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RECOVERY OF PHYTOCHEMICAL RICH FRACTIONS FROM WINEMAKING BYPRODUCTS FOR NOVEL FOOD APPLICATIONS

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ABSTRACT

The food industry is facing the significant challenge of developing new ingredients and foods addressing prevention of the major chronic diseases, while optimizing processing technologies in order to achieve a sustainable use of natural resources. Grape pomace, the byproduct of winemaking, is available on a large scale and is comprised of various compounds associated with potential health benefits. In this context, the overall PhD project aimed at developing an integrated strategy for recovery and reuse of value added fractions of winemaking byproducts in the food system.
Grape pomace samples, differing for the varieties and winemaking conditions were collected from winemaking processes and evaluated for valuable components, i.e, dietary fibre, total phenolics, soluble and insoluble proanthocyanidins (*n*-butanol/HCl assay), individual phenolic compouns (UPLC-DAD-MS), tocopherol and tocotrienol contents (HPLC with fluorimetric detection). This led to the design of a comprehensive recovery plan, bringing about the overall value of winemaking byproducts.

The aim of targeting a specific health functionality by grape phenolics was tackled in chapter 1, focused on their protective effects on hyperglyacemia induced damage. In the current scenario, prevention of diabetes complications is of utmost importance due to an upsurge in the prevalence of this disease among the world population (with an estimation of 300 million cases that could be registered by 2030). *In vivo* study has shown that phenolics can prevent the leading cause of hyperglycemia damage, i.e., non-enzymatic protein glycaton, most likely via radical scavenging, metal chelation and carbonyl trapping. However to target this effect, there is a need for phenolic fractions with high concentration and efficiency. An *in vitro* model system using fructose as glycating agent and bovine serum albumin as target was set up. The protective ability of grape skin phenolics was evaluated with both a novel methodological approach by 2D-SDS/IEF-PAGE and quantification of the formation of advanced glycation end-products by fluorescence measurements. Structural modifications of BSA upon glycation, as evidenced by changes in pI and M*r* were found to be inhibited by the grape skin extracts. The antiglycation activity ranking was: quercetin-3-O-glucoside > malvidin-3-O-glucoside > catechin > procyanidin A2 >> aminoguanidine (reference drug). Despite variability in phenolic content (in the range 4.6-53.6 g gallic acid equivalents (GAE)/kg) and composition among grape skin extracts, upon equalization of the phenolic content, similar antiglycation properties were observed. Grape skins (I_{50} in the range 9.2-20 µg GAE/mL) were more efficient than the commercial nutraceutical products Leucoselect® (I_{50} 38 µg GAE/mL) and Pycnogenol® (I_{50} 34 µg GAE/mL) and hence could provide cost-effective antiglycation agents, to be used either as food ingredients or as nutraceutical preparations.

The task of designing new foods by the incorporation of grape skins, which can deliver both dietary fibers and phenolics, was examined in chapter 2. Grape skins have been proposed as ingredients for "solid" or "gel-like" foods, but knowledge on their possible application in complex food fluids that could represent low caloric vehicle for value added ingredients is lacking. Hence, a tomato puree added with grape skins was studied as a model application and a production process was designed to increase potential health benefit of this product with maximum consumers' liking.
To optimize grape skin ingredient incorporation in tomato puree, six formulations (3% dietary fibre and ~ 0.7% proanthocyanidins) varying in the particle size (assessed by a laser granulometer) were considered in parallel. Stabilization was performed by either an intensive autoclave treatment or an optimized microwave treatment achieving six decimal reductions of the target microorganism (*Alicylobacillus acidoterrestris*). Upon processing, major changes in the fortified purees were both decrease in proanthocyanidin solubility (by ~ 40%, probably due to interaction with tomato protein) and decrease in flavonol glycosides with parallel increase of their aglycones. However, phenolic contents, reducing capacity and antiglycation activity remained ~ 3 times higher in all the fortified purees than in the controls. These chemical parameters were related to particle size distribution, since with decreasing surface-weighed mean diameter, d(3,2), proanthocyanidin solubility increased. The

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addition of grape skins played a major role on the color (measured by Hunter colorimeter), storage (G') and loss (G'') moduli, complex viscosity (studied by means of dynamic oscillatory measurements) and Bostwick consistency of the formulations. A sensory test based on consumers' preference disclosed that the formulation having the maximum appreciation was that incorporated with the smallest particle sizes. Liking ratings were found to be inversely correlated to the volume-weighted mean diameter, d(4,3) values. The overall results obtained from this methodological approach represent a basis for the optimization of fibrous byproducts incorporation into complex food fluids.

Besides use of grape skins as source of antiglycation and fortifying agents, utilization of grape seeds which forms a major part of the pomace is of importance in the recovery strategy. Chapter 3 focused on assessment of supercritical-CO_2 (SC-CO_2) extraction for the recovery of oil, as a "green technology" alternative to *n*-hexane extraction and mechanical extraction. Extraction by SC-CO_2 can be a sustainable technology, if the process is carried out at optimum operating conditions ensuring high yield and in a sufficient extractor volume. The breakeven point, which makes the process economically sustainable, is also linked to the quality of the oil obtained. Besides oil, the defatted seeds rich in phenolics were also considered as a relevant co-product of this process. Hence, grape seed phenolics were studied as inhibitors of starch digestion enzymes, which is the first relevant step to control blood glucose level. Extraction by SC-CO_2 provided the same oil yield as that of *n*-hexane extraction (10.1-16.6 $g_{oil}/100g_{seeds}$). ^{1}H-NMR spectra showed that diacylglycerols and oxidized lipids were present only in trace amounts. The levels of tocols were in the range: 355-559 mg/kg higher than those of the oils obtained by *n*-hexane extraction and similar to those of oils obtained by mechanical extraction. Tocotrienols, which possess high antioxidant and antiproliferative properties were the prominent compounds present. In the defatted grape seeds, levels of phenolics one order of magnitude higher than those of the grape skins were observed (49-277 g GAE/kg). The anthocyanin rich grape seed extracts showed the highest inhibitory effectiveness towards α-glucosidase (I_{50} 47 µg GAE/mL ~ half than that of the drug acarbose). Inhibitory effectiveness towards α-amylase activity was similar among grape varieties, with I_{50} values comparable to that of acarbose and correlated to proanthocyanidin contents.

The overall results could pave the way for recovery and value-addition of winemaking byproducts for food and nutraceutical uses, including grape skins as a source of antiglycation agents or as dietary antioxidant fibre, grape seeds as a source of tocol rich oil, defatted grape seeds as a source of phenolics.

RIASSUNTO

L'industria alimentare sta fronteggiando le significative sfide di sviluppare nuovi ingredienti e alimenti progettati per prevenire le principali malattie croniche e, nello stesso tempo, ottimizzare i processi e utilizzare le risorse in una prospettiva di sostenibilità. Le vinacce, sottoprodotto della vinificazione, sono disponibili in grandi quantità e comprendono diversi composti associati a potenziali effetti benefici. In questo contesto, il progetto di tesi ha avuto l'obiettivo di progettare una strategia integrata per il recupero di frazioni ad alto valore aggiunto dai residui di vinificazione e il loro riutilizzo nel sistema alimentare.
I campioni di vinacce, diversi per varietà e condizioni di vinificazione, sono stati recuperati da processi di vinificazione e analizzati per determinare il contenuto di composti ad alto valore aggiunto, quali la fibra dietetica, i fenoli totali, le proantocianidine solubili e insolubili (idrolisi con *n*-butanolo/HCl), singoli composti fenolici (UPLC-DAD-MS), tocoferoli e tocotrienoli (HPLC con detector fluorimetrico). Questa caratterizzazione ha portato a progettare un completo piano di recupero per ottimizzare il potenziale valore di questo residuo di vinificazione.

L'obiettivo di focalizzare una specifica funzionalità dei polifenoli dell'uva, benefica per la salute, è stato studiato nel capitolo 1, riguardante la capacità di prevenire i danni dovuti all'iperglicemia. Nell'attuale scenario, la prevenzione delle complicanze del diabete è di importanza fondamentale a causa di una allarmante diffusione di questa malattia nell'intera popolazione mondiale (con una stima di 300 milioni di casi che potrebbero essere registrati entro il 2030). Uno studio *in vivo* ha mostrato che i fenoli possono prevenire la causa primaria dei danni dovuti all'iperglicemia, cioè la glicazione proteica per via non enzimatica, mediante le loro proprietà di scavenging di radicali, chelazione di metalli e disattivazione di carbonili. Tuttavia, per raggiungere questo effetto, sono necessarie frazioni fenoliche concentrate ed efficienti. E' stato quindi messo a punto un modello di studio *in vitro* che utilizza fruttosio come agente glicante e albumina di siero bovino (BSA) come proteina target. L'attività protettiva dei fenoli della buccia d'uva è stata valutata con un nuovo approccio metodologico mediante 2D-SDS/IEF-PAGE e quantificata mediante la misura della fluorescenza dei prodotti di glicazione avanzata. I danni strutturali a carico della BSA in seguito alla glicazione, come evidenziato da modificazione di pI e M*r*, sono stati inibiti dagli estratti di bucce d'uva. La scala di efficacia per l'attività antiglicante è risultata: quercitina-3-O-glucoside > malvidina-3-O glucoside > catechina > procianidina A2 >> aminoguanidina (farmaco di riferimento). Nonostante la variabilità nel contentuto fenolico (nell'intervallo 4.6-53.6 g di acido gallico equivalenti (GAE)/kg) e nel profilo fenolico degli estratti di bucce d'uva, le proprietà antiglicanti degli estratti normalizzati per il contenuto fenolico sono risultati simili. Gli estratti di buccia (I_{50} nell'intervallo 9.2-20 µg GAE/mL) sono risultati più efficienti che i prodotti nutraceutici commerciali Leucoselect® (I_{50} 38 µg GAE/mL) e Pycnogenol® (I_{50} 38 µg GAE/mL) e pertanto possono fornire composti antiglicanti da utilizzare sia come prodotti nutraceutici che come ingredienti alimentari.

L'obiettivo di formulare nuovi alimenti con bucce d'uva, valorizzando sia il contenuto di fibra dietetica che di antiossidanti, è stato sviluppato nel capitolo 2. Le bucce d'uva sono state precedentemente proposte come ingredienti per alimenti solidi o gelificati, ma non c'è informazione su come applicarle in un fluido complesso, che potrebbe rappresentare un veicolo a basso valore calorico per i composti ad alto valore aggiunto dell'uva. In questo contesto, è stata studiata una purea di pomodoro formulata con bucce d'uva come applicazione modello ed è stato progettato il processo produttivo per aumentare le potenzialità benefiche di questo prodotto con il massimo gradimento da parte del consumatore. Per ottimizzare l'aggiunta delle bucce d'uva nella purea di pomodoro sono state considerate in parallelo sei formulazioni (con il 3% di fibra dietetica e lo 0.7% di proantocianidine), diverse per la distribuzione granulometrica (valutata mediante un granulometro laser). La stabilizzazione è stata ottenuta mediante un trattamento intensivo in autoclave o un trattamento ottimizzato con le microonde per ottenere sei riduzioni decimali di un microorganismo target

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(*Alicyclobacillus acidoterrestris*). Le principali modificazioni indotte dal processo sono state sia la diminuzione sia della solubilità delle proantocianidine (fino al 40%, probabilmente a causa dell'interazione con le proteine) che del contenuto di glicosidi flavonolici, con parallelo aumento dei corrispondenti agliconi. Tuttavia il contenuto fenolico, la capacità riducente e l'attività antiglicante sono rimaste ~ 3 volte superiori nei prodotti fortificati rispetto che nel controllo. Questi parametri chimici sono risultati in relazione con la distribuzione granulometrica; infatti la solubilità dei fenoli è aumentata con la diminuzione del valore medio del parametro d(3,2). L'aggiunta delle bucce d'uva ha avuto un effetto notevole sul colore (misurato con colorimetro di Hunter), sui moduli elastico (G') e viscoso (G''), sulla viscosità complessa (studiati con analisi di reologia fondamentale) e sulla consistenza di Bostwick delle formulazioni. Un test sensoriale di gradimento ha rilevato che la formulazione avente il massimo gradimento era quella con la minor granulometria. I punteggi di gradimento sono risultati inversamente correlati al valore medio del parametro d(4,3). Nell'insieme i risultati ottenuti da questo approccio metodologico potrebbero aprire la strada all'ottimizzazione dell'incorporazione di sottoprodotti fibrosi in fluidi complessi.

Oltre all'uso delle bucce d'uva come fonti di antiglicanti e agenti fortificanti, nella strategia di recupero è di importanza l'utilizzo si semi, che costituiscono una percentuale significativa delle vinacce. Il capitolo 3 è focalizzato sull'estrazione con CO_2 supercritica (SC-CO_2) per il recupero dell'olio, come "tecnologia verde", in alternativa alle estrazioni con *n*-esano o meccanica. L'estrazione con SC-CO_2 può essere una tecnologia sostenibile se il processo è condotto in condizioni operative ottimali, assicurando un'elevata resa e con un estrattore di adeguata capacità. Il punto cruciale, che rende il processo economicamente sostenibile, è anche legato alla qualità dell'olio ottenuto. Inoltre, oltre all'olio, i semi sgrassati ricchi in fenoli sono stati considerati come un co-prodotto rilevante di questo processo. Infatti i fenoli dei semi sono stati studiati per la loro capacità di inibitozione di enzimi che degradano l'amido, che rappresenta il primo stadio per controllare il livello di glucosio nel sangue. L'estrazione con SC-CO_2 ha mostrato la stessa resa che l'estrazione con *n*-esano (10.1-16.6 g oilo/100 g di semi). Gli spettri ^{1}H-NMR hanno mostrato che i diacilgliceroli e i lipidi ossidati erano presenti solo in tracce. I livelli di tocoli erano nel range 355-559 mg/kg, maggiori dei livelli di tocoli negli oli ottenuti mediante estrazione con *n*-esano e simili a quelli degli olio ottenuti con estrazione meccanica. I tocotrienoli, che possiedono attività antiossidante e antiproliferativa, sono risultati i composti principali presenti. Nei semi sgrassati è stato osservato un livello di fenoli di un ordine di grandezza superiore rispetto a quello delle bucce (49-277 g GAE/kg). Gli estratti di semi ricchi in antocianine hanno mostrato la maggior efficacia di inibizione nei confronti dell'α-glucosidasi (I_{50} 47 µg GAE/mL ~ metà di quella dell'acarbosio). L'efficacia di inibizione nei confronti dell'α-amilasi si è rivelata simile tra le varietà di uva, con valori di I_{50} simili a quelli dell'acarbosio e correlati ai contenuti di procianidine.

Nell'insieme i risultati possono fornire indicazioni per il recupero e la valorizzazione dei residui di vinificazione per usi alimentari e nutraceutici, includendo le bucce come fonti di agenti antiglicanti o come fibra dietetica antiossidante, i semi come fonti di olio ricco in tocoli e i semi sgrassati come fonti di fenoli.

PREFACE

The global food industry is facing an uphill task with respect to utilization of byproducts generated from large-scale food processing systems (Russ et al., 2004). The winemaking industry generates a substantial amount of grape pomace, which comprises of valuable fractions having food applications, i.e., grape seed oil, grape skins and defatted grape seeds. The recovery of grape pomace would at the same time reduce the environmental impact imparted by waste and provide an extra income for wine makers. In fact, grape pomace can be considered more as a resource than as waste as demonstrated by numerous researches for its recovery, such as: a) Composting of grape pomace for its use as soil conditioners; b) Removing heavy metals from industrial effluents by using a grape pomace sludge as an adsorbent; c) Production of pullulan by using grape pomace *via* fermentation with *Aerobasidium pullulans*; d) Production of xylanase and pectinase by *Aspergillus awaori via* fermentation of grape pomace (Botella et al., 2007); e) Extraction of natural antioxidants (Spigno et al., 2007) and production of fibers (Valiente et al., 1995), which exhibit potential health benefits.

One of the promising applications for the food sector is the recovery of phenolic and fiber-rich fractions from these resources, which could be used as bioactive food ingredients (Saura-Calixto, 2011). In fact, the "French paradox" initiated numerous studies that focused on the antioxidant and health promoting effects of plant secondary metabolites and revealed the inhibition of human low-density lipoprotein oxidation by grape and wine phenolics (Teissedre et al., 1996). Because of the close relationship between antioxidants and dietary fibre in grape pomace and their common fate in the gut, it has been proposed that these food components have a joint role in prevention of human diseases (Perez-Jimenez et al., 2008). *In vivo* studies on human adults have demonstrated that grape pomace has a positive effect in the prevention of cardiovascular diseases (Perez-Jimenez et al., 2008). Further studies have demonstrated the ability of the grape-derived extracts exerting protective effects against chronic diseases such as cancer, Alzheimer's disease, atherosclerosis and diabetes (Vislocky et al., 2010). An anti-diabetes effect was demonstrated when grape-derived extracts with high amounts of proanthocyanidins were supplemented into the diet of high fructose fed rats (Dandona et al., 2005) and also when grape polyphenols were administered in type-2 diabetes patients (Hokayem et al., 2013). The outcome of these studies supported the ability of grape phenolics in improving insulin resistance and suppressing oxidative stress.

In this framework, the aim of the thesis was thus focused towards the development of a utilization strategy for winemaking byproducts based on phytochemical recovery. In particular, due to changing lifestyle and nutritional habits, there is an upsurge in the prevalence of diabetes with an estimation of 300 million cases that could be registered by 2030 among the world population (Anhe et al., 2013). Hence, prevention of hyperglycemia induced damage is of utmost importance in the current scenario. While some phenolic sources have been proposed along with drugs for prevention of hyperglycemia damage, the identification of a cost-effective source is still a challenge. This led to the design of chapter 1 dealing with inhibitory effects of grape skin phenolics against structural damage induced by glycation on target protein giving further impetus and knowledge towards prevention of hyperglycemia damage leading to diabetes complications.

Studies addressing the health-related properties of phenolics associated with dietary fibers have shown a positive effect on the prevention of cardiovascular diseases and cancer (Saura-Calixto, 2011). A large portion of non bioaccessible antioxidants associated with the dietary fibers are known to have essential physiological functions in the human body. Thus grape-derived fractions can have numerous benefits when used as food ingredients. These multiple effects formed the rationale behind taking up chapter 2, where innovative fruit-based product wereconsidered as a "vehicle" for the delivery of phytochemical rich fractions recovered from winemaking byproducts. This study could lead to development of new foods with improved functional properties. Technological challenges arising from the fortification with

fibrous fractions i.e., the choice of the model food and the level of incorporation; the particle size of the fibrous fraction to be used; the verification of the processing effect on phenolic content and the assessment of consumers' liking were addressed.

One of the major components of grape pomace present in considerable amount is seeds, which are a source of high value oil rich in unsaturated fatty acids and tocols. Tocols possess vitamin E activity and numerous health promoting functions, i.e., antioxidant, anti-inflammatory, anti-thrombotic and anti-proliferative effects and protection against damage caused by various pollutants (Constantinides et al., 2006). Hence recovery of grape seed oil could be of value, while the traditional recovery process is *n*-hexane extraction, mechanical pressing has a limited application due to its low oil yield. This formed the basis for chapter 3 to assess supercritical CO_2 extraction of grape seed oil built within the principles of green technology, to attain maximum oil yield and attain high tocol levels. Grape seed phenolics were known to be utilized as sources of additives in foods and possess many health beneficial effects (Jayaprakasha et al., 2003; Perumalla et al., 2011). Hence, the left over grape seed residues rich in phenolics were studied for their inhibitory effects against starch digestive enzymes, which is one of the critical steps in controlling blood glucose levels under diabetic conditions.

Main scientific goals of the thesis aimed towards the development of an integrated recovery strategy from winemaking byproducts for novel food applications were:

1. Targeting hyperglycemia induced damage on protein through grape skin phenolics
2. Design of a fruit-based food formulated with grape skin antioxidant dietary fibre and process optimization.
3. Assessment of supercritical-CO_2 oil extraction from grape seeds by evaluation of tocol content and potential uses of the defatted co-product.

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1. TARGETING HYPERGLYCEMIA INDUCED DAMAGE ON PROTEIN THROUGH GRAPE SKIN PHENOLICS

1. Targeting hyperglycemia induced damage by grape skin phenolics

1.1. INTRODUCTION

The process of non-enzymatic protein glycation plays a major role in a number of biochemical abnormalities associated with progression of hyperglycemia complications and age-related pathologies, which have become a challenge due to increase of diabetic elderly people (Saraswat et al., 2009). The pattern of protein glycation *in vivo* is complex. This process is initiated by the reaction of glucose or fructose with amino groups of proteins to form Schiff bases, which then undergo an Amadori rearrangement. These Amadori products formed degrade further to form reactive α-dicarbonyls such as methylglyoxal, glyoxal and 3-deoxyglucosone. These harmful compounds can also be formed through other routes such as polyol pathway and lipid peroxidation. The covalent cross-linking between proteins and α-dicarbonyl compounds leads to formation of heterogenous group of protein-bound moieties known as advanced glycation endproducts (AGEs) (Figure 1). AGEs are present in the target proteins with large amounts of lysine residues and long half-lives such as collagen, serum albumin, elastin, myelin, low density lipoprotein, plasminogen activator, fibrinogen etc. These glycated proteins lose their functionality with an increased resistance to removal by proteolytic enzymes. In addition, AGE receptors are expressed on many cell types, and their interactions can trigger intracellular signalling and promote oxidative stress, thus causing tissue damage ultimately leading to diabetes complications. Intracellular AGE formation can also lead to quenching of nitric oxide and impaired function of growth factors (Engelen et al., 2013; Singh et al., 2001).

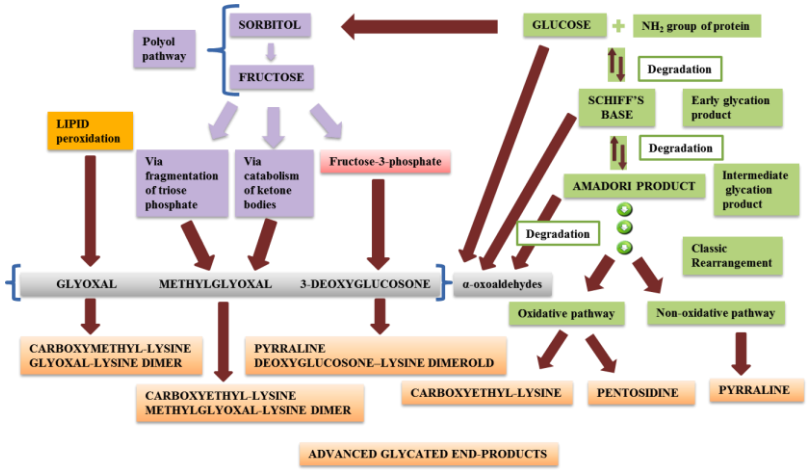


Figure 1.1. Schematic representation of non-enzymatic protein glycation resulting in formation of AGEs through polyol pathway, lipid peroxidation and degradation of Amadori products. Details of these degradation patterns have been reported elsewhere (Singh et al., 2001).

There are a range of synthetic AGE inhibitors discovered so far, but most of them were withdrawn after the clinical trials due to poor pharmacokinetics and safety concerns (Kawanishi et al., 2003; Manzanaro et al., 2006). Hence pharmacologically active principles derived from natural sources has raised interest and phenolics, especially flavonoids, which were known to act as radical scavengers, metal chelators and carbonyl trapping agents were found to exert antiglycation activity (Matsuda et al., 2003). Many phenolic-rich spices and herbs were regarded as natural sources of antiglycation agents, and could be used either as food ingredients or as nutraceutical preparations (Saraswat et al., 2009). The structural requirements of flavonoids for maximum antiglycation and antioxidant activity are found to be in part the similar (Matsuda et al., 2003).

The anti-diabetic efficiency of grape polyphenols was tested in type-2 diabetic patients, resulting in improved insulin resistance and suppressed glycooxidative stress, i.e., formation of thiobarbituric acid–reactive substances and protein carbonylation (Hokayem et al., 2013). This study supported the *in vitro* phenolic properties demonstrating that the *in vivo* targets are reached and protected. In this context,

grape skins derived from winemaking byproducts could be valuable sources for the production of grape phenolics. Red grape skins recovered from winemaking could be advantageous due to the presence of major polyphenol compounds i.e., anthocyanins possessing high antioxidant properties. Even though white grape skins lack anthocyanins, they could have additional advantages with respect to previously considered antiglycation agents, since they possess higher stability (Rohn et al., 2007) and thus being suitable for incorporation into various food systems. A critical point for phenolic recovery from winemaking byproducts is that the composition of grape pomace differs widely in the phenolic content depending on grape variety, vineyard location and winemaking procedures, while the food industry requires standardized ingredients to be incorporated into specifically designed foods for consumers. Another point to be considered is that the antiglycation efficacy should be compared to that of a reference drug i.e., a synthetic inhibitor such as aminoguanidine. The current study focused on screening of grape skins recovered from winemaking byproducts to assess if they can be proposed as natural sources of antiglycation agents. In this context, the aims were designed to: 1) characterize the phenolic content of grape skins by UPLC-DAD-MS and evaluate *in vitro* reducing capacity; 2) acquire knowledge on the inhibitory effect of phenolics against structural damage induced by glycation on the target protein; 3) assess the antiglycation efficiency of the extracts with respect to both catechin and aminoguanidine as reference compounds and define how to overcome the differences in raw material quality.

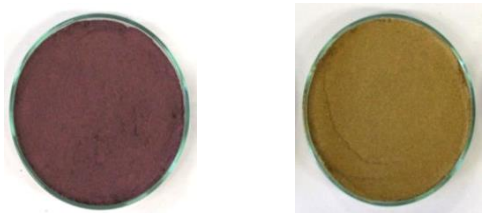
1.2. MATERIALS AND METHODS

1.2.1. Chemicals

The 3-O-glucosides of malvidin, cyanidin, delphinidin, peonidin, petunidin, quercetin, kaempferol; 3-O-glucoronides of quercetin and kaempferol; quercetin-3-rhamnoside; kaempferol-3-O-galactoside and aglycones of quercetin and kaempferol were purchased from Polyphenols (Sandes, Norway). Pycnogenol® (proanthocyanidins from pine bark) was obtained from Horphag (Geneva, Switzerland). Procyanidin A2 and Leucoselect® (proanthocyanidins from grape seeds) were obtained from Indena (Settala, MI, Italy). All other standards and chemicals were purchased from Sigma-Aldrich (Milan, Italy).

1.2.2. Grape pomace

Nineteen batches of grape pomace were recovered from different winemaking processes at wineries located in Northern Italy. The collection plan involved ten red grape varieties, namely: Barbera (BA), Croatina (CR), Freisa (FR), Dolcetto (DO 1 and DO 2, processed by two different wineries), Grignolino (GR), Neretto (NR), Nebbiolo (NE), Pinot Nero (PI 1 and PI 2, processed by two different wineries) and nine white grape varieties, namely: Chardonnay (CH 1 and CH 2 processed by different wineries), Moscato (MO), Muller Thurgau (MT), Nascetta (NA), Erbaluce (ER), Arneis (AR), Riesling (RI 1 and RI 2 processed by different wineries). At the winery, the pomace samples were sieved with a 5 mm sieve to separate the skins from the seeds and were frozen to inhibit microbial growth. Then, the samples were transported frozen to the lab, dried at 50 ^{o}C for about 8 h, milled and sieved to obtain fractions having particle sizes in the range of 125-250 µm (Figure 1.2). The samples were stored under vacuum, in the dark, at 4 ^{o}C, until performing characterization studies.



Barbera skin powder **Chardonnay skin powder**

Figure 1.2. Grape skin powder samples obtained from winemaking byproducts.

1.2.3. Moisture content

Moisture content of the grape skin powders was determined in triplicate by drying in a vacuum oven at 70 °C and 50 Torr for 18 h (AOAC, 1990).

1.2.4. Grape skin extract (GSE)

1.2.4.1. Red GSE

For anthocyanin extraction, ~ 100 mg of red grape skin powder was added with 8 mL methanol:water:HCl (80:20:0.1, v/v/v) and continuously stirred for 2 h at room temperature. The mixture was centrifuged at 10,000 *g* for 10 min, the supernatant recovered and the solid residue was re-extracted using 6 mL of the same solvent twice. The three supernatants were pooled, dried under vacuum at 35 °C and the residue was suspended in 10 mL of methanol:water:HCl (20:80:0.1, v/v/v). The extracts were centrifuged at 2,000 *g* for 1 min before analytical characterisation. For flavonols extraction, ~ 2 g of red grape skin powder was extracted four times with methanol:water:HCOOH (80:20:0.1, v/v/v) and the mixture was treated as described above. The final residue was suspended in 30 mL of the diluent. Extractions were performed in duplicate.

1.2.4.2. White GSE

For flavonol extraction, ~ 2 g of white grape skin powder was added with methanol:water:HCOOH (80:20:0.1, v/v/v) and stirred continuously for 2 h at room temperature. The mixture was centrifuged at 10,000 *g* for 10 min, the supernatant recovered and the solid residue was re-extracted using 6 mL of the same solvent twice. The supernatants were pooled and the extracts were centrifuged at 2,000 *g* for 1 min before analytical characterisation. Extractions were performed in duplicate.

1.2.5. Chromatographic analysis

1.2.5.1. Anthocyanins in red GSE

Chromatographic analysis was performed with an UPLC mod. Acquity (Waters, Milford, MA, USA) equipped with a mod. E-lambda photodiode array detector (Waters) and a triple quadrupole mass spectrometer mod. Quattro micro (Waters), operated by Masslynx 4.0 software (Micromass) with Quan-Optimize option for fragmentation study. A 2.6 µm Kinetex C_{18} column (250 x 4.6 mm; Phenomenex, Castel Maggiore, Italy) was used for the separation at a flow-rate of 1.8 mL/min. The sample and the column were maintained at 40 and 20 °C, respectively. The separation was performed by means of a linear gradient elution. Eluents were: (A) 0.1% trifluoroacetic acid; (B) acetonitrile:trifluoroacetic acid 0.1% (30:70, v/v). The gradient was as follows: 14% B for 15 min, 14-20% B in 10 min, 20-32% B in 10 min, 32-50% B in 10 min; 50-90% B in 3 min; 90% B for 3 min. Data acquisition was performed as described previously (Del Bo et al., 2010). Briefly, DAD analysis was carried out in the range of 200-700 nm with peak integration at 520 nm. Mass spectrometer was operated in positive full-scan mode in the range 200-800 *m/z*. The capillary voltage was set to 3.5 kV, the cone voltage was 20 V, the source temperature was 130 °C, the desolvating temperature was 250

°C and nitrogen was used as carrier gas. The calibration curve was obtained with malvidin-3-O-glucoside and results were expressed as milligrams of malvidin-3-O-glucoside equivalents per kilogram of dry product.

1.2.5.2. Flavonols in red GSE

Analysis was performed with the equipment as described before. A 1.7 µm BEH C_{18} column (150 x 2.1 mm; Waters) was used for the separation at a flow-rate of 0.55 mL/min. The column was maintained at 55 °C and the separation was performed by means of a linear gradient elution. Eluents were: (A) 0.1% formic acid; (B) 0.1% formic acid in acetonitrile. The gradient was as follows: 5-20% B in 9 min, 20-35% B in 3 min, and then 80% B for 3 min. Chromatographic data were acquired from 200 to 450 nm and integrated at 354 nm. Mass spectrometer operated in negative full-scan mode in the range 100-1000 m/z. The capillary voltage was set to 3 kV, the cone voltage was specific for each compound, the source temperature was 130 °C, the desolvating temperature was 300 °C and nitrogen was used as carrier gas. The calibration curve was obtained with a quercetin-3-O-glucoside solution and results were expressed as milligrams of quercetin-3-O-glucoside equivalents per kilogram of dry product.

1.2.5.3. Flavonols in white GSE

Chromatographic analysis was performed with an HPLC DAD model 2996 (Waters) equipped with a model 600 pump (Waters), operated by Empower software (Waters, Vimdrone, Italy). A 2.6 µm Kinetex C_{18} column (150 x 4.6 mm; Phenomenex, Castel Maggiore, Italy) was used for separation at a flow rate of 1.8 mL/min. The column was maintained at 60 °C and the injection volume was 50 µL. The separation was performed by means of a linear gradient elution. Eluents were: (A): 0.1% formic acid; (B): acetonitrile. The gradient was as follows: from 5% to 20% B in 12 min, 20% to 30% B in 1 min, 30% to 90% B in 5 min; 90% B for 7 min; 5% B in 1 min; 5% B for 5 min. Chromatographic data were acquired from 200 to 450 nm and integrated at 354 nm. Standard compounds, namely quercetin-3-O-glucuronide, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, kaempferol-3-O-galactoside, kaempferol-3-O-glucuronide, kaempferol-3-O-glucoside, quercetin and kaempferol were used as reference compounds. The calibration curve was obtained with quercetin-3-O-glucoside solution and results were expressed as milligrams of quercetin-3-O-glucoside equivalents per kilogram of dry product.

1.2.6. Total phenolics

The Folin-Ciocalteu assay was performed on the GSE and on the reference nutraceutical products, namely Pycnogenol® and Leucoselect®, according to a procedure described by Singleton et al. (1990). The reaction mixture contained 6.0 mL of distilled water, 0.5 mL of the extracts diluted with methanol:water:HCl (80:20:0.1, v/v/v) for red GSE and 0.5 mL of the extracts diluted with methanol:water (80:20, v/v) for white GSE or 0.5 mL of the reference products dissolved in methanol, 0.5 mL of Folin-Ciocalteu reagent and 3 mL of 10% Na_2CO_3. The mixtures were incubated for 90 min at room temperature and then the absorbance was recorded at 760 nm against a blank with no extract addition by an UVdec-610 spectrophotometer (Jasco, Lecco, Italy). For each extract, 2-4 dilutions were assessed in triplicate. A calibration curve was built using gallic acid as reference compound. Total phenolics were expressed as grams of gallic acid equivalents (GAE) per kilogram of dry product.

1.2.7. Proanthocyanidins

Proanthocyanidins were determined according to the method of Porter et al. (1986). Briefly, 1 mL of the diluted extract was added to 6 mL of *n*-butanol:HCl (95:5, v/v) and 0.2 mL of 2% $NH_4Fe(SO_4)_2.12 H_2O$ in 2 M HCl. Hydrolysis was carried out at 95 °C for 40 min. The reaction mixtures were cooled and the absorbance was recorded at 550 nm against a blank made as for the sample but incubated at room temperature. For each extract, 2-4 dilutions in respective diluents were assessed in triplicate. Proanthocyanidin amount was determined by using 0.1736 (mg/mL) as conversion factor (Travaglia et al., 2011). The results were expressed as grams per kilogram of dry product.

1.2.8. Ferric ion reducing antioxidant power (FRAP)

The FRAP assay was performed according to a procedure described by Benzie et al. (1996). Briefly, FRAP reagent was prepared by adding 25 mL of 300 mM acetate buffer, pH 3.6; 2.5 mL of 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3$. The reaction mixture contained 0.4 mL of extracts diluted with methanol:water:HCl (80:20:0.1, v/v/v) for red GSE; methanol:water (80:20, v/v) for white GSE and 3 mL of FRAP reagent. The increase in absorbance at 593 nm was evaluated after 4 min of incubation at 37 ^{o}C against a blank with no extract addition. For each extract, 2-4 dilutions were assessed in triplicate. A methanolic solution of $FeSO_4.7H_2O$ was used for calibration. Results were expressed as millimoles of Fe(II) sulfate equivalents per kilogram of dry product.

1.2.9. *In vitro* antiglycation activity using bovine serum albumin (BSA)/fructose model system

A BSA-monosaccharide *in vitro* model system was chosen to study the efficacy of the following antiglycation agents: diluted red GSE (3-4 dilutions with methanol:water:HCl, 80:20:0.1, v/v/v), diluted white GSE (3-4 dilutions with methanol:water, 80:20, v/v) and methanolic solutions of Leucoselect®, Pycnogenol®, catechin, aminoguanidine, malvidin-3-O-glucoside, quercetin-3-O-glucoside and procyanidin A2 (5-10 dilutions in triplicate).

The BSA/fructose model system was performed according to the procedure as described by McPherson et al. (1988) with modifications. Briefly, the reaction mixture contained 0.9 mL of 200 mM phosphate buffer, pH 7.4 added with 0.02% sodium azide, 0.3 mL of 50 mg/mL BSA in phosphate buffer, 0.3 mL of 1.25 M fructose in phosphate buffer and 0.1 mL of each antiglycation agent. A control mixture with 0.1 mL of methanol instead of the antiglycation agent and a blank mixture with 0.3 mL of phosphate buffer instead of fructose solution and 0.1 mL of methanol instead of the antiglycation agent were run in parallel. The mixtures were incubated at 37 ^{o}C for 3 days in the dark. After incubation, 1.6 mL of 20% trichloroacetic acid was added to the reaction mixture before centrifugation at 10,000 *g* for 10 min. The supernatant was discarded and the precipitate was re-dissolved in 1.6 mL of phosphate buffer for fluorescence measurement (Wang et al., 2011) on a LS55 Luminescence Spectrometer (Perkin-Elmer, Monza, Italy) with an excitation/emission wavelength pair of 350/420 nm and 5 nm slit width, read against phosphate buffer. Percentage of inhibition was calculated by the following equation:

$$\%\, Inhibition = 100 - 100\, x\, \frac{(FL_S - FL_b)}{(FL_c - FL_b)} \qquad (1.1)$$

Where:

FL_s = fluorescence intensity of the mixture with the antiglycation agent,

FL_b = fluorescence intensity of the blank mixture,

FL_c = fluorescence intensity of the control mixture.

Data points of concentration versus % inhibition were plotted and an estimate of inhibition of protein glycation by 50% (I_{50}) was selected, since this level of inhibition occurred within the linear range of all antiglycation agents and standards evaluated. Antiglycation activity was reported as millimoles of catechin equivalents (CE) per kilogram of dry product and as millimoles of aminoguanidine equivalents (AE) per kilogram of dry product, calculated with reference to the dose-response curve built with catechin and aminoguanidine respectively. The antiglycation efficacy index (I_{50}) was calculated as the amount of phenolics in the assay mixture required to inhibit the glycation reaction by 50% (µg GAE per milliliter for GSE).

1.2.10. 2D-Isoelectric focusing/sodium dodecyl sulphate-polyacrylamide gel electrophoresis (IEF/SDS-PAGE)

The BSA-fructose reaction mixtures containing the antiglycation agent, the relative control mixture without the antiglycation agent and native BSA incubated alone were analysed by 2D-IEF/SDS-

PAGE. Prior to electrophoresis, these samples were precipitated with an equal volume of 20% trichloroacetic acid (TCA), and the mixture was centrifuged at 5,000 *g* for 10 min. The precipitate was collected), washed with cold acetone and dissolved in a solution containing 7 M urea and 2% 3-[(3 chloroamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS). IEF was performed on 7 cm, pH 3–10 linear immobilised pH gradient (IPG) strips (Biorad, Milan, Italy). The strips were rehydrated overnight in a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM dithiothreitol (DTT) and 2% IPG buffer pH 3-10 containing 10 µg of the protein sample. Focalisation was carried out at 8500 Vh, with a maximum of 2500 V at 20 ^{o}C, using a Multiphor II electrophoresis unit (GE Healthcare, Milan, Italy). Prior to the second dimension (SDS-PAGE), strips were incubated in equilibration buffer (375 mM Tris/HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol) with 65 mM DTT for 15 min, then with 245 mM iodoacetamide in the same buffer but without DTT for 15 min. SDS-PAGE was carried out as described by Laemmli (1970) on 12% polyacrylamide gels. Polypeptide spots were stained with Coomassie Brilliant Blue. The Mrs. of the polypeptides were determined by comparison with a standard protein solution, which contained phosphorylase b (94 kDa), BSA (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa). To calculate the relative spot volume, gels were digitalised in an Epson Expression 1680 Pro Scanner (Epson Corp., Long Beach, CA, USA) and analysed with Imagemaster 2D Platinum software (GE Healthcare, Milano, Italy).

1.2.11. Statistical analysis of data

Experimental data were obtained in triplicate analyzed by one-way ANOVA using the least significant difference (LSD) as a multiple range test, and by linear regression analyses using Statgraphics 5.1 (STCC Inc.; Rockville, MD). Results are reported as average ± standard error (SE).

1.3. RESULTS AND DISCUSSION

1.3.1. Characterization of phenolics and FRAP values

The total phenolic content of GSE ranged from 4.6 (CH 1) to 53.6 (FR) g GAE/kg (Sri Harsha et al., 2013; Sri Harsha et al., 2014), and red GSE had higher phenolic content than white GSE as shown in figure 1.3. Wide inter- and intra-varietal variability in the phenolic content ($p < 0.05$) was observed among different grape varieties. The reason could be due to different geographical location of the vineyards; genetic factors and varied climatic and winemaking conditions (Montealegre et al., 2006). For red GSE, the results were similar to that observed for pomace of red grape varieties by Rockenbach et al. (2011) and Deng et al. (2011). The values of white GSE were at a similar order of magnitude as observed in the phenolic content of the skins recovered from vinification of the Muller Thurgau and Morio Muscat cultivars (Deng et al., 2011). For a comparative study, the phenolic content of seven herbs and spices (18-82 g GAE/kg) were plotted and the values of phenolic content of some red GSE were found in this range of these well-known plant sources of phenolics (Dearlove et al., 2008).

The reducing capacity of the extracts measured by FRAP assay ranged from 26.0 (CH 1) to 511.0 (FR) mmol Fe(II) Eq/kg (Sri Harsha et al., 2013; Sri Harsha et al., 2014) and as expected red GSE had higher FRAP values than white GSE (Figure 1.3). Since both Folin-Ciocalteu reaction and FRAP assays are electron transfer based reactions, although occurring at different pH values, a highly positive and a significant correlation was observed between values for total phenolics and reducing capacity ($R = 0.9681$, $p < 0.01$) (Figure 1.3). Similar trends of correlation were also observed in different plant species (Yang et al., 2002).

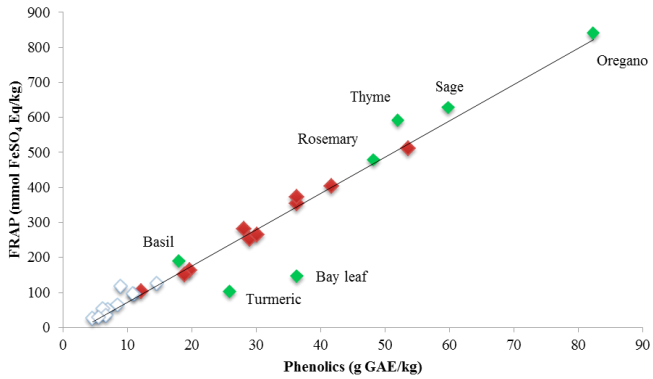


Figure 1.3. Plot of FRAP values vs phenolic content of different varieties of red GSE (◆) and white GSE (◇). Data comparison with herbs and spices reported by Dearlove et al. (2008) (◆)

Total anthocyanin content ranged from 2.5 (NE) to 13.8 (FR) g malvidin-3-O-glucoside Eq/kg for red GSE (Sri Harsha et al., 2013), while white GSE lack anthocyanins. Similar values were found in different pomace samples of red grape skins studied by Kammerer et al. (2004), Ruberto et al. (2007) and Rockenbach et al. (2011). However, exceptionally high anthocyanin contents were found to be about 132 g/kg in Cabernot Mitos skins (Kammerer et al., 2004), 45 g/kg in Nerello Cappuccio vintage (Ruberto et al., 2007) and about 29 g/kg in Bordeaux vintage (Rockenbach et al., 2011).

The main anthocyanins of red grape skin samples characterized by UPLC-DAD-MS were the 3-O-glucosides of five common anthocyanidins: cyanidin, peonidin, petunidin, delphinidin and malvidin (Figure 1.4). Additionally, six minor anthocyanin compounds were detected and identified as *p*-coumaryl-glucoside derivative of cyanidin, peonidin, petunidin and malvidin, and caffeoyl-glucoside and acetyl-glucoside of malvidin. An unidentified compound, which could be a delphinidin derivative was also present. Similar compounds and their derivatives were identified by Kammerer et al. (2004)

and Ruberto et al. (2007) in red grape skins. In general, malvidin-3-O-glucoside was found to be the prevalent compound in all the samples, except for the FR samples, in which major compound was peonidin-3-O-glucoside (Sri Harsha et al., 2013). The chromatogram of BA for individual anthocyanin characterization is shown in the Figure 1.4 as a representative variety.

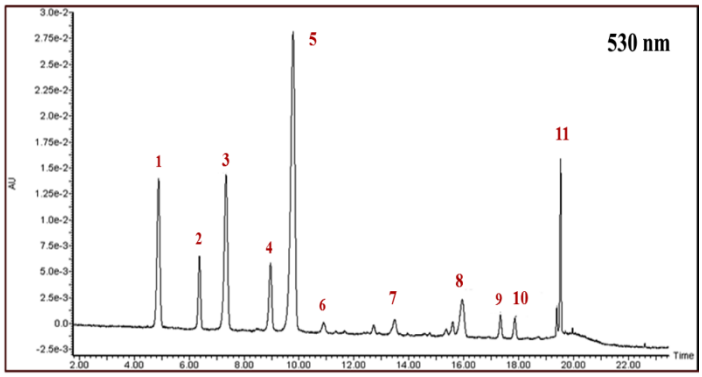


Figure 1.4. UPLC-DAD chromatogram for anthocyanin identification in Barbera (BA) skin extract. Major anthocyanin peaks were identified to be: 1) Delphinidin-3-O-glucoside; 2) Cyanidin-3-O-glucoside; 3) Petunidin-3-O-glucoside; 4) Peonidin-3-O-glucoside; 5) Malvidin-3-O-glucoside. Minor anthocyanin peaks were identified to be: 6) Delphinidin derivative; 7) Malvidin-acetyl-glucoside; 8) Cyanidin-*p*-coumaroyl glucoside; 9) Petunidin-*p*-coumaroyl glucoside; 10) Peonidin-*p*-coumaroyl glucoside; 11) Malvidin-*p*-coumaroyl glucoside.

Total flavonols content ranged from 0.3 (NR) to 2.6 (CR) g quercetin-3-O-glucoside Eq/kg for red GSE and the six main flavonol constituents were identified as quercetin, kaempferol, and their 3-O-glucosides and 3-O-glucuronides. Additionally, two quercetin glucoside isomers and one isomer of quercetin glucuronide were also detected. Quercetin was found to be the prevalent compound in all the samples, except for the FR sample, in which it was quercetin-3-O-glucoside (Sri Harsha et al., 2013). The individual flavonol identification of BA is shown in figure 1.5.

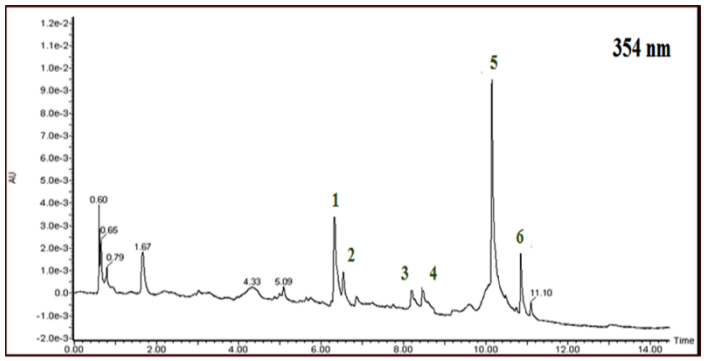


Figure 1.5. UPLC-DAD chromatogram for flavonol identification in Barbera (BA) skin extract. Major flavonol peaks were identified to be: 1) Quercetin-3-O-glucoronide; 2) Quercetin-3-O-glucoside; 3) Kaempferol-3-O-glucoronide; 4) Kaempferol-3-O-glucoside; 5) Quercetin; 6) Kaempferol.

For white GSE, the total flavonol content ranged from 0.31 (CH 2) to 1.89 (AR) g quercetin-3-O-glucoside Eq/kg and the constituents were identified as quercetin, kaempferol, and their 3-O-glucosides and 3-O-glucuronides, kaempferol-galactoside and quercetin-rhamnoside (Sri Harsha et al., 2014). These compounds were also identified in other grape skin samples with a similar range as

observed by Kammerer et al. (2004), Ruberto et al. (2007), Rockenbach et al. (2011). The amount of flavonols found in grape skins recovered from winemaking were found to be higher than those found in the skins of fresh grape varieties and also sixty commonly consumed fresh fruits, vegetables and nuts (Montetealegre et al., 2006; Harnly et al., 2006). The chromatogram of CH for individual flavonol characterization was shown in figure 1.6 chosen as a representative variety.

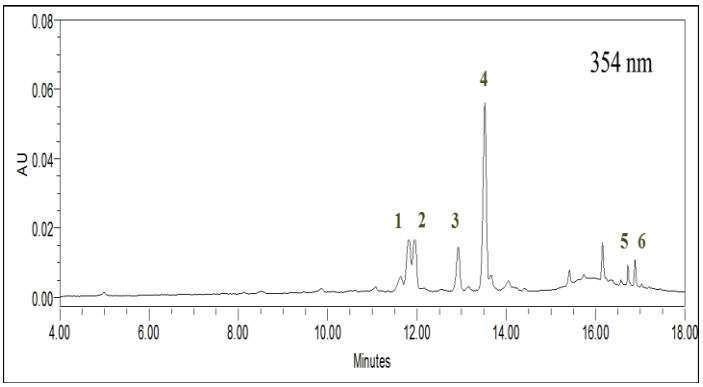


Figure 1.6. HPLC-DAD chromatogram for flavonol identification in Chardonnay (CH) GSE. Major flavonol peaks were identified to be: 1) Quercetin-3-O-glucoside; 2) Quercetin-3-O-glucoronide; 3) Kaempferol-3-O-galactoside; 4) Kaempferol-3-O-glucoside and Kaempferol-3-O-glucuronide; 5) Quercetin; 6) Kaempferol.

The values of proanthocyanidins measured after depolymerisation with *n*-butanol/HCl were in the range of 1.9 (CH 1) to 51.1 (FR) g/kg (Sri Harsha et al., 2013; Sri Harsha et al., 2014). This range is similar to that obtained in the study by Travaglia et al. (2011), and a significant correlation was also observed between the results obtained with the *n*-butanol/HCl assay and UPLC determination ($p < 0.05$). A study of 37 cultivars of *Vitis vinifera* L. revealed that skin proanthocyanidins (ranging between 1.16-39.0 g/kg) are comprised of catechin, epicatechin, epicatechin gallate and epigallocatechin units, with a great variability in the polymeric degree and total content among the varieties. The mean polymerization degree for red grape skin proanthocyanidins was 27.0 for BA, 9.5 for CR, 10.0 for DO, 10.4 for FR, 6.54 for NE and 11.8 for PI and for white grape skin proanthocyanidins was 11.3 for AR, 12.1 for CH, 13.2 for MO and 9.83 for NA (Travaglia et al., 2011). In this study, a significant correlation was observed between values for soluble proanthocyanidins and total phenolics ($R = 0.977$, $p < 0.05$) as shown in figure 1.7. Moreover, ~ 60% of phenolic compounds were found to be proanthocyanidins.

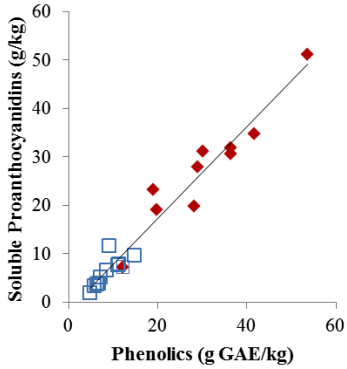


Figure 1.7. Correlation established between soluble proanthocyanidin content (g/kg) and total phenolic content (g GAE/kg) of different varieties of red GSE (◆) and white GSE (□).

1.3.2. Antiglycation activity

Hyperglycemia induced damage in diabetic patients or elderly people is caused by a non-enzymatic protein glycation leading to formation of irreversible AGEs. This process ultimately causes structural and functional changes in the proteins leading to chronic complications in diabetes like nephropathy, retinopathy, neuropathy, angiopathy etc (Singh et al., 2001). Glycation reaction usually occurs when the amino groups are either close to the imidazole moiety or part of lysine doublet. Proximity ($\approx 5A^{o}$) of an aminogroup to an imidazole moiety is the strongest predictor of susceptibility to glycation (Acosta et al., 2000). In this context, our objective focused on inhibition of structural damage induced by glycation on a target protein by grape skin phenolics. BSA was chosen as a target protein, since it is an analogue of human serum albumin bearing 90 % homology and also due to the fact that it is relatively abundant in the serum and can be glycated at multiple sites (Wautier et al., 2001). Fructose was chosen as glycation agent in the model reaction system. In fact, inhibition of protein glycation mediated by fructose is of relevance in disease prevention. Fructose is synthesized through the oxidation of sorbitol in the organs and upon increase in the supply of glucose, the sorbitol pathway becomes active leading to accumulation of fructose in ocular lens, peripheral nerves, blood vessels and red blood cells. Moreover, the formation of AGEs at intracellular level is more attributable to fructose as the reaction rate is approx. ten times faster than glucose considered as a least reactive sugar (Schalkwijk et al., 2004).

Several studies have used different techniques for the characterisation of target proteins following non-enzymatic glycation, which includes detection of the AGE fluorescence intensity, spectrophotometric evaluation of the level of fructosamine (Jariyapamornkoon et al., 2013) and immunological detection of Nε-(carboxymethyl)-lysine (Jariyapamornkoon et al., 2013; Tsuji-Naito et al., 2009). Protein oxidation was examined by spectrophotometric evaluation of protein carbonyl content and free thiol group content (Jariyapamornkoon et al., 2013). SDS-PAGE is also used to demonstrate the occurrence of crosslinking, especially the formation of dimers (McPherson et al., 1988; Wang et al., 2009). In this study, a 2D-IEF/SDS-PAGE approach was employed. The rationale behind adopting this approach is that at the very early stage of glycation, a decrease in the number of free amino groups occurs as a consequence of Maillard reactions and there could be a shift in protein isoelectric point (pI) towards more acidic pH values. At a later stage of glycation, the protein crosslinking is accelerated by increase in the number of dicarbonyl compounds and the formation of dimers or high molecular weight compounds is expected.

Figure 1.8 illustrates the results obtained with respect to measurement of fluorescence intensity and 2D-IEF/SDS-PAGE. When native BSA was incubated alone, the isoforms (with M*r* of 66 kDa) showed pI values in the range 4.5-5.5 (panel A). The relative control mixture i.e., the glycated BSA formed upon reaction with fructose did run as a single discrete spot, with pI more anionic than non-glycated protein, of about 3.5 (panel B). A simultaneous increase in fluorescence can be observed in figure 1.8a. This shows that glycation strongly changed the charge-based behaviour of the protein. A second spot with higher Mr. (125 kDa) was visible with the same pI, which is in a good agreement with the theoretical Mr. of the dimeric form of BSA (Peters, 1996). The amount of the dimeric form with respect to the monomeric form was relatively low (approximately 2%, as determined by densitometric analysis of the gel), confirming that protein crosslinking is a relatively slow process occurring at pH 7.4 and 37 ^{o}C. On the contrary, most of protein moieties displayed different pI values with respect to native BSA. It can be hypothesised that not only formation of covalent crosslinks, but also the loss in positive charges could damage the protein structure and functionality.

1. Targeting hyperglycemia induced damage by grape skin phenolics

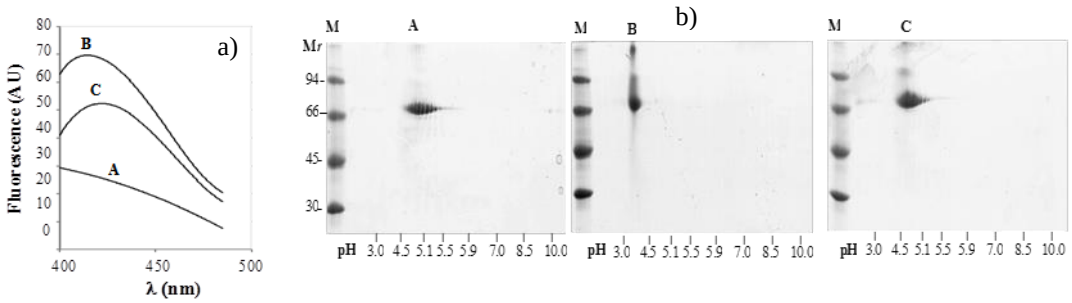


Figure 1.8. a) Fluorescence (λex 350 nm) and b) 2D-IEF/SDS-PAGE profiles of 9.4 mg/mL BSA in 0.2 M phosphate buffer, pH 7.4 (panel A); as in A, in the presence of 0.23 M fructose (panel B); as in A in the presence of 0.23 M fructose and 50 mg/mL of grape skin extract from the Arneis (AR) variety (panel C). M represents markers. Incubation was carried out for 10 d at 37 °C. M*r* is expressed as kDa.

In the BSA-fructose reaction mixture containing the antiglycation agent, an overall inhibition of the reaction was apparent (panel C). The electrophoretic pattern was similar to that shown in panel A. The pI of the protein was in the range 4.4-5.2. A decrease in the spot volume of the dimeric form was noted, strongly indicating the effectiveness of grape skin components to inhibit protein crosslinking and charge variation due to glycation (Figure 1.8b). A decrease in the fluorescence with respect to the control reaction can be observed (Figure 1.8a) when grape skin phenolics were present in the reaction mixture. Phenolics were known to act as radical scavengers, metal chelators and carbonyl trapping agents (Ho et al., 2010). In addition, phenolics can bind to BSA through hydrogen bonds and electrostatic interactions (Skrt et al., 2012) thus hindering the reaction of BSA with fructose. For the quantification of AGEs formation to determine the antiglycation activity, the development of non-tryptophan fluorescence was evaluated during the time course of the reaction. The efficacy of inhibition was expressed with reference to catechin, as flavanols are generally considered as reference antiglycation agents (Nakagawa et al., 2002; Peng et al., 2008) and aminoguanidine, a synthetic inhibitor, which is known to inhibit formation of AGEs by quenching dicarbonyl compounds, thus preventing diabetes induced risk (Thornalley, 2003). Significant differences in antiglycation abilities were observed in different grape skin samples, with red GSE ranging from 118 (GR) to 480 (FR) mmol CE/kg; while white GSE ranged from 35 (MO) to 103 (AR) mmol CE/kg (Table 1.1). Considering aminoguandine as a reference compound, the efficacy values were found six times higher than the CE, due to lower efficacy of this drug with respect to catechin. The red grape varieties which are rich in anthocyanins were found more effective than white grape varieties in terms of antiglycation activity. Commercial nutraceutical standards Leucoselect® (derived from grape seeds) and Pycnogenol® (derived from pine bark) exhibited higher antiglycation activity values of 2591 ± 34 and 2858 ± 131 mmol CE/kg respectively as expected since both of these compounds are purified products.

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Table 1.1. Antiglycation activity of GSE recovered from winemaking, evaluated with reference to catechin (mmol CE/kg) in the BSA/fructose model system

Red grape varieties	Antiglycation activity	White grape varieties	Antiglycation activity
BA	$371^{de} \pm 16$	CH 1	$39^{a} \pm 3$
CR	$443^{ef} \pm 5$	CH 2	$43^{a} \pm 3$
FR	$480^{f} \pm 27$	RI 1	$74^{cd} \pm 4$
DO 1	$380^{de} \pm 48$	RI 2	$83^{cde} \pm 8$
DO 2	$325^{cd} \pm 33$	NA	$69^{bc} \pm 7$
NE	$261^{bc} \pm 18$	AR	$103^{e} \pm 10$
PI 1	$236^{abc} \pm 6$	ER	$89^{de} \pm 5$
PI 2	$294^{cd} \pm 24$	MO	$35^{ab} \pm 8$
GR	$118^{a} \pm 5$	MT	$100^{e} \pm 5$
NR	$178^{ab} \pm 24$		

Data are average ± SE. Different letters within the same column (a-f) indicate significant differences (LSD; $p < 0.05$).

Previous studies revealed that a correlation exists between phenolic content and antiglycation capacities (Dearlove et al., 2008; Peng et al., 2008). In this study, among the grape skin samples correlation coefficient of 0.9561 ($p < 0.01$) was established with total phenolic content and antiglycation capacity for the BSA/fructose model system (Figure 1.9).

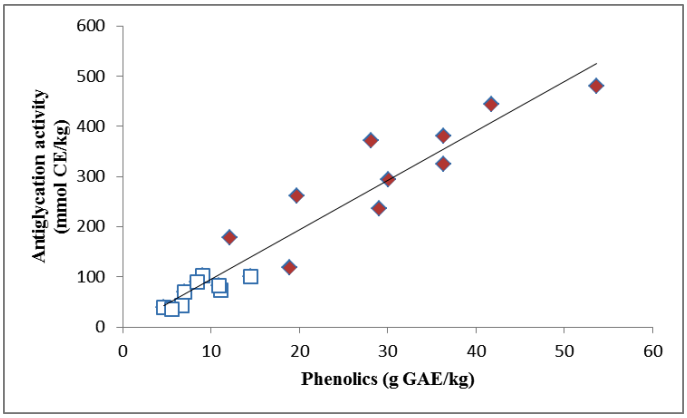

Figure 1.9. Correlation established between values for antiglycation activity (mmol CE/kg) and total phenolic content (g GAE/kg) of different varieties of red GSE (◆) and white GSE (□).

However, the antiglycation activity not only depends on the quantity of the phenolics, but also on the phenolic composition of the extract. For instance, rosemary infusion was found to have stronger antiglycation activity than that of green tea, when infusions were compared at the same phenolic content (Ho et al., 2010). Hence, in order to verify the efficacy of the phenolic pool of the grape skin samples, the antiglycation activity was expressed as the concentration of phenolics that is required to inhibit protein glycation by 50% (I_{50}). This approach has already been used to study the efficacy of different anti-glycating agents (Dearlove et al., 2008; Ho et al., 2010). In this study, I_{50} values for the red GSE ranged between 9.2 and 15 µg GAE/mL and for white GSE the values ranged between 11.6 and 19.9 µg GAE/mL (Figure 1.10). The variability in the I_{50} values among the red GSE is slightly less compared to white GSE, suggesting that the samples had similar antiglycation effectiveness with good "phenolic quality" despite differences among the varieties and winemaking procedures. This result is quite important to standardize the efficacy of grape skin extracts in order to overcome the differences

in raw material quality.

For a comparative study with respect to the antiglycation efficacy of GSE, I_{50} values of few herbs and spices, which were considered to be efficient inhibitors of protein glycation, were plotted and the values for rosemary, sage and cinnamon were found to be 9.6, 10.0 and 12.2 µg GAE/mL (Figure 1.10), respectively (Dearlove et al., 2008). An interesting point to note is that grape skin extracts obtained from a cost-effective raw material such as winemaking byproducts were shown to possess similar antiglycation effectiveness as that of well-known sources of dietary antiglycation agents (Saraswat et al., 2009; Ho et al., 2010).

The commercial nutraceutical preparations Leucoselect® and Pycnogenol® exhibited higher I_{50} values of 38.0 and 34.2 µg GAE/mL respectively than those of GSE, suggesting that the pool of antioxidants present in the grape skins is more efficient than the proanthocyanidins that are present in these products. The reason could most likely be due to the additional presence of anthocyanins and flavonols in the grape skins.

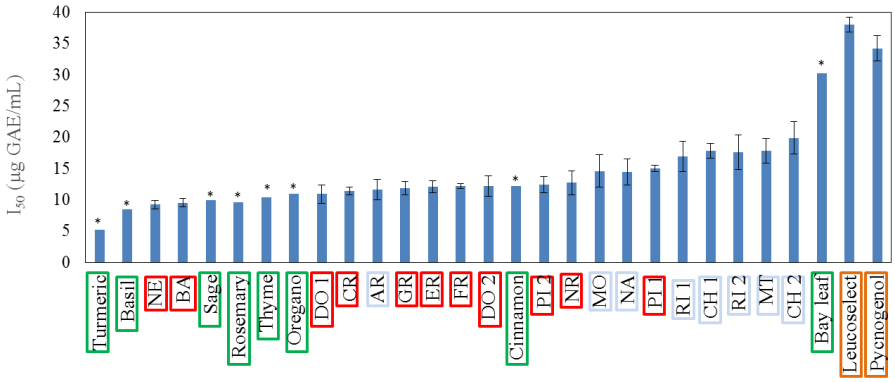

Figure 1.10. I_{50} values for red and white GSE, commercial nutraceutical preparations (Leucoselect® and Pycnogenol®) and the dietary antiglycation agents proposed by Dearlove et al., 2008 (indicated by asteriscs).

Besides, some standard compounds were also tested to calculate the antiglycation efficacy index using BSA/fructose model system. Quercetin-3-O-glucoside was more efficient than catechin. In general, flavonols are more efficient than flavanols according to Wu et al. (2005). It was also demonstrated that malvidin-3-O-glucoside had intermediate activity with respect to quercetin-3-O-glucoside and catechin. These results support the importance of grape skin products as sources of antiglycation agents. The flavanol procyanidin A2 was less efficient than the monomer catechin if results were expressed with reference to flavanol units. This result could be related to the difficulty or inefficiency of higher molecular weight molecules to inhibit glycation. Similarly, a previous study has shown that proanthocyanidins' antioxidant activity decreases with increasing molecular weight due to their steric hindrance (Prior et al., 2005). Aminoguanidine was found to be the least effective inhibitor of protein glycation. Hence, overall antiglycation efficacy ranking of standard compounds was as follows: quercetin-3-O glucoside > malvidin-3-O glucoside > catechin > procyanidin A2 > aminoguanidine. On the contrary, the antioxidant activity towards the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was similar among these compounds (Kähkönen et al., 2003), confirming that the structural requirements for high antiglycation activity are not the same as that of antioxidant activity.

Bioaccessibility studies support the hypothesis that phenolics present in foods can reach cellular targets, even at lower concentrations than those administered. A study by Manach et al. (2005) investigated the kinetics and extent of polyphenol absorption among adults and observed recovery of these compounds in micro- or nanomolar concentrations in the plasma. Indeed, Hokayem et al. (2013) reported that 2g/day intake of grape polyphenols inhibit fructose-induced oxidative stress leading to diabetes. Hence, grape phenolics can potentially play a role in inhibition of glyco-oxidative stress linked to hyperglycemia complications.

1.4. CONCLUSIONS

2D electrophoresis provided a clear evidence of fructose-induced structural damage occurring in the BSA target protein. In particular, IEF gave a better understanding than SDS-PAGE on the glycation process. When dimer/monomer ratio was only 2%, the extent of charge variation was notable, suggesting that protein functionality had been largely affected. Thus grape skin phenolics were able to protect the target protein from both charge variation that occurs at the beginning of the glycation process and crosslinking that occurs at a later stage.

Non-tryptophan fluorescence measurements proved that most of the GSEs had good ‘‘phenolic quality’’, as indicated by similar strength of antiglycation ability evaluated with reference to GAE. Grape skin phenolics showed antiglycation activity higher than those of commercial nutraceutical preparations. The antiglycation efficacy ranking of standard compounds proved that grape skin phenolics were more efficient than the synthetic inhibitor aminoguanidine. Hence, despite differences in variety, climate, location of the vineyard and winemaking procedures, by standardizing the phenolic content of the grape skins, it could be possible to obtain extracts having efficient antiglycation properties.

Compared to other plant phenolics, i.e., cinnamon, sage and rosemary, which were found to be efficient antiglycation agents, grape skin phenolics are more cost-effective. Results of this study raise interest in the use of cost effective bioresources such as byproducts of grape vinification as a dietetic antiglycation agents and provide a platform to develop new specifically-designed foods targeting the wellbeing of diabetic and elderly people.

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2. DESIGN OF A FRUIT-BASED FOOD FORMULATED WITH GRAPE SKIN ANTIOXIDANT DIETARY FIBRE AND PROCESS OPTIMIZATION

2. Fruit-based food formulated with grape skin antioxidant dietary fibre

2.1. INTRODUCTION

A promising application of the food sector is the recovery of polyphenol and fiber-rich fractions with potential health benefits from grape pomace, which could be used as bioactive food ingredients. Previous chapter has described the alternative use of grape skins as cost effective resources of antiglycation agents, while this chapter deals with the development of new fruit-based product through the incorporation of recovered grape skin ingredients.

Grape pomace contains both phenolics and dietary fibres, thus has been referred to as ‘‘antioxidant dietary fibre’’. Due to the close relationship between antioxidant and dietary fibre, and their common fate in the gut, it has been proposed that these food components have a joint role in prevention of human diseases (Perez-Jimenez et al., 2008). Increasing fibre content in foods is a relevant strategy to prevent the occurrence of chronic diseases. In fact, numerous health benefits have been associated with an increased intake of dietary fibre, including reduced risk of coronary heart disease, diabetes, obesity, and some forms of cancer (Mann et al., 2009; Perez-Jimenez et al., 2008; Lopez-Oliva et al., 2010; Hokayem et al., 2013; Scheiber et al., 2001). The development of foods that provide additional health benefits beyond basic nutrients is also a trend in the fruit processing industry (Augusto et al., 2011). In fact, incorporation of fibre-rich byproducts, which are inexpensive, non-caloric bulking agents can bring about an increase in viscosity, provide ability to form gels and/or act as emulsifiers, modify texture, taste and colour in the new foods developed. On the other hand, food formulation with fibre-rich ingredient results in changes in overall sensorial properties, and hence fibre addition needs to be tailored according to the consumers’ liking (Palzer, 2009; Walker et al., 2014).

Recently, applications of winemaking byproducts as food ingredients in new functional foods have been investigated to increase the nutritional value and/or to modulate the physical properties of chicken hamburgers (Sáyago-Ayerdi et al., 2009), rye bread (Mildner-Szkudlarz et al., 2011), cheese (Han et al., 2011), ice cream (Sagdic et al., 2012), wheat biscuits (Mildner-Szkudlarz et al., 2013), fish (Riberio et al., 2013; Pazos et al., 2005), yogurt and salad dressing (Tseng et al., 2013). However, there is no information on the effects of addition of grape pomace derived ingredients on complex food fluids such as tomato puree.

Tomato is one of the most important vegetable products especially in the Mediterranean diet and it is mainly consumed as processed products, i.e., paste, concentrate, ketchup, salsa, etc. Rheological properties of tomato-based products could to be optimized by using thickening agents (such as dietary fibres) to improve tomato consistency and quality as demonstrated by Anthon et al. (2010); Bayod et al. (2011); Juszczak et al. (2013); Moelants et al. (2013); Moelants et al. (2014). One major aspect that can determine the product consistency and quality and ultimately affects the rheological properties is the particle size. Particle size distribution (PSD) of a food suspension affects texture, i.e., perceivable homogeneity, consistency, compartmentalization of nutrients and sensory-active compounds, which means the rate of their release during processing and eating. In particular, the surface-weighted mean diameter, d(3,2) affects the kinetics of release of various compounds from the solid matrix (Walstra, 2003), while the volume-weighted mean diameter, d(4,3) is the most significant parameter in relation to the perceptible texture of the product (Imai et al., 1998).

Fortification of foods with fibrous ingredients however, poses many technological challenges that have to be addressed i.e., choice of the incorporation level, need for process optimization, particularly with reference to re-design of the pasteurization step without affecting the stability of the new ingredient and assessment of consumers’ liking.

In the current study, these technological challenges were taken into consideration in order to gain information that could be of importance for the prospective use of recovered winemaking byproducts for sustainable food innovation, especially with reference to complex fluids.

2. Fruit-based food formulated with grape skin antioxidant dietary fibre

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

Standards of catechin, quercetin-3-O-rutinoside (rutin), quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempferol-3-O-galactoside, kaempferol-3-O-glucuronide, kaempferol-3-O-glucoside, quercetin, kaempferol and naringenin were purchased from Extrasynthese (Lyon, France). The integrated total dietary fibre assay procedure kit was purchased from Megazyme International Ireland Ltd. (Bray, Ireland). All other chemicals were purchased from Sigma-Aldrich Italia (Milan, Italy).

2.2.2. Grape skins (GS)

Grape pomace sample of the Chardonnay variety was kindly provided by a winery located in Northern Italy. At the winery, grapes were pressed with separation of grape solids and must. Grape stalks were separated by a mechanical destemmer and the remaining material was sieved (with a 5 mm sieve) to separate the GS from the seeds and frozen to inhibit microbial growth. GS were transported frozen to the lab and dried at 50 ^{o}C for about 8 h and finely milled by a rotor mill (Cross Beater Mill, Retsch GmbH, Haan, Germany) at room temperature. The powders obtained were sieved by using the Octagon Digital sieve shaker (Endecotts Ltd., United Kingdom), with three certified sieves (openings: 125, 250 and 500 μm), under continuous sieving for 10 min at amplitude 8. Three granulometric fractions were collected, namely: GSL (250μm < GSL ≤ 500μm), GSM (125μm < GSM ≤ 250μm) and GSS (GSS ≤ 125μm). These fractions were stored under vacuum, in the dark, at 4 ^{o}C.

2.2.3. Tomato puree processing at industrial plant

Two tomato puree samples, namely PV (smooth puree) and PR (rough puree) were provided by a tomato processing company. At the industrial plant, tomatoes were homogenised and heated to ~ 95 ^{o}C by steam injection to inactivate endogenous enzymes (hot-break). The homogenate was then passed hot through a 0.5 mm-screen (PV) or a 1 mm-screen (PR) pulper/finisher to remove seeds and skin fragments and deareated under vacuum. The finished purees were then concentrated at 80 ^{o}C under reduced atmospheric pressure using a tubular heat exchanger (the final moisture contents were 89.1 ± 0.2 and 89.8 ± 0.2 for PV and PR, respectively) and the solid content increased from 5.0 % to 9.9 %. The purees were then aseptically stored in a tank under nitrogen for 6 months before bottling. After bottling, the purees were autoclaved at 115 ^{o}C for 5.5 min (Figure 2.1).

2.2.4. Lab scale preparation of fortified tomato purees

A 3.2 g sample of the GSL, GSM and GSS fractions was added to 96.8 g of the PV and PR tomato purees to achieve 3% fibre content in the final product (Figure 2.2). Each puree was filled into different glass bottles (250 mL capacity). The incorporation of GS derived fractions into tomato puree, requires the design of an effective heat treatment, since the presence of spores in GS cannot be ruled out. The pH values of these products were in the range of 4.1-4.3. To achieve pasteurization of low-pH foods, the most heat resistant microorganism and common spoilage microorganism found in these foods i.e., *Alicyclobacillus acidoterrestris* has been proposed as a process target (Silva et al., 2004). A set of the bottled fortified purees and their corresponding control purees were then submitted to microwave heating. Microwave heating of puree is very fast, and hence can be representative of an optimized continuous industrial treatment. During heating, the temperature of the tomato puree was monitored continuously by using a thermocouple set in the geometric centre of one of the bottles (the slowest heating point) to calculate the pasteurization effect (Earle et al., 2003).

2. Fruit-based food formulated with grape skin antioxidant dietary fibre

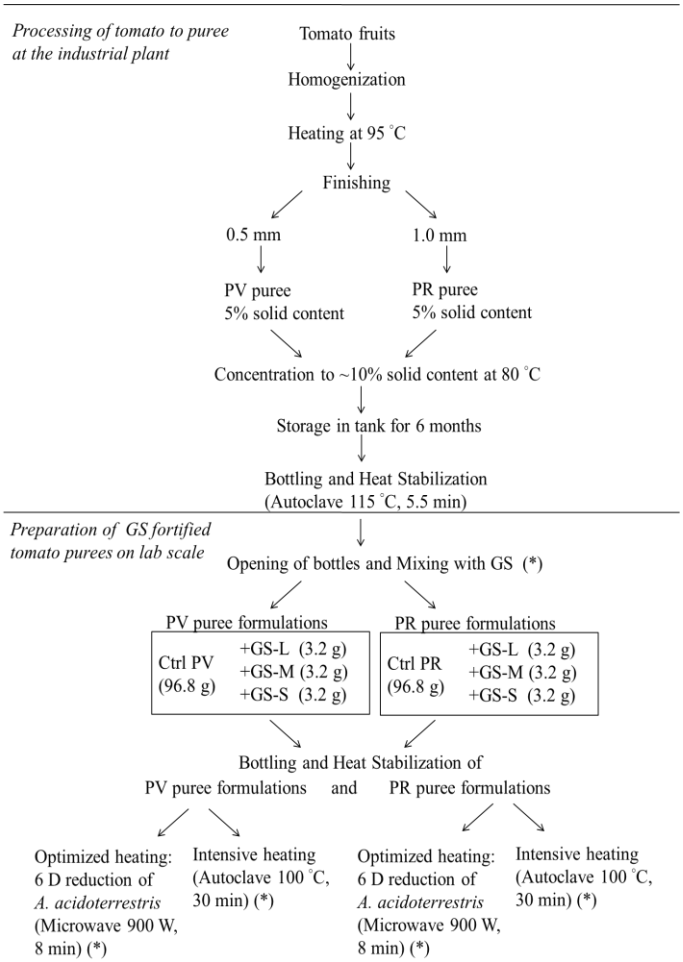

Figure 2.1. Schematic representation of tomato processing to fortified purees. Top section includes industrial processing of PV and PR purees. Bottom section includes lab scale mixing of PV and PR purees with GS fractions followed by either microwave treatment or autoclave treatment. Asterisks (*) indicate the sampling points. Abbreviations: PV, smooth puree (≤ 0.5 mm); PR, rough puree (≤ 1 mm); GS, granulometric fractions of dried grape skins: GSL (250μm < GSL ≤ 500μm), GSM (125μm < GSM ≤ 250μm) and GSS (GSS ≤ 125μm).

Figure 2.2. PV and PR tomato purees formulated with grape skins (GS) ingredient.

Different heating conditions were tried and the resulting time/temperature curves were obtained. D values for the target microorganism were calculated as a function of temperature using the Bigelow's model, as reported below:

$$D = D_{ref} 10^{(T_{ref} - T)/z} \quad (2.1)$$

Where for the target microorganism, D_{ref} = 1.5 min, T_{ref} = 95 °C and z = 7 °C (Bevilacqua et al., 2011). The 1/D values were then plotted as a function of time and the resulting curves were then integrated to evaluate the total decimal reductions (Silva et al., 2004). Microwave conditions were then chosen in order to achieve six decimal reductions (6D) for the target microorganism (8 min at 900 W).
In parallel, another set of bottled fortified purees was submitted to autoclave treatment (100 °C, 30 min). Discontinuous and intensive autoclave treatments are also common in tomato processing industry (Figure 2.1).

2.2.5. Moisture, protein, fat, carbohydrate and fibre

Moisture content of tomato purees and grape skins was determined in triplicate by drying in a vacuum oven at 70 °C and 50 Torr for 18 h. Protein and fat contents were measured according to AOAC official methods of analysis (Tseng et al., 2013). Glucose and fructose were determined as described by Lavelli et al. (2006). Fibre contents were determined by the Megazyme total dietary fibre assay procedure (based on AOAC 991.43).

2.2.6. Sample extraction

For GS extraction, an aliquot of 1 g was weighed in duplicate, added with 20 mL methanol:water:formic acid (70:29.9:0.1, v/v/v) and extracted for 2 h at 60 °C with continuous stirring. The mixture was centrifuged at 10,000 *g* for 10 min, the supernatant recovered and the solid residue was re-extracted using 10 mL of the same solvent. The supernatants were pooled.
For tomato puree extraction, 3.75 g was weighed in duplicate and added to 1.9 mL of water, 7 mL of methanol and 0.3 mL of formic acid (in order to use the same medium as for the grape skin fractions, taking into account the amount of water present in the puree). The same extraction method for the grape skins was used for the tomato purees. Extracts were stored at -20 °C until analytical characterisation.

2.2.7. Chemical characterisation

HPLC-DAD analysis for flavonols, proanthocyanidins, FRAP assay and determination of antiglycation activity by BSA/fructose model system were performed as described in the materials and methods section of chapter 1.

2.2.8. Physical characterisation

2.2.8.1. Particle size distribution (PSD)

The analysis of PSD of tomato purees in the range of 0.2-1000 μm, GS granulometric fractions and their mixtures was performed according to the specifications reported in the international standard ISO 13320 (2009) using a Malvern 2000 Laser granulometer (Malvern instruments Ldt., Malvern, UK) equipped with a single laser source at λ = 633 nm. Deionised water was used as the dilution medium (1:500 ratio) in order to avoid multiple scattering phenomena and excessive obscuration of the laser source; the temperature was set at 25 ± 1 °C. The refractive index for water used as dispersant medium was set to 1.33, and in order to model the angular intensity distribution function of scattered light, the Fraunhofer model was used described as:

$$\frac{I(\theta)}{I_0} = \frac{1}{k^2 l_a^2} \alpha^4 \left[\frac{J_1 \alpha \sin\theta}{\alpha \sin\theta}\right]^2 \quad (2.2)$$

where: $I(\theta)$ is the angular intensity distribution of light scattered by particles, Io the intensity of the incident unpolarized light, k is the wavenumber, l_a is the distance from scattering object to detector, α is the dimensionless size parameter ($\alpha = \pi x n_m/\lambda$, with x, particle size, n_m, refractive index of the medium, λ wavelength of illuminating light source in vacuum), J_1 is first order Bessel function, θ is the scattering angle with respect to forward direction.

Fraunhofer approximation is valid for these samples because their particle sizes were much larger than λ (ISO 13320, 2009). Calculations for PSD and its descriptors were made using the instrument's software General Purpose Model, assuming a spherical particle shape with normal calculation sensitivity. The descriptors considered were the surface-weighted mean diameter (μm), i.e., d(3,2), also called Sauter mean diameter and the volume-weighted mean diameter (μm) or d(4,3), defined as:

$$d(3,2) = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \; ; \; d(4,3) = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3} \qquad (2.3)$$

Where d_i is the i-th diameter class and n_i is the respective number of particles per unit volume

2.2.8.2. Bostwick consistency

Consistency was determined using a Bostwick consistometer (LS 100, Labo-Scientifica, Parma, Italy). Measurements taken after both 30 s and 60 s gave the same information. Results were expressed as distance travelled by the sample (cm) through the trough in 60 s.

2.2.8.3. Rheological properties

The fundamental rheological properties of raw and fortified tomato purees were studied by means of dynamic oscillatory measurements performed on a Physica MCR300 Rheometer (Anton Paar GmbH, Graz, Austria), supported by the software Rheoplus/32 (v. 3.00, Physica Messtechnik GmbH, Ostfildern, Germany). Parallel plate geometry (25 mm diameter, 2 mm gap) was used, with corrugated plates to prevent sample slippage. The temperature was regulated at 25 °C by using a circulating bath and a controlled peltier system. After loading, the excess sample was trimmed off, and before starting the tests, the sample was allowed to rest for 5 min to relax stress. A moisturizing external chamber was used to prevent moisture loss during measurements. The fundamental rheological properties were studied within the linear viscoelastic region, as determined by the amplitude sweep performed in the range of 0.001-300% strain, at a constant frequency of 1 Hz. Frequency sweep was carried out under a constant strain of 0.1% in the range of frequencies 0.1-10 Hz to calculate the elastic modulus (G', Pa), the viscous modulus (G'', Pa) and complex viscosity (η^*, Pa·s), defined as:

$$\eta* = \sqrt{\frac{(G')^2 + (G'')^2}{\omega}} \qquad (2.4)$$

Where ω is the angular oscillatory frequency (rad/s) .

2.2.8.4. Colorimetric parameters

Colour evaluation was performed in quadruplicate with a Chroma meter II (Konica Minolta, Osaka, Japan), which provides the Hunter L^*, a^*, and b^* coordinates, representing: lightness and darkness (L^*), redness ($+a^*$), greenness ($-a^*$), yellowness ($+b^*$), and blueness ($-b^*$). The chromameter was calibrated with a white standard. The head of the colorimeter was placed directly on the bottom of the bottles containing the tomato purees for colour measurement.

To study the total variation in colour, ΔE_T was calculated, as indicated by the following equation:

$$\Delta E_T = \sqrt{(a^* - a^*_{UH})^2 + (b^* - b^*_{UH})^2 + (L^* - L^*_{UH})^2} \qquad (2.5)$$

Where a^*, b^*, and L^* are the values of the colorimetric parameters of heat-treated purees and a^*_{UH},

b^*_{UH}, and L^*_{UH} are the values of the colorimetric parameters of the corresponding unheated tomato puree.

2.2.9. Sensory test

Eighty-six consumers (44 males, 42 females, between 19–68 years with mean age 28) participated in the sensory study performed at the University of Pollenzo (Italy). They had received an invitation and were selected based on their interest and availability. All tests were conducted individually and social interaction among the subjects was not permitted. The experimenter verbally introduced the consumers to the computerised data collection procedure (FIZZ Acquisition software, version 2.46A, Biosystèmes, Courtenon, France). The consumers' test was organised in two sub-sessions. In the first sub-session, participants evaluated a set of six fortified tomato purees. In the second sub-session, a set of the control unfortified purees was tested. Fortified and control purees were analysed in different sub-sessions to limit the contrast effect (Meilgaard et al., 2006).

The samples (20 g) were offered to the consumers in completely randomized order within the two sessions, at 50 ± 1 ^{o}C in coded, opaque white plastic cup (38 mL) hermetically sealed with a clear plastic lid. For each sample, consumers accurately stirred the tomato puree using a plastic teaspoon, observed its appearance and tasted a full teaspoon of the product. Then, consumers rated overall liking, liking for colour and texture on a nine-point hedonic scale ranging from 'dislike extremely' (1) to 'like extremely' (9). A 30 s gap between each sample was enforced by the computerised system. Consumers were required to eat unsalted crackers and rinse their mouth with still water during the gap interval. A 10 min gap was enforced between the two sub-sessions. Preference tests were performed in individual booths under white light. The overall evaluation took between 25 to 35 min.

2.2.10. Statistical analysis of data

Experimental data were obtained at least in triplicate and analysed by one-way ANOVA using the least significant difference (LSD) as a multiple range test, and by linear regression analysis using Statgraphics 5.1 (STCC Inc.; Rockville, MD, USA). Results were reported as average ± standard deviation (SD). Liking data (overall liking, liking for colour and texture) from consumers were independently submitted to a two-way ANOVA model, assuming sample and subject as main effects, by performing LSD ($p < 0.05$).

2.3. RESULTS AND DISCUSSION

2.3.1. Chemical characterisation

2.3.1.1. Product and process design

Three granulometric fractions of Chardonnay GS (in the range 125-500 µm) and two tomato purees of different particle sizes (0.5 and 1 mm) were used in combined formulations. The compositional profiles of GS and tomato purees were first characterised in order to choose the level of incorporation (Table 2.1). The results will be explained in detail with respect to PV tomato puree and its formulations which are chosen as a representative samples, while any major variations in the PR formulations will be presented in the text.

Table 2.1. Chemical composition of PV tomato puree and grape skins (GS) of Chardonnay variety (g/kg).

Parameter	PV	GSL	GSM	GSS
Protein	12.0 ± 0.1	91.5^{a} ± 0.4	97.5^{ab} ± 0.2	103.4^{b} ± 0.9
Soluble sugars	49 ± 1	169^{a} ± 1	169^{a} ± 1	169^{a} ± 1
Fat	1.0 ± 0.1	39.5^{a} ± 0.2	55.2^{b} ± 0.3	68.6^{c} ± 0.2
Total dietary fibre	15 ± 1	484^{a} ± 2	509^{a} ± 1	485^{a} ± 4
Soluble proanthocyanidins		26.0^{a} ± 1.5	31.8^{b} ± 1.4	34.0^{b} ± 1.7
Insoluble proanthocyanidins		125^{a} ± 3	171^{b} ± 14	169^{b} ± 22
Total Flavonols	0.067 ± 0.001	0.59^{a} ± 0.06	0.59^{a} ± 0.03	0.61^{a} ± 0.03

Data are average ± SD. Different letters within in the same row (a-c) among the GS fractions indicate significant differences (LSD, $p < 0.05$).

Dietary fibre content of GS was ~ 500 g/kg. Among phenolics, insoluble proanthocyanidins were the major components. For both soluble and insoluble proanthocyanidins, the contents were found to be lower for GSL than GSM and GSS, indicating lower solubility for the GSL fraction (Table 2.1). Flavonols were comprised of quercetin-3-O-glucuronide, quercetin-3-O-glucoside, quercetin, kaempferol-3-O-galactoside, kaempferol-3-O-glucuronide, kaempferol-3-O-glucoside and kaempferol. The dietary fibre content of tomato puree was 15 g/kg. Tomato lacks proanthocyanidins. The main flavonoids found in the puree were rutin and naringenin.

The level of GS addition into the puree was then chosen to have 3% fibre content in the final products (3.2 g of grape skins added to 96.8 g of tomato puree). Hence, the purees can be labelled as a "fibre-source" according to the EC Regulation 1924/2006. Furthermore, in an *in vivo* study, Perez-Jimenez et al. (2008) have demonstrated that the intake of grape antioxidant dietary fibre (5.25 g of dietary fibre and 1.06 g of proanthocyanidins in the supplemented dose) significantly reduces the biomarkers of cardiovascular risk. Based on GS fibre and proanthocyanidin contents, a 175 g dose of the fortified purees, that could be a recommended daily dose in the Mediterranean diet, can provide 5.25 g of dietary fibres and around 1 g of proanthocyanidins (soluble and insoluble). Hence, positive *in vivo* effects of these purees can be hypothesised, while food matrix effect on the bioavailability of food components cannot be ruled out.

The incorporation of grape skin derived fractions into a semi-liquid food, such as tomato puree, requires the design of an effective heat treatment. *A. acidoterrestris*, a thermoacidophilic, non-pathogenic and spore forming bacterium has been proposed as a target microorganism often found in fruit juices, including tomato puree and white grape juice (Silva et al., 2004). A plot of conditions to achieve 6D reductions i.e., time (min), temperature (^{o}C) and 1/D (min^{-1}) for the target microorganism during the microwave treatment of tomato puree can be seen in figure 2.3.

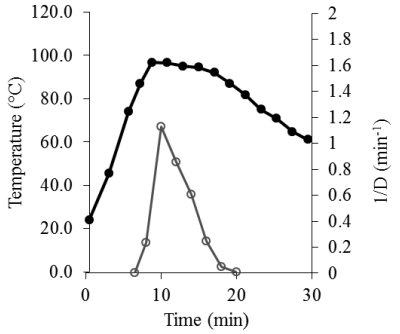

Figure 2.3. Temperature (●) and 1/D values (o) for the target microorganism *A. acidoterrrestris* of tomato puree during microwave treatment.

2.3.1.2. Effect of processing on antioxidant compounds

Mixing of the purees with the GSL, GSM and GSS skin fractions at room temperature affected soluble proanthocyanidin contents, which were lower than that calculated based on the proanthocyanidin content of grape skins, with 54-56% recovery (Table 2.2). Peng et al. (2010) found that in bread made with a proanthocyanidin-rich grape seed extract, the observed antioxidant activity increases less than what was expected. They explained the reason could be due to either interactions of proanthocyanidins with food components to produce insoluble molecules, or to thermal degradation. In this study at the mixing stage, thermal degradation can be ruled out. Hence, lower proanthocyanidin recovery can be ascribed to their interaction with tomato components, such as proteins or polysaccharides, to produce high molecular weight aggregates, through hydrogen bonding or hydrophobic interactions (Pinelo et al., 2006).

Table 2.2. Soluble proanthocyanidin contents (mg/kg) of PV tomato puree formulated with grape skins (GS) fractions after mixing, microwave and autoclave treatment.

	Soluble proanthocyanidins		
Puree	**Mixing**	**Microwave**	**Autoclave**
PV			
PVGSL	355^{ax} ± 6 (54)	348^{ax} ± 1 (53)	455^{ay} ± 81 (69)
PVGSM	446^{bx} ± 17 (55)	411^{ax} ± 45 (51)	629^{by} ± 65 (78)
PVGSS	487^{bx} ± 35 (56)	468^{ax} ± 44 (54)	668^{by} ± 19 (77)

Data are average ± SD. Percent recovery is indicated in parenthesis. Different letters within the same row (x,y) and column (a,b) are significantly different (LSD, $p < 0.05$).

FRAP values of the fortified purees increased approximately by twofold. However, as observed for proanthocyanidins, the increase in FRAP values were only 63-66% of that calculated considering the values grape skin fractions (Table 2.3).

Table 2.3. Reducing capacity values as measured by FRAP assay (mmolFe(II) Eq/kg) of PV tomato puree formulated with grape skins (GS) fractions after mixing, microwave and autoclave treatment.

	FRAP		
Puree	**Mixing**	**Microwave**	**Autoclave**
PV	$2.68^{ax} \pm 0.22$	$2.60^{ax} \pm 0.18$	$2.75^{ax} \pm 0.15$
PVGSL	$5.16^{bx} \pm 0.04$ (64)	$5.35^{bx} \pm 0.15$ (66)	$6.27^{by} \pm 0.38$ (77)
PVGSM	$5.89^{cx} \pm 0.07$ (63)	$5.93^{cx} \pm 0.04$ (64)	$6.95^{bcy} \pm 0.23$ (75)
PVGSS	$6.35^{cx} \pm 0.30$ (66)	$6.02^{cx} \pm 0.18$ (63)	$7.50^{cy} \pm 0.45$ (78)

Data are average ± SD. Percent recovery is indicated in parenthesis. Different letters within the same row (x,y) and column (a-c) are significantly different (LSD, $p < 0.05$).

Microwave treatment did not show any effect on the flavonols and naringenin (not shown). Similar result was found by Capanoglu et al. (2008) where pasteurisation at 98 ^{o}C did not change rutin and naringenin contents in tomatoes. Additionally, microwave treatment did not have an effect on the proanthocyanidin contents or FRAP (Tables 2.2, 2.3).

Table 2.4. Phenolic contents (mg/kg) in the PV tomato puree formulated with grape skins (GS) after autoclave treatment.

	Puree formulation			
Phenolics	**PV**	**PVGSL**	**PVGSM**	**PVGSS**
Q-ud	$10.71^{a} \pm 0.44$ (81)	$10.91^{a} \pm 1.91$ (85)	$10.59^{a} \pm 0.62$ (82)	$10.49^{a} \pm 0.96$ (82)
Q-rut	$55.89^{b} \pm 0.34$ (95)	$53.59^{a} \pm 0.05$ (94)	$52.42^{a} \pm 1.07$ (92)	$53.61^{a} \pm 0.98$ (94)
Q-gln		$2.93^{a} \pm 0.18$ (80)	$3.05^{a} \pm 0.29$ (84)	$3.05^{a} \pm 0.03$ (84)
Q-glc		$2.97^{a} \pm 0.96$ (97)	$2.88^{a} \pm 0.74$ (94)	$3.03^{a} \pm 0.03$ (99)
Q	$0.85^{a} \pm 0.01$	$6.77^{b} \pm 0.04$ (1590)	$6.67^{b} \pm 0.85$ (1567)	$7.10^{b} \pm 0.99$ (1669)
Total Q-der	$67.45^{a} \pm 0.79$ (93)	$77.17^{a} \pm 3.14$ (100)	$75.60^{a} \pm 3.57$ (98)	$77.28^{a} \pm 3.15$ (100)
K-gal		$2.10^{a} \pm 0.49$ (95)	$2.02^{a} \pm 0.27$ (91)	$1.97^{a} \pm 0.12$ (89)
K-gln+K-glc		$6.81^{a} \pm 1.45$ (70)	$7.22^{a} \pm 0.46$ (74)	$7.23^{a} \pm 0.36$ (74)
K		$1.79^{a} \pm 0.05$ (325)	$2.23^{a} \pm 0.02$ (404)	$1.95^{a} \pm 0.04$ (354)
Total K-der		$10.70^{a} \pm 0.71$ (86)	$11.46^{a} \pm 0.22$ (92)	$11.15^{a} \pm 0.17$ (89)
Total flavonols	$67.45^{a} \pm 0.79$ (93)	$87.9^{b} \pm 3.9$ (98)	$87.1^{b} \pm 3.8$ (97)	$88.4^{b} \pm 3.3$ (100)
Nar	$45.53^{a} \pm 0.72$ (90)	$45.99^{a} \pm 0.89$ (94)	$44.60^{a} \pm 0.36$ (91)	$44.63^{a} \pm 0.01$ (91)

Data are average ± SD. Percent recovery is indicated in parenthesis. Q-ud, unidentified quercetin derivative; Q-rut, Rutin; Q-gln, Quercetin-3-O-glucuronide; Q-glc, Quercetin-3-O-glucoside; Q, Quercetin; K-gal, Qaempferol-3-O-galactoside; K-gln, Kaempferol-3-O-glucuronide; K-glc, Kaempferol-3-O-glucoside; K, Kaempferol; Nar, Naringenin. Different letters within the same row (a,b) are significantly different (LSD, $p < 0.05$).

Upon autoclave treatment, quercetin and kaempferol glycosides and glucuronides decreased, while the corresponding aglycones increased. However, the recovery was ~ 100% when the sum of quercetin derivatives was considered and ~ 90% for the sum of kaempferol derivatives (Table 2.4). This means that the prevalent modification occurring during autoclave treatment was deglycosylation. Rohn et al. (2007) found that during the roasting process of model flavonols (180 ^{o}C, 60 min), quercetin glycosides were degraded and produced quercetin as the major degradation product and hence can be regarded as a stable end-product. Naringenin content was above 90%, with lower retention for the unfortified purees than for the fortified purees (Table 2.4).

Autoclave treatment also affected proanthocyanidin contents, which increased in the fortified puree with respect to the mixing (Table 2.2). In parallel, FRAP values increased in the fortified purees (Table 2.3). The intense thermal treatment could have weakened the binding between proanthocyanins and other food components (Pinelo et al., 2006), or it could have promoted proanthocyanidin depolymerisation (Chamorro et al., 2012), thus leading to increased proanthocyanidin solubility.

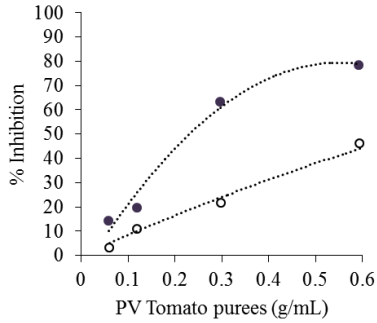

Figure 2.4. Dose-response curve for the inhibition of protein glycation by autoclaved PV puree (○) and their formulation with the GSS fraction (●). Formulations with GSM and GSL fractions behaved similarly.

Upon heat treatment, the dose-dependent antiglycation activity *in vitro* of the fortified purees remained higher than the controls measured with BSA/fructose model system, as can be seen in the figure 2.4. The I_{50} values of the fortified purees were found to be three fold lower with respect to the control puree. These new purees developed could have the potential ability to act as dietary factors in the prevention of complications relating to hyperglycaemia.

2.3.2. Physical characterisation

2.3.2.1. Effect of processing on PSD

Tomato puree generally forms structured suspensions consisting of cells and/or cell wall material dispersed and arranged in a liquid matrix phase, which is comprised of soluble materials such as polysaccharides, i.e., pectins and sugars, and some proteins (Bayod et al., 2011).

In the current study, assuming the particle shape to be spherical, two descriptors i.e., surface-weighed mean diameter, d(3,2) and volume-weighed mean diameter, d(4,3) were chosen to calculate the mean size. As shown in Table 2.5, after the mixing step, it was possible to notice that d(3,2) appeared to be very much influenced by the addition of the GSS fraction, while the variations induced by GSL and GSM were minor and not significant for PVGSL puree. A similar trend was observed for the mean d(4,3) diameter, which highlighted considerable changes for PVGSS puree (Table 2.5).

The effect of heat treatment on the PSD was observed as a decrease of the d(3,2) for PVGSS puree, but not for PVGSM and PVGSL purees. The d(4,3) parameter, which is mostly affected by the largest particles, increased significantly for the purees added with GSM and GSL fractions, but it did not show significant changes for those added with the GSS fraction. Hence, heat treatments caused both particle fragmentation, as shown by decreased d(3,2) and further aggregation, as observed by increased d(4,3) (Table 2.5).

Table 2.5. Surface-weighted mean diameter (d(3,2), μm) and Volume-weighted mean diameter (d(4,3), μm) of PV tomato puree formulated with grape skins (GS) fractions and control puree after mixing, microwave and autoclave treatments.

	d(3,2)			d(4,3)		
Puree	**Mixing**	**Microwave**	**Autoclave**	**Mixing**	**Microwave**	**Autoclave**
PV	$122.8^{cdx} \pm 0.4$	$122.1^{cx} \pm 1.8$	$123.6^{dx} \pm 0.5$	$323.5^{cx} \pm 1.0$	$327.7^{bxy} \pm 5.6$	$331.1^{cy} \pm 1.1$
PVGSL	$116.0^{bcx} \pm 3.8$	$115.2^{bcx} \pm 0.8$	$113.5^{cx} \pm 0.1$	$335.7^{dx} \pm 4.6$	$349.5^{cy} \pm 2.2$	$348.3^{dy} \pm 0.9$
PVGSM	$110.6^{bx} \pm 4.3$	$109.7^{bx} \pm 14.5$	$99.9^{bx} \pm 0.6$	$317.4^{bx} \pm 4.1$	$326.6^{by} \pm 6.7$	$323.4^{bxy} \pm 1.6$
PVGSS	$93.0^{ay} \pm 2.9$	$82.0^{ax} \pm 0.4$	$81.3^{ax} \pm 0.6$	$311.0^{ax} \pm 3.2$	$312.0^{ax} \pm 1.6$	$314.2^{ax} \pm 3.5$

Data are average ± SD. Different letters within the same column (a-d) or row (x,y) indicate significant differences (LSD, $p < 0.05$).

2.3.2.2. Effect of processing on Bostwick consistency

The flow properties of tomato products referred to as the gross viscosity or the consistency, are typically evaluated using a Bostwick consistometer. In this measurement, the distance the product flows in a given time is measured. More viscous juices flow shorter distances and thus, higher Bostwick values indicate a lower consistency. Bostwick value is a fundamental quality parameter for tomato derivatives and loss of consistency has to be considered as a negative effect (Anthon et al., 2010).

PV tomato puree subjected to concentration step and stored in an aseptic tank had low consistency values of 8.7 cm before heat treatment (Table 2.6). Indeed, during tomato concentration, there is a loss of consistency, which is greater for tomato paste. One proposed mechanism for loss in consistency is that the high osmotic and ionic strength in the paste causes changes in the polymeric materials in juice particles, altering the interactions between these particles (Anthon et al., 2010).

Upon mixing, the total solid content increased from 9.9 to 12.6% and a statistically significant rise in consistency was observed in PV formulations (Table 2.6). Interestingly consistency values in the fortified purees were in the range 5.3-6.0 cm showing that the particle size played a major role in consistency changes. In fact mixing, PVGSS puree exhibited higher increase than PVGSL puree, while PVGSM puree exhibited an intermediate behaviour (Table 2.6).

Table 2.6. Bostwick consistency (cm in 60 s) of PV tomato puree formulated with grape skins (GS) fraction after mixing, microwave and autoclave treatments.

	Bostwick consistency		
Puree	**Mixing**	**Microwave**	**Autoclave**
PV	$8.7^{cy} \pm 0.4$	$7.4^{cx} \pm 0.5$	$7.8^{dx} \pm 0.4$
PVGSL	$6.0^{bz} \pm 0.4$	$5.4^{by} \pm 0.2$	$4.8^{cx} \pm 0.2$
PVGSM	$5.9^{bz} \pm 0.1$	$4.8^{ay} \pm 0.0$	$4.2^{bx} \pm 0.1$
PVGSS	$5.3^{az} \pm 0.1$	$4.6^{ay} \pm 0.2$	$3.7^{ax} \pm 0.1$

Data are average ± SD. Different letters within the same column (a-c) and row (x,y) indicate significant differences (LSD, $p < 0.05$).

Upon both the microwave and the autoclave treatments, a further statistically significant increase in consistency was observed in the fortified purees and their corresponding control, especially in PVGSS puree (Table 2.6). In a previous study, it was hypothesized that modifications that contribute to increased component solubilisation, such as decreased particle size (as observed for PVGSS) and a rise in temperature, cause an increase in viscosity (Cordoba et al., 2012). In addition, a larger surface (as observed for PVGSM and PVGSS) might enable more physical entanglements of polymers and additional interactions (Moelants et al., 2014).

2.3.2.3. Effect of processing on rheological properties

The oscillatory frequency sweep tests were performed at a constant strain of 0.1%, which was found to be within the linear viscoelastic region for all the control and fortified purees, in the frequencies 0.1 - 10 Hz (Figure 2.5). The critical parameters that influence the rheological properties of tomato concentrate are the tomato variety, sieve pore size, processing temperature and the volume fraction of solids (Juszczak et al., 2013). All tomato purees showed low viscoelastic behaviour, as G' (storage modulus) dependency of oscillatory frequency was very small. Values of G' were always higher than those of G'' (loss modulus), which indicates that tomato purees had dominant elastic properties rather than viscous behaviour. Thus, the products can be classified as weak gels according to Rao (1999). This behaviour is typically observed in fruit products, as reported for peach puree (Massa et al., 2010), peach juice with fibres (Augusto et al., 2011) and tomato concentrates (Bayod et al., 2011).

Variation in G' and G'' of PV tomato puree formulated with GS fractions and control puree upon mixing was shown in figure 2.5. After addition of the GS fractions, similar mechanical spectra were observed with respect to the controls, but G' and G'' were both increased sharply, most likely due to

increased solid content in the puree. Similar effect was observed by Tiziani et al. (2005) upon addition of soy protein to tomato juice. The reason was explained as enhanced non-covalent aggregation among the macromolecules that increased the stability of the suspension. The values of G', calculated at 1 Hz, increased from 407 ± 57 Pa to ~ 935 ± 47 Pa and G" values increased from 61 ± 10 Pa to ~ 151 ± 21 Pa in the control PV puree and in the GS formulated PV purees respectively (Figure 2.5). Estimation of these parameters was not precise enough to evidence possible changes due to heat treatments.

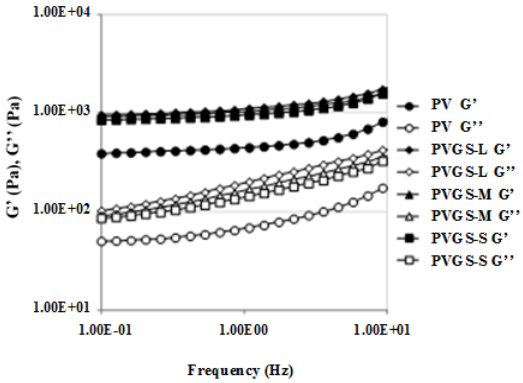

Figure 2.5. Frequency Sweep Test: Shift in the storage modulus (G', Pa) and loss modulus (G'', Pa) of PV tomato puree formulated with grape skins (GS) fractions with respect to control puree after mixing.

Values for η*, were then calculated at 1 Hz, to further investigate the effect of GS addition to tomato puree (Figure 2.6). These values increased from 60 ± 9 Pa.s in the control PV puree to ~ 134 ± 11 Pa.s in the GS formulated PV purees Hence, significant increase in η* values were observed when the control purees were added with the various GS fractions.

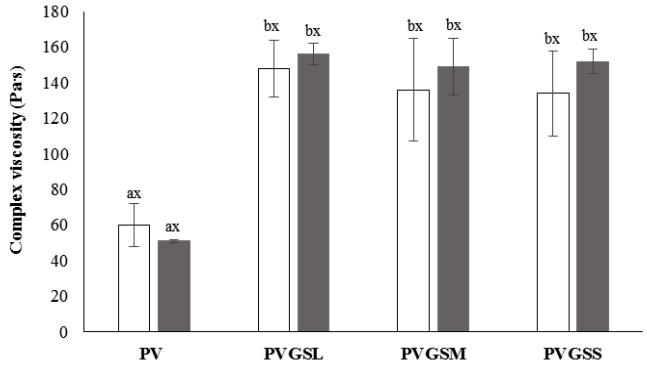

Figure 2.6. Complex Viscosity (η*, Pa.s) values (at 1 Hz) of control PV puree and PV purees formulated with grape skins (GS) after mixing (□) and autoclave treatments (■). Different letters among control puree and puree formulations (a,b) indicate significant differences (LSD, $p < 0.01$).

In the fortified purees, η* values appeared to be correlated with d(4,3) values ($R = 0.86$, $p < 0.01$) (Figure 2.7). Even if the exact nature of the interactions among solids of the GS fortified tomato purees cannot be derived, this result shows that by changing the particle size of GS ingredient in the range below 0.5 mm, major physical changes occur in the resulting suspension. There is no information in the literature on the optimal particle size of GS to be incorporated in a food fluid, but some information exists for other food matrices.

2. Fruit-based food formulated with grape skin antioxidant dietary fibre

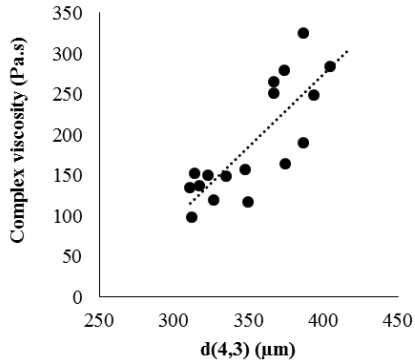

Figure 2.7. Correlations between d(4,3) (µm) and complex viscosity (Pa.s) for the PV and PR purees formulated with grape skins after mixing, microwave and autoclave treatments.

GS with particle sizes of 0.18 mm was selected for the fortification of yogurt and salad dressing, which caused a positive increase in viscosity (Tseng et al., 2013). On the contrary, GS had a negative effect on the instrumental texture of fish, and this effect was ascribed to the large granulometry, i.e., particle sizes ≤ 1 mm (Riberio et al., 2013). For meat products, GS having particle sizes ≤ 0.5 mm were selected, which did not cause changes in tenderness (Sáyago-Ayerdi et al., 2009). Other studies have evidenced positive effect of using GS as an ingredient to improve gel properties in milk for cheese manufacturing (Han et al., 2011) and to enhance the hardness and gumminess of a rye bread (Mildner-Szkudlarz et al., 2011), however the granulometry of the ingredient was not specified.

2.3.2.4. Effect of processing on colorimetric parameters

Colour is one of the most important quality characteristics for the tomato processing industry, since it has a marked influence on consumers' preference. Grape fibre addition is also expected to modify food colour, as previously observed for GS added to chicken breast hamburger (Sayago-Ayerdi et al., 2009) and to an ice cream formulation (Sagdic et al., 2011).

Table 2.7. Colorimetric parameters *L** and *a** of control PV puree and PV puree formulated with grape skins (GS) fractions after mixing, microwave and autoclave treatments.

	L*			***a****		
Puree	**Mixing**	**Microwave**	**Autoclave**	**Mixing**	**Microwave**	**Autoclave**
PV	$42.4^{by} \pm 0.1$	$42.4^{by} \pm 0.1$	$41.3^{ax} \pm 0.1$	$16.8^{cy} \pm 0.1$	$15.0^{xc} \pm 0.1$	$16.5^{cy} \pm 0.1$
PVGSL	$41.4^{ax} \pm 0.1$	$41.7^{ax} \pm 0.1$	$43.8^{by} \pm 0.1$	$12.3^{ax} \pm 0.1$	$14.0^{by} \pm 0.1$	$14.0^{ay} \pm 0.1$
PVGSM	$42.2^{bx} \pm 0.1$	$43.2^{cy} \pm 0.1$	$44.1^{cz} \pm 0.1$	$12.5^{ax} \pm 0.1$	$13.0^{ay} \pm 0.1$	$14.5^{bz} \pm 0.1$
PVGSS	$45.0^{cx} \pm 0.1$	$44.4^{dy} \pm 0.1$	$45.8^{dz} \pm 0.1$	$12.9^{bx} \pm 0.1$	$13.0^{ax} \pm 0.1$	$14.3^{by} \pm 0.1$

Data are average ± SD. Different letters within the same column (a-d) and row (x-z) indicate significant differences (LSD, $p < 0.05$).

Variations in *L** and *a** values were observed in tomato purees upon mixing with GS fractions (Table 2.7), whereas changes of *b** values were minimum (not shown). Variation in *L** and *a** parameters were higher in the PVGSS formulation than that of PVGSM and PVGSL. Higher *L** values in the purees added with GSS fraction could probably be due to backscattering of particles with diameter less than 30 µm, leading to white colour to the suspensions (Walstra, 2003). After the heat treatments, the *a** values increased for all the fortified samples, and moderately decreased for the controls. *L** values increased in the fortified puree. This result could be an effect of increase of small particles, as observed from decrease in d(3,2).

The total colour difference (ΔE_T) was also calculated using the sample before heat treatment as a reference. Values between 1.0 and 3.1 were observed for the microwaved purees, whereas values

between 2.0 and 3.0 were observed for the autoclaved purees (Lavelli et al., 2014). Accordingly, ΔE_T values in the range 2.42-3.79 were found by Giner et al. (2013) upon tomato juice pasteurization. These values indicate that the extent of colour modification in the fortified purees was low. In fact, in a previous study, a ΔE_T value of 5 was considered as a threshold for acceptable colour variation during tomato sterilization (Zanoni et al., 2003).

2.3.3. Sensory test

The prospective use of fibrous fractions in developing new functional tomato purees needs to be evaluated not only from an analytical point of view, but also by exploring the sensory acceptability of the formulations. In fact, consumers base their choices more on pleasantness rather than perceived healthiness (Lähteenmäki, 2006). So there is a need to study, if the physico-chemical changes occurring in the fortified purees are perceivable to consumers, by performing a liking test which provides an overall estimate of the acceptability. Since variations in particle sizes of fruit puree influences the texture (Moelants et al., 2013) and processing of fruit puree can affect colour (Lavelli et al., 2011), liking ratings for texture and colour were also investigated. The average liking ratings expressed by all 86 consumers for overall acceptability, colour and texture of the analysed tomato purees are reported here. Consumers rated the unfortified purees highly in terms of overall acceptability (6.9 ± 1.8 for PR; 6.7 ± 1.9 for PV), liking for colour (7.4 ± 1.7 for PR; 7.2 ± 1.7 for PV) and texture (7.0 ± 1.8 for PR; 6.8 ± 1.7 for PV) (Lavelli et al., 2014). The addition of the GS fractions to the tomato purees decreased the ratings for all the sensory parameters ($p < 0.05$). This effect could be explained considering the consumers familiarity with the unfortified samples which are commercially available and are under regular use, and they were not previously exposed to the fortified samples. As it is known, the level of familiarity for a food strongly influences its acceptability by the consumer and repeated exposure to the same taste of a food can increase liking for it (Wardle et al., 2008). Regarding the overall liking, average ratings of the fortified samples corresponded approximately to the central value of the scale (5 = neither like nor dislike). PVGSL, PVGSM and PVGSS were significantly preferred (5.3 ± 1.9) than PRGSL (4.6 ± 2.1) ($p < 0.05$) (Lavelli et al., 2014). Indeed liking rating inversely correlated with d(4,3) values ($R = -0.794$, $p < 0.01$), suggesting preference for formulations added with smallest particles (Figure 2.8).

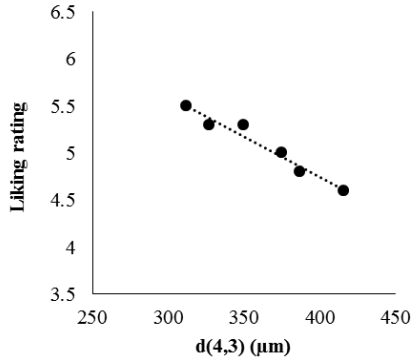

Figure 2.8. Correlations between d(4,3) (µm) and complex viscosity (Pa.s) for the PV and PR purees formulated with grape skins subjected to microwave treatment.

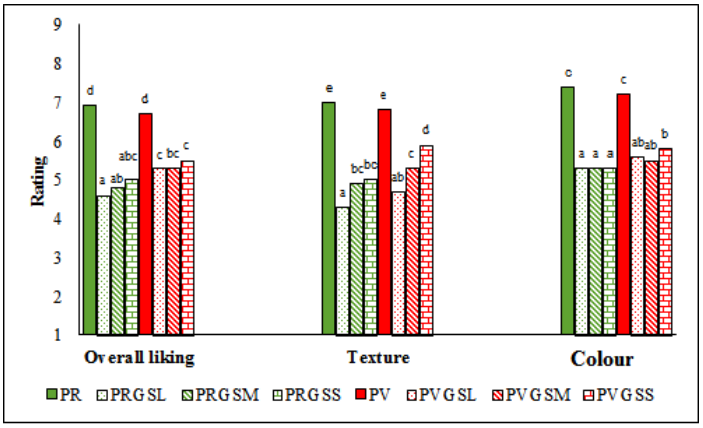

Figure 2.9. Overall liking and liking for colour and texture for PV and PR purees formulated with grape skins (GS) fractions subjected to microwave treatment.

Concerning the texture, as the particle size decreased, liking increased. This tendency was more evident for the PV formulations. Average ratings of liking for colour were all above the central value (5). The only significant difference in colour was observed for PVGSS, which was rated higher than the PR formulations (Lavelli et al., 2014). A plot of overall liking and the liking for colour and texture of PV and PR tomato purees formulated with GS fraction can be seen in figure 2.9.

2.4. CONCLUSIONS

Tomato purees formulated with GS could be considered more beneficial to improving potential health properties than the conventional purees, due to their much higher content of fibres and phytochemicals. Indeed, tomato is rich in lycopene, but it does not contain proanthocyanidins and hence the addition of GS improved its antioxidant and antiglycation properties *in vitro*. Upon processing, major changes in the fortified purees were both decrease in proanthocyanidin solubility and hydrolysis of flavonol glycosides. However, phenolic contents, reducing capacity and antiglycation activity remained ~ 3 times higher in all the fortified purees than in the controls.

The varying particle sizes of puree formulations had a statistically significant effect on proanthocyanidin solubility. This phenomenon could affect the potential nutritional and functional properties of the new formulations. In tomato purees formulated with GS, Bostwick consistency, G', G'' and η* increased. Indeed, GS particles can fill the space between tomato fragments trapping more water and create new entanglements causing more frictions resulting in varied flow properties.

Particle size also played a major role in the consumers' preference for the formulation. PVGSS, having the smallest particle sizes, had the maximum appreciation, with similar liking ratings to those of the control puree. Thus, this innovative functional puree can have a positive feedback by consumers.

The overall results obtained from this methodological approach could pave way for the optimization of fibrous byproducts incorporation into complex food fluids.

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3. ASSESSMENT OF SUPERCRITICAL-CO_2 OIL EXTRACTION FROM GRAPE SEEDS BY EVALUATION OF TOCOL CONTENT AND POTENTIAL USES OF THE DEFATTED CO-PRODUCT

3. Recovery of grape seed oil and phytochemicals

3.1. INTRODUCTION

Previous chapter has described the alternative use of grape skin ingredients as cost effective resources of antiglycation agents and their possible application as fortifying agents in the food industry. Another major component of grape pomace is grape seeds which constitute 38-52% of this byproduct (Maier et al., 2009).

Grape seeds are an excellent source of high value oil rich in therapeutically beneficial compounds such as sterols, fatty acids and tocols. The traditional extraction process for grape seed oil recovery is by *n*-hexane extraction, while mechanical pressing has a limited application because it is characterized by a low oil yield (Crews et al., 2006). Supercritical CO_2 (SC-CO_2) extraction can serve as an alternative green technology with respect to the traditional liquid solvent technique and could achieve a comparable oil yield. The physico-chemical properties of supercritical fluids are intermediate between those of a gas and a liquid and hence they can diffuse faster compared to the conventional solvents making supercritical fluid extraction ideal for higher extraction in less time. Moreover, CO_2 as a solvent is a non-toxic, non-flammable, non-polluting and cheap substance; and no solvent traces remain in the extracted oil. One of the drawbacks of this technique is the greater cost of investment linked to the supercritical technology. However, the operating costs can be lowered, since the process does not require a post extraction treatment. Therefore, the total costs are comparable to conventional systems, if the process is carried out at standard operating conditions and in a sufficient extractor volume (Sovová et al., 1994; Goodarznia et al., 1998) considering that the capital amortization sharply decreases when capacity increases (Perrut et al., 2000). In a design and feasibility study, the amount of exhausted grape pomace produced in a specific geographical region was considered and a SC-CO_2 plant with two extractors in series operating in the counter-current mode was sized accordingly, to simulate the extraction process under varying operational conditions. Energy inputs, investment and processing costs were then estimated and the proposed industrial application was found economically interesting (Fiori et al., 2010). Encouraging results concerning the scale-up of the SC-CO_2 process for grape seed oil extraction were also obtained by Prado et al. (2010).

SC-CO_2 process has been proposed to extract tocopherols and tocotrienols from various byproducts and unconventional sources for their use as nutraceuticals (Pereira et al., 2010; Kagliwal et al., 2011). In fact, both tocopherols and tocotrienols possess vitamin E activity, with numerous functions i.e., antioxidant, anti-inflammatory, anti-thrombotic effects and protection against damage caused by various pollutants (Constantinides et al., 2006). Tocopherols were found to be potent compounds in increasing superoxide dismutase (SOD) and nitric oxide synthase (NOS) activities. Tocotrienols play distinctive roles in suppressing the production of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG CoA) and also possesses *in vitro* anti-proliferative activity against various cancer cell lines (Constantinides et al., 2006; Choi et al., 2009).

Grape seeds contain high concentrations of phenolic compounds which include condensed tannins, flavanols, flavonols and phenolic acids (Sun et al., 1999). Hence, another application to be performed after SC-CO_2 extraction could be recovery of grape seed phenolics from the exhausted residue (Perez-Jimenez et al., 2008; Perumalla et al., 2011). Grape seed phenolics possess antioxidant, antibacterial, antiviral, anti-tumor, antidiabetic and anti-inflammatory properties (Jayaprakasha et al., 2003; Perumalla et al., 2011). Indeed, grape seed phenolics were proposed as functional food ingredients, since their bioavailability and efficiency in inhibiting oxidative stress was also evidenced *in vivo* (Quiñones et al., 2013).

In this study, the defatted and non-defatted grape seed phenolics were tested for their inhibitory ability towards starch digestion enzymes, which is a key step towards controlling blood glucose levels. *In vitro* studies have shown inhibitory effects of phenolic extracts of various fruits (Mc Dougall et al., 2005a) and unconventional plant sources (Palanisamy et al., 2011; Satpathy et al., 2011) on both α-glucosidase and α-amylase, the main enzymes involved in starch digestion. These studies have concluded that use of phenolics to treat diabetes by multiple mechanisms is a promising prospect. Although the results from dietary human interventions are still scarce, there are a few *in vivo* studies

reported with animal models. The antioxidant-rich grape pomace extract was found to suppress post-prandial hyperglycaemia in diabetic mice, most likely due to α-glucosidase activity inhibition (Hogan et al., 2010). da Silva et al. (2014) found that extracts rich in procyanidins inhibited α-amylase activity in rats, thus leading to prevention of post-prandial increase in blood glucose level. This application of grape seed phenolics could further increase the efficiency of winemaking from a bio-economy perspective.

A major challenge for the recovery of grape seeds from winemaking byproducts is the variability among these resources, which vastly depends on grape variety, climate, cultivation and winemaking conditions. Conversely, for the industrial exploitation of grape seeds, the oil quality should be demonstrated with reference to a standard (*Codex Alimentarius*). Hence, taking these variations under consideration, characterization studies were carried out for six grape varieties over two harvesting years (2011 and 2012).

Seeds obtained from six grape varieties were investigated with the following aims: a) Comparison of SC-CO_2 extraction technology with respect to traditional methods and their effects on oil yield, fatty acid composition and tocol content over two production years; b) Evaluation of the effect of SC-CO_2 oil extraction on the total phenolic content of grape seeds; c) Determination of the efficiency of the grape seed phenolic extracts on inhibition of starch digestive enzymes *in vitro* with respect to the conventional pharmaceutical drug acarbose.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

Carbon dioxide (4.0 type, purity greater than 99.99%) used as a supercritical solvent was purchased from Messer (Padova, Italy). *n*-Hexane for the atmospheric pressure extraction was purchased from Sigma Aldrich (Milano, Italy). R-tocopherol isomers and R-tocotrienol isomers were obtained from VWR International PBI (Milano, Italy). Rat intestinal acetone powder (N1377-5G), *p*-nitrophenyl α-D-glucoside (*p*-NPG), acarbose, porcine pancreatic α-amylase, type VI-B (A3176), *p*-nitrophenyl α-D-maltopentaoside (*p*-NPGP) and all other chemicals were purchased from Sigma Aldrich (Milan, Italy).

3.2.2. Grape seeds

Three red grape pomace samples of Barbera (BA), Nebbiolo (NE), Pinot Noir (PI) and three white grape pomace samples of Chardonnay (CH), Moscato (MO), Muller Thurgau (MT) were obtained from winemakers in Northern Italy, for the two production years of 2011 and 2012. At the winery, stalks were separated from the seeds and skins. The mixture of seeds and skins was taken to the laboratory and stored at -20 ^{o}C before drying. The samples were dried at 55 ^{o}C for 48 h, and then the skins and seeds were separated by means of vibrating sieves (Figure 3.1). Dried grape seeds were milled using a grinder (Sunbeam Osterizer blender, Boca Raton, USA). To avoid overheating, the sample was flaked for 10 s, then grinding was halted and the sample was shaken for another 10 s, and the milling process was continued. Each variety was then separated into two batches. One batch was subjected to defatting by employing SC-CO_2 extraction process. Another batch was characterized prior to oil extraction. To standardize phenolic extraction, defatted samples (DF) and non-defatted samples (NDF) were then sieved by using the Octagon Digital sieve shaker (Endecotts Ltd., United Kingdom), with three certified sieves (openings: 125, 250 and 500 μm), by continuous sieving for 10 min at amplitude 8. The medium fraction (≥125 μm ≤ 250 μm) was collected and stored under vacuum in dark at 4^{o} C.

3. Recovery of grape seed oil and phytochemicals

Barbera seeds **Chardonnay seeds**

Figure 3.1. Grape seeds of red (Barbera (BA)) and white (Chardonnay (CH)) varieties recovered from winemaking byproducts.

3.2.3. Extraction of grape seed oil

3.2.3.1. Supercritical-CO_2 extraction

The supercritical extraction equipment (Proras, Rome, Italy, shown in figure 3.2) and procedure were previously described (Fiori, 2007). Referring to the original assembly (Fiori, 2007), the system was further improved by the introduction of amini Cori-Flow digital mass flow meter (Bronkhorst, Ruurlo, The Netherlands) placed on the liquid CO_2 line upstream of the CO_2 pump; the CO_2 consumption was totalized and recorded during the experiments by this additional flow meter. Another improvement is represented by the utilization of a tailor made spacer in order to place the extraction basket close to the exit of the extraction vessel, which assures meaningful measurement of the extraction kinetics (Fiori, 2007). The extraction basket utilized in this study had an internal volume of 0.1 L and, for each test, batches of about 65 g of grape seeds were placed in the basket and utilized for the extraction. Pressure and temperature were kept constant during the different tests. The tests were performed at a pressure of 500 bar and a temperature of 50 ^{o}C and the solvent flow rate was fixed at about 8 g/min. After extractions, the particle size distribution of the exhausted grape seeds was evaluated by utilizing sieves having different mesh sizes placed in a vibrating device (Automatic Sieve Shaker D406control, Auckland, New Zealand).

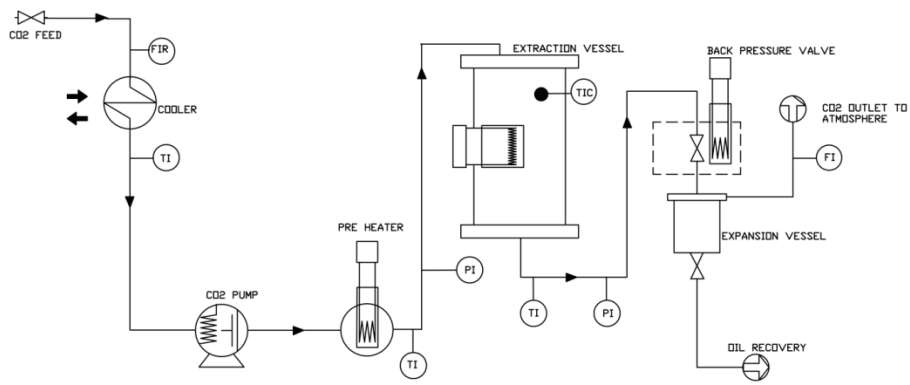

Figure 3.2. Scheme of SC-CO_2 pilot plant extraction by Fiori et al. (2007).

3.2.3.2. Soxhlet extraction

Soxhlet extraction was performed in a SER 148/3 (Velp Scientifica, Usmate, Italy) solvent extractor, which works according to the Randall technique with three samples in parallel. Batches of 10 g of

milled grape seeds were placed in each extraction thimble and the relevant extraction cup was filled with 60 mL of *n*-hexane. The Randall technique foresees the sample inside the thimble to be immersed in the boiling solvent (in the present case at 69 °C, the boiling temperature of *n*-hexane at atmospheric pressure). The immersion step was followed by a washing step, where the extraction was completed according to the standard Soxhlet technique. The immersion and the washing steps lasted for one and 3 h, respectively. Solvent recovery was made in rotary evaporator (Heidolph, Schwabach, Germany) at a reduced pressure of 335 mbar, bath temperature of 40 °C and rotation speed of 30 rpm.
Fat evaluation allowed all measurements to be referred to dry weight subtracted by fat during the analysis of DF and NDF grape seed extracts.

3.2.3.3. Mechanical extraction

The mechanical extraction was performed by means of a press machine (Galdabini, PMA/10, Cardano al Campo, Italy) equipped with a stainless steel punch and a stainless steel high strength specimen holder specially built for this purpose in the workshop at the University of Trento (Italy). The ground seeds were placed in the holder and the press machine applied a force to the punch growing up to a maximum value of 100 kN. Oil surfaced from the edges of the punch was collected and used for analysis.

3.2.4. Analysis of grape seed oil

3.2.4.1. Nuclear magnetic resonance (NMR) and Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

The qualitative analysis of the crude oils was carried out by ^{1}H-NMR and MALDI-TOF-MS techniques at the University of Trento (Italy).
^{1}H-NMR spectra were recorded on a Bruker-Avance 400 MHz NMR spectrometer (Bruker Inc., Bremen, Germany) operating at 400.13 MHz for ^{1}H-NMR and at 100.61 MHz for ^{13}C-NMR by using a 5 mm BBI probe with 90° proton pulse length of 9 μs (transmission power of 0 db) with a delay time between acquisitions of 30 s. All spectra were taken at 25 °C in $CDCl_3$ (700 μL, 50-100 mM solution). The chemical shift scales (δ) were calibrated on the residual signal of $CDCl_3$ at δ_H 7.26 ppm.
MALDI-TOF was a model Ultraflex (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser and with a reflectron. The acceleration voltage was set at 20 kV. For desorption of the components, a nitrogen laser beam (λ = 337 nm) was focused on the template. The laser power level was adjusted to obtain high signal-to-noise ratios, while ensuring minimal fragmentation of the parent ions. All measurements were carried out in the delayed extraction mode, allowing the determination of monoisotopic mass values (*m/z*; mass-to-charge ratio). After crystallization at ambient conditions, positive ion spectra were acquired in the reflectron mode, giving mainly sodiated adducts ($[M+Na]^+$). Samples were directly applied onto the stainless-steel spectrometer plate as 1 μL droplets, followed by the addition of 1 μL of 2,5-dihydroxybenzoicacid (DHB) (0.5 M in CH_3OH). Every mass spectrum represents an average of about 100 single laser shoots.

3.2.4.2. Chromatographic analysis

3.2.4.2.1. Fatty acid methyl esters (FAMEs)

The transesterification was carried in basic media on 200 μL of crude oil, at room temperature, by adding 5 mL of a 0.5 M solution of KOH in methanol for 3 h avoiding any contamination with water and was monitored using TLC (*n*-hexane/ethyl acetate 93:7 v/v). After neutralization of the basic solution with sulphuric acid and in vacuum evaporation of the organic solvents (Rotovapor, Heidolph, Schwabach, Germany), FAMEs were isolated by flash chromatography on Silica gel with *n*-hexane/ethyl acetate gradient elution (initial fractions), whilst oxidized lipids and phytosterols were eluted later and were not further analyzed.
A Thermo-Finnigan Trace GC Ultra (Thermoquest, Rodano, Italy), equipped with a flame ionization

detector (FID) and a Trace DSQ quadrupole mass spectrometer, was used to carry out the GC-MS analysis of FAMEs. DB-WAX (30 m x 0.250 mm x 0.50 µm) was used as a chromatographic column. The temperatures of the injector and detector were kept constant at 250 ^{o}C and 280 ^{o}C, respectively. The flow-rate of the carrier gas (He) was 1.4 mL/min. The source and the transfer line were kept at 300 ^{o}C. The detector gain was set at 1.0×10^5 (multiplier voltage: 1326 V). For every chromatographic run, 1.0 µL of sample solution was injected. The oven program was set with an initial temperature of 50 ^{o}C held for 1 min, followed by a linear ramp from 50 to 200 ^{o}C at 25 ^{o}C/min and from 200 to 230 ^{o}C at 3 ^{o}C/min. The final temperature of 230 ^{o}C was held for 19 min. The source filament and the electron multiplier were switched off during the initial 5 min to avoid the detection of the solvent front. Mass spectra were recorded both with 70 eV electron impact ion (EI) and chemical ionization (CI) ion sources. The mass range scanned was from *m/z* 50 to *m/z* 500 at 500 amu/s. Data were collected and processed with Xcalibur (version 1.4). FAMEs were identified by comparing their retention times with those of a reference solution run at identical GC conditions and by matching the MS spectra with the MS-library implemented in the GC apparatus. GC analysis was performed in duplicate and the results were expressed as the percentage of total fatty acids (mean FID area ratio).

3.2.4.2.2. Tocols

Grape seed oil was diluted with *n*-hexane to a final concentration of 10 mg/mL and directly analyzed for tocol content by HPLC in triplicate. The HPLC equipment consisted of a model 600 HPLC pump (Waters, Vimodrone, Italy) coupled with a model X-20 fluorimetric detector (Shimadzu, Milan, Italy) operated by Empower software (Waters). A sample volume of 50 µL was injected. Chromatographic separation of the compounds was achieved with the normal phase method of Panfili et al. (2003). In brief, a 250 x 4.6 mm i.d., 5 µm particle size, Kromasil Si column (Phenomenex, Torrance, CA) was used. The mobile phase was *n*-hexane/ethyl acetate/acetic acid (97.3:1.8:0.9 v/v/v) at a flow-rate of 1.6 mL/min. Fluorimetric detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm.

3.2.5. Analysis of defatted (DF) and non-defatted (NDF) grape seeds

3.2.5.1. Moisture content

Moisture content of DF and NDF grape seed samples was determined in triplicate by drying in a vacuum oven at 70 ^{o}C and 50 Torr for 18 h.

3.2.5.2. Sample extraction

About 1 g of grape DF and NDF seed samples was extracted in duplicate with 16 mL methanol/0.1% HCl, for 2 h at room temperature with continuous stirring. The mixture was centrifuged at 10,000 *g* for 10 min, the supernatant recovered and the solid residue was re-extracted using 12 mL of the same solvent twice. The three supernatants were pooled and stored in the dark at -20 °C until performing characterization studies.

3.2.5.3. Total phenolics, proanthocyanidins and ferric ion reducing antioxidant power (FRAP)

The Folin-Ciocalteu and proanthocyanidins determination and FRAP assay were performed as described in materials and methods section of chapter 1.

3.2.5.4. Anthocyanins

Anthocyanin content was analyzed as described previously (Lee et al., 2005). In brief, each extract was diluted in both 0.025 M potassium chloride buffer, pH 1.0, and 0.4 M sodium acetate pH, 4.5. Dilution factor was chosen until absorbance at 520 nm was within the linear range. The absorbance of sample diluted with pH 1.0 and pH 4.5 buffer was then determined at both 520 and 700 nm versus distilled water. For each extract, 2 dilutions were assessed in triplicate. Anthocyanin concentration was expressed as mg of cyanidin-3-glucoside equivalents (CyE) per kilogram of dry product, as follows:

$$CyE\ (mg/kg) = [(A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}]*MW*d*1000/\varepsilon*C \quad (3.1)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), d is the dilution factor, ε is the molar extinction coefficient of cyanidin-3-glucoside (26,900 $M^{-1}cm^{-1}$), C is the concentration of the extract (kg/L). HPLC-DAD-MS analysis of anthocyanins was performed as described previously (Sri Harsha et al., 2013).

3.2.5.5. *In vitro* α-glucosidase and α-amylase inhibition

A crude α-glucosidase solution was prepared from rat intestinal acetone powder. Briefly, 200 mg of rat intestinal acetone powder was dissolved in 4 mL of 50 mM ice cold phosphate buffer (pH 6.8) and sonicated for 15 min at 4 ^{o}C. The suspension was vortexed for 20 min and then centrifuged at 10,000 *g* at 4 ^{o}C for 30 min. The resulting supernatant was used for the assay. For the α-glucosidase activity assay, 650 µL of 50 mM phosphate buffer, pH 6.8; 100 µL of the enzyme solution and 50 µL of DF or NDF grape seeds extract were added in eppendorf tubes and pre-incubated for 5 min at 37 ^{o}C. Then, 200 µL of 1mM *p*NPG was added as substrate and the mixture was further incubated at 37 ^{o}C for 25 min.

For the pancreatic α-amylase assay, 650 µL of 50 mM phosphate buffer, pH 6.8, 200 µL of the enzyme solution (10 µM in the same buffer) and 50 µL of DF or NDF grape seeds extract were added in eppendorf tubes, pre-incubated for 5 min at 37 ^{o}C. Then, 200 µL of 1mM *p*NPGP was added to the tubes as substrate and the mixture was further incubated at 37 ^{o}C for 55 min.

For both the enzymatic reactions, the assay mixture was centrifuged at 10,000 *g* for 3 min and the absorbance of the clear supernatant was recorded at 405 nm. The control was run by addition of the diluent (methanol/0.1% HCl) replacing the sample. A sample blank and a control blank were run without addition of substrate and without addition of both substrate and sample, respectively.

Acarbose was used as a control for both enzymatic reactions. Percentage of inhibition was calculated using the formula:

$$\%\ Inhibition = [(A_c - A_{cb}) - (A_s - A_{sb})/ (A_c - A_{cb})] \times 100 \quad (3.2)$$

Where A_c, A_{cb}, A_s and A_{sb} are the values of absorbance of the control, control blank, sample and sample blank, respectively.

A dose-response curve was built for samples and acarbose and results are reported as the amount of sample/standard that inhibits reaction by 50% (I_{50}).

3.2.6. Statistical analysis of data

Experimental data were obtained at least in duplicate and analyzed by one-way ANOVA with the least significant difference (LSD) as a multiple range test using Statgraphics 5.1 (STCC Inc.; Rockville, MD). Results were reported as average ± standard deviation (SD) or ± standard error (SE) as specified.

3.3. RESULTS AND DISCUSSION

3.3.1. Extraction kinetics

Figure 3.3 shows kinetic curves of oil extraction from grape seeds by SC-CO_2 for all the six varieties: the yield, expressed as gram of extracted oil per gram of seeds, is reported as function of extraction time in minutes. A high pressure of 500 bar was selected for a faster extraction rate and minimum solvent consumption. The choice of operating condition at 50 ^{o}C was in accordance with typical supercritical fluid extraction temperatures utilized for the recovery of tocol from grape seeds and other sources (Beveridge et al., 2005; Pereira et al., 2010).

In figure 3.3, the curves show a typical linear trend up to 80 min, due to the extraction of oil that is released by the breakdown of the oil-bearing cells during the grinding of the grape seeds. Extraction curves mostly overlap in their linear part, and the small differences could be due to a slightly different solvent flow rate (controlled by a manual valve) in the various tests. This is followed by flattening of the curves due to the decrease in the extraction rate, since the oil outside the oil-bearing cells was completely extracted and the solvent began to recover the oil still contained in the cells. The slow phase extraction was different among grape varieties due to different oil contents and lasted from 80 min to about 160 min. Extraction curves were modelled by means of the approximate form of the broken and intact cell model by Sovová (1994). The model fitted the experimental data to a very satisfactory extent and a reference value for the internal mass transfer parameter was found as representative for all the six varieties: $k_s a_p = 10^{-4} s^{-1}$, which is useful for process scaling-up (Fiori et al., 2014).

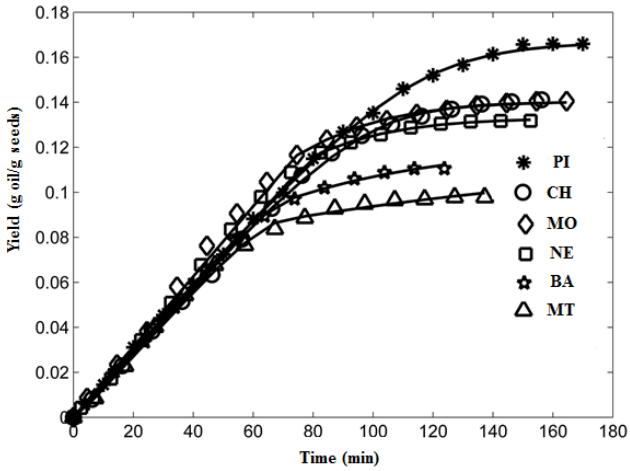

Figure 3.3. SC-CO_2 extraction kinetics: experimental points and modelled curves for the six grape varieties. Pressure equal to 500 bar, temperature equal to 50 ^{o}C, harvesting year 2012.

3.3.2. Characterisation of grape seed oil

3.3.2.1. Oil yield

SC-CO_2 extractions were performed at least twice and *n*-hexane extractions were repeated at least three times for each variety and harvesting year. The surface-weighed mean diameter of the milled particles used for extraction was lower than 0.5 mm in all the cases. The oil yield ranged between 10.1% MT (SC-CO_2, 2012) and 16.6% CH (*n*-hexane, 2011) (Fiori et al., 2014). An oil yield of 3.95-12.4% was reported for grape cultivars (Fernandes et al., 2013), while Passos et al. (2009) found oil yields of 11.5% and 16.5% with and without enzymatic treatment before SC-CO_2 extraction, respectively. Da Porto et al. (2013) reported 14% oil yield using Soxhlet and ultrasound assisted extraction. In general,

oil yield depends on several factors, such as: the variety, the environmental factors that influence the ripening of the grapes, the type of seed pre-treatment, the extraction technique adopted, the type of solvent used and operating conditions applied. The yields obtained with *n*-hexane were ~ 10% higher than those obtained with SC-CO_2 for only 5 out of 12 grape seed batches, while for the remaining, the oil yields were not significantly different ($p < 0.05$). The effect of harvesting year on the yield of SC-CO_2 extraction process was statistically significant (up to 25% variability) for 2 out of 6 grape seed batches (Fiori et al., 2014). Similar variations in oil yield in different harvesting years were observed by Agostini et al. (2012).

3.3.2.2. Qualitative analysis by ^{1}H-NMR and MALDI-TOF

The crude grape seed oil samples obtained by SC-CO_2 extraction were analyzed by ^{1}H-NMR. This allows for the obtainment of overall chemical composition of the samples (Figure 3.4). ^{1}H-NMR spectra showed that all the extracts were largely dominated by triacylglycerols (TAGs, ~ 98%), with presence of minor amounts of 1,2 diacylglycerols (1-2% of 1,2 DAGs) and oxidized lipids (0.1-0.3% as hydroperoxides). The presence of DAGs was established by the presence of ^{1}H-doublet signal at δ_H 3.72 ppm attributable to proton at sn-2 position, whilst oxidized lipids showed the characteristic olefinic protons of the conjugated diene system at δ_H 6.56, 5.98 and 5.76 ppm. The presence of unsaturated ω-3 lipids is near or below the NMR detection limit (≤ 0.5%) as confirmed by the presence in the ^{1}H-NMR spectrum of a weak triplet at δ_H 0.969, a structural feature for homo-allylic Me group in unsaturated ω-3 fatty chains. Finally, the presence of phytosterols (mainly β-sitosterol) was established to represent only a minor contribution (0.2-0.5%) to the overall composition of these oil extracts. No significant differences were noticed in the relative amounts of these minor metabolites (DAGs, oxidized lipids and phytosterols) with respect to major TAGs components in the different samples. The integration of the ^{1}H-NMR signals attributable to lipids with different number of unsaturations were performed to establish the quantitative distribution among saturated (SFA), mono-unsaturated (MUFA) and di-unsaturated (DUFA) acyl chains on the glycerol backbone. The relative amount of SFA ranged between 8.7-12.8, DUFA ranging from the lowest limit of CH (70.3%) to the highest of MT (74.9%) and MUFA ranging from the highest limit of CH (19.0%) to the lowest limit of NE (14.3%) (Fiori et al., 2014).

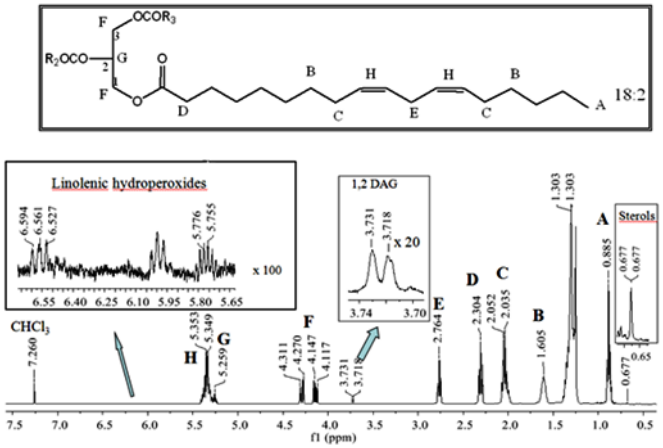

Figure 3.4. ^{1}H-NMR spectrum of Moscato (MO) grape seed oil by SC-CO_2 extractions. Letters represent the attribution of ^{1}H-NMR signals to specific protons of the linoleic acyl chain reported in the top box.

The above data obtained from ^{1}H-NMR was further supported by MALDI-TOF mass spectral data. In fact, most of the major TAGs contained the linoleic (18:2) acyl chain. A total of 7 TAGs were identified among which trilinolein (LLL) was the most abundant detected as Na^+ adduct at *m/z* 901.8.

Among the others, triolein (OOO) and palmitoyl-diolein (POO) did not contain any linoleic chains. The major TAGs found were: PLL (16:0,18:2,18:2)detected at *m/z* 877.8, POL (16:0,18:1,18:2) at *m/z* 879.8, POO (16:0,18:1,18:1) at *m/z* 881.8, LLL (18:2,18:2,18:2) at *m/z* 901.8, OLL(18:1,18:2,18:2) at *m/z* 903.8, OOL (18:1,18:1,18:2) at *m/z* 905.8 and finally OOO (18:1,18:1,18:1) at *m/z* 907.8 (Fiori et al., 2014).

3.3.2.3. Quantitative analysis of FAMEs

A complete analysis of the acyl chains diversity was carried out on FAMEs obtained by alkaline trans-esterification followed by Silica gel flash chromatography. The last step in the chromatographic process implied that only FAMEs deriving from TAGs and DAGs (~ 98% of the overall oil content) were analysed. Oxidized linoleic acid (deriving from hydrolysis of oxidized TAGs) and phytosterols had higher polarity on silica column and hence were not present in chromatographic fractions containing the FAMEs. Figure 3.5 reports a chromatogram depicting the fatty acid profile of MO grape seed oil, where the retention time of the various assigned peaks is evidenced.

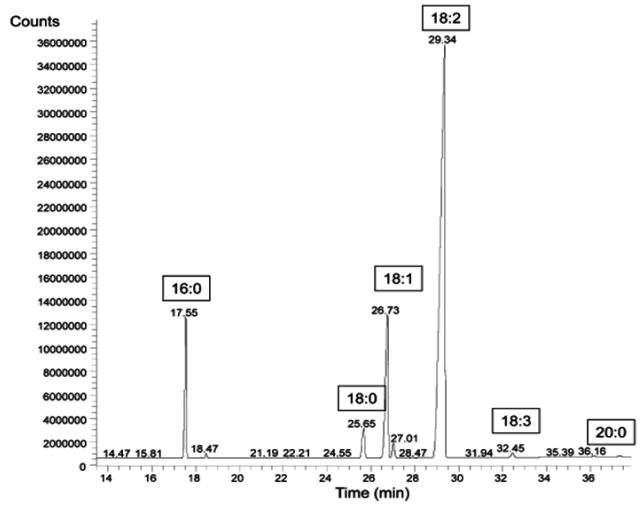

Figure 3.5. Fatty acid profile of Moscato (MO) grape seed oil by SC-CO_2 extraction as analysed by GC-FID. EI-MS spectra data was used to assign the reported peaks above.

In general, the major fatty acids identified in grape seed oils were linoleic acid (C18:2 ω6, 70.4-74.3%), oleic acid (C18:1 ω9, 13.6-16.8%), palmitic acid (C16:0, 6.53-8.89%), and stearic acid (C18:0, 2.84-4.16%) (Fiori et al., 2014). The amounts of these major fatty acids were in the intervals of values indicated for grape seed oil in the *Codex Alimentarius* standard, which however are much wider than those observed in this study. Other fatty acids in minor amounts detected in grape seed oils were myristic acid (C14:0), heptadecanoic acid (C17:0), linolenic acid (C18:3 ω3), arachidic acid (C20:0), eicosenoic acid (C20:1 ω9), eicosadienoic acid (C20:2 ω6). The fatty acid contents of grape seed oils extracted by SC-CO_2 did not vary significantly ($p < 0.05$) with respect to those of oils extracted by mechanical pressure. As a representative variety, BA was chosen to show the fatty acid composition (Table 3.1).

Table 3.1. Fatty acid composition (%) of the oil obtained from Barbera (BA) grape seeds subjected to SC-CO_2 extraction.

Fatty acid	Percentage
C14:0	0.073 ± 0.004
C16:0	6.660 ± 0.2
C17:0	0.047 ± 0.003
C18:0	4.040 ± 0.02
C18:1 (ω-9)	16.000 ± 0.4
C18:2 (ω-6)	71.700 ± 0.1
C18:3 (ω-3)	0.470 ± 0.01
C20:0	0.140 ± 0.01
C20:1 (ω-9)	0.130 ± 0.01
C20:2 (ω-6)	0.035 ± 0.004

Data are average ± SD.

3.3.2.4. Tocol content

The total content of tocols in grape seed oils obtained from different varieties by extraction with supercritical CO_2, *n*-hexane and mechanical extraction is shown in Table 3.2.

Table 3.2. Total tocol contents (mg/kg) of grape seed oils obtained from six grape varieties by SC-CO_2, *n*-hexane and mechanical extraction (harvesting year 2012)

Variety	Tocol content		
	SC-CO_2 extraction	***n*-Hexane extraction**	**Mechanical extraction**
BA	500^{b} ± 41	342^{a} ± 20	516^{b} ± 34
CH	381^{b} ± 34	263^{a} ± 32	398^{b} ± 11
MO	355^{b} ± 53	143^{a} ± 4	329^{b} ± 18
NE	559^{b} ± 8	442^{a} ± 49	520^{ab} ± 18
PI	436^{a} ± 14	436^{a} ± 89	438^{a} ± 86
MT	378^{a} ± 12	333^{a} ± 20	359^{a} ± 15

Data are average ± SD. Different letters within the same row (a,b) indicate significant differences (LSD, $p < 0.05$) due to the extraction technology.

The total tocol contents of the six grape seed oils extracted by SC-CO_2 ranged between 355 (MO) and 559 (NE) mg/kg. According to the *Codex Alimentarius*, the levels of tocopherols and tocotrienols in crude grape seed oil are in the range of 240-410 mg/kg. Based on this standard, NE and BA oils had higher total tocol contents, while the other varieties were in a similar range (Table 3.2). The oil extracted by *n*-hexane showed a lower content of tocols as compared with SC-CO_2 extraction. The results obtained for mechanical extraction showed that tocol content were more similar to those obtained by extraction with SC-CO_2: the values were between 329 mg/kg (MO) and 520 mg/kg (NE). It should be noted that mechanical extraction is considered as a process with minimal impact on oil quality (Crews et al. 2006).

Irrespective of the extraction technology, the content of tocols was highest in NE and BA, while it was lowest in MO (Table 3.2). Crews et al. (2006) reported a wide range for tocol contents in grape seed oils extracted with *n*-hexane (63-1208 mg/kg) following a survey of winemaking sites in France, Italy and Spain, which are the major grape producers in the world. However, information is scarce with reference to tocol contents of oils extracted by SC-CO_2.

Table 3.3. Tocopherol and tocotrienol contents (mg/kg) of grape seed oils obtained from six grape varieties and in different harvesting years (2011 and 2012) extracted by SC-CO_2.

		Tocol				
Variety	**Year**	**α- Tocopherol**	**α-Tocotrienol**	**γ-Tocopherol**	**γ-Tocotrienol**	**Total**
BA	2011	$166^{a} \pm 17$	$154^{a} \pm 13$	$33^{a} \pm 5$	$114^{a} \pm 12$	$467^{a} \pm 46$
	2012	$196^{a} \pm 6$	$97^{a} \pm 42$	$55^{a} \pm 2$	$151^{a} \pm 3$	$500^{a} \pm 41$
CH	2011	$62^{a} \pm 7$	$137^{a} \pm 15$	$14^{a} \pm 1$	$95^{a} \pm 15$	$309^{a} \pm 28$
	2012	$68^{a} \pm 6$	$122^{a} \pm 11$	$21^{b} \pm 1$	$170^{b} \pm 17$	$381^{a} \pm 34$
MO	2011	$126^{a} \pm 7$	$114^{b} \pm 8$	$30^{a} \pm 3$	$106^{a} \pm 3$	$376^{a} \pm 15$
	2012	$131^{a} \pm 14$	$81^{a} \pm 13$	$33^{a} \pm 6$	$110^{a} \pm 21$	$355^{a} \pm 53$
NE	2011	$118^{a} \pm 7$	$189^{a} \pm 12$	$39^{a} \pm 1$	$129^{a} \pm 9$	$475^{a} \pm 26$
	2012	$157^{a} \pm 21$	$170^{a} \pm 5$	$53^{b} \pm 4$	$179^{b} \pm 4$	$559^{a} \pm 8$
PI	2011	$113^{b} \pm 6$	$105^{b} \pm 2$	$29^{b} \pm 1$	$152^{a} \pm 4$	$399^{a} \pm 11$
	2012	$79^{a} \pm 9$	$82^{a} \pm 7$	$23^{a} \pm 4$	$253^{b} \pm 2$	$436^{b} \pm 14$
MT	2011	$77^{b} \pm 10$	$129^{a} \pm 17$	$24^{b} \pm 2$	$176^{a} \pm 21$	$406^{a} \pm 44$
	2012	$51^{a} \pm 2$	$98^{a} \pm 4$	$18^{a} \pm 2$	$212^{a} \pm 4$	$378^{a} \pm 12$

Data are average ± SD. Different letters within the same column (a,b), for the same variety and between the years of collection, indicate a significant difference (LSD, $p < 0.05$).

Beveridge et al. (2005) observed higher tocol contents in grape seed oils extracted by SC-CO_2 from BA (701 mg/kg) and PI (606 mg/kg) than those observed in the current study. These differences could be due to different geographical origin and maturity stage of these varieties and on different handling of seeds after collection. In fact, in the study by Beveridge et al. (2005), grape pomace was freeze-dried and butylated hydroxyl toluene was added to the oils to prevent oxidation, whereas in this study a cost-effective air-drying was selected with no addition of additives. Beveridge et al. (2005) also found that most of the oils extracted by SC-CO_2 had similar tocol contents with respect to those extracted by *n*-hexane, but for some varieties SC-CO_2 extraction was more efficient. Mechanical extraction was not considered.

In order to take into consideration the effect of harvesing year on tocol level, data collection was done in both 2011 and 2012 (Table 3.3). In general, the harvesting year had no effect on total tocol content of the oils except for PI, where the tocol content was significantly lower in 2011 (by 10%) than in 2012 ($p < 0.05$) (Table 3.3). Hence, similar tocol contents could be forecasted in the future harvesting years.

Regarding tocol composition of the oils, the major tocol compounds were identified to be α-tocotrienol, γ-tocotrienol, α-tocopherol and γ-tocopherol, whereas the δ and β-isomers were below the limit of detection for all the oils (2 mg/kg) (Figure 3.6). γ-tocotrienol was found to be the prevalent tocol for all the varieties characterized (Table 3.3). γ-tocotrienol is a promising antioxidant compound for prevention of both cardiovascular disease and cancer (Constantinides et al., 2006) and hence grape seed oils could find applications in the nutraceutical, food and cosmetic industries.

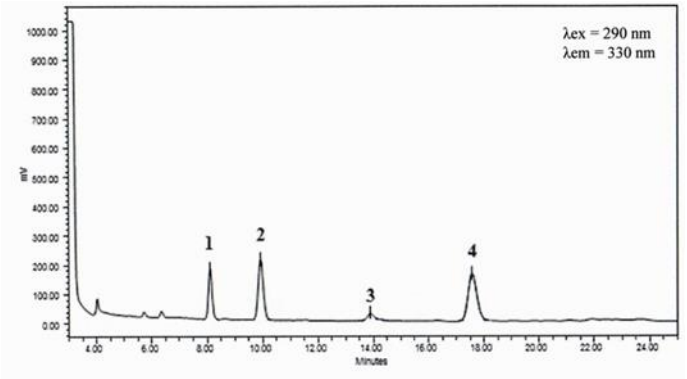

Figure 3.6. HPLC chromatogram for tocol identification in Muller Thurgau (MT) grape seed oil. The tocol peaks were identified to be: 1) α-Tocopherol; 2) α-Tocotrienol; 3) γ-Tocopherol; 4) γ-Tocotrienol.

3.3.3. Characterisation of defatted (DF) and non-defatted (NDF) grape seeds

3.3.3.1. Phenolic content

After the grape seed oil was extracted by SC-CO_2, the exhausted grape seeds (DF) and a batch of NDF grape seeds obtained from the six grape varieties were characterised for their phenolic content, reducing capacity and inhibitory efficiency against starch digestive enzymes for a possible use in the control of blood glucose level.

Both DF and NDF grape seed extracts showed significant variability among the samples for their total phenolic content (Table 3.4). Total phenolics ranged between 49 (NE) and 277 (PI) g GAE/kg for DF, while for NDF, the values ranged between 42 (BA, NE) and 233 (PI) g GAE/kg. Differences in total phenolics were mainly attributable to the sample rather than to the SC-CO_2 treatment (Table 3.4). These differences observed could be influenced by genetic factors, pedo-climatic factors, exposure to the sunlight, geographical location, the type of soil and winemaking conditions. However, the concentration of total phenolics were found one order of magnitude higher than the grape skins (Sri Harsha et al., 2013; Sri Harsha et al., 2014). The phenolic content of most of the grape seed extracts was found approximately double than those of medicinal plants, herbs and spices already considered as sources of natural inhibitors of starch digestion enzymes (Ranilla et al., 2010). For grape varieties that are known to possess a relatively low phenolic content in the skins (i.e, CH), the discovery of relatively high phenolic content in the seeds could open up promising applications. This suggests that the characterization of grape seeds could allow for a better use of the residues of vinification for some of the varieties little considered. For the grape seeds treated with SC-CO_2 with moderate operating conditions, the white grape seed samples has resulted in minimal changes in phenolic content (except for MO which decreased by 8%), whereas for red grape seed samples, an increase in the content of the phenolics was observed (Table 3.4). This could be due to removal of unsaturated apolar components by SC-CO_2, thus allowing for a better stability of phenolics in the matrices.

Table 3.4. Total phenolic (g GAE/kg d.w.), proanthocyanidin (g/kg d.w.) and anthocyanin (g CyE/kg d.w.) contents of extracts of defatted (DF) and non-defatted (NDF) grape seeds recovered from winemaking byproducts.

Variety	Total phenolics		Proanthocyanidins		Anthocyanins	
	Treatment					
	DF	NDF	DF	NDF	DF	NDF
BA	$74^{by} \pm 1$	$42^{ax} \pm 1$	$59^{by} \pm 5$	$36^{ax} \pm 2$	$3.22^{cy} \pm 0.02$	$1.46^{cx} \pm 0.08$
NE	$49^{ay} \pm 1$	$42^{ax} \pm 1$	$24^{ax} \pm 3$	$19^{ax} \pm 1$	$0.23^{ax} \pm 0.02$	$0.19^{ax} \pm 0.02$
PI	$277^{fy} \pm 7$	$233^{cx} \pm 7$	$182^{dx} \pm 4$	$217^{dy} \pm 6$	$1.04^{by} \pm 0.02$	$0.89^{bx} \pm 0.01$
CH	$164^{cx} \pm 4$	$175^{bx} \pm 11$	$136^{cx} \pm 6$	$150^{bx} \pm 6$		
MT	$241^{ex} \pm 9$	$231^{cx} \pm 5$	$208^{ex} \pm 14$	$182^{cx} \pm 16$		
MO	$215^{dx} \pm 6$	$259^{dy} \pm 7$	$117^{cx} \pm 1$	$141^{bx} \pm 13$		

Data are avaerage ± SE. For NDF samples, calculations are referred to dry weight subtracted from fat content. Different letters within the same column (a-e) and in the same row (x,y) indicate significant differences (LSD, $p < 0.05$).

The major components of grape seed phenolics were the proanthocyanidins, ranging between 19 (NE) and 217 (PI) g/kg (Table 3.4). As expected, these compounds showed a similar trend of variation as that observed for phenolics. Proanthocyanidins have been well characterized and found to be comprised of catechin, epicatechin and several galloylated and non-galloylated flavan-3-ol compounds (up to 251 different molecules) (Rockenbach et al., 2012; Ping et al., 2012).

Another class of grape phenolics are anthocyanins that were present in the seeds of the red varieties BA, NE and PI. These anthocyanin-rich varieties showed a markedly lower amount of anthocyanins in the NDF samples than in the DF samples (Table 3.4) except for NE. A recent patent was focused on the extraction of anthocyanins from grape seeds (Bi et al., 2014), whilst in general anthocyanins are not reported among grape seed phenolics (Kammerer et al., 2004). HPLC analysis revealed that the main anthocyanins present in the extracts were the 3-O glucosides of five common anthocyanidins: cyanidin, peonidin, petunidin, delphinidin and malvidin, as those observed in the grape skins (Sri Harsha et al., 2013).

FRAP values varied between 446 (NE) and 2260 (MT) mmol Fe (II) Eq/kg for the defatted seed samples and between 383 (NE) and 2336 (MO) mmol Fe (II) Eq/kg for the non-defatted seeds. A similar trend was observed with respect to the total phenolic content, where seeds treated with SC-CO_2 for white grape seed samples varied only for MO extract, while the red grape seed samples treated with SC-CO_2 showed an increase in FRAP values compared to NDF extracts of BA, NE. It could also be observed that the antioxidant properties of the seeds are an order of magnitude higher than those observed in the skins (Sri Harsha et al., 2013).

3.3.3.2. α-glucosidase inhibition

Intestinal α-glucosidase plays a key role in carbohydrate digestion and subsequent blood glucose absorption, and hence inhibition of α-glucosidase provides a metabolic target for managing diabetes thus improving post-prandial glucose control (Casirola et al., 2006). Rat intestine is used as a source for mammalian α-glucosidase activity, i.e., exohydrolysis of 1-4 α-glucosidic linkage, due to the presence of two intestinal brush border membrane-bound glycohydrolases: maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI). Given that MGAM and SI genes arose from duplication and divergence of an ancestral gene (Nichols et al., 2003), these enzymes are similar in sequence. MGAM only hydrolyses 1-4 α-glucosidic linkage and its natural substrates are maltose, amylopectin, amylase, starch and glycogen. SI hydrolyses 1-4 α-glucosidic linkage and 1-6 α-glucosidic linkage and its natural substrates are sucrose, isomaltose and maltose. Both MGAM and SI are able to hydrolyze *p*NPG (Williamson, 2013).

A variety of naturally derived phytochemicals, especially phenolic compounds present in different plant sources such as strawberries, blueberries, blackcurrants, green tea etc. have been found to be involved in the inhibition of α-glucosidase (McDougall et al., 2005a; Matsui et al., 2001; McDougall et al., 2005b).

3. Recovery of grape seed oil and phytochemicals

The DF and NDF extracts were tested for their efficiency against α-glucosidase enzyme. The dose-dependent inhibition of grape seed extract against α-glucosidase activity was observed in all the grape seed extracts. The most efficient extract was BA DF, with an I_{50} value of 0.63 mg/mL (Table 3.5). The main factor for variation of α-glucosidase inhibition effectiveness was the variety, while the effect of oil extraction was low. For all the varieties, oil extraction had no effect except for BA which showed less inhibitory properties in the NDF sample than in the DF samples.

The I_{50} values were then expressed with reference to the phenolic content, to evidence the efficacy of the phenolic pool. On a phenolic content basis, the values were found to be 47 and 37 µg GAE/mL for the anthocyanin-rich extracts BA DF and BA NDF respectively, while this index was much higher for the other grape seed extracts, in the range ~ 150 (CH) - 270 (PI) µg GAE/mL (Table 3.5).

A commercially available oral α-glucosidase inhibitor acarbose prescribed to treat type 2 diabetes (Chiasson et al., 2002) was chosen as a standard. The I_{50} for the therapeutically used inhibitor, i.e., acarbose was 100 µg/mL, and it was lower with respect to most of the grape seed varieties, except for the anthocyanin-rich extracts of BA which is around two fold higher. Interestingly, in a screening study performed on sixteen culinary herbs to test their efficacy as source of inhibitors of α-glucosidase, only Vietnamese mint and Green tea have been found to be effective, with I_{50} values of 186 and 92.8 µg GAE/mL, respectively (Kee et al., 2013), i.e., higher than the anthocyanin rich extracts.

Isolated anthocyanins have been shown to play a major role in α-glucosidase inhibition both *in vitro* and *in vivo* (McDougall et al., 2005a; Adisakwattana et al., 2011). Additionally, non-anthocyanin inhibitors such as flavanol compounds with gallate side chains have shown a particularly high affinity towards α-glucosidase enzyme *in vitro* (Gamberucci et al., 2006; Liu et al., 2011). Interactions of proanthocyanidins with this enzyme have further been confirmed by differential scanning calorimetry (Barret et al., 2013).

Table 3.5. α-glucosidase inhibitory activity of phenolic extracts of defatted (DF) and non-defatted (NDF) grape seeds recovered from winemaking byproducts.

	α-glucosidase inhibition			
Variety	**I_{50} (mg/mL)**		**I_{50} (µg GAE/mL)**	
	DF	**NDF**	**DF**	**NDF**
BA	$0.63^{ax} \pm 0.02$	$0.88^{ay} \pm 0.03$	$47^{ay} \pm 2$	$37^{ax} \pm 1$
NE	$1.64^{dx} \pm 0.03$	$1.48^{ex} \pm 0.02$	$80^{ay} \pm 2$	$62^{bx} \pm 1$
PI	$0.99^{cx} \pm 0.09$	$1.02^{dx} \pm 0.02$	$274^{ex} \pm 17$	$238^{ex} \pm 4$
CH	$0.97^{cx} \pm 0.03$	$0.94^{bcx} \pm 0.03$	$159^{bx} \pm 4$	$165^{cx} \pm 3$
MT	$0.80^{bx} \pm 0.02$	$0.89^{abx} \pm 0.03$	$192^{cx} \pm 7$	$206^{dx} \pm 8$
MO	$1.02^{cx} \pm 0.03$	$0.98^{cdx} \pm 0.02$	$221^{dx} \pm 4$	$241^{ex} \pm 10$

Data are average ± SE. For NDF samples, calculations are referred to dry weight subtracted from fat content. Different letters within the same column (a-e) and in the same row (x,y) indicate significant differences (LSD, $p < 0.05$).

3.3.3.3. α-amylase inhibition

α-amylase is an endo-acting enzyme, which hydrolyses the 1-4 α-glucosidic linkage but cannot hydrolyze the 1-6 α-glucosidic linkage (Williamson, 2013).

The dose-dependent inhibitory effect of grape seed extracts was observed with respect to α-amylase activity. Among grape seeds samples, the I_{50} values expressed on a weight basis varied from ~ 0.20 (MT, PI) - 0.43 (BA NDF) mg/mL (Table 3.6). The differences among samples were mainly attributable to the variety rather than the effect of oil extraction. On a phenolic content basis, the I_{50} values varied between ~ 20 (NE DF and NE NDF) and ~ 70 (MO DF and MO NDF) µg GAE/mL, with the highest efficacy in the anthocyanin-rich extracts (Table 3.6). The I_{50} of acarbose was 30 µg/mL. Hence, grape seeds in general exhibited similar effectiveness as that of acarbose. Significant correlations were found between total phenolic/proanthocyanidin contents and α-amylase inhibitory activity, with $R = 0.901$ ($p < 0.01$) and $R = 0.947$ ($p < 0.01$) respectively.

Table 3.6. α-amylase inhibitory activity of phenolic extracts of defatted (DF) and non-defatted (NDF) grape seeds recovered from winemaking byproducts.

	α-amylase inhibition			
Variety	**I_{50} (mg/mL)**		**I_{50} (μg GAE/mL)**	
	DF	**NDF**	**DF**	**NDF**
BA	$0.32^{cx} \pm 0.01$	$0.43^{dy} \pm 0.01$	$24^{ay} \pm 1$	$18^{ax} \pm 1$
NE	$0.35^{cx} \pm 0.01$	$0.39^{cy} \pm 0.01$	$17^{ay} \pm 1$	$16^{ax} \pm 1$
PI	$0.19^{ax} \pm 0.03$	$0.20^{ax} \pm 0.01$	$52^{bcx} \pm 7$	$46^{bx} \pm 1$
CH	$0.26^{bx} \pm 0.01$	$0.27^{bx} \pm 0.01$	$43^{bx} \pm 1$	$47^{by} \pm 1$
MT	$0.19^{ax} \pm 0.01$	$0.19^{ax} \pm 0.01$	$47^{by} \pm 1$	$44^{bx} \pm 1$
MO	$0.28^{bx} \pm 0.01$	$0.27^{bx} \pm 0.01$	$60^{cx} \pm 2$	$70^{cx} \pm 2$

Data are average ± SE. For NDF samples, calculations are referred to dry weight subtracted from fat content. Different letters within the same column (a-e) and in the same row (x,y) indicate significant differences (LSD, $p < 0.05$)

While anthocyanins played a major role in α-glucosidase activity, proanthocyanidins have been demonstrated to inhibit α-amylase activity (Soares et al., 2009; Gonçalves et al., 2011; Liu et al., 2011; Barrett et al., 2013). Interestingly, proanthocyanidin extracts have been demonstrated to diminish the post-prandial glycemic level in rats after starch administration (da Silva et al., 2014).
It is worth noting that grape phenolics have been demonstrated to inhibit the glyco-oxidative stress-linked damage (Farrar et al., 2007; Wang et al., 2011; Sri Harsha et al., 2013). Hence, grape phenolics can potentially possess two complementary pathways against hyperglycemia complications, i.e., inhibition of starch digestion enzymes and inhibition of the formation of advanced glycation end-products.

3.4. CONCLUSIONS

A green technology based on SC-CO_2 extraction was studied to recover grape seed oils from winemaking byproducts. Oil yields were majorly dependent on the variety rather than the harvesting year or technology applied (*n*-hexane vs SC-CO_2). It is worth noticing that from ^{1}H-NMR analysis, it was possible to gain useful information regarding the low level of oxidized lipids. Oils extracted by SC-CO_2 had similar quality, in terms of fatty acid and tocol contents, compared to mechanical extraction, while the level of tocols were higher compared to *n*-hexane extraction. The level of tocols were found to be similar in the two harvesting years and the values were higher with those indicated in the *Codex Alimentarius*. SC-CO_2 extraction technique can thus lead to the obtainment of high quality oil with minimum degradation to the compounds.
Defatted grape seeds were relevant co-product of SC-CO_2 oil extraction. Extraction of oil was advantageous for the recovery of anthocyanin-rich extracts from these matrix. Even though anthocyanins were present in minor amounts with respect to proanthocyanidins, their recovery could efficiently improve specific functionalities, for instance α-glucosidase inhibitory activity. The variety-dependent concentration of phenolics had a minimal effect on the α-amylase inhibitory efficacy (phenolic content was correlated to α-amylase inhibition effectiveness). On the contrary, grape variety had a profound influence on the α-glucosidase inhibitory efficacy, where the anthocyanin-rich extracts were found more effective than the anthocyanin-free extracts. The inhibitory activities towards both these enzymes could be interesting in order to reduce the postprandial glycemic response and prevent damage caused by hyperglycemia.
Thus, the overall results support the potential use of tocol-rich grape seed oil extracted by SC-CO_2 and grape seed phenolics in food, nutraceutical and cosmetic industries.

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4. CONCLUSIONS AND FUTURE PERSPECTIVES

4. Conclusions and future perspectives

4.1. CONCLUSIONS

The global food industry is facing the uphill task of developing strategies for optimal use of natural resources, which includes efficient utilization of byproducts generated from large-scale processing systems. At the same time, there is a need to develop a healthy food supply, addressing wellbeing and disease prevention. Bridging these aims is a challenging area of research which was a priority in this study.

The project was focused on grape pomace i.e., the main byproduct of winemaking with the aim of developing a value addition based recovery strategy. Grape pomace is comprised of various compounds having numerous potential health benefits. New knowledge was obtained on the multifunctional properties of these byproducts and technological advancements applicable for the recovery of valuable bioactive phytochemicals along with their re-introduction into the food chain. This section summarizes short overall conclusion statements for the three chapters discussed:

Chapter 1 (Outcome - Phenolics from grape skins as antiglycation agents):

Protein glycation is the root cause of diabetes complications. A 2D electrophoresis approach provided a clear evidence that the grape skin phenolics were able to inhibit the charge based variation that occurs at the beginning of the glycation reaction and protein crosslinking that occurs at a later stage. Fluorescence measurements evidenced that grape skin extracts had good "phenolic quality" being more efficient than standard synthetic inhibitors and commercial nutraceutical preparations. These results suggest their possible use as cost effective resources of natural antiglycation agents alternatively to synthetic compounds, which is one of the most relevant target in disease prevention.

Chapter 2 (Outcome - Antioxidant dietary fibre of grape skins as a fortifying food ingredient):

Fibrous grape skin was used as an ingredient for incorporation into a model tomato puree to enhance its nutritional value. Processing conditions led to increased phenolic content, reducing capacity and antiglycation activity, and simultaneously, changed physical characterstics i.e., color, Bostwick consistency, and rheological parameters, such as storage and loss moduli and complex viscosity. By varying particle size of the formulations, physico-chemical parameters could be modulated. Formulations added with smallest particle size showed maximum consumers' compliance. Thus the overall results indicate that tomato puree could be a low caloric "vehicle" for delivering grape skin phytochemicals and fibers to the consumers, thus contributing to a novel product innovation in the food industry.

Chapter 3 (Outcome - Oil from grape seeds as a source of tocols and unsaturated fatty acids and defatted grape seeds as sources of phenolics):

A green technology based on supercritical CO_2 extraction yielding efficient oil recovery was found to achieve higher extraction of tocols than *n*-hexane. ^{1}H-NMR analysis revealed that in the oil extracted by supercritical CO_2 only trace amounts of oxidized lipids were present. Tocotrienols, identified as major tocols, are known to possess relevant health promoting effects. Phenolics present in high levels in the defatted grape seeds were found to have high antioxidant activity along with specific functionalities, such as α-glucosidase and α-amylase inhibitory activities.

4.2. FUTURE PERSPECTIVES

Need for a multidisciplinary approach:

Adding value to food byproducts by transforming them into a source of functional compounds and ingredients for a healthy food supply is a challenging, but a conceivable research area. To fulfill such an ambitious aim, an interdisciplinary research involving food technologists, nutritionists and economists is required.

4. Conclusions and future perspectives

This study has focused on winemaking as a model industry that generates potentially valuable byproducts. Results have provided a platform to develop applications of winemaking byproducts for processing new ingredients and foods targeting the wellbeing of diabetic and elderly people.

To develop the valorization process further, major points that have to be addressed are briefly outlined below.

- **Task to validate effectiveness *in vivo***

Key inputs of this study were the health benefits of grape phenolics and fibre that have recently demonstrated that these components can exert beneficial effects in the gut lumen or reach and protect cell targets *in vivo*. On the other hand, when these components are incorporated in a complex food matrix, their interaction with food could decrease their effectiveness. Hence, *in vivo* assessment of new foods incorporating recovered ingredients is necessary to ultimately clarify if the designed new food matrix can efficiently deliver grape phytochemicals.

- **Task to assess sustainability**

Creating economic value from byproducts is linked to the nutritional value of the products recovered. Simultaneously, another priority of the food industry is to operate from a bioeconomic perspective and hence, there is also a need to study the life cycle assessment of the proposed recovery strategy to evaluate the feasibility of its scaling-up.

- **Task to comply with regulation, ensure consumer information and protection**

Despite food legislation promotes recycling of food byproducts, a recovery strategy based on value addition and re-uses in the food chain requires further regulatory issues. Functional foods are on the boundary between foods and drugs, and hence their regulation still proves difficult. In any case, consumer protection must have priority over economic interests, and health claims need to be substantiated by standardized, scientifically sound and reliable studies.

APPENDIX 1: LIST OF PAPERS, ORAL COMMUNICATIONS AND POSTERS

Peer-reviewed publications

1. Fiori, L., Lavelli, V., Duba, K. S., **Sri Harsha, P. S. C**., Mohamed, H. B., & Guella, G. (2014). Supercritical CO_2 extraction of oil from seeds of six grape cultivars: modelling of mass transfer kinetics and evaluation of lipid profiles and tocol contents. *Journal of Supercritical Fluids, 94,* 71-80.
2. **Sri Harsha, P. S. C**., Lavelli, V., & Scarafoni, A., (2014). Protective ability of phenolics from white grape vinification by-products against structural damage of bovine serum albumin induced by glycation. *Food Chemistry, 156,* 220-226.
3. Lavelli,V., **Sri Harsha, P. S. C**., Torri, L., & Zeppa, G. (2014). Use of winemaking by-products as an ingredient for tomato puree: The effect of particle size on product quality. *Food Chemistry, 152,* 162-168.
4. **Sri Harsha, P. S. C**., Gardana, C., Simonetti, P., Spigno, G., & Lavelli, V. (2013). Characterization of phenolics, *in vitro* reducing capacity and anti-glycation activity of red grape skins recovered from winemaking by-products. *Bioresource Technology, 140,* 263-268.
5. Lavelli, V., Kerr, W., & **Sri Harsha, P. S. C**. (2013). Phytochemical stability in dried tomato pulp and peel as affected by moisture properties. *Journal of Agricultural and Food Chemistry, 61,* 700-707.

Research papers communicated

1. Lavelli, V., **Sri Harsha, P. S. C**., Mariotti, M., Marinoni, L., & Cabassi, G. Tuning physical properties of tomato puree by fortification with grape skin antioxidant dietary fibre. *Food and Bioprocess Technology* (Under review).
2. Lavelli, V., **Sri Harsha, P. S. C**., & Fiori, L. Screening grape seeds recovered from winemaking waste as sources of reducing agents and mammalian α-glucosidase and α-amylase inhibitors. *International Journal of Food Science and Technology* (Under review).
3. **Sri Harsha, P. S. C**., Mesias, M., Lavelli, V., & Morales, F.J. Grape skin extracts recovered from winemaking by-products as a source of trapping agents for reactive carbonyl species. *Journal of Science of Food and Agriculture* (Under review).

Conference and symposia contributions (Presenting author is underlined)

Oral presentations

1. Lavelli, V., & **Sri Harsha, P. S. C**. "Matching the variability among winemaking by-products with the purpose to develop phenolic extracts having synergic effects in glycaemia control". *In:* Total Food, Science and Technology for the Economic and Sustainable Exploitation of Agri-Food Chain Wastes and Co-products, organized by Institute of Food Research, Norwich, UK, 11-13 November, 2014.
2. **Sri Harsha, P. S. C**. "Recovery of phytochemical rich-fractions from grape pomace for new applications in the food system". *In*: XIX Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Bari, September 24–26, 2014.
3. Marinoni, L., Gardana, C., **Sri Harsha, P. S. C**., Lavelli, V., & Simonetti, P. Characterization of phenolics in red grape skins recovered form winemaking by-products employable as a food ingredient or as a nutraceutical preparation. *In:* Comprendere e Applicare i LARN, organized by Società Italiana di Nutrizione Umana (SINU), Florence, Italy, 21-22 October, 2013.
4. Lavelli, V., & **Sri Harsha, P. S. C**. "Targeting glyco-oxidative stress through phenolics extracted from winemaking by-products - a strategy to prevent hyperglycaemia complications". *In:* 12th International Conference on Oxidative stress, Redox States and

Antioxidants, organized by International Society of Antioxidants (ISANH), Paris, France, 3-4 July, 2013.

5. **Sri Harsha, P. S. C**., Lavelli, V., Gardana, C., & Simonetti, P. "Protein Glycation Inhibitory Activity of Winemaking By-products: Prospects for Developing Cost-effective and Value-added Food ingredients". *In:* Eurofoodchem XVII, organized by Food Chemistry Division of EuCheMS, the European Association of Chemical and Molecular Sciences, Turkey, Istanbul, 7-10 May, 2013.

Posters

1. **Sri Harsha, P. S. C.,** Lavelli, V., Mariotti, M., & Torri, L. "Upgrading winemaking by-products as innovative ingredients for tomato puree: from process and product design to the evaluation of consumers' liking". *In*: Bio-based Technologies in the Context of European Food Innovation Systems, 27th EFFOST Annual Meeting, organized by European Federation of Food Science and Technology (EFFOST), Bologna, Italy, 12-15 November, 2013.
2. **Sri Harsha, P. S. C**. "In vitro screening of winemaking by-products to develop sustainable food ingredients with potential to prevent hyperglycaemia complications". *In*: XVIII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Universities of Padova and Udine, Conegliano, Italy, 25-27 September, 2013.
3. **Sri Harsha, P. S. C**., & Lavelli, V. "Screening among byproducts of wine making, tomato- and apple processing for selection of efficient natural inhibitors against hyperglycemia complications". *In*: 7th ISANH Congress on Polyphenols Applications, Bonn, Germany, 6-7 June, 2013.
4. **Sri Harsha, P. S. C**. "Phytochemical-rich fractions recovered from wine-making by-products as ingredients for new sustainable fruit-based foods. *In*: XVII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bologna, Cesena, Italy, 19-21 September, 2012.

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