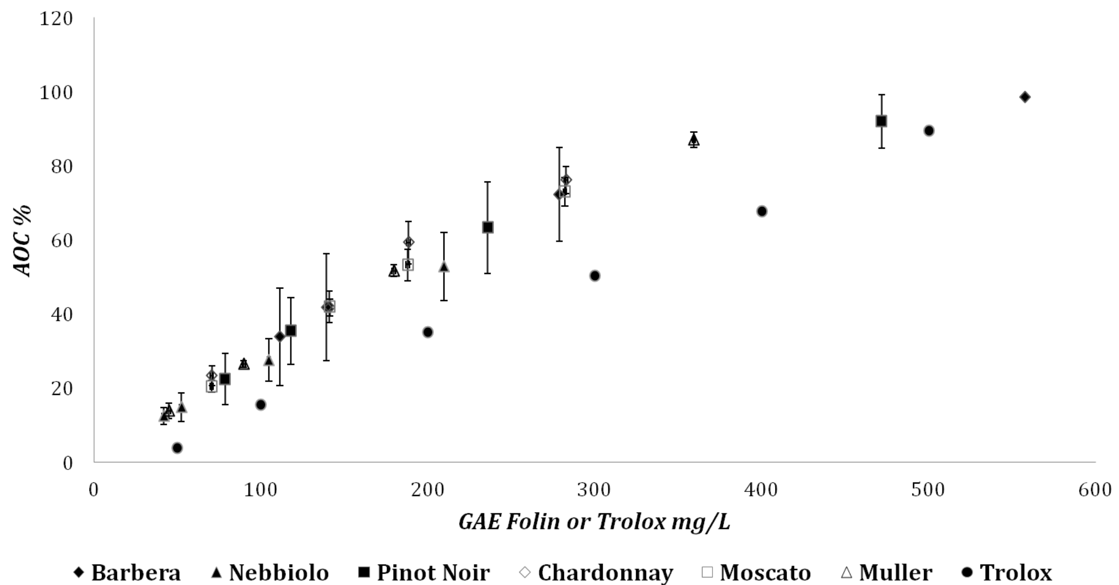


Figure 4.5. Antioxidant capacity (AOC% according to the ABTS assay) of extracts from 2012 skins, as a function of total phenols concentration (GAE_{Folin}). Error bars represent ± SD.

4.1.2.2.3 Minerals content

The 2012 extract were also analyzed for the minerals content as done on the raw materials. The concentration values were elaborated as performedfor the phenolic compounds, expressing the results as extraction yields (Table 4.5).

Table 4.5. Minerals extraction yields from 2012 grape skins. Results are reported as mean ± s.d. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test (p< 0.01).

Variety	*K (g/kg$_{dm}$)*	*Ca (g/kg$_{dm}$)*	*Na (mg/kg$_{dm}$)*	*Cu (mg/kg$_{dm}$)*	*Fe (mg/kg$_{dm}$)*	*Zn (mg/kg$_{dm}$)*
Chardonnay	688.19±1.02^{a}	15.87±0.04^{a}	2.53±1.40^{b}	1.06±0.40^{bc}	1.18±0.04^{ab}	1.14±0.10^{a}
Moscato	295.39±0.23^{c}	5.55±0.02^{c}	4.94±0.37^{ab}	1.02±0.01^{bc}	1.21±0.10^{ab}	1.10±0.12^{a}
M. Thurgau	620.52±56.37^{ab}	6.23±0.04^{c}	6.28±1.05^{a}	2.46±0.14^{a}	1.26±0.02^{a}	1.12±0.04^{a}
Barbera	70.57±0.23^{e}	4.43±0.05^{c}	4.24±0.17^{b}	1.05±0.12^{bc}	1.15±0.07^{ab}	1.00±0.05^{ab}
Nebbiolo	193.31±46.85^{d}	6.78±1.96^{c}	5.98±0.32^{a}	1.35±0.03^{b}	1.07±0.01^{b}	0.88±0.04^{b}
Pinot noir	575.83±8.05^{b}	11.28±2.80^{b}	5.85±0.56^{a}	0.74±0.05^{c}	1.08±0.08^{b}	0.96±0.09^{ab}

Potassium was confirmed as the most abundant element in the extract, followed by Na and Ca. Comparing Table 4.5 with Table 4.2, the recovery of the initial mineral amount can be easily calculated. The percentage of K recovery was not high (always below 25 %) and very variable, ranging from 1.05 % (Barbera) to 24.75 % (Pinot noir). Na recovery exceeded 60 % for Barbera, Chardonnay and Nebbiolo while was < 10 % for the other varieties. 10 – 20 % of the initial Fe and Cu was extracted in all the samples (higher values were obtained from Pinot Noir and Chardonnay). Almost 90 % of Zn was recovered for Barbera, Moscato and M. Thurgau, and only 45 % for the other varieties.

These differences could not be attributed to the wine-making process (fermented vs. unfermented skins), or to the grape type (red vs. white) or cultivar (e.g. Barbera showed the highest recovery of Zn and Na, but not of K).

4.1.2.3 Phenolic extracts from 2013 grape skins

4.1.2.3.1 Phenolic profile

For the 2013 vintage, only the red grape extracts were obtained and characterized (Table 4.6).

Table 4.6. Phenolics recoveries from 2013 grape skins by conventional solvent extraction. Results are reported as mean ± s.d. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test ($p<0.01$).

Variety	TPC_{280} $g_{GAE}/100g_{dm}$	TPC_{Folin} $g_{GAE}/100g_{dm}$	TAC $mg_{WAE}/100g_{dm}$	CA $g_{CAE}/100g_{dm}$	TFA $mg_{QE}/100g_{dm}$	TT $g_{TT}/100g_{dm}$
Barbera	1.74±0.07^{b}	4.21±0.78^{b}	1054.66±40.07^{a}	0.30±0.00^{a}	195.91±3.26^{a}	2.66±0.08^{b}
Nebbiolo	1.01±0.08^{c}	1.95±0.11^{c}	118.54±1.37^{b}	0.14±0.00^{b}	96.76±2.37^{b}	2.02±0.03^{c}
Pinot Noir	2.07±0.09^{a}	6.18±0.19^{a}	67.49±6.19^{c}	0.13±0.00^{b}	75.59±1.13^{c}	8.36±0.12^{a}

CA: Cinnamic Acids; CAE: caffeic acid equivalents; GAE: gallic acid equivalents; QE: quercetin equivalents;.TAC: Total Anthocyanins Content; TT: Total Tannins; TFA: Total Flavonols Content; TPC_{280}: Total Phenolic Compounds at based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; WAE: wine anthocyanins equivalents.

As already observed for the 2011 and 2012 samples, Barbera extract was the richest in anthocyanins and flavonols, while Pinot noir differentiated for the higher total phenols content (mainly according to the Folin index) and tannins content.

4.1.2.3.2 Antioxidant capacity

For the 2013 extracts, the antioxidant capacity of the extracts was evaluated with the ABTS and FRAP test, while the superoxide anion radical scavenging activity was replaced by the ORAC assay.

In the ABTS test, all the varieties showed almost comparable activity and superior to the standard Trolox (Figure 4.6).

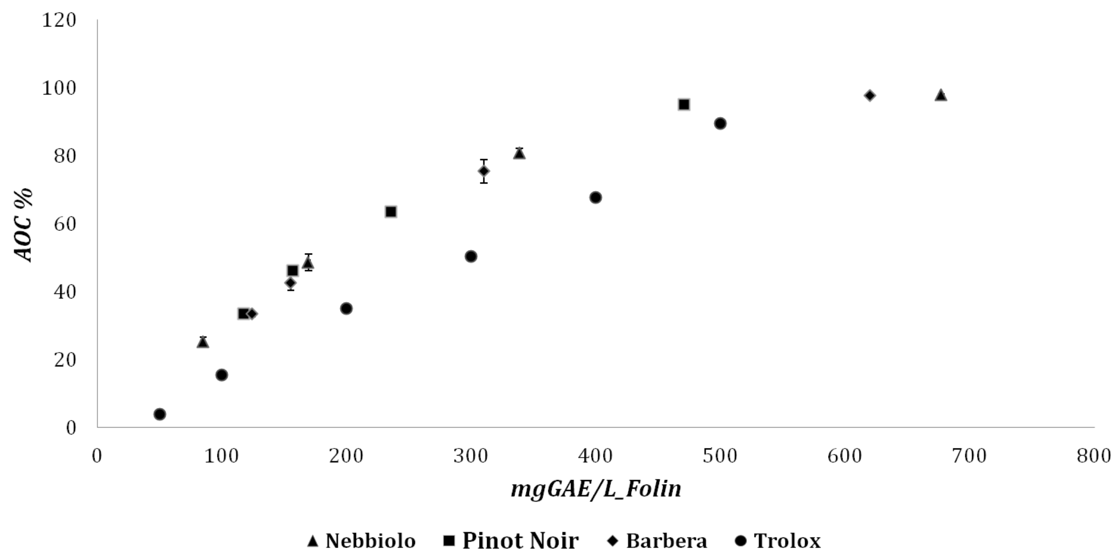


Figure 4.6. Antioxidant capacity (AOC% according to the ABTS assay) of extracts from 2013 skins, as a function of total phenols concentration (GAE_{Folin}). Error bars represent ± SD.

The highest reducing power (Figure 4.7), tested using FRAP, was observed for Nebbiolo as already found for the 2012 extracts, even though no statistically significant difference was observed between Pinot Noir and Nebbiolo. The lowest value was obtained for Barbera variety.

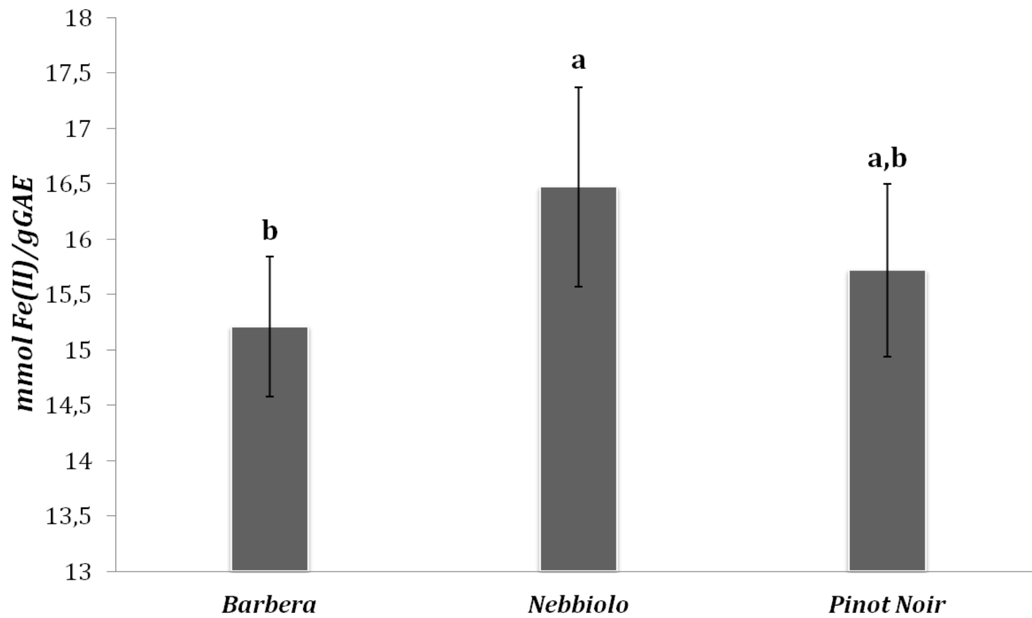


Figure 4.7. Reducing power (FRAP test) of 2013 skin extracts expressed as $mmol_{Fe(II)}/g_{GAE-Folin}$. Error bars indicate ± SD. Superscripts with different letters on the bars show significant differences according to ANOVA and Tukey's post-hoc test. ($p < 0.01$)

The results of the ORAC assay are reported in Figure 4.8. Partly in contrast with the FRAP test, the highest ORAC value was obtained for Pinot Noir, and the lowest for Nebbiolo. Similar values have been obtained by Ky et al. (2014) on six different French grapes varieties.

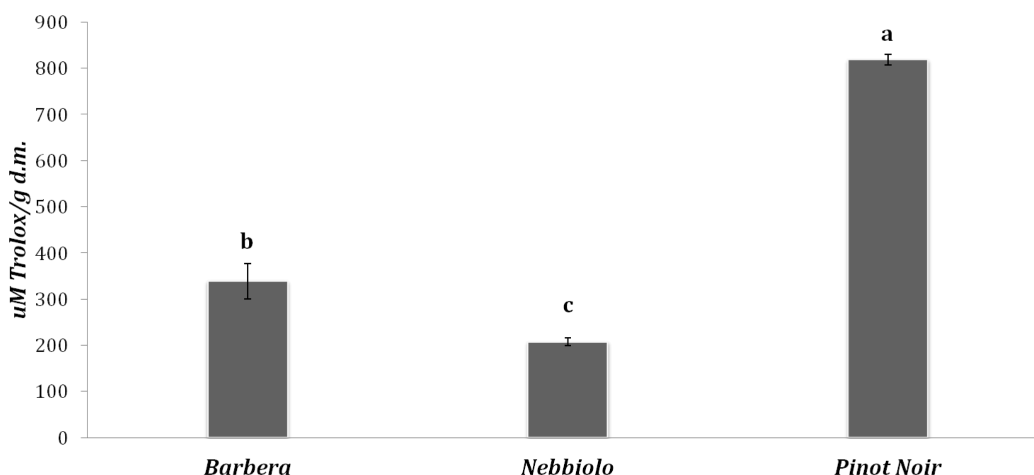


Figure 4.8. ORAC assay results for 2013 skins extract. Superscripts with different letters on the bar show significant differences according to ANOVA and Tukey's post-hoc test. ($p < 0.01$). Error bars indicate ± SD.

4.1.2.4 Glucose and Fructose content

Table 4.7 reports the reducing sugars yields for the 2012 and 2013 extracts.

Table 4.7. Glucose and fructose recovery for the 2012 and 2013 conventional solvent extractions. Results are reported as mean ± SD. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test ($p < 0.01$).

Year	Variety	Glucose ($g/100\ g_{dm}$)	Fructose ($g/100\ g_{dm}$)	Total ($g/100\ g_{dm}$)
2012	*Barbera*	0.64 ± 0.01^{f}	0.41 ± 0.01^{e}	1.04 ± 0.02^{f}
	Nebbiolo	2.16 ± 0.03^{d}	1.55 ± 0.04^{c}	3.71 ± 0.07^{d}
	Pinot Noir	1.38 ± 0.09^{e}	0.95 ± 0.13^{d}	2.33 ± 0.22^{e}
	Moscato	2.55 ± 0.02^{c}	1.63 ± 0.02^{c}	4.18 ± 0.04^{c}
	M. Thurgau	3.23 ± 0.08^{b}	2.09 ± 0.05^{b}	5.33 ± 0.10^{b}
	Chardonnay	3.37 ± 1.00^{b}	1.90 ± 0.13^{b}	5.27 ± 0.07^{b}
2013	*Barbera*	0.40 ± 0.01^{g}	0.45 ± 0.01^{f}	0.85 ± 0.01^{g}
	Nebbiolo	0.16 ± 0.01^{h}	0.21 ± 0.03^{g}	0.37 ± 0.02^{h}
	Pinot Noir	10.68 ± 0.08^{a}	10.21 ± 0.02^{a}	20.89 ± 0.07^{a}

As expected, the sugars yield was higher from unfermented varieties (Chardonnay, Pinot Noir and M. Thurgau) than fermented ones. However, the values obtained with the 2012 skins significantly changed in 2013, with a definitely higher (up to 10 times more) yield in the case of Pinot noir sample.

The presence of sugars should not be a problem for purification in case of extracts food application, however it was reported they could exert antagonistic or

synergistic effect on the total antioxidant capacity of intrinsic polyphenols (Bolling et al., 2013).

4.1.2.5 Influence of year on grape skins extract

4.1.2.5.1 Phenolic profile

The comparison of the extracts phenolic profiles obtained from the red grape skins collected in different years, is reported in Table 4.8.

Table 4.8. Phenolic profiles of the 2011, 2012 and 2013 red grape skins extracts. Error bars indicate ± s.d. Same letter under each parameter, indicates means not statistically different according to ANOVA analysis and Tukey's post-hoc test (p< 0.01).

	2011	2012	2013
Barbera			
TPC_{280} (g_{GAE}/100 g_{dm})	1.78 ± 0.36^{b}	2.07 ± 0.17^{a}	1.74 ± 0.07^{a}
TPC_{Folin} (g_{GAE}/100 g_{dm})	4.54 ± 1.02^{a}	3.55 ± 0.33^{c}	4.21 ± 0.78^{b}
TAC (mg_{WAE}/100 g_{dm})	862.26 ± 180.53^{b}	1200.66 ± 185.51^{a}	1054.66 ± 40.07^{ab}
CA (g_{CAE}/100 g_{dm})	0.12 ± 0.01^{b}	0.31 ± 0.013^{a}	0.30 ± 0.00^{a}
TFA (mg_{QE}/100 g_{dm})	244.95 ± 35.60^{a}	230.40 ± 11.55^{a}	195.91 ± 3.26^{b}
TT (g_{TT}/100 g_{dm})	3.02 ± 0.57^{a}	3.18 ± 0.25^{a}	2.66 ± 0.08^{a}
Nebbiolo			
TPC_{280} (g_{GAE}/100 g_{dm})	1.23 ± 0.13^{a}	0.87 ± 0.19^{b}	1.02 ± 0.08^{b}
TPC_{Folin} (g_{GAE}/100 g_{dm})	2.19 ± 0.36^{a}	1.52 ± 0.26^{b}	1.95 ± 0.11^{a}
TAC (mg_{WAE}/100 g_{dm})	106.19 ± 3.78^{b}	54.41 ± 4.22^{c}	118.54 ± 1.37^{a}
CA (g_{CAE}/100 g_{dm})	0.12 ± 0.01^{a}	0.09 ± 0.01^{b}	0.14 ± 0.00^{a}
TFA (mg_{QE}/100 g_{dm})	103.87 ± 24.08^{a}	73.19 ± 11.05^{b}	96.76 ± 2.38^{a}
TT (g_{TT}/100 g_{dm})	1.90 ± 0.13^{a}	1.73 ± 0.22^{a}	2.02 ± 0.03^{a}
Pinot Noir			
TPC_{280} (g_{GAE}/100 g_{dm})	2.88 ± 0.31^{a}	2.95 ± 0.53^{a}	2.07 ± 0.09^{b}
TPC_{Folin} (g_{GAE}/100 g_{dm})	7.07 ± 1.15^{a}	6.45 ± 0.67^{b}	6.18 ± 0.16^{c}
TAC (mg_{WAE}/100 g_{dm})	171.06 ± 3.94^{a}	99.72 ± 4.39^{b}	67.49 ± 6.19^{c}
CA (g_{CAE}/100 g_{dm})	0.16 ± 0.01^{a}	0.18 ± 0.03^{a}	0.13 ± 0.00^{b}
TFA (mg_{QE}/100 g_{dm})	107.82 ± 10.88^{a}	87.06 ± 6.28^{b}	75.59 ± 1.13^{c}
TT (g_{TT}/100 g_{dm})	8.34 ± 0.33^{a}	5.86 ± 0.15^{b}	8.36 ± 0.12^{a}

CA: Cinnamic Acids; CAE: caffeic acid equivalents; GAE: gallic acid equivalents; QE: quercetin equivalents;.TAC: Total Anthocyanins Content; TT: Total Tannins; TFA: Total Flavonols Content; TPC_{280}: Total Phenolic Compounds at based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; WAE: wine anthocyanins equivalents.

Generally, it can be commented that the year slightly influenced the content of phenolic compounds, but the influence was not the same for all the varieties and phenolic class. For example, 2012 was better for anthocyanins recovery from Barbera, but not for the recovery of any phenolic classes from Pinot Noir and Nebbiolo. Important from a standardization point of view, the year did not

influence the overall phenolic profile (in terms of ratio between the different phenolic groups) for all the investigated varieties.

4.1.2.5.2 Antioxidant capacity

Regarding the antiradical activity evaluated with the ABTS test, the year influenced the extracts properties only for the Barbera skins (and slightly for the Nebbiolo) (Figure 4.9). For fermented skins, the activity of residual phenolic compounds also might have been influenced by the applied fermentation conditions in the winery, which was not possible to evaluate.

On the contrary, the FRAP value was influenced by the year only for the Nebbiolo extracts (Figure 4.10).

It was not possible to compare the results obtained in different years for the superoxide test and for the ORAC assay.

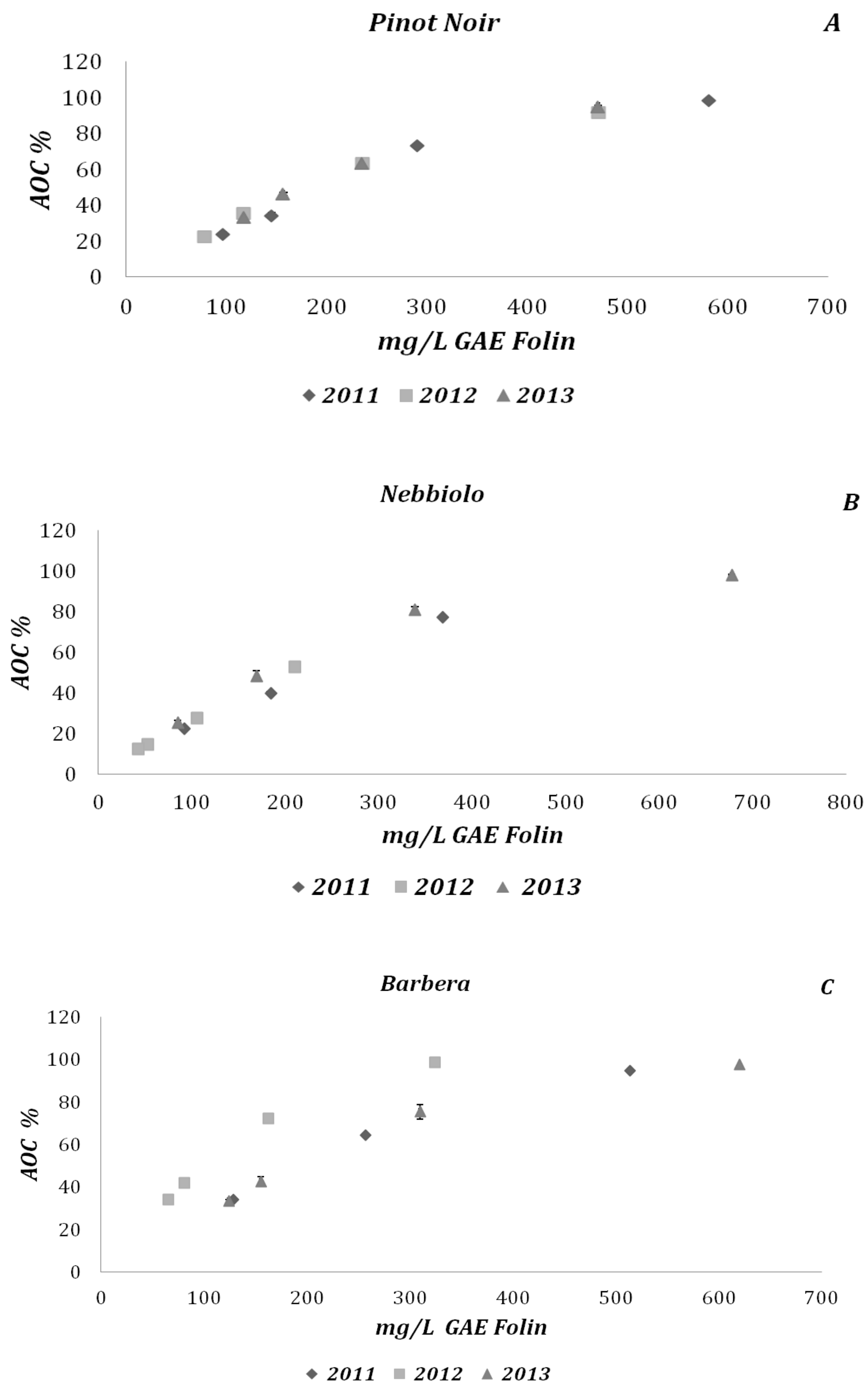


Figure 4.9. Influence of the year on the antioxidant activity (AOC% based on ABTS assay) of red grape skins extracts for the varieties (A) Pinot Noir, (B) Nebbiolo and (C) Barbera. Error bars indicate ± SD.

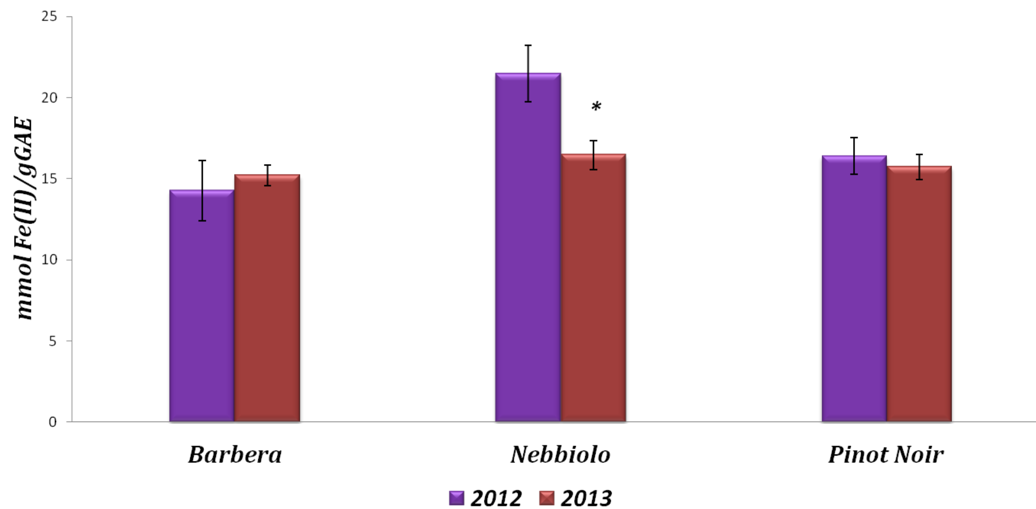


Figure 4.10. Influence of the year on the reducing power (FRAP assay) of red grape extracts. The symbol * indicates a significant difference between the years for the same variety (t Test; p < 0.01).

4.2 Process intensification

4.2.1 CGAs characterization

CGAs stability results of the three different investigated Tw20 concentrations are reported in Table 4.9 and Figure 4.11, showing that both $T^{1/2}$ and ε increase with increasing surfactant concentration.

In particular, the lowest stability was observed for Tw20 1mM which is a concentration very close to the reported 0.08 mM CMC in water (Kim & Hsieh, 2001). CGAs generated from Tw20 20mM and 10mM showed comparable behaviours with a higher stability for 20mM which was, then, selected for the following experiments. Higher concentrations were not tested since, as reported by Dermiki et al. (2009) and Jarudilokkul et al. (2004), concentration of Tw20 higher than 20mM does not have any significant effect on gas hold up and T½.

Table 4.9. Evaluation of CGAs stability (as gas hold up and T½) as a function of Tw20 concentration and ethanol content. Results are expressed as mean ± SD. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

Tw20 (mM)	*Ethanol (%)*	*Gas Hold Up*	*T½ (sec)*
1	0	0.49 ± 0.00^{c}	173 ± 1.00
10	0	0.65 ± 0.01^{b}	523 ± 1.00
20	0	0.71 ± 0.01^{a}	701 ± 4.00
19	3	0.72 ± 0.00^{a}	725 ± 15.00
16	12	0.71 ± 0.01^{a}	767 ± 31.00
10	30	0.66 ± 0.00^{b}	960 ± 0.00

The presence of ethanol clearly increases the foam stability without influencing the gas hold up.

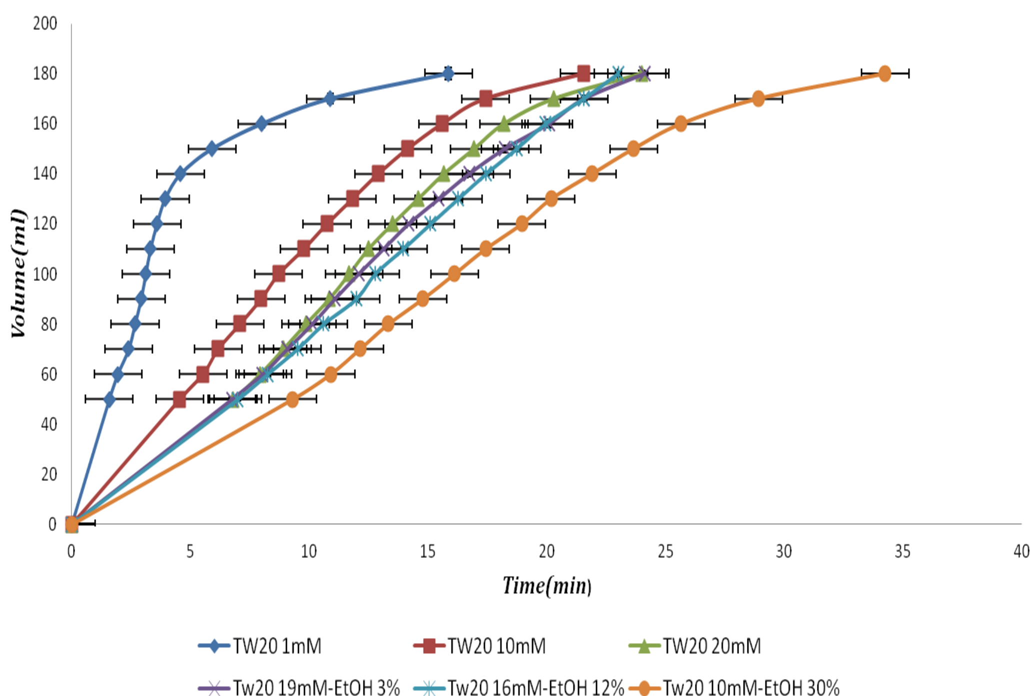


Figure 4.11. Stability of CGAs (trend of volume of drained liquid Vs. time) generated from Tw20 solutions at different concentrations without and with ethanol. Results are expressed as mean value. Error bars indicate ± SD.

This effect is most probably due to the chemical structure of Tw20. In the Tw20 structure there is a fatty acid chain that is more soluble in ethanol than water. The complete solubilisation of Tw20 in ethanol produces a change in the foam structure. The solution with 30 % ethanol showed the highest $T^{1/2}$ value with the complete collapse occurring 10 min later than for the other solutions. Comparing

the CGAs obtained from ethanol solutions, the lower ε for the 30 % ethanol, indicates a smaller bubble dimension. CGAs containing ethanol therefore, appeared more compact and collapsed more slowly than the CGAs generated without ethanol.

The obtained results are very interesting, especially if compared with the results obtained by Dermiki et al.(2009) and by Spigno & Jauregi (2005) with another surfactant (the cationic surfactant CTAB). In fact, these authors observed that the presence of ethanol destabilized the CGAs structure with a consequent lower stability of the foam. This effect was explained by the fact that addition of alcohol causes localized reduction of the surface tension to exceptionally values with subsequent bubbles rupture (Save & Pangarkar, 1994).

4.2.2 Application A

The application A consists in a partial and/or complete replacement of ethanol with different concentrations of the food grade surfactant Tw20 for direct extraction of phenolic compounds from DMWS. The results, in terms of total phenolic compounds and total anthocyanins recovery, are reported in Table 4.10.

Table 4.10. Extraction yields in total phenolic compounds and total anthocyanins content from Barbera skins for the trials of Surfactant – Application A. The results are reported as mean ± SD. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test. ($p < 0.01$).

Trial	*Tw20 (mM)*	*Ethanol (%)*	*RE of TPC_{280} (mg_{GAE}/ g_{dm})*	*RE of TPC_{Folin} (mg_{GAE}/ g_{dm})*	*RE of TAC (mg_{WAE}/ g_{dm})*	*RE_{Folin}/ RE_{WAE}*	*RE_{Folin}/RE_{280}*
A	20	0	$4.31±0.54^{d}$	$4.05±0.26^{c}$	$2.59±0.09^{c}$	1.56	0.94
B	10	30	$8.90±0.74^{c}$	$13.64±1.20^{b}$	$5.47±0.99^{ab}$	2.49	1.53
C	0	60	$24.58±1.17^{a}$	$24.35±2.77^{a}$	$6.42±0.90^{ab}$	3.79	0.99
D	20	60	$12.70±0.13^{b}$	$20.54±0.21^{a}$	$6.98±0.00^{a}$	2.94	1.62
E	20	30	$8.86±0.09^{c}$	$11.76±0.82^{b}$	$5.77±0.43^{ab}$	2.04	1.33
F	10	60	$13.13±0.04^{b}$	$20.11±0.65^{a}$	$7.19±0.13^{a}$	2.80	1.53
G	10	30	$7.88±0.21^{c}$	$10.59±0.43^{b}$	$4.99±0.00^{b}$	2.12	1.34

GAE: gallic acid equivalents; RE: Recovery; TAC: Total Anthocyanins content; TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; WAE: wine anthocyanins equivalents.

The yields obtained with 60 % ethanol (reference treatment) were lower than those reported in Table 4.8 for 2012 Barbera skins (35.5 $mg_{GAE\text{-}Folin}$/g and 12 mg_{WAE}/g). This is due to the fact that in these trials, extraction was carried out only for 1 h instead of 2.

From Table 4.10, the following comments are possible:

- 60% ethanol was confirmed the best solvent to get the highest extraction of both total phenols and anthocyanins compared with the other solvents tested. Addition of Tw20 did not significatly influence the phenols recovery.
- Trials B and G were carried out with the same solvent composition. However in trial B, the Tw20 and ethanol concentration was 10 mM and 30 %, respectively, these were obtained by mixing equal volumes of solutions 20 mM Tw20 and 60 % ethanol; whilst in trial G Tw20 was directly dissolved in ethanol 30 %. Regarding the recovery , the first option is to be preferred. All the other trials (C-F), however, were carried out as for trial G.
- When extraction was already performed only with an aqueous Tw20 solution (trial A), TPC yield was definitely lower (6 times) than for the reference trial C. Also anthocyanins recovery was lower, but only 3 times lower. This would confirm a particular affinity of Tw20 for anthochyanins, as previously observed (Spigno et al., 2014).
- The higher Tw20 affinity for WAE than for other phenolic compounds is also enforced by the RE_{Folin}/RE_{WAE} ratio, since increasing the Tw20 concentration led to reduced ratios.
- Also the RE_{Folin}/RE_{GAE} ratio can be considered as indication of change in the phenolic profile of the extract or in the oxidation status. For example a higher ratio can indicate a reduced oxidation of the recovered compounds (the Folin index is more influenced by the oxidative status of the molecules than the total phenol index) but also a different composition of the extract (molar extinction coefficient for absorption at 280 nm is not the same for all the phenolic compounds). Strangely the ratio was the lowest and the same when only

aqueous ethanol or only aqueous Tw20 solutions were used, while it increased when Tw and ethanol were used together.

- An optimization of the solvent composition and/or study of extraction kinetics for the different solvent compositions could be exploited to obtain extracts enriched in anthocyanins or in other phenolic compounds.

Papaioannou & Karabelas (2012) have studied lycopene recovery from tomato peel under mild conditions, assisted by enzymatic pre-treatment and in presence of non-ionic surfactant. They tested the lipophylic Span20 surfactant with excellent results in term of lycopene recovery, estimated as four and ten times greater compared to simple enzymatic pre-treatment and untreated peels, respectively. In agreement with this literature work, our results suggest as the direct application of surfactant solution could be a low cost pre-treatment method. For example, from the results in Table 4.10, it might be useful to carry out a first extraction with Tween 10mM / Ethanol 30% to recover all the anthocyanins together with half of the other phenolic compounds, followed by extraction with only ethanol 60 % to recover the remaining phenolic compounds.

Antioxidant capacity (ABTS assay) of all the obtained extracts (with reference to Table 4.10) are reported in Figure 4.12.

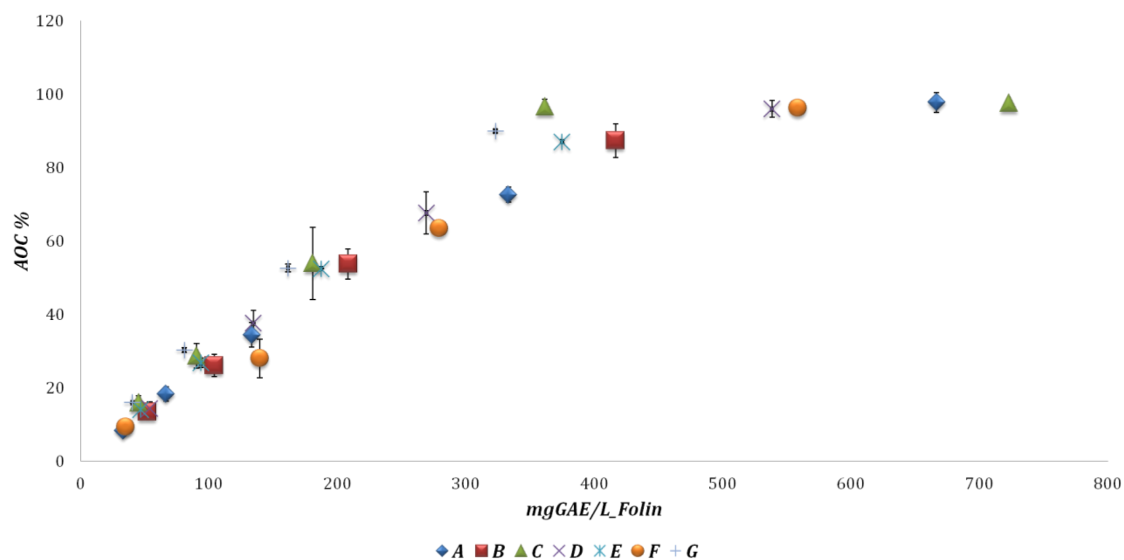


Figure 4.12. Antioxidant capacity (AOC% based on ABTS test) of the different extracts of the trials of Surfactant Application A: (A) Tw20 20mM- EtOH 0% (B) Tw20 10mM-EtOH 30% (C) EtOH-Water 60/40 v/v(D) Tw20 20mM-EtOH 60% (E) Tw20 20mM-EtOH 30% (F) Tw20 10mM-EtOH 60% (G) Tw20 10mM-EtOH 30%. Error bars indicate ± SD.

The higher antioxidant capacity was observed for the solvent C (reference trial with ethanol 60 %) and solvent G (10 mM Tw20 and 30 % ethanol). However all the extracts slightly differed for their activity without a clear relation with the solvent composition.

4.2.3 Application B

The Application B consists in a integrated extraction – purification step by direct application of CGAs after a previous CSE step.

Table 4.11 reports the recovery of phenolic compounds calculated based on Eq. (3.2) using the volume and concentration of aphron phase instead of extract. The values are lower than those observed with conventional solvent extraction (Table 4.8) or in the trials of Application A (Table 4.10), since in this case the conventional solvent extraction was carried out with magnetic stirring instead of using the semi-industrial scale mixer. Since we had to produce small volumes of suspensions extract-skins, it was not possible to use the semi-industrial Silverson Mixer.

Table 4.11. Recovery of phenolic compounds in the trials of Application B. Results are reported as average ± SD. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test. ($p < 0.01$).

Ethanol Suspension /CGAs Ratio (v/v)	TPC_{280}		TPC_{Folin}		TAC	
	RE (mg_{GAE}/g_{dm})	mg_{GAE} in the Aphron phase	RE (mg_{GAE}/g_{dm})	mg_{GAE} in the Aphron phase	RE (mg_{WAE}/g_{dm})	mg_{WAE} in the Aphron phase
1/9	$3.63±0.12^{b}$	$33.77±1.14^{b}$	$6.05±0.23^{b}$	$56.23±2.18^{b}$	$1.49±0.00^{c}$	$13.87±0.01^{ab}$
1/12	$6.37±0.46^{a}$	$44.43±3.24^{a}$	$10.07±0.86^{a}$	$70.19±6.03^{a}$	$1.93±0.01^{b}$	$16.48±0.01^{a}$
1/24	$7.40±0.27^{a}$	$25.77±0.94^{c}$	$11.04±0.88^{a}$	$38.45±3.07^{c}$	$2.60±0.12^{a}$	$9.07±0.44^{b}$

TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content; GAE: gallic acid equivalents; WAE: wine anthocyanins equivalents

The statistically highest TPC recovery (both as Folin index and total phenol index) was obtained with at least a 1/12 suspension/CGAs ratio, while the highest TAC recovery with the 1/24 ratio.

However, if we consider the total amounts of phenolic compounds retained in the aphron phase in the different trials (Table 4.11), the highest values were those for the 1/12 ratio

Application B was thought for a double potential aim: 1) to integrate the extract-solids separation step with a purification/fractionation step; 2) to further increase the recovery of phenolic compounds from grape skins.

In the first case, other parameters are important for the evaluation of a CGAs-based separation process:

- Selectivity (SE): indicates the affinity of a compound for the aphron phase, and is calculated as the ratio of the compound concentration in the aphron phase to that in the liquid phase.
- Enrichment factor (EF): indicates the concentration of the separated compounds in the aprhron phase, and is calculated as the ratio of the compound concentration in the aphron phase to that in the initial solution fed into the column.

Values of SE and EF for the different phenolic compounds are reported in Table 4.12. Values of these parameters were calculated using the average concentration values, therefore statistical analysis was not carried out.

Table 4.12 Selectivity (SE) and enrichment factors (EF) for the CGAs separation process of Application B.

Ethanol Suspension /CGAs Ratio (v/v)	SE TPC_{280}	EF $TPCC_{280}$	SE TPC_{Folin}	EF TPC_{Folin}	SE TAC	EF TAC
1/9	1.47	0.18	1.51	0.19	1.50	0.20
1/12	1.57	0.16	1.57	0.18	1.48	0.16
1/24	1.20	0.06	1.33	0.06	1.08	0.06

Results showed that the applied process did not lead to a selective separation of anthocyanins from other phenolic compounds, neither to an enrichment of the initial extract into the aphron phase (the latter was inevitably due to the use of a concentrated solution as feeding of the flotation column).

In order to better compare our results with those of previous CGAs application on phenolic extracts (Spigno et al., 2014), the process recovery was calculated according to equations 3.11 and 3.12 (Figures 4.13 and 4.14).

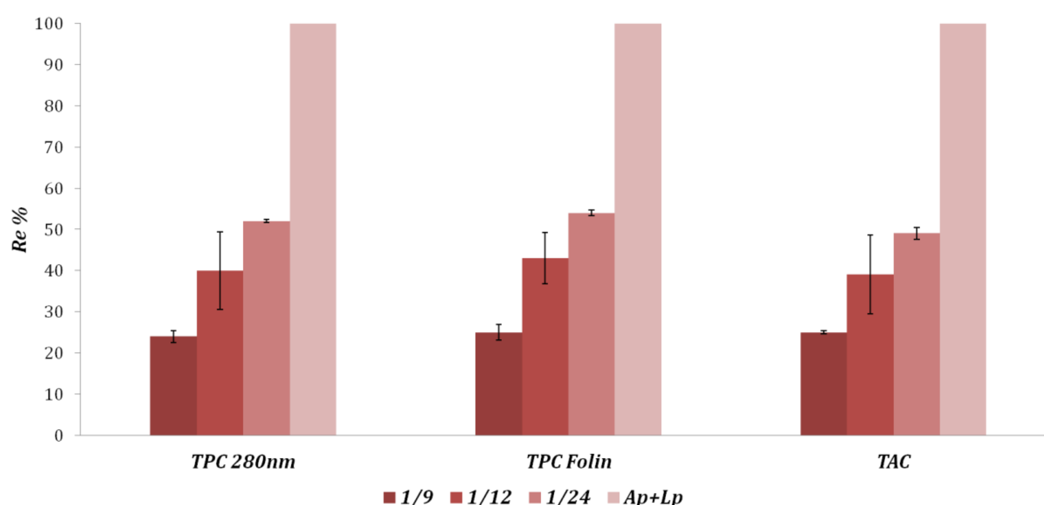


Figure 4.13. Phenolic compounds recovery for the CGAs Application B process. Values have been calculated according to Equation 3.11. Error bars indicate ± SD. TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content.

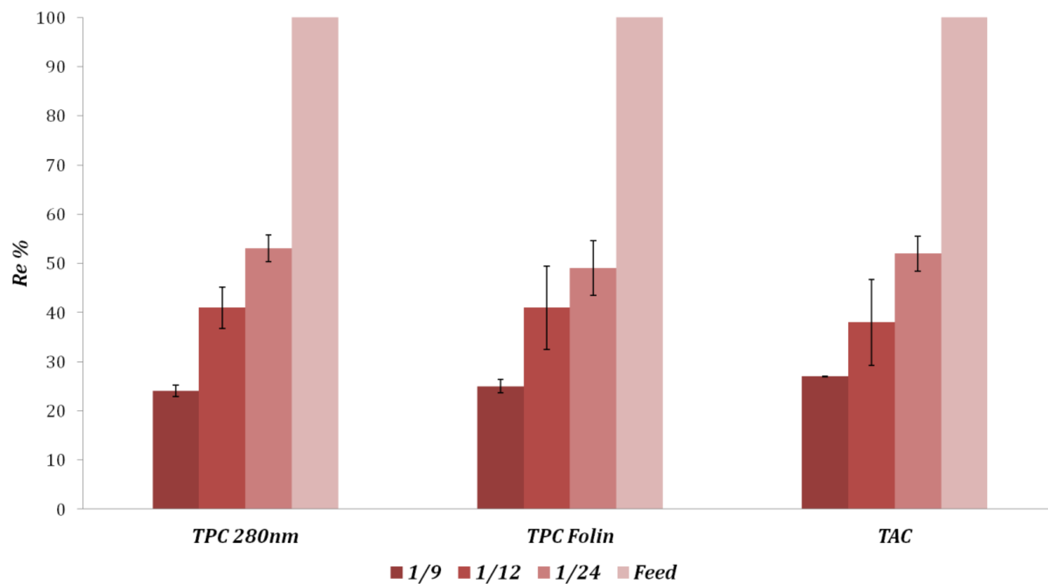


Figure 4.14. Phenolic compounds recovery for the CGAs Application B process. Values have been calculated according to Equation 3.12. Error bars indicate ± SD. TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content.

Better recoveries, up to 76-78 % were achieved by Spigno et al. (2014) using Tw20 10mM and a 1/22 volumetric ratio. A too high surfactant concentration (20 mM) in our trials, selected because of the higher foam stability, might have prevented a good interaction between micelles and phenolic compounds.

However, looking in the literature for other purification/fractionation methods, it was found that Negro et al. (2003) purified a Negro Amaro skins ethanol extract by solid phase extraction with a C18 column. After purification, the recovered anthocyanins corresponded to the 35% of the total initial anthochyanins content. Jampani et al. (2014) tested the anthochyanins adsorption and desorption capacity of seven different resins. The highest anthochyanins desorption ratio (87 %) was observed for Amberlite XAD7HP, but the elution required the application of organic solvent. Our CGAs process allowed the recovery of the 50% of the total anthocyanins in the extract without requiring application of additional organic solvents, therefore it could be a real low cost alternative purification method.

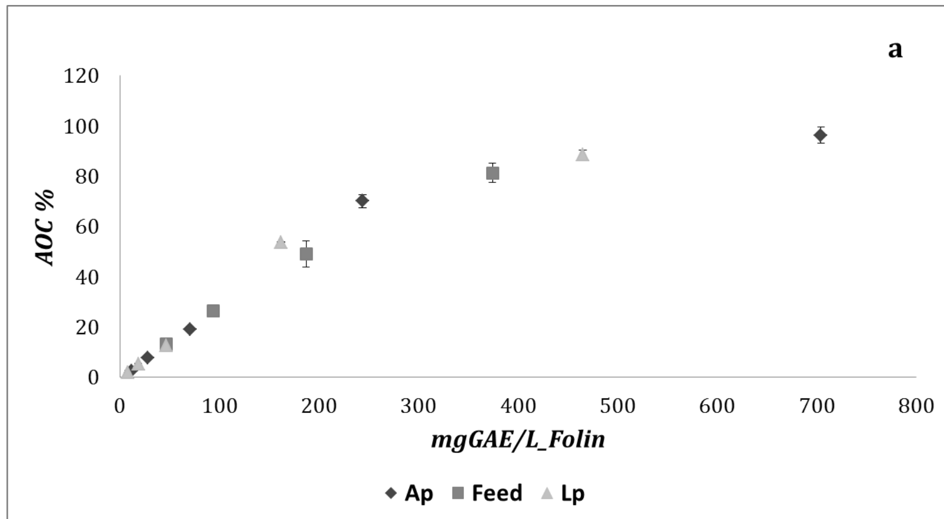
Table 4.13. CGAs Application B: comparison of total amount of phenolic compounds recovered at the end of the process (aphron + liquid phase) and total amount loaded into the column with the ethanol suspension. The symbol * indicates a significant difference. Statistical analysis was performed with t Test ($p < 0.01$).

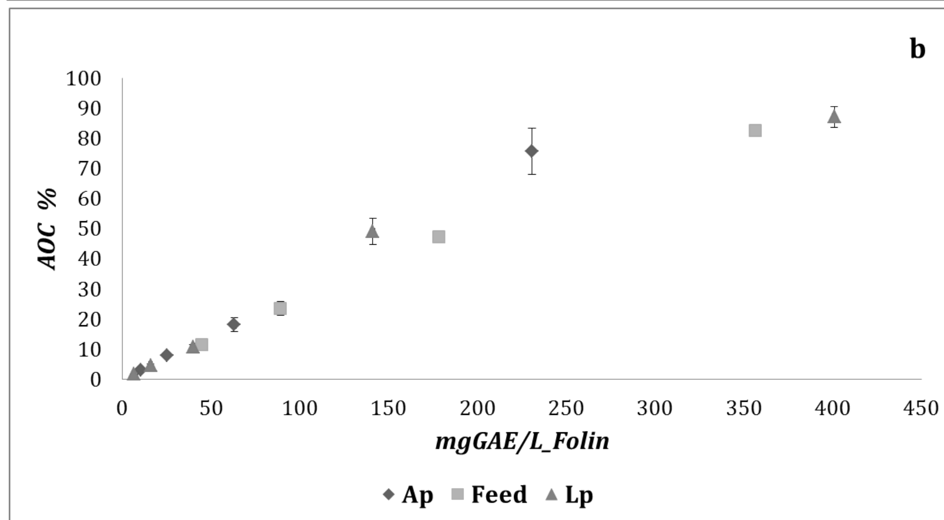
Extract-CGAs Volumetric Ratio	*TPC_{280} (mgGAE)*	*TPC_{Folin} (mgGAE)*	*TAC (mgWAE)*
1/9			
Ap+Lp	137.99±1.07	224.79±3.11	55.98±0.62*
Feed	138.29±2.50	222.63±9.63	51.93±1.33
1/12			
Ap+Lp	102.67±7.24	161.14±8.17	38.58±0.62
Feed	108.65±12.61	155.04±1.36	40.48±4.10
1/24			
Ap+Lp	49.81±1.57	71.05±6.37	18.45±1.28
Feed	48.88±0.76	78.14±1.05	17.28±0.24

TPC_{280}: Total Phenolic Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content. GAE: gallic acid equivalents; WAE: wine anthocyanins equivalents.

Analysis of Figures 4.13 and 4.14 also suggests that the second potential aim of this CGAs application, that was additional phenols extraction from the grape skins, was not achieved. In fact, if the sum of the phenolic compounds recovered in the aphron and liquid phase is calculated (in order to apply Equation 3.10) and compared with the total amount contained in the initial extract suspension, it can be seen that the two values almost coincide (Table 4.13). Only for the 1/9 volumetric ratio and for total anthocyanins, it was found a significantly higher amount. Further studies would, then, be necessary for a better evaluation of additional extraction during the CGAs application step and to understand the CGAs behaviour in the presence of solid particles like grape skins.

The antioxidant capacity (ABTS test) of the recovered aphron and liquid phases was evaluated and compared with that of the ethanol suspension loaded into the column (Figure 4.15).
As previously observed (Spigno et al., 2014), the process did not modify the antiradical capacity of the extract.





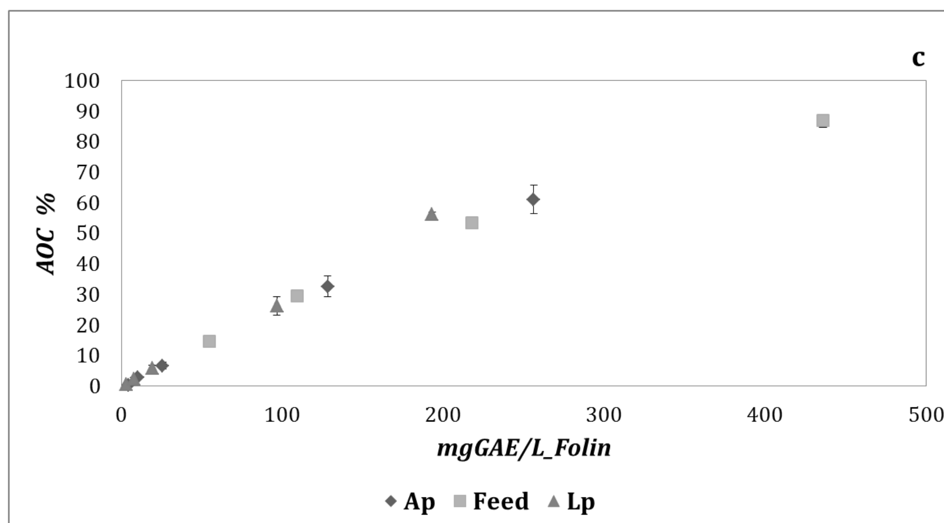


Figure 4.15. Antioxidant capacity (AOC% based on ABTS assay) of the feed, aphron phase and liquid phase of the trials for CGAs Application B, at different volumetric ratios: 1/9(a); 1/12 (b); 1/24 (c). Error bars indicate ± SD

The comparison of the results obtained by the CGAs application B with the results present in literature is very difficult due to the absence of published works based on CGAs generated with a non-ionic surfactant in presence of solid particles.
As previous commented, Spigno et al. (2014) tested the CGAs generated with the Tw20 at the concentration of 10 mM on a real phenolic extract obtained from grape skins. They tested different Extract-CGAs volumetric ratios and achieved the maximum recovery (above 70 %) of TPC and TAC at the 1/24 volumetric ratio.
This comparison suggests that the optimal Tw20 concentration is 10mM but since the presence of the skins inside the column would have reduced the microfoam stability a higher Tw20 concentration was selected to perform the trials. At higher surfactant concentration the CGAs stability increases but mass transfer resistance also increases due to presence of more surfactant at aqueous‑organic phase interface, coming out in a reduced transfer of phenolic compounds to internal phase (Das et al., 2008).

4.3 Encapsulation process

For the spray-drying experiments, the conventional extract had to be concentrated in order to remove ethanol and then Tw20 20 mM was used to restore the initial volume.
Formation of a sticky dark red precipitate (Figure 4.16) after the Tw20 addition was observed, therefore the liquid phenolic extract was separated and analyzed again for the TPC_{280}, TPC_{Folin} and TAC. The results are reported in Table 4.14.

Figure 4.16. The sticky dark red precipitate observed Tw20 addition to the initial Barbera skins extract.

Table 4.14. Phenolic profile of conventional Barbera extract before(pre) and after (post) Tw20 addiction. The symbol * indicates a significant difference between the values under the same parameter. Statistical analysis was performed with t Test ($p < 0.01$).

Extract	*TPC_{280} (mg_{GAE}/g_{dm})*	*TPC_{Folin} (mg_{GAE}/g_{dm})*	*TAC (mg_{CyN}/g_{dm}.)*	*Total solids (mg/mL)*	*Density (g/cm^3)*
Pre	14.04±0.06*	26.48±0.01*	1.93±0.05	17.99±0.00*	0.924±0.01*
Post	8.62±0.03	17.55±1.85	2.09±0.20	28.79±0.75	0.997±0.00

TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content; GAE: gallic acid equivalents; CyN: Cyanidin – 3 – glucoside equivalents.

In spite of the red-blue color of the precipitate, the total anthocyanins content of the extract remained constant. Actually, the applied pH differential method determines only the monomeric anthocyanin pigment, while polymerized anthocyanin pigments are not measured. The precipitate probably contained only polymeric anthocyanins and not monomeric one. This could be exploited to achieve a selective separation, even though the precipitate could be solubilized only with acetone (acetonitrile and ethanol could not solubilize it).

Total phenolic content (both as total phenols index and Folin index), on the other hand, clearly showed a reduction after the precipitation. Total solids content increased after precipitation. However, this increase is the result of the increase

due to Tw20 concentration (based on Tw molecular weight, 20 mM corresponds to 24 mg/mL) and the decrease due to precipitation. Also the increase in density is due to the substitution of the initial extract medium (ethanol 60 %) with aqueous Tw20.

In spite of the precipitate, specific antioxidant capacity (AOC% as a function of Folin index concentration) of the extract did not change after Tw20 addition (Figure 4.17).

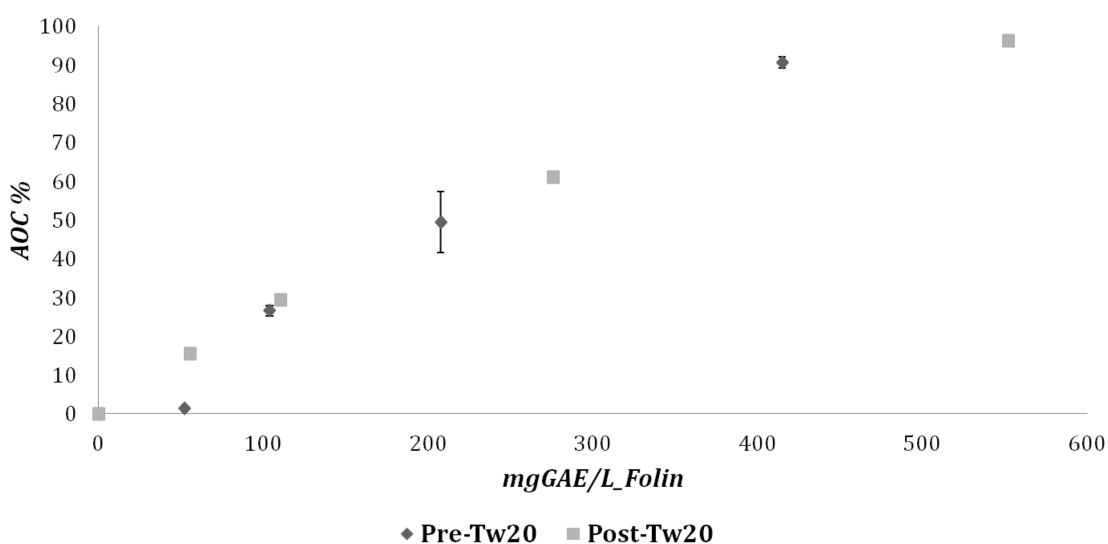


Figure 4.17. Antioxidant capacity (AOC% based on ABTS assay) of Barbera extract before and after Tw20 addition. Error bars indicate ± SD

Spray-drying of the extract-surfactant mixture with addition of maltodextrins as wall material, gave the process yield reported in Table 4.15. Recovery was calculated comparing the initial total solids and phenolic compounds amount in the extract, with those in the final powders.

Table 4.15. Recovery of wet powder, total phenolic compounds, total anthocyanins and water activity of the powders obtained from spray-drying of Barbera skins extract with Tw20 and maltodextrins. Results are reported as mean ± SD. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

Molar ratio MD/GAE	***Wet Powder recovery (Re%)***	***TPC_{280} (Re%)***	***TPC_{Folin} (Re%)***	***TAC (Re%)***	***a_W***
3.85	$71±1.25^{a}$	$90±0.11^{a}$	$49±0.87^{b}$	$55±5.55^{a}$	$0.110±0.01^{a}$
2.44	$73±0.2^{a}$	$75±0.15^{b}$	$57±1.86^{a}$	$47±1.67^{b}$	$0.163±0.04^{ab}$
1.28	$26±3.32^{b}$	$26±2.72^{c}$	$18±2.30^{c}$	$12±0.40^{b}$	$0.249±0.05^{bc}$
0.64	$25±2.45^{b}$	$24±2.04^{c}$	$19±1.91^{c}$	$15±3.74^{a}$	$0.334±0.02^{c}$

TPC_{280}: Total Phenolc Compounds based on total phenol at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content; a_w: water activity.

As already observed in the spray-drying of a Barbera skins extract without surfactant (Duserm Garrido, 2012), maltodextrins addition allowed high wet powder recovery. Recovery is always below 100 %, since part of the powder is lost on the walls of the frying chamber and of the exit cyclone (Figure 4.18). Product lost was unacceptable (> 70 %) when the amount of maltodextrins was reduced below a 2.44 MD/GAE molar ratio. Furthermore, under a 2.44 MD/GAE molar ratio, the water activity of the final powders was higher. Such a_W values are not critical for the shelf-life of the product in terms of microbial growth, since the minimum a_W at which microorganisms can grow (the value under which physiological activities necessary for cell division are depressed) is 0.60 (the minimum for growth of most bacteria is approximately 0.87, while halophilic bacteria could grow at a_W as low as 0.75) (Beuchat et al., 2013). Anyway, shelf-life problem would occur in terms of caking phenomena.

Figure 4.18. Cyclone system at the spray-drying exit for powder collection (trial with Barbera skins extract enriched in Tw20).

The encapsulation efficiency, expressed as percentage of phenols and anthocyanins recovered at the end of the encapsulation process from the total antioxidant compounds in the feed solution, is strictly related to the efficiency in terms of powder recovered. Comparing the total phenols recovery (based on total phenols index) with the wet powder yield, it can be assumed that the composition of the powder lost in the cyclone is the same as that of the collected powder. However, the lower measured recovery for total phenols index and total anthocyanins, indicates a certain thermal degradation of total phenols and of monomeric pigments during the process (carried out at 150 °C). Of course, maltodextrins addition inevitably decreases the purity of the final extract in terms of phenolic content, but definitely improve the powder water solubility (Table 4.16).

Table 4.16. Total phenols and anthocyanins content, and water solubility index (WSI) of spray-dried Barbera skins extract with surfactant and maltodextrins. Results are reported as mean ± SD. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

Molar ratio MD/GAE	TPC_{280} (mg_{GAE}/g_{wp})	TPC_{Folin} (mg_{GAE}/g_{wp})	TAC (mg_{CyN}/ g_{wp})	WSI (%)
3.85	13.27±0.25^{d}	15.99±0.49^{d}	2.20±0.26^{b}	93±0.14^{a}
2.44	14.95±0.01^{c}	25.25±0.75^{c}	2.55±0.08^{b}	90±0.03^{b}
1.28	21.78±0.49^{b}	33.25±0.86^{b}	2.87±0.27b	89±0.56^{c}
0.64	28.81±0.38^{a}	51.39±0.47^{a}	4.75±0.20^{a}	88±0.59^{c}

***TPC_{280}*: Total Phenolic Compounds based on total phenol index at 280nm; *TPC_{Folin}*: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content; GAE: gallic acid equivalents; CyN: Cyanidin – 3 – glucoside equivalents**

Obviously, also the colour is affected by the maltodextrins addition (Figure 4.19): the powder produced with a low quantity of MD is dark violet coloured, very similar to the colour of the original grape skin extract; while the powder produced with the highest quantity of MD is light pink coloured.

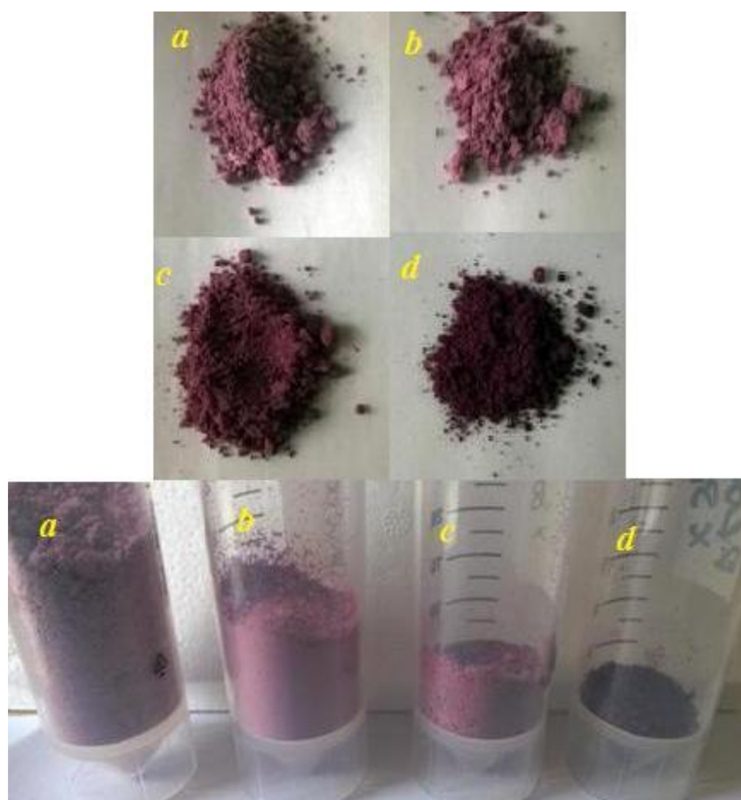


Figure 4.19. Barbera skins extract powders produced by spray drying at different MD/GAE molar ratios: (a) 3.85 (b) 2.44 (c) 1.28 (d) 0.64.

Addition of MD at different ratios did not modify the specific antioxidant capacity (based on ABTS test) of the final powders, also in comparison with the initial extract (Figure 4.20). This is apparently in contrast with what previously commented about the lower phenols recovery compared to total solids recovery. However, Figure 4.20 reports the antiradical capacity as a function of the Folin index, therefore it means that the phenolic compounds that still are measured with the Folin assay, have the same initial ABTS reducing capacity.

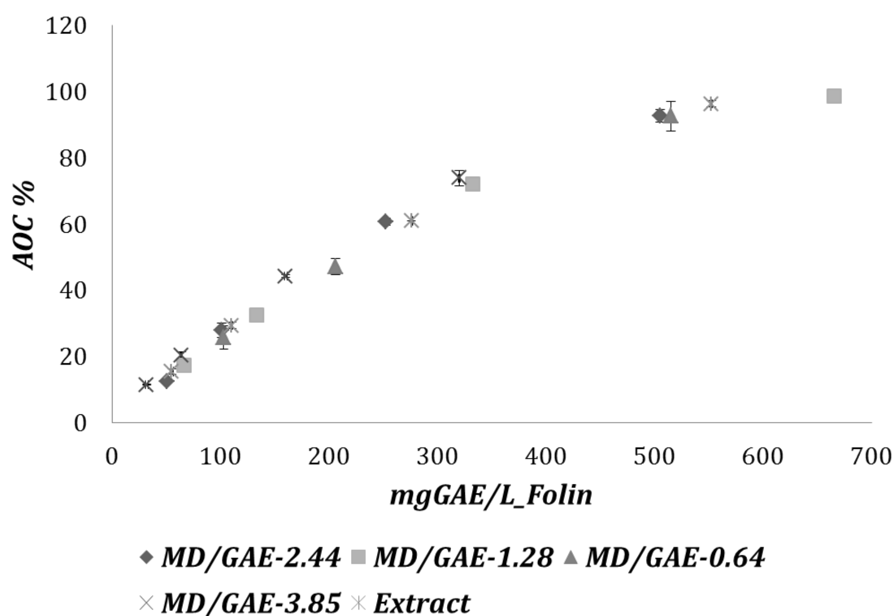


Figure 4.20. Antioxidant capacity (AOC% based on ABTS assay) of the different spray-dried Barbera skins extracts with Tw20 and maltodextrins in comparison with AOC% of initial extract. Error bars indicate ± SD.

The comparison of our results with the literature was very difficult because there are only a few researches focused on phenols encapsulation in presence of surfactant as a wall material.

Some interesting results were obtained by Sansone et al.(2011) who encapsulated quercetin and naringenin with a solution composed by Tween 85 and other wall materials such as cellulose acetate phthalate and carboxymethylcellulose. With Tween they obtained an encapsulation efficiency up to 90% and a process efficiency up to 69%. Furthermore, the surfactant enhanced the dissolution rate of

the flavonoid release (from 80% to 100%) in simulated intestinal fluid and also stabilised the antioxidant activity during 12 months of storage. Spray-drying conditions were not the same as in our trials and the authors worked with pure phenolic compounds, while we used a natural and complex real mixture.

To better evaluate the influence of surfactant presence, additional powders at molar ratio of 2.44 and 0.64 were prepared at the same conditions but without Tw20 (Tables 4.17-4.18, Figure 4.21).

Table 4.17. Total phenols and anthocyanins content, and water solubility index (WSI) of spray-dried Barbera skins extract with or without surfactant and maltodextrins. Results are reported as mean ± SD. * indicates means statistically different for the same parameter and molar ratio, according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

MD/GAE molar ratio	***Tw20***	***TPC_{280} (mg_{GAE}/g_{wp})***	***TPC_{Folin} (mg_{GAE}/g_{wp})***	***TAC (mg_{CyN}/g_{wp})***	***WSI (%)***
2.44	*No*	25.48±0.28*	38.21±0.64*	2.76±0.11	86±0.16*
2.44	*Yes*	14.95±0.01	25.25±0.75	2.55±0.08	90±0.03
0.64	*No*	56.02±2.13*	87.66±1.12*	5.63±0.06	74±0.88#
0.64	*Yes*	28.81±0.38	51.39±0.47	4.75±0.75	88±0.59

TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content; GAE: gallic acid equivalents; CyN: Cyanidin – 3 – glucoside equivalents.

Table 4.18. Recovery of wet powder, total phenolic compounds, total anthocyanins and water activity of the powders obtained from spray-drying of Barbera skins extract with Tw20 and maltodextrins. Results are reported as mean ± SD. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

Molar ratio MD/GAE	***Tw20***	***Wet Powder recovery (Re%)***	***TPC_{280} (Re%)***	***TPC_{Folin} (Re%)***	***TAC (Re%)***	***a_W***
2.44	*No*	80±1.67*	88±0.00*	80±3.23*	93±5.50*	0.132±0.01
2.44	*Yes*	73±0.20	75±0.15	57±1.86	47±1.66	0.163±0.04
0.64	*No*	91±1.05#	86±2.08#	81±0..93#	85±2.12#	0.187±0.00#
0.64	*Yes*	25±2.45	24±2.04	19±1.92	15±3.74	0.334±0.02

TPC_{280}: Total Phenolc Compounds based on total phenol index at 280nm; TPC_{Folin}: Total Phenolic Compounds based on Folin Index; TAC: Total Anthocyanins Content.

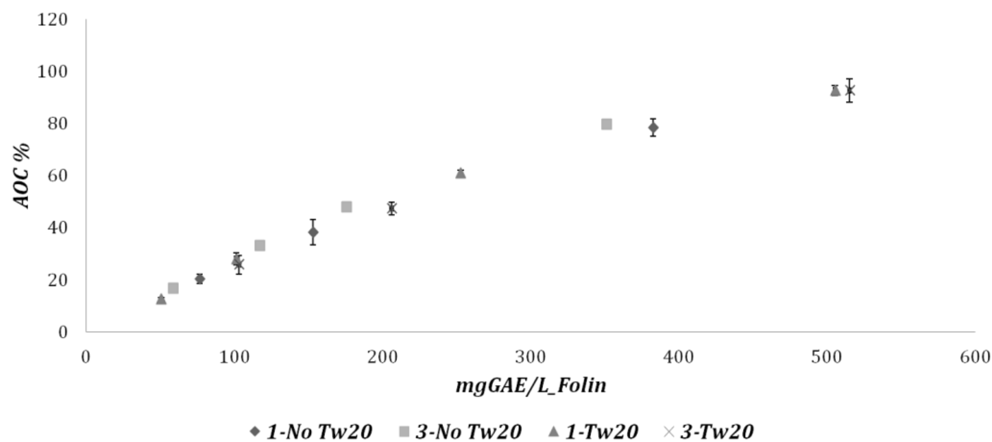


Figure 4.21. Antioxidant capacity (AOC% based on ABTS assay) of the different spray-dried Barbera skins extracts with/without Tw20 and maltodextrins. (1: 2.44 MD/GAE molar ratio; 3: 2.44 MD/GAE molar ratio). Error bars indicate ± SD.

On average, Tw20 addition worsened the spray-drying process performance since:

- total phenols and anthocyanins recovery was definitely lower (the powder was very sticky and remained attached on the cyclone walls);
- a_w was higher;
- powder purity was lower, except for the monomeric pigment content.

The only positive effect of Tw20 was an increase in water solubility, whilst no effect was observed on the antioxidant capacity.

Adhikari et al. (2009) studied the effect of low molecular weight surfactant, such as Tween80, on the powder recovery in spray drying of highly sticky sugar-rich food in presence of proteins. They underlined the difficulty in obtaining high powder recovery from sticky solutions. Addition of Tween80 caused the powder recovery to drop down further to almost zero, due to the phenomena called "Orogenic Displacement model" where the non-ionic surfactant dislodges the protein from the droplets surface.

Application of surfactants for spray drying of bioactive compounds still remains worth of investigation because they could mainly modify/improve the solubility and release, but more studies are required to optimize the dosage and formulations.

5. Conclusions

In relation to the two main aims of this research, the following conclusions can be summarized.

- ***Phenolic antioxidant extracts from waste grape skins by conventional solvent extraction***

The phenolic profile of the extract is influenced by the grape variety but not by the year which, anyway, can influence the phenolic yields. Wine-making process can obviously influence the antioxidants recovery, since unfermented skins are richer than fermented ones. However, variety influence can be higher than process influence for some specific compounds. For example, extracts from fermented Barbera waste skins contained more anthocyanins than extracts from unfermented Pinot noir skins. Fermentation reduces, of course, the amount of residual sugars which, depending on the target application of the isolated phenolic compounds, might need to be removed in a downstream purification process.

Regarding the antioxidant capacity of the obtained extracts, results were different depending on the adopted evaluation assay. For the same grape variety, the results were influenced by the year only in case of Barbera, but it is difficult to state if this effect was actually due to the year or to some slight modifications of the fermentation process in the winery. In general, from the point of view of large-scale production of grape skin extracts, screening of antioxidant capacity should be performed in order to prepare mixtures of raw materials able to provide final extract with standardized functionality, rather than producing extract from single variety and mixing in a second step the obtained extracts.

- ***Surfactant application for process intensification***

Different strategies based on the use of a food-grade surfactant (Tween 20), with different aims, were applied. In general, obtained results were not the expected and hoped ones, althought some interesting results were anyway obtained.

The use of Tw20 as extraction enhancer did not allow to reduce the amount of ethanol in a conventional solvent extraction without also reducing the total phenols yield. However, results suggest that solvent composition and extraction process could be optimized in order to exploit the surfactant different affinity for

different phenolic classes (higher affinity for anthocyanins) to achieve a selective extraction.

When the surfactant was used in the form of colloidal gas aphrons, its direct application to the ethanol extract – exhausted skins mixture did not allow to collect all of the phenolic compounds in the final separated aphron phase, or to selectively separate anthocyanins from other phenolic compounds. However, compared to the efficiency and costs of other separation and purification technologies, the CGAs approach could be further investigated and optimized to improve its efficiency.

Finally, when Tw20 was used, together with maltodextrins, as encapsulating material for the spray-drying of Barbera extracts, it first caused the formation of a solid precipitate (the composition of which should be better investigated to understand if this was due to a selective interaction of surfactant-phenols) and, after, it reduced the powder and phenols yields since it increased powder stickiness. Since water solubility of the extract was definitely enhanced by the surfactant, further optimization of the process and of the wall material formulation appear worth of further investigation.

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