UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI FARMACIA

Dipartimento di Scienze Farmaceutiche "Pietro Pratesi"

DOTTORATO DI RICERCA IN CHIMICA DEL FARMACO
INDIRIZZO ANALISI FARMACEUTICA, BIOFARMACEUTICA E TOSSICOLOGICA
CICLO XXIV

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF POLYPHENOLS FROM *UVA DI TROIA AD ACINO PICCOLO* SEEDS AND SKINS FOR THE DEVELOPMENT OF NEW NUTRITIONAL SUPPLEMENTS

(CHIM/08)

Tesi di Dottorato di:
Dott.ssa DARIA CATALANO
Matr. Nr. R08313

TUTOR:
Ch.mo Prof. VENIERO GAMBARO

COORDINATORE:
Ch.mo Prof. ERMANNO VALOTI

A.A. 2010-2011

Ai miei genitori

ABSTRACT

The aim of this Ph.D. project was to study the phenolic composition of *Uva di Troia ad acino piccolo* (*Uva di Troia* with small berry) seeds and skins in relation to the vinification process, in order to create a new nutritional supplement based on the benefits of the phenolics extracted. This grape biotype represents an autochthonous *Vitis vinifera* L. grape variety of Apulia region (South Italy) and is supposed to have significant levels of polyphenols and a great wine aging potential.

Grape samples were collected at four different fermentation stages (from no fermentation to complete fermentation), called *thesis*.

The extraction of seeds was performed with a multi-step extraction by maceration either with ethanol or acetone in water and the extracts obtained were characterized by Reversed Phase Liquid Chromatography coupled to Diode Array Detector (RPLC-DAD). Finally, extracts were successfully purified with Ethyl acetate.

On the other hand, skins were subjected to a single step extraction with methanol and the extracts were analyzed by RPLC-UV; only *Thesis 1* skin extract was also purified using a synthetic adsorbent resin.

Data obtained show that the phenolic content of both grape seeds and skins decreases from the beginning of fermentation to the end of the process; these results are related to the extraction of the active compounds by the must during vinification.

Moreover, *Uva di Troia ad acino piccolo* seeds represent a rich font of Flavan-3-ols and further studies will be conducted to produce new nutraceuticals based on this vegetable matrix. Particularly, *Thesis 2* seeds represent the best fraction because the partial fermentation allows the concomitant production of wine and of the polyphenolic phytocomplex.

ACKNOWLEDGEMENTS

I express all my gratitude to all of those I had the pleasure to work with during this project, Prof.ssa Gabriella Roda, my faculty members and, especially, the research group directed by Prof. Veniero Gambaro.

This work would not have been possible without the support of Dr. Sergio Fontana, general manager of Farmalabor Srl, who provided us grape samples and the opportunity to contribute to this important project.

I am really grateful also to Dr. Giuseppe Mustich for the important help given to me in developing grape extraction and purification techniques, as well for the human support.

Finally, I also extend my heartfelt thanks to my family.

TABLE OF CONTENTS

1. INTRODUCTION

1. INTRODUCTION

The Ph.D. project reported herein is born from the partnership between the University of Milan and Farmalabor Srl, an Apulian pharmaceutical society with base in Canosa di Puglia (BAT Province, Apulia, Italy). Farmalabor has obtained a financial support by Apulia Region institution in its operative programme 2007/2013 for the development of a project, whose title is: "*Valorizzazione delle qualità salutistiche dell'Uva di Troia ad acino piccolo per la produzione di integratori nutrizionali*", that means "Valorisation of the beneficial properties of *Uva di Troia with small berry* for the production of nutritional supplements". In order to realize this project, Farmalabor has also created an experimental vineyard of *Uva di Troia ad acino piccolo*.

Uva di Troia represents an autochthonous *Vitis vinifera* L. grape variety of Apulia region and can exist as two different biotypes in relation to the berry size. The small berry biotype, i.e. *Uva di Troia ad acino piccolo* is also called "canosina" because of the city of Canosa where this variety is today cultivated. This particular grape biotype is nowadays considered unproductive from the oenological point of view, thus, its cultivations are going to be replaced with more productive vineyards. However, recent studies confirmed significant levels of polyphenols in this kind of grape and a great wine aging potential (Suriano *et al.*, 2005).

Phenolic compounds are plant secondary metabolites and they are synthesized to counteract diverse biological and biochemical situations of stress. They are widely present in the human diet and are responsible for many organoleptic characteristics of grape and its derivatives. Their concentration and composition in grapes depend on the cultivar and are influenced by viticultural and environmental factors, such as climate conditions, maturity stage and production area (Cavaliere *et al.*, 2008). Polyphenols exhibit beneficial effects on human health thanks to the strong free radical scavenging and antioxidant activity, as well as cardioprotective, anticarcinogenic, anti-inflammatory, antimicrobial and estrogenic properties, all characteristics that will be further described in detail.

1.1 POLYPHENOLS

Phenolic compounds, also known as *polyphenols*, constitute a big class of secondary metabolites ubiquitous in the plant kingdom, where they are synthesized to accomplish diverse biological and biochemical activities. As secondary metabolites, their synthesis is not just involved in the sustenance, growth and development of the plant, but they are produced in order to defence the organism by abiotic and biotic stresses, such as nutritional deficiency, drought, pollutants, adverse climatic conditions, pathogens, insects, and phytophagy (Nicoletti *et al.*, 2008). Furthermore, beyond the protection against predation, phenolic compounds can restrict the growth of neighbouring plants (He *et al.*, 2008).

The phenolic composition is highly variable qualitatively and quantitatively and depends on various factors, such as the vegetable family, genetic factors, environmental conditions and maturity stage. Their presence is also essential for the plant reproduction because they represent the main responsible for odour and pigmentation, which are attractive characteristics for insects and other pollinating animals.

Phenolic compounds can be divided into two major groups, depending on their chemical structure: non-flavonoid and flavonoid compounds.

1.1.1 Non-flavonoids

This group of compounds includes not only those *simple phenols* characterized by a single aromatic ring, such as hydroxybenzoic and hydroxycinnamic acids and their tartaric esters, but also more complex compounds. Among the latter category are included Stilbenes (e.g. *trans*-Resveratrol) and also hydrolysable tannins, which refer to gallic or ellagic acid-based mixtures, also called gallotannins or ellagitannins, respectively. Fig. 1 shows the chemical structures of the main classes of non-flavonoid compounds.

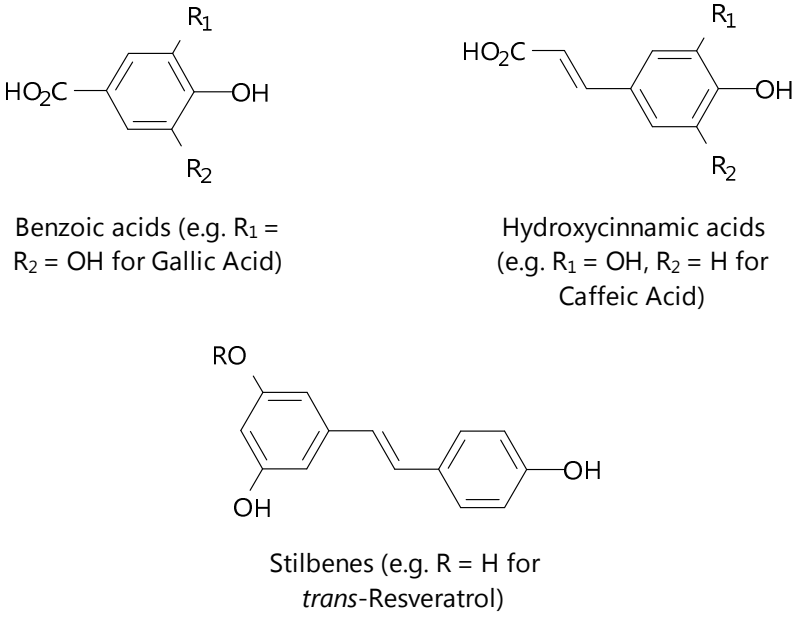


Figure 1: Chemical structures of the main classes of non-flavonoid polyphenols

Hydroxycinnamates are ubiquitous in fruits and in all plant tissues and can be found also as quinic esters, but in grapes they exist only as esters of tartaric acid, especially the tartaric ester of caffeic acid (i.e. caftaric acid) is found (Waterhouse, 2002).

Moreover, the ester form of gallic acid represents the most important and common benzoic acid found in fruit sources, such as fresh grapes, while the free acid is more frequent in wines, due to the hydrolysis of the gallate esters of hydrolysable tannins and condensed tannins.

1.1.2 Flavonoids

Flavonoids derive biosynthetically from phenylalanine and are found widespread throughout the plant kingdom. To flavonoids belong those molecules having a common C_6-C_3-C_6 flavone skeleton where the three-carbon bridge linking the two phenylic groups is cyclised with an oxygen atom, forming an heterocyclic pyranic ring. Commonly, the pyranic ring is referred to as the C-ring, the fused aromatic ring as the A-ring and the phenyl constituent as the B-ring, as shown in Fig. 2.

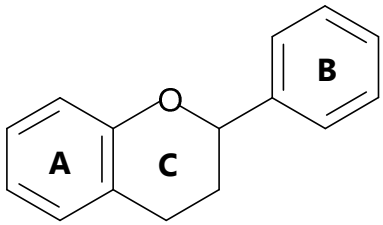


Figure 2: Flavonoids chemical structure

Ring A is formed by the condensation of three moles of malonyl-coenzyme A coming from the glycolytic pathway. Rings B and C also derive from glucose metabolism, but via the Shikimate pathway (Herrmann, 1995) through the aminoacid phenylalanine, which is firstly converted to cinnamic acid and then to coumaric acid. The condensation and subsequent intramolecular cyclization of the *p*-coumaric acid CoA with three moles of malonyl CoAs produce a Naringenin chalcone. This step is catalyzed by the enzyme chalcone sinthase (CHS). Naringenin chalcone then goes under isomerization spontaneously or with the help of the high-stereoselective enzyme chalcone isomerase (CHI) to produce the (2S)-flavanone Naringenin with the C ring closed. Naringenin can be further oxidized at the B ring to eriodictyol or pentahydroxyflavanone by flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H), respectively. These three molecules constitute the substrate for other enzymes, e.g. the flavone sinthase (FS) which produces Flavones, otherwise the flavanone 3-β-hydroxylase (F3H), which leads to the corresponding dihydroflavonols (Fig. 3) and, afterwards, to Flavonols and Leucoanthocyanidins (Merken and Beecher, 2000).

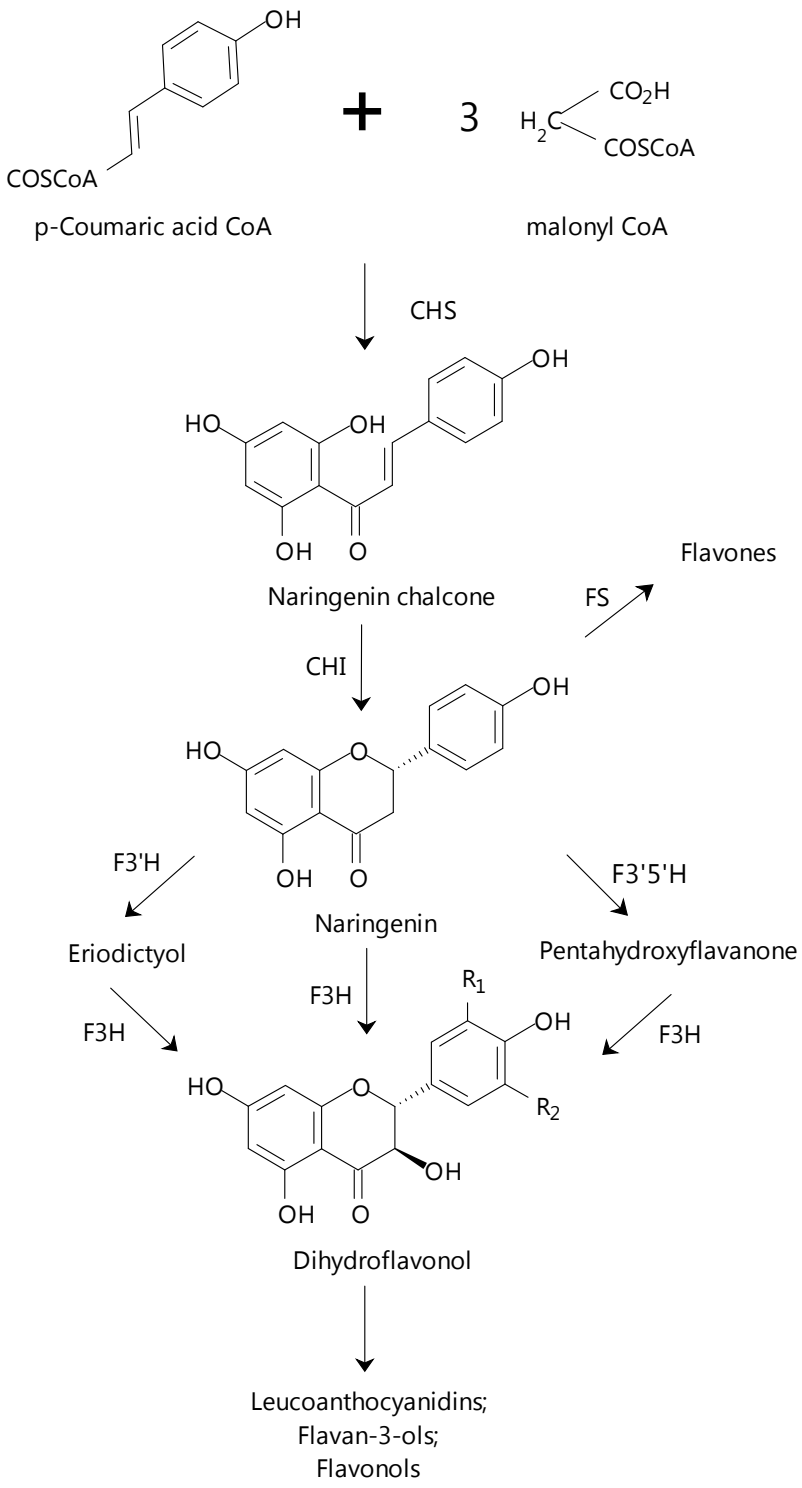


Figure 3: Flavonoids biosynthesis

Therefore, the benzopyranic C-ring can be differently oxidized, leading to several groups of flavonoids which differ each other also for the substitution pattern on the other rings. Hydroxyl, methoxyl and glycosyl groups represent the most common primary substituents, but more complex structures are also found (Cheynier, 2005).

The most important classes belonging to flavonoids are: Flavones, Isoflavonoids, Flavanones, Flavonols, Anthocyanidins and Flavanes, whose structures are shown in Fig. 4.

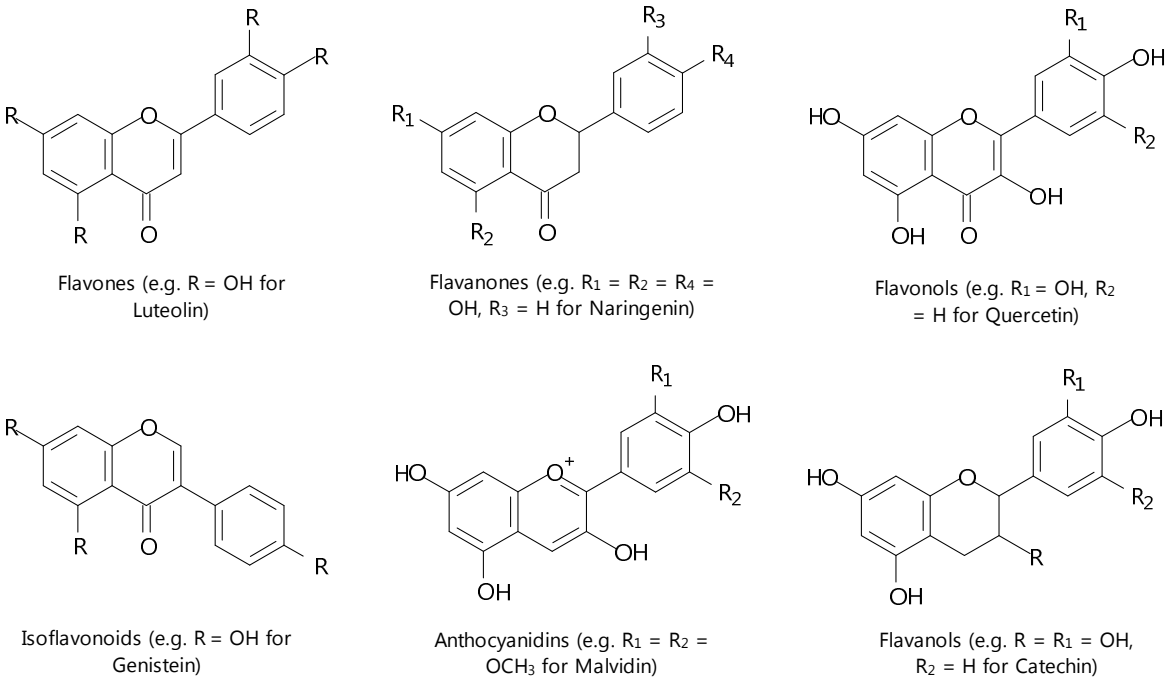


Figure 4: Chemical structure of the main classes of flavonoid polyphenols

An important feature of flavonoid compounds regards ultraviolet (UV) absorption, in fact they show two characteristic absorption bands. Band I has a range comprised between 300 and 560 nm and arises from the B-ring, while Band II is related to the A-ring and has a maximum in the 240-285 nm range. For example, Anthocyanidins present Band II and Band I absorption maxima in the ranges 265-275 nm and 465-560 nm, respectively (Fig. 5).

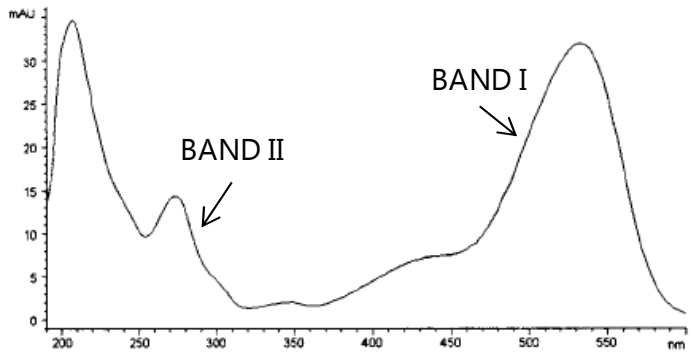


Figure 5: UV/VIS spectra of the anthocyanidin Delphinidin (Font: Merken and Beecher, 2000)

1.2 GRAPE POLYPHENOLS

Grape is a non-climacteric fruit, specifically a berry, that belongs to the genus *Vitis*. Three main species of grape exist: European grapes (*Vitis vinifera*), North American grapes (*Vitis labrusca* and *Vitis rotundifolia*) and French hybrid grapes.

Vitis vinifera is native to the Mediterranean region, central Europe and southwestern Asia and is cultivated widespread in Europe, Asia and Americas. The plant is a liana with a flaky bark and the leaves are alternate, palmately lobed and broad. The fruit is a berry and can be green, red or purple (Fig 6).

Grapes contain a wide variety of nutrient elements, such as minerals, carbohydrates, fibres and vitamins, but also polyphenols as important phytochemicals.

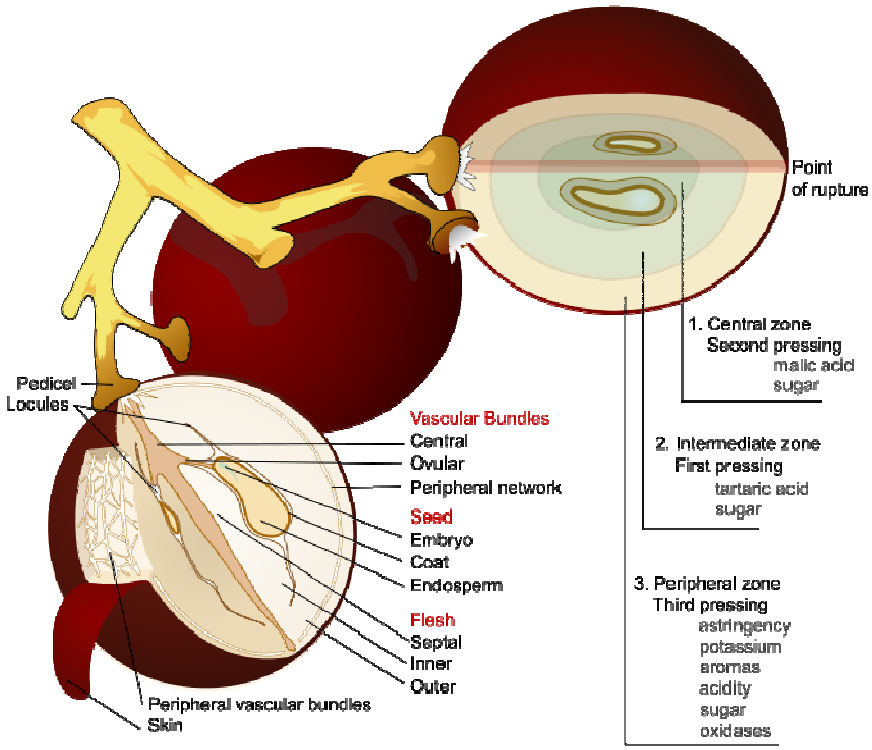


Figure 6: Anatomy of a grape (Font: www.wikipedia.org)

Particularly, as reported by the *National Institute of Food and Nutrition Research* (Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione: INRAN), grapes belonging to *Vitis vinifera* L. variety are constituted by the elements listed in Table 1.

CHEMICAL COMPOSITION AND ENERGETIC VALUES FOR 100 g OF FRESH GRAPE (*Vitis vinifera* L.)		
Chemical composition	**Energy / 100g**	**Notes**
Edible matter (%):	94	
Water (g):	80.3	
Proteins (g):	0.5	
Lipids (g):	0.1	
Cholesterol (mg):	0	
Available carbohydrates (g):	15.6	Depending on ripeness, cultivar and climate factors
Amid (g):	0	
Soluble sugars (g):	15.6	
Total fibre (g):	1.5	
Alcohol (g):	0	
Energy (kcal):	61	
Energy (kJ):	257	
Sodium (mg):	1	
Potassium (mg):	192	
Iron (mg):	0.4	
Calcium (mg):	27	
Phosphorus (mg):	4	
Copper (mg):	0.27	
Zinc (mg):	0.12	
Thiamine (mg):	0.03	
Riboflavin (mg):	0.03	
Niacin (mg):	0.4	
Vitamin A retinol eq. (µg):	4	
Vitamin C (mg):	6	
Vitamin E (mg):	traces	

Table 1: Chemical composition and energetic values for *Vitis vinifera* L. (Font: INRAN)

The above values are related to the whole berry of the grape, but the energetic and chemical composition and distribution change with the grape variety and with the organs and tissues of the plant taken into consideration.

For instance, skins, leaves, stems, pulp and seeds of the *Vitis vinifera* variety are differently characterized, especially in terms of grape polyphenols. These molecules

are responsible for many organoleptic characteristics of grape and, consequently, of wine and other derivatives. Their concentration and composition in grapes also depends on the cultivar and so it is influenced by viticultural and environmental factors, such as climate conditions, maturity stage and production area (Cavaliere *et al.*, 2008).

As a matter of fact, while Anthocyanidins are almost exclusively located in red grape skins, where give colour characteristics, Flavan-3-ols (monomeric catechins, oligomeric and polymeric proanthocyanidins and their gallates) are mainly located in grape seeds and are responsible for wine flavour, structure, astringency and bitterness.

Hence, the localization of phenolic compounds in plant cells strictly depends on their physicochemical characteristics, i.e. the stereochemistry and/or the grade of polymerization. It has been proposed that polyphenols interact with cell wall polysaccharides through hydrogen bonds or hydrophobic interactions; moreover, phenols can also be found in the cytoplasm entangled in vacuoles or associated with the cell nucleus (Pinelo *et al.*, 2006).

Anyway, the four classes of grape polyphenols that have attracted most attention in the area of nutraceutical and functional foods for their pharmacological activity, are: Flavonols, Anthocyanidins, Flavanols (Catechins and Proanthocyanidins) and Stilbenes.

1.2.1 Flavonols

Flavonols are found in a wide variety of foods and beverages derived from vegetable sources. Therefore, grape berries represent a rich font of this class of compounds, which is exclusively located in skins. Their role in grape skins may be related to the protection against UV rays coming from sun, in fact some studies confirmed an increment of flavonols biosynthesis subsequent the exposition of the berry skins to the sunlight (Pereira *et al.*, 2006).

Flavonols are usually found in plants as *O*-glycosides, where the sugar (e.g. Glucose, Glucuronides) is linked to the oxygen of the hydroxyl group at position 3 of the C-

ring (Fig. 7). Glycosylation increases flavonols polarity and thereby the water solubility, allowing storage in plant cell vacuoles.

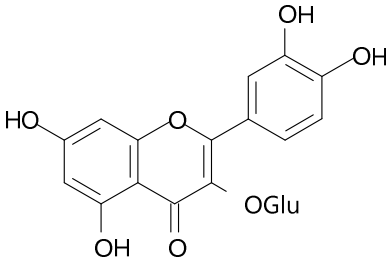


Figure 7: Isoquercitrin or Quercetin-3-*O*-β-D-glucosyde

In grape berries, the most common flavonols encountered are the glycoside forms of Quercetin, Myricetin and Kaempferol (Fig. 8).

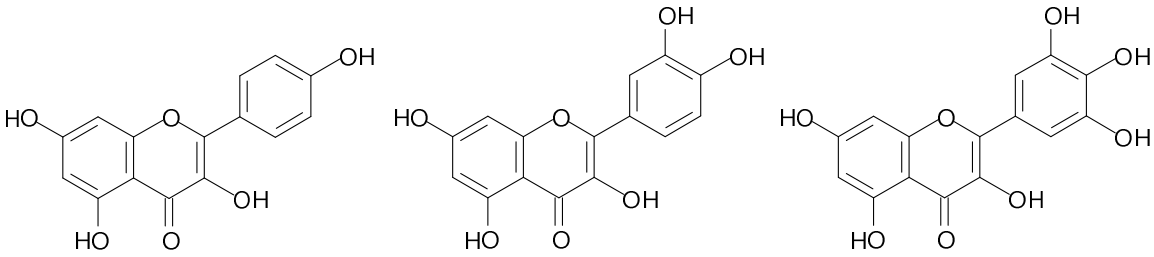


Figure 8: Flavonols

The synthesis of Flavonols starts from the dihydroflavonol structures described in paragraph 1.1.2 thanks to the activity of the enzyme flavonol synthase (FLS), which oxidizes the C-ring of each Dihydroflavonol to the corresponding Flavonol.

1.2.2 Anthocyanidins

Anthocyanidins (from Greek ἀνθός = flower and κυανός = blue) are water-soluble pigments which provide the colour in red grape berries and wine, but are also present in many other plants and foods. They play a distinct role in the attraction of animals for pollination and seed dispersal, and may be important factors in the resistance of plants to insect attack (Kong *et al.*, 2003).

Food industries have always used antocyanins as food colourants, but in recent decades this class of compounds is studied for the antioxidant properties as potential nutritional supplements and medicines.

The term Anthocyanidin refers to the aglycone form, but this underivatized structure is never found in grapes or wine, except in trace quantities, because of its instability. The term Anthocyanin, instead, indicates the glycoside form of Anthocyanidins that occurs naturally in grapes. Glucose is the most common encountered sugar, followed by galactose, rhamnose, xylose and arabinose, whereas glucuronic and galacturonic acids are rare to be found. Particularly, in *Vitis vinifera* varieties, the glycosylation appears almost exclusively at the 3-position (Fig. 9), even if also 3,5-diglucoside forms exist in other species (Downey and Rochfort, 2008).

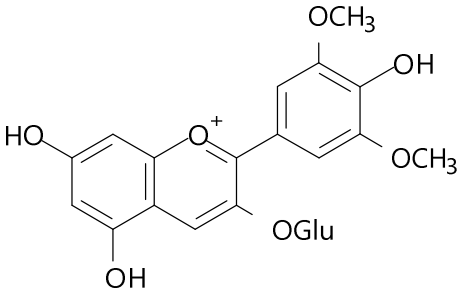


Figure 9: Oenin or Malvidin-3-*O*-glucoside

The most found Anthocyanins in red grape skins and red wine are the glycoside forms of: cyanidin, malvidin, peonidin, petunidin and delphinidin, whose structures are shown in Fig. 10.

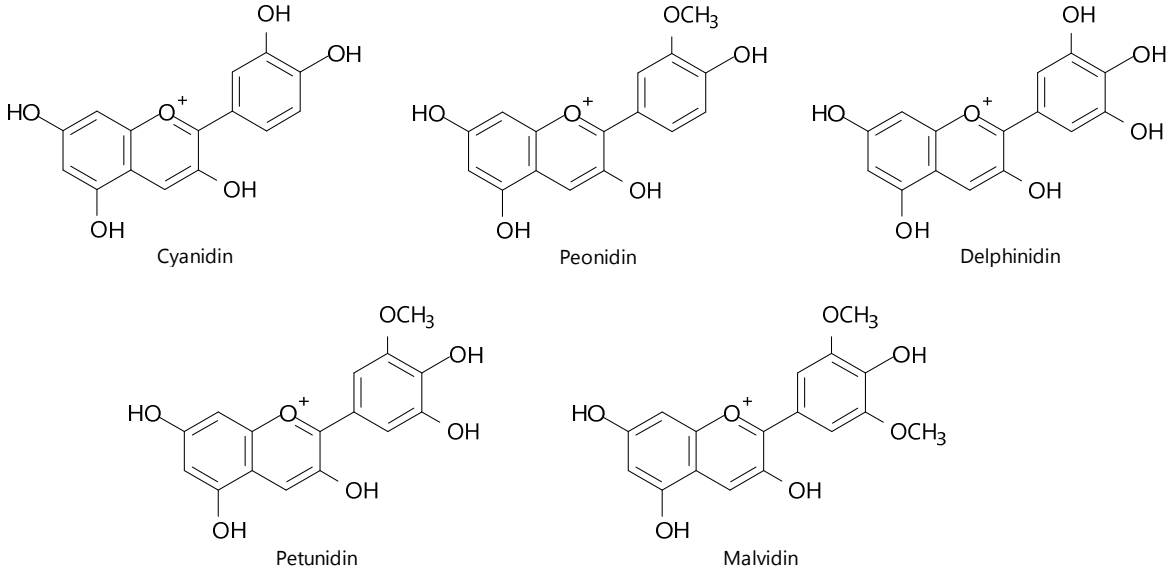


Figure 10: Anthocyanidins

From a chemical point of view, the chromophore properties are related to the fully conjugated 10 electron A-C ring π-system, but the characteristic colour is strongly affected by the pH of the solution in which these molecules are solubilised (e.g. wine). In fact, at very low pH values all the anthocyanin molecules are in the red flavylium ion form, while at very high pH values a colourless carbinol pseudobase is formed (Fig. 11).

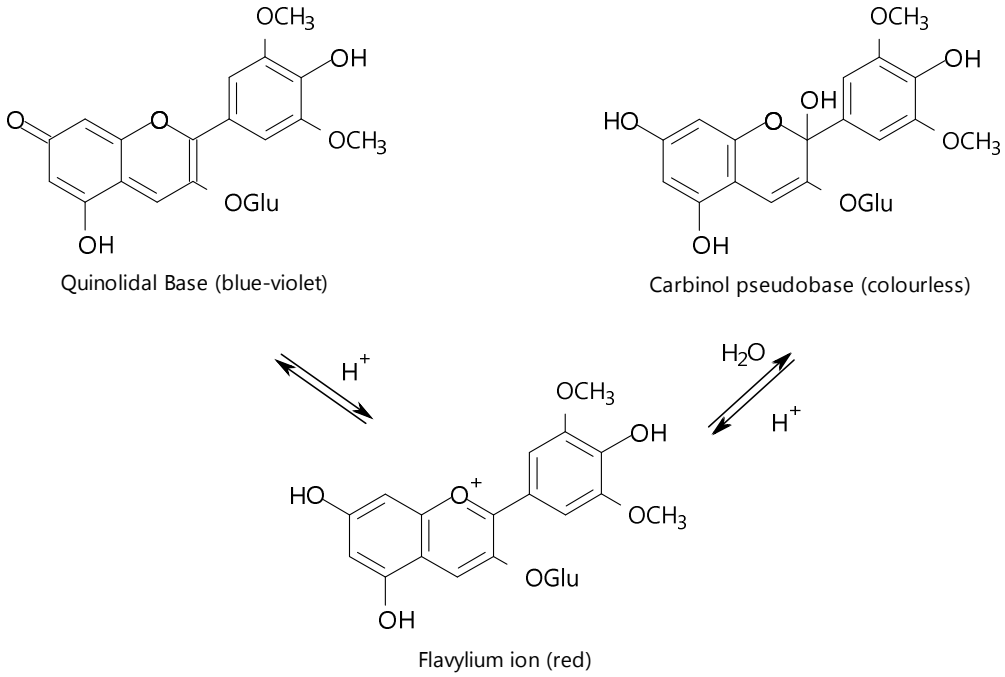


Figure 11: pH dependence of Anthocyanin colour

These pigments are almost exclusively located in grape skins, where they are implicated in the reproduction of the plants, because of their capability of attracting animals involved in pollination and seed dispersal. They can also act as a natural sunscreen, together with Flavonols, protecting cells from UV light damage and stress (Yong Ju and Howard, 2003).

The biosynthesis of Anthocyanidins begins from the reduction of the Dihydroflavonols to the corresponding colourless Leucoanthocyanidins, a reaction that is catalyzed by the stereospecific enzyme dihydroflavonol-4-reductase (DFR). The 2R,3S,4S-Leucoanthocyanidins formed are then oxidized to the corresponding coloured Anthocyanidins by the enzyme anthocyanidin synthase (ANS), also known as leucoanthocyanidin dioxygenase (LDOX). Additionally, the unstable Anthocyanidins

can be also bound to a sugar at the 3-*O*-position with formation of an oxygen-carbon acid-labile acetal bond, to yield the final stable anthocyanins (Fig. 12). The glycosylation occurs thanks to the enzyme UDP-glucose:anthocyanidin/flavonoid 3-glucotransferase (UFGT) and makes the flavonoid less reactive, more polar and thereby more water soluble, so that it can prevent cytoplasmatic damage and allows the accumulation in the cell vacuole (Cavaliere *et al.*, 2008). Furthermore, one or more of the sugar hydroxyls can be esterified with an acid, such as acetic, malonic, sinapic, p-coumaric, caffeic or ferulic acids, giving acyl-glycosilated Antocyanidins.

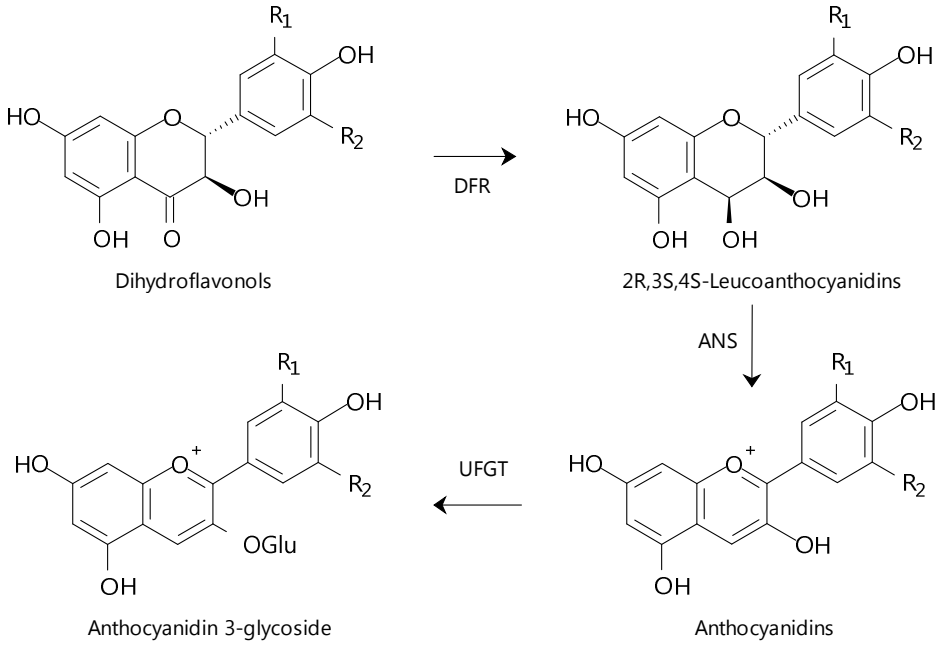


Figure 12: Anthocyanin synthesis

1.2.3 Flavan-3-ols

Flavan-3-ols represent the most abundant class of flavonoids in grapes, found both in skins and seeds, and also widespread throughout the plant kingdom. The name derives from the location of the hydroxyl group on the C-ring, which is consequently located at the 3 position.

As already mentioned above, Flavan-3-ols are mainly located in grape seeds to provide protection against predation and microbial pathogens. In grape derivatives, such as wine, they are responsible for flavour, clarity, structure, astringency and

bitterness. Furthermore, the composition of polymeric flavanols (i.e. Proanthocyanidins) is correlated with wine stability and aging.

The term Flavan-3-ol is commonly referred to catechins, i.e. monomeric units, but includes also oligomers and polymers composed of elementary units, which are called Proanthocyanidins (PAs) or *condensed tannins*. PAs name derives from the fact that the extension units of these molecules, under strong acidic conditions, are hydrolyzed and released as the corresponding anthocyanidins.

Flavan-3-ol monomers differ structurally each other according to the stereochemistry of the carbons on the C-ring, in fact, the carbons at positions 2 and 3 are two stereogenic centres. In grapes, the most common chiral intermediates in the flavonoid pathway are characterized by a *R* configuration at the carbon 2, while the carbon 3 can be either *S* or *R*, i.e. 2*R*,3*S*-Flavan-3-ols or 2*R*,3*R*-Flavan-3-ols.

Another important difference among these molecules is linked to the hydroxylation pattern on the B ring and this feature leads to a wide range of complex structures. The oxidation state depends on the activity of the enzymes cytochrome P450 monooxygenases flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H), described in paragraph 1.1.2, that catalyze the formation of the 3' and 3',5'-hydroxyl groups, respectively. Therefore, Procyanidins designate oligomers and polymers with a 3',4' hydroxylation pattern in the extension units, whose monomers are represented by (+)-Catechin and (-)-Epicatechin. Propelargonidins, instead, are characterized by monomers with a 4' hydroxylation pattern, such as (+)-Afzelechin and (-)-Epiafzelechin, while Prodelphinidins designate those oligomers and polymers composed by monomers with a 3',4',5' hydroxylation pattern, such as (+)-Gallocatechin and (-)-Epigallocatechin (Fig. 13).

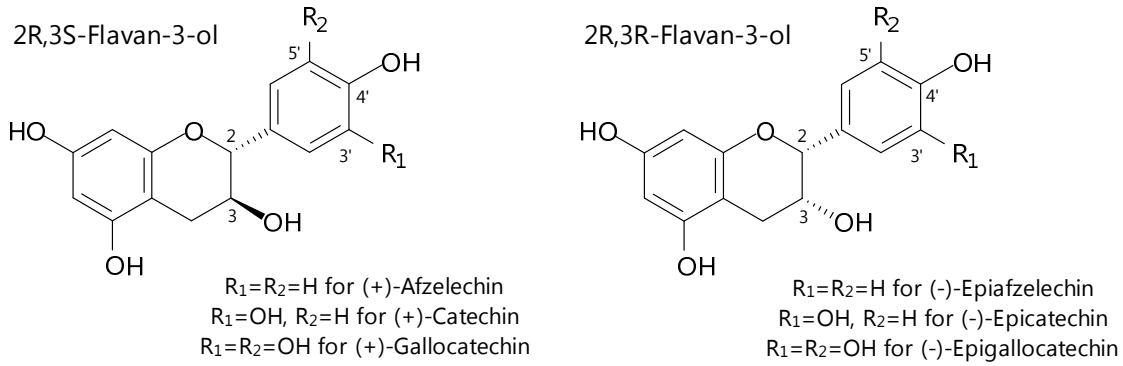


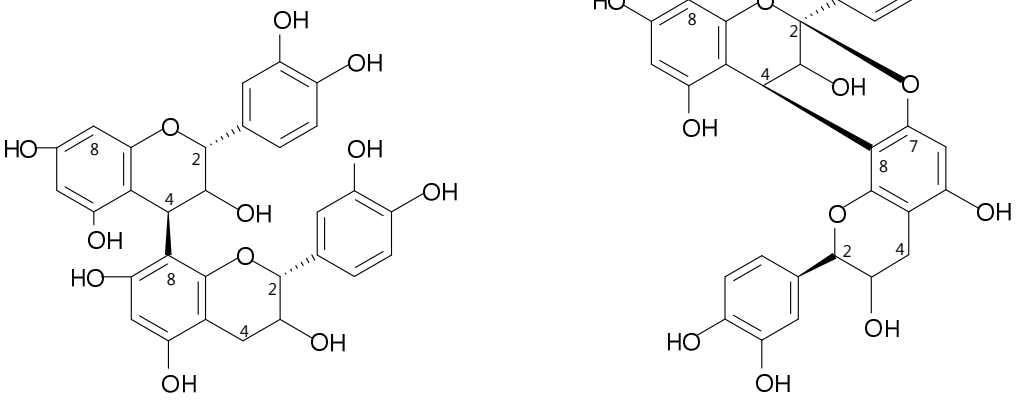
Figure 13: Flavan-3-ol monomers

Monomeric flavan-3-ols can be further modified by methyl, acyl or glycosyl substituents, with the latter being the predominant one at the 3 or 5-hydroxyl group of the C-ring. However, glycosylated PAs are more rarely detected in plants than other glycosylated flavonoids, such as flavonols.

In grapes and wine, the most common flavan-3-ol monomers are (+)-Catechin and (-)-Epicatechin, while (-)-Gallocatechin and (-)-Epigallocatechin are identified in traces. Moreover, Catechins can often be found as gallate ester with the esterification occurring at the 3 position of the *epi*-series only (Waterhouse, 2002). (-)-Epicatechin is also the most common extension unit of grape Procyanidins (PC), while Catechin is less frequent, together with Epicatechin gallate.

In PCs, the linkage of successive monomeric units occurs usually between the C4 of the upper unit and the C8 of the lower unit, which is the starter one, and this single interflavan bond can be either α or β. This kind of bond is categorized as B-type, as well as another linkage that is less frequent: the C4→C6 interflavan linkage. For example, among dimers, Procyanidins B1, B2, B3 and B4 are linked by the C4→C8 interflavan bond, while Procyanidins B5, B6, B7 and B8 are linked by the C4→C6 linkage.

On the other hand, flavan-3-ol monomers can also be linked together with two interflavan bonds, as happens for the A-type Procyanidins, where the single units are connected also through a linkage between the C2 of the upper unit and C5 or C7 of A-ring lower unit (Fig. 14).



Procyanidin B (dimer 4β→8) **Procyanidin A** (dimer 4β→8;2β→*O*→7)

Figure 14: A-type and B-type Procyanidins

The degree of polymerization is another important variable, since the great heterogeneity encountered in grape seeds. In fact, 14 dimeric, 11 trimeric, and one tetrameric PCs have been identified in grape seed extracts and nine of these PCs are esterified with one or two gallic acid molecules attached to an Epicatechin unit Moreover, 18 additional galloylated and non-galloylated procyanidin polymers, up to octamers, were identified (Fuleki and Ricardo da Silva, 2003). Oligomers and polymers which contain only the C4→C8 or the C4→C6 interflavan linkage are listed as B-type PCs, while those containing also C2→*O*→7 either C2→*O*→5 are categorized as A-type PCs.

Recent studies suggest that the degree of polymerization, the stereochemistry and the number of hydroxyl groups can affect the biological activities of this class of compounds (Xie and Dixon, 2005).

Regarding monomeric flavanol biosynthesis, is well known that these compounds are products of a branch pathway of the anthocyanidin biosynthesis. Leucoanthocyanidins formed by DFR activity are consequently subjected to ANS/LDOX activity to yield Anthocyanidins (see paragraph 1.2.2) or to the action of leucoanthocyanidin reductase (LAR) in order to form (2*R*,3*S*)-flavan-3-ols. On the other hand, the *epi*-series is formed thanks to the enzyme anthocyanidin reductase (ANR), which converts Anthocyanidins into (2*R*,3*R*)-flavan-3-ols.

On opposite, the polymerization mechanism that leads to PC oligomers and polymers is still not well known, but it's commonly held that ANR activity is fundamental, because Epicatechin represent PCs predominant extension unit and also a common starter unit. An accepted route supports the formation of 2*R*,3*R*-quinone methide addition units from Epicatechin or the 2*R*,3*S*-quinone methides from Catechin, maybe through the catalysis of the enzyme polyphenol oxidase (PPO). The quinones could then be converted to carbocations via a flav-3-en-3-ol intermediate. Consequently, the electrophilic carbocations could undergo nucleophilic attack by Epicatechin or Catechin to produce condensed dimers and oligomeric B-type PCs. A similar mechanism has been proposed for A-type PCs formation (Dixon *et al.*, 2004; He *et al.*, 2008) (Fig. 15).

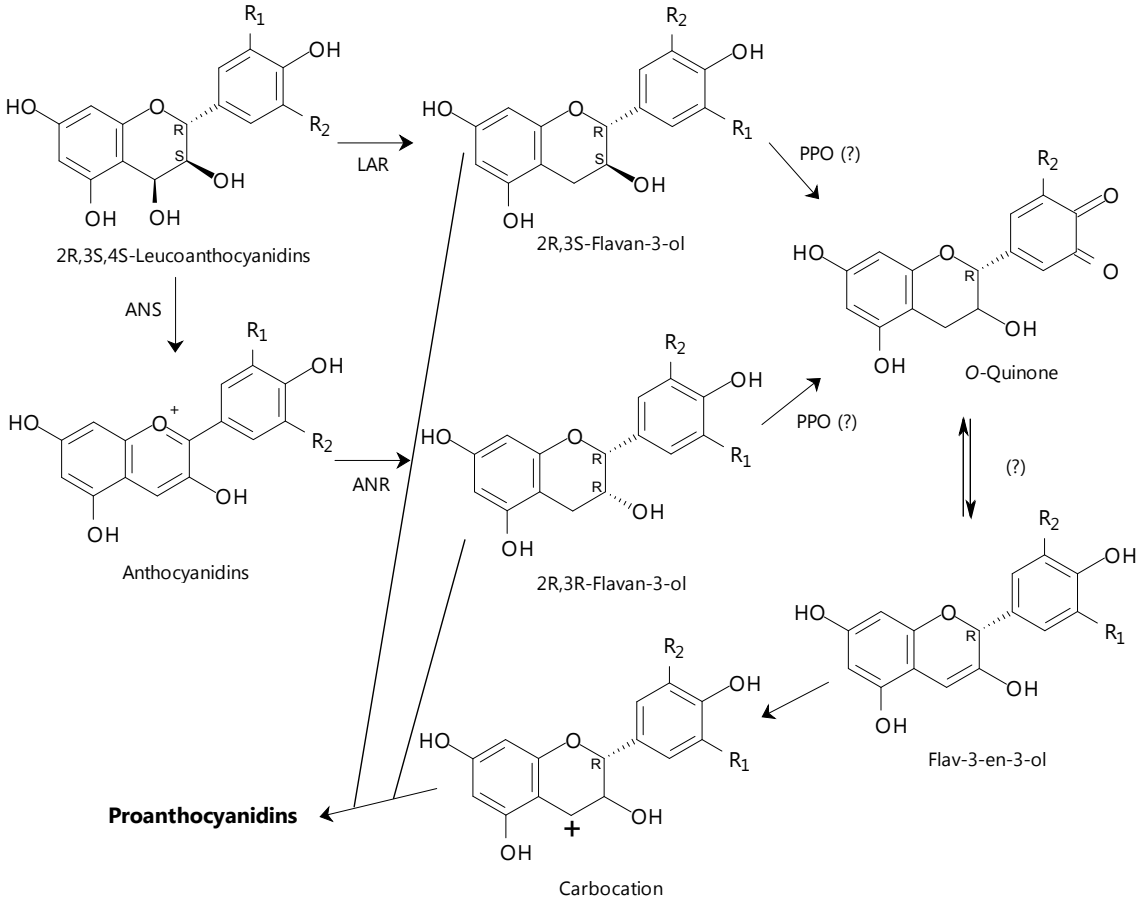
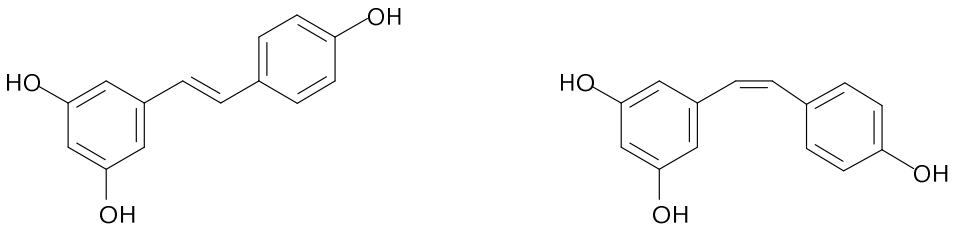


Figure 15: Putative mechanism for PAs polymerization

1.2.4 Stilbenes

Stilbenes are plant secondary metabolites consisting of two phenol moieties linked by a two carbon bridge. The basic stilbene structure is widespread in plants, but diverse species-specific substitution patterns exist. This class of compounds is present in soft tissues as phytoalexins, whose accumulation in plant cell represents the most frequently encountered defense response to biotic and abiotic stress. These compounds are synthesized, for example, in response to microbial or fungal attack or to ultraviolet radiations (Hammerbacher *et al.*, 2011).

Particularly, in *Vitis vinifera* grape varieties, the principal stilbene elicited by a situation of stress is Resveratrol, which exists naturally in two isomeric forms: *cis*-Resveratrol and *trans*-Resveratrol (Fig. 16). The latter is more stable and predominantly occurs, especially in red grape skins; it also represents the form more biologically active on human health.



trans-Resveratrol *cis*-Resveratrol

Figure 16: Resveratrol isomeric forms

In wine, both isomers are found, while in grapes only the *trans* form is detected, since the isomerization is attributed to light exposure. Moreover, Resveratrol can also be encountered in the 3-glycosylated form, which is referred to as Polydatin or Piceid (Resveratrol-3-*O*-β-D-glucopyranoside) and is present in quantities comparable to free Resveratrol in grape berries and wines (Fig. 17).

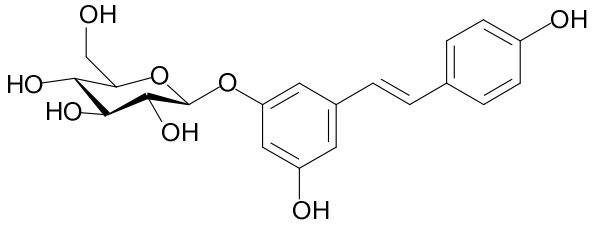


Figure 17: *trans*-Piceid chemical structure

trans-Resveratrol (3,5,4'-trihydroxystilbene) represents an end product of the phenylpropanoid pathway. It is obtained by the condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA in a reaction catalyzed by the enzyme stilbene synthase (STS). This reaction leads to a linear tetraketide intermediate, which is subjected to an intramolecular aldol condensation with loss of CO_2 to achieve ring closure (Austin *et al.*, 2004) (Fig. 18).

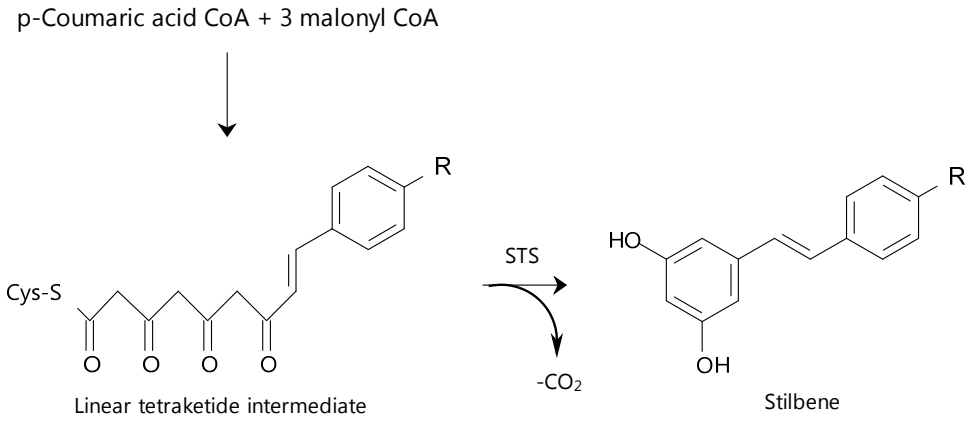


Figure 18: Stilbene synthesis

STS belongs to a large enzymatic family, whose best known representative in plants is chalcone synthase (CHI), catalyst of the first step in flavonoid biosynthesis and previously described in paragraph 1.1.2.

Furthermore, several oxidation products of *trans*-resveratrol have been identified, such as oligomers and more highly polymerized polymers known as viniferins. The most common viniferin detected in grape and wine is represented by ε-viniferin, i.e. *trans*-resveratrol dimer.

The mechanism of viniferin biosynthesis is still not well elucidated, but it's held that peroxidase is the plant enzyme involved in the oxidation of *trans*-resveratrol and its transformation into viniferins (Santamaria *et al.*, 2011).

1.3 HEALTH EFFECTS

Plant-derived polyphenols are increasingly receiving attention for their nutritional value as dietary supplements.

Indeed, a number of large-scale epidemiological studies have demonstrated that this large family of phytochemicals exhibits physiological functions which can result in benefits for human health, as showed by the so-called "French Paradox". This expression has been coined because of the relatively low incidence of coronary heart diseases in French population, in spite of a typical diet rich in saturated fats, a common risk factor for this kind of diseases. This relationship may be associated to the large consumption of wine by French people. In the beginning, it was believed that alcohol was the primary causal factor between wine consumption and reduced heart disease risk, because of its activity in raising the concentration of high-density lipoproteins (HDL) in blood (Stoclet *et al.*, 2004). However, it has been reported that moderate intake of other alcoholic beverages doesn't show the same protection offered by red wine, which may have a beneficial effect that is additive to that of alcohol (Grønbæk *et al.*, 2000). Consequently, the protective effects of red wine are ascribed to the presence of high concentrations of phenolic compounds that originate from grapes.

Phenolic compounds exert their health-promoting effects thanks to their antioxidant, cardioprotective, anti-inflammation, anticancer and antimicrobial activities. Furthermore, recent studies have reported evidences that polyphenols may display anti-allergic properties and also modulation of human cell receptors.

1.3.1 Antioxidant activity

The interest in the investigation of new antioxidant substances has significantly increased in recent years. The reason for that is mainly associated to the industrial use as food preservatives of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), because of their potential adverse effects on human health, e.g. the cancerogenic effect of BHA (Shahidi, 2000). At the same

time, antioxidants have also been of interest in the clinical field because they protect the human organism against oxidative stress, e.g. damage caused by reactive oxygen species (ROS), as well as those of nitrogen (RNS) and chlorine (RCS). Therefore, synthetic antioxidants should be replaced by natural inhibitors of oxidation originated from plants, such as grape phenolic compounds.

The antioxidant activity of polyphenols is correlated to the chemical structure of this class of compounds, and consequently to their free radical scavenging capacity and metal chelating properties.

Up to date, several methods are employed to evaluate the antioxidant power of these substances, such as the DPPH (1,1-diphenyl-2-picryhidrazyl) test, ORAC (Oxygen Radical Absorbance Capacity) assay, FRAP (Ferric Reducing Antioxidant Power) assay and TBARS (Thio-Barbituric Acid Reactant Substances) method. These tests have shown a huge variability in the antioxidant content of different grape tissues and cultivars, thus related to the influence of viticultural and environmental factors on phenolics biosynthesis, as well as the extraction procedure (Rubilar *et al.*, 2007; Mullen *et al.*, 2007).

The *in vivo* generation of free radicals can cause damage to human nucleic acids, lipids and proteins, but the electronic configuration of polyphenols can inhibit this process. Therefore, the number of hydroxyl groups and their position are important for the antioxidant capacity of phenolic compounds, which is linked to the formation of stable phenoxyl radicals. For this reason, the substitution of hydroxyl groups with methoxyl or glycosyl groups inhibits their antioxidant activity.

Specifically, the cathecol group (1,2-dihydroxy benzene) of polyphenols readily reacts with free radical reactive oxygen species to form a semi-quinone radical which is very stable because of the adjacent oxygen anion and so cuts off the reaction chain. Thanks to its stability, the phenoxyl radical can now react with another semi-quinone radical to produce a quinone and a phenol, through a disproportionation reaction (Fig. 19). Otherwise, two semi-quinone radicals could couple in order to generate oligomeric compounds through a nucleophilic addition (Aron and Kennedy, 2008).

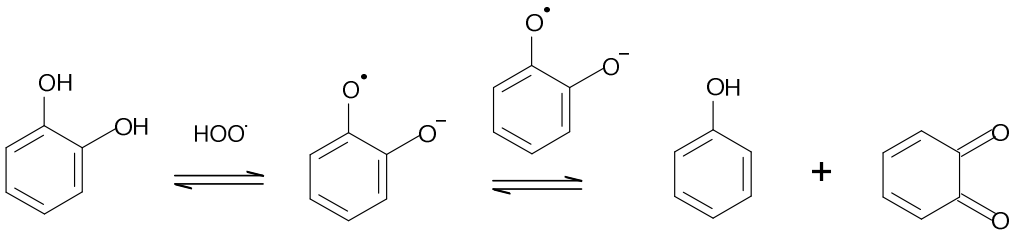


Figure 19: Anti-radical activity of phenolic compounds

Between the different classes of flavonoid compounds, flavanols represent the best antiantioxidants, especially the procyanidin dimers with *ortho*-diphenols. This feature could be related to the stabilization of the phenoxyl radicals through hydrogen bonding (Amico *et al.*, 2008). *In vitro* studies previously carried out also demonstrated that procyanidins exert a direct radical scavenging activity against peroxyl and hydroxyl radicals and show good affinity towards superoxide anion with an IC_{50} value of 5,64 x 10^{-6} M (Maffei Facino *et al.*, 1996). Procyanidins action is carried out at the surface of the phospholipid bilayer, due to their low hydrophobic character; on the contrary, quercetin inhibits superoxide anion formation by preventing NADPH-oxidase activity within the cell structure (Carini *et al.*, 2001; Dávalos *et al.*, 2009).

Moreover, again Maffei Facino *et al.*, verified that the antioxidant action of procyanidins is also ascribed to their metal chelating properties. In fact, they show a strong sequestering ability towards iron and copper which leads to the formation of stable complexes with a favorable stoichiometric binding ratio (Fe^{2+}/PC 2:1; Cu^{2+}/PC 4:1). These complexes display good stability constants (log K≈9) and prevent the action as metal catalysts for $HO^{\bullet}$ development through the Fenton and Haber-Weiss reactions.

Finally, *in vitro* and *in vivo* studies confirmed that cathecol-type flavonoids, such as oligomeric procyanidins, catechin and quercetin, prevent α-tocopherol degradation, spare Vitamin E from consumption and enhance Vitamin E antioxidant activity through a synergistic interaction (Maffei Facino *et al.*, 1998; Zhao *et al.*, 2011).

Another important phenolic compound which exhibits a strong antioxidant activity is *trans*-Resveratrol. Some studies suggest different antioxidant mechanisms based on competition with coenzyme Q, scavenger activity against oxygen radicals (peroxyl and

hydroxyl radicals) and also inhibition of lipid peroxidation induced by Fenton reaction products (Alarcón de la Lastra and Villegas, 2007).

1.3.2 Cardiovascular protective action

Oxidative stress, free radicals, cholesterol and smoking strongly concur to the onset of cardiovascular diseases, such as cardiomyopathy, ischemic heart disease and atherosclerosis.

Thanks to their beneficial effects on circulatory disorders, Procyanidins from *Vitis vinifera* seeds are successfully employed as active ingredients for the treatment of capillary fragility, peripheral chronic venous insufficiency and microangiopathy of the retina (Flamini, 2003). Furthermore, anthocyanin extracts obtained from *Vaccinium myrtillus* have been administrated to reduce capillary permeability and fragility.

Indeed, the antioxidant activity of grape polyphenols interferes in the pathogenesis of cardiovascular diseases, leading to a possible explanation for the "French Paradox", but several studies have also demonstrated the involvement of other protective mechanisms.

Actually, phenolic compounds, especially Procyanidins and *trans*-Resveratrol, exert radical scavenging activity (hydroxyl, peroxynitrite and peroxylipid radicals), thus quenching exogenous harmful radicals. This action is valuable for the protection of blood vessels, but, moreover, it has been verified that phenolics increase the tonicity and resistance of capillary walls. This feature is a result of an aspecific binding of polyphenols to the components of the elastic fibers, collagen and elastin, so diminishing the degradative action of collagenase and elastase (Gabetta *et al.*, 2000).

In addition, Procyanidins, Anthocyanins and *trans*-Resveratrol retard the development and progression of the atherosclerotic process by suppressing the oxidation of low density lipoproteins (LDL), modulating the metabolism of lipids and reducing cholesterol levels, reducing platelet activation and aggregation (Leifert and Abeywardena, 2008a; Ghiselli *et al.*, 1998). It has also been verified that grape seed and red wine polyphenol extracts significantly decrease *in vitro* cholesterol uptake, independently of polyphenol antioxidant activity (Leifert and Abeywardena 2008b).

Another important feature of Procyanidins is related to their direct non-competitive inhibiting action over those enzymes, located in the endothelial cells, that are implicated in the onset of the oxy radical cascade and in the turn-over of the components of the extracellular matrix surrounding the capillary walls. In fact, Maffei Facino *et al.* (1994) have also demonstrated *in vitro* that xanthine oxidase, and therefore superoxide ion formation, is strongly inhibited by Procyanidins, as well as other lysosomal enzyme systems, such as elastase, collagenase, hyaluronidase and β-glucuronidase.

Together with the antioxidant capacity and the inhibitory actions on endothelial enzymes, polyphenols exert a key role in the protection against myocardial post-ischaemic damage and in cardiovascular disease also through the preservation of the coronary endothelium-dependent relaxant function (Maffei Facino *et al.*, 1999). The principal modes of action involved are represented by: the increased bioavailability of nitric oxide (NO) thanks to their antioxidant activity, the enhanced NO release by endothelial NO synthase (eNOS), the increased prostacyclin PGI_2 production, and the reduced vascular constriction mediated by endothelin-1 (Aldini *et al.*, 2003) (Fig. 20).

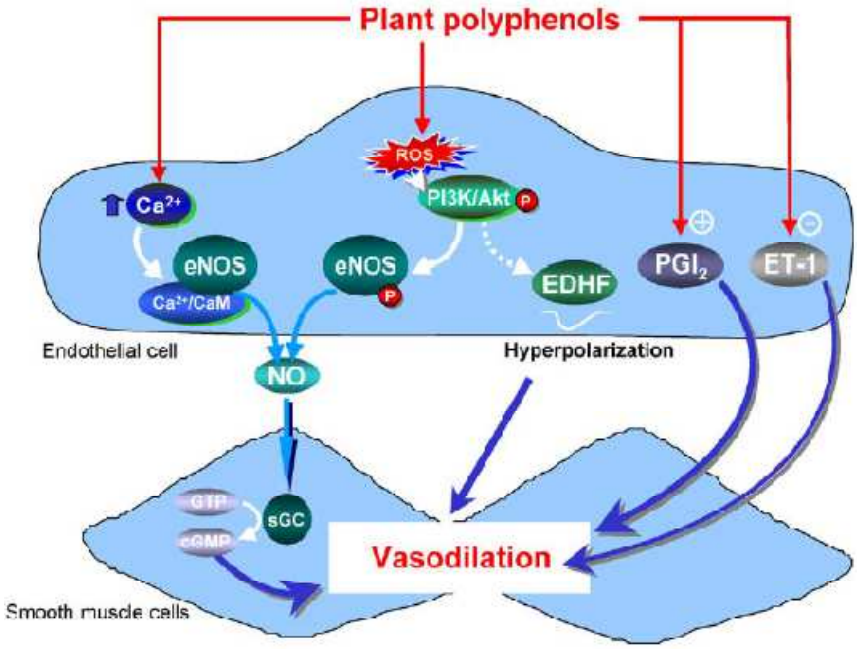


Figure 20: Endothelium-dependent effects of plant polyphenols (Font: Stoclet *et al.*, 2004)

Moreover, another mechanism of action involved in the vasorelaxation is represented by the inhibition of 5-phosphodiesterase activity, accomplished by anthocyanins (Dell'Agli *et al.*, 2005).

1.3.3 Anti-inflammatory activity

Polyphenolic beneficial health effects can be also correlated to their anti-inflammatory activity, which has been widely investigated in recent years, although the mechanism of action has not already been cleared.

It has been verified, for instance, that oral intake or topical administration of grape seed Proanthocyanidins or Resveratrol inhibits UVB radiation-induced edema, erythema, COX-2 (ciclooxygenase-2) activation, infiltration of inflammatory leukocytes and myeloperoxidase activity in *in vivo* models (Nichols and Katiyar, 2010). In addition, oligomeric Procyanidins protect neutrophils from degranulation, thanks to a membrane-stabilizing effect, thus preventing neutrophils adhesion and activation during inflammatory events (Carini *et al.*, 2001).

Other studies revealed that polyphenol extracts dose dependently inhibit the activity of the enzyme 5-lipoxygenase, which catalyzes the oxidation of essential fatty acids to the corresponding conjugated hydroperoxides. In living cells, they are responsible for inflammation and are very active against biomolecules to induce cell death. More specifically, Procyanidins suppress phospholipid hydroperoxides formation at the bilayer of the cell membranes, maybe via a complexation mechanism involving electrostatic interactions between the nucleophilic phenol group of oligomeric catechins and the cationic polar head of phospholipids (Carini *et al.*, 2000).

Furthermore, polyphenols such as quercetin, quercitrin, resveratrol and catechin are active in rheumatoid arthritis models and against inflammatory bowel disease or gastric mucosal inflammation and ulceration. The mechanisms are not well elucidated, but may include antiproliferative or proapoptotic actions, general immune depression, inhibition of neutrophil infiltration and reduction in the levels of histamine, tumour necrosis factor (TNF) and phospholipase-A_2 (González *et al.*, 2011).

Therefore, grape phenolics probably act also via the modulation of proinflammation factors, such as the inhibition or reduction of the adipokine and cytokine gene expression and transcription (Xia *et al.*, 2010).

1.3.4 Anticancer activity

In recent decades, it has been shown an inverse correlation between the dietary consumption of fruits and vegetables and cancer incidence and many epidemiological studies support with evidences the anticancer activity of grape extracts and products.

The mechanisms of action suggested are different, but they are closely related to those involved in the cardiovascular protection.

For instance, grape polyphenols are implicated in the regulation of angiogenesis, a complex process characterized by the degradation of extracellular matrix followed by the maturation of new blood vessels. It has been verified that polyphenols strongly inhibit Matrix metalloproteinases-2 (MMP-2) expression and activation and also prevent the expression of induced VEGF (Vascular Endothelial Growth Factor) in vascular smooth muscle cells. Furthermore, Leifert and Abeywardena (2008b) demonstrated that red wine polyphenol and grape seed extracts significantly inhibit cell proliferation in cancer cells with the concomitant increment in the level of caspases-mediated apoptosis. Resveratrol is also able to limit migration and proliferation of endothelial cells by preventing the progression through S and G_2 phase, as well by increasing the expression of the tumour suppressor gene protein p53 and of the cyclin-dependent kinase inhibitor p21 (Oak *et al.*, 2005). Recent *in vitro* studies have also demonstrated the synergistic effect of Resveratrol and Quercetin against glioma cell lines via the induction of apoptosis (Gagliano *et al.*, 2010).

Another putative target of the anticancer action of polyphenols is represented by Nuclear factor-kappa B (NF-κB), a nuclear transcription factor which protects cells from apoptotic stimuli and, thus, it is involved in signal transduction pathways related to cancer and inflammation. It has been verified that polyphenols, especially flavan-3-

ols and Resveratrol, inhibit constitutive and induced NF-κB, as well as other transduction signalling pathways and genes related to cell cycle and apoptosis, such as PI3K (phosphoinositide 3-kinase), MAPK (mitogenactivated protein kinase), c-myc and Bcl-2 (Dixon *et al.*, 2004; Alarcón de la Lastra and Villegas, 2007).

In addition, polyphenols prevent DNA damage and are active as hormone metabolism regulators, so they can be used for the treatment of hormone-related cancers, e.g. breast cancer.

Resveratrol shows a modulated estrogenic activity, but it also acts as an antagonist for estrogen receptors (ER) α and β in dependence of their expression. Particularly, it doesn't increase ERE (Estrogen Response Elements) activity in the presence of estradiol in normal bone cells, while competitively inhibits its effect in breast cancer cells (Veprik *et al.*, 2011). On the other hand, procyanidin B dimers are effective in suppressing androgen-dependent tumour growth by strongly inhibiting aromatase activity and, thus, *in situ* estrogen biosynthesis (Eng *et al.*, 2003).

1.3.5 Other activities

Grape polyphenols act also as antibacterial and antiviral agents and exert beneficial effects towards metabolic disorders.

As a matter of fact, these compounds inhibit the growth and the microbial activity of various pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*, via diverse mechanisms of action. The activity against *Streptococcus mutans*, for example, is linked to the inhibition of the enzymes glucosyltransferases B and C and F-ATPase (Thimothe *et al.*, 2007). Otherwise, these compounds can form complexes with cell walls to disrupt bacterial envelopes or can exert their antimicrobial activity thanks to the anti-inflammatory and antioxidant properties (Kurek *et al.*, 2011).

Anyway, the natural preservative and antimicrobial activities of polyphenols may be exploited for food preservation, thus improving storage and safety of food products.

On the other hand, phenolic compounds are useful for the treatment of other pathologies that are associated to oxidative stress and chronic inflammation, such as

type-2 diabetes, asthma and obesity. For example, plasmatic levels of the inflammatory biomarker C-reactive protein (CRP) have been shown to be significantly higher in obese populations, but the administration of grape seed procyanidins counteracts this event (Hogan *et al.*, 2010).

Furthermore, different researchers have analyzed the potential benefits of flavonoids as anti-allergic substances for the treatment of asthma, allergic rhinitis and atopic dermatitis. Recent investigations demonstrated that the administration of flavonoids leads to the downregulation of serum immunoglobulin E (IgE) levels (Kaneko *et al.*, 2010). At the same time, Quercetin and *trans*-Resveratrol are effective at inhibiting human eosinophil activation, recruitment and degranulation (Rogerio *et al.*, 2007; Tan and Lim, 2009).

Finally, it has been shown that polyphenolic extracts also exhibit hepatoprotective and neuroprotective effects (Nassiri-Asl and Hosseinzadeh, 2009).

1.3.6 Bioavailability and metabolism of polyphenols

The bioavailability of polyphenols is important for their effectiveness on human health, but the wide variability among the different subclasses may lead to a harder characterization of the individual components.

Small molecules, such as catechin monomers and PC dimers, can be easily absorbed by the gut into the bloodstream after oral ingestion, whereas higher molecular weight polyphenols pass through the digestive system unabsorbed, otherwise are poorly absorbed. The high weight polymeric Procyanidins perhaps are first degraded into low molecular weight metabolites thanks to the action of the intestinal microflora (Aron and Kennedy, 2008).

Generally, once absorbed after oral intake, phenolic compounds are subjected to intraluminar and hepatic first-pass metabolism, which reduces bioavailability, and to serum protein binding, i.e. albumin with an affinity that depends on the B-ring hydroxylation in case of flavonoids. Furthermore, *in vitro* experiments revealed that flavonoid glycosides have much lower permeability than aglycons and can also be extruded by membrane proteins (Gónzalez *et al.*, 2011).

Phase II metabolism leads to glucuronidation, methylation and/or sulfation of these compounds. These conjugated forms result to be predominant in plasma and may retain some biological activity. For instance, it has been shown that resveratrol metabolites, such as Piceatannol (3,5,3',4'-tetrahydroxystilbene) and sulfates derivatives, maintain part of resveratrol antioxidant, anticancer and anti-inflammatory properties (Delmas *et al.*, 2011). Polyphenol metabolites are then excreted especially by kidneys, but also by lungs, as well as in the bile and feces.

Anyway, in order to improve the bioavailability of phenolic compounds, many strategies have been investigated in recent years, complexation with phytosomes being one of the most important. The phytosome technology is based on intermolecular bonding between a polyphenol mix and a phospholipid preparation, mainly constituted by phosphatidylcholine, thus facilitating the access to the bloodstream. One of the best polyphenol phytosome known is represented by the pharmaceutical preparation Leucoselect™-Phytosome™ (Indena S.p.A., Milan, Italy; European Patent 0275224; US Patent 4, 963, 527; 2 hard, gelatine capsules), a highly standardized grape seed extract prepared at a ratio of one part of grape seed polyphenols and three parts of phosphatidylcholine by weight. Many studies have verified the high safety and tolerance of this food supplement. In addition, it doesn't compromise the beneficial health effects of grape seed polyphenols on human health (Parris, 2009).

1.4 UVA DI TROIA

Grape fruit contains a large availability of nutrient elements, such as polyphenols, whose nature and composition depends on the grape variety and on the cultivar. Phenolic compounds represent a very important class, thanks to their beneficial pharmacological activity on human health. Because of the great variability among the phenolic compounds in grape varieties, knowledge of the chemical composition of each grape biotype is critical for understanding its biological properties, as well as the characteristics of the specific derivatives, such as wine and juices.

Uva di Troia is a grape variety native to Southern Italy, particularly to the north area of Apulia region. It is believed that this variety originates from Asia Minor, but other studies suppose that the name derives from the town of Troia, in the Province of Foggia (Apulia). *Uva di Troia* grape is mainly cultivated in the north part of Bari province, but also along Apulian coasts.

From the botanical point of view, leaves are middle-size, pentagonal, five-lobed, green, opaque with light green nervatures and a hairless upper side. The bunch is quite big, compact, simple or winged, with a middle-size berry that is spheroidal and regular; the peel is pruinose, thick and the colour is black-violet with blue tints. The ripening time is middle-tardy.

However, because of the high ampelographic diversity, three different clones and two biotypes of *Uva di Troia* are known to exist, such as the "ruvese" and the "canosina" biotypes.

The latter is also referred to as *Uva di Troia ad acino piccolo*, i.e. *Uva di Troia* grape with a small berry, that is due to the berry size of this biotype. It is believed that this particular variety is the oldest one, but nowadays it is considered unproductive and, thus, it is not widespread in the Apulian territory, but it is still cultivated nearby the city of Canosa di Puglia (BAT Province, Apulia). Nevertheless, recent investigations have shown that *Uva di Troia ad acino piccolo* has a great wine aging potential and, especially, that it's characterized by a definitely high phenolic content, above all the

flavonol content (Tarricone and Suriano). This feature may be related to the small size of the berry because of its influence on phenolics extraction. In fact, a small berry implies a minor pulp/skin ratio, with the consequence that the maceration of the grape in the must leads to a better extraction of the phenolic components with respect to a middle-size berry (Suriano *et al.*, 2005).

2. AIM OF THE PROJECT

2. AIM OF THE PROJECT

In the last two decades, according to Food and Agriculture Organization (FAO), a large increase of production and consumption of grape has been registered around the world, thanks to its beneficial effects on human health. As a consequence, vineyards that have been always considered unproductive are going to be replaced by those more productive, for instance *Uva di Troia ad acino piccolo* grapevine is nowadays rarely cultivated because of the characteristic small berry, although its relatively high phenolic content and wine potential.

In order to limit the abandon of this particular grapevine, the Apulian pharmaceutical society Farmalabor Srl has proposed and obtained a financial support by Apulia Region Institution for the project *"Valorisation of the beneficial properties of Uva di Troia with small berry for the development of nutraceutical supplements"*. Consequently, the objective of this project was to enhance the production of this autochthonous grape biotype and the growth of the entire supply chain using technologies with low environmental impact. Moreover, Farmalabor has also created an experimental vineyard of *Uva di Troia ad acino piccolo*, whose first harvest took place in October 2011 (Fig. 21). Grapes coming for this crop will be soon investigated in order to be compared with those used for the research herein described.

Figure 24: Farmalabor experimental vineyard of *Uva di Troia ad acino piccolo*

Hence, the aim of the work reported herein was to study the phenolic composition of different samples of *Uva di Troia ad acino piccolo* seeds and skins in relation to the fermentation and vinification process, through the development of appropriate extraction and purification techniques.

Different grape phenolic characterization methods have been widely investigated in recent years, ranging from High Performance Liquid Chromatograghy (HPLC) coupled to spectrophotometric detectors (UV or Diode Array Detector (DAD)), fluorescent detectors or Mass Spectrometry (MS), as well as Capillary Electrophoresis (CE) (Gómez-Alonso *et al.*, 2007; Priego Capote *et al.*, 2006); each technique gives us information about grape phenolic content from a point of view that is secondary to the purpose of the investigation.

Particularly, HPLC represents the analytical technique most employed for the separation and characterization of grape phenolic compounds, whereas reversed phase columns, almost exclusively composed of a C18 stationary phase are the most common columns used for the determination of such phenolics. For what concerns the mobile phase, an acid is often added to the solvents, being formic acid or trifluoroacetic (TFA) acid the most used. The acidic phase is necessary to control the protonic equilibrium of the analytes. In fact, a low pH value is able to keep analytes carboxyl and hydroxyl groups in the protonated form, thus avoiding the simultaneous presence of differently ionized forms of polyphenols and improving the hydrophobic interactions with the C18 stationary phase (Nicoletti *et al.*, 2008).

Moreover, also various extraction procedures have been investigated and described, as well as the role of ripening and of viticultural factors over the polyphenolic composition and concentration (Geny *et al.*, 2003; Fuleki and Ricardo da Silva, 2003). Another variable that has been explored is represented by grape fermentation. Actually, the winemaking processing and the storage influence the phenolic content of wine and consequently its antioxidant activity (Sun *et al.*, 2011).

Methanol, ethanol and acetone variously mixed with water represent the most common solvents used for the extraction of grape seeds and skins by maceration,

while HPLC-UV constitutes the analytic technique of choice (Mané *et al.*, 2007; Careri *et al.*, 2003). Besides maceration and LC analysis, also Solid Phase Extraction (SPE) and Gas Chromatography have been investigated for phenolic extraction and characterization (Soleas *et al.*, 1997).

However, there aren't exhaustive studies concerning *Uva di Troia ad acino piccolo* grape biotype and the direct influence of fermentation on its phenolic content.

Grape phenolic composition not only affects the sensory characteristics of grape products, but also the health of the consumer, so it is closely related to the concept of functional food or nutraceutical, which is any substance that is a food or part of a food and provides health benefits.

Therefore, the final objective of this research is to understand the best extraction and analytical conditions to develop a new nutraceutical product based on the benefits of *Uva di Troia ad acino piccolo* natural active ingredients, thus leading to the valorisation of this autochthonous Apulian grapevine.

Nevertheless, polyphenols are not completely extracted during winemaking procedures and grape residues constitute a very abundant source of these phenolic compounds. Hence, the ideal extraction conditions chosen for the development of the nutritional supplement should preserve at the same time wine production, thus leading to the total exploitation of the cultivar and limiting the remaining solid waste.

In order to achieve this goal, we have studied different extraction and purification procedures, as well as the chromatographic conditions, for the recovering and characterization of polyphenols from *Uva di Troia ad acino piccolo* grape tissues. Particularly, we have analyzed the phenolic content of four different fractions of grape seeds and skins, called *thesis*, collected at four different fermentation stages (from no fermentation to complete fermentation).

Considering that the extraction of phenolics depends first on the dissolution of the active principles in the plant matrix and then on their diffusion in the external medium, the least time consuming extraction technique is represented by extraction via maceration with organic solvents for grape seeds, as well for skins.

Thus, in this work two solvents mixtures were tested for grape seeds extraction: 70:30 ethanol/water and 70:30 acetone/water. These solvents are food compatible and don't affect human health according to the Official Journal of the European Union[1], as they can be used *"in compliance with good manufacturing practice for all uses"*, and therefore for the production of nutritional supplements. Then, grape seeds extracts were analyzed by LC-DAD technique, using a Reversed Phase C18 column and a binary solvent gradient. The differences in the phenolic content between fresh and dry seeds were also evaluated with the purpose of determine if any possible degradation of the active products might occur after the drying procedure.

Once established the best chromatographic conditions, grape seeds extracts were also purified, using Ethyl Acetate as organic solvent for a liquid-liquid extraction (LLE), with and without adding salt, i.e. sodium chloride NaCl.

Then, grape skins coming from each *Thesis* were extracted by maceration and analyzed by reversed phase LC-UV, too. The extraction was performed with methanol as organic solvent, in order to achieve the best phenolic recovering, before the chromatographic separation. Methanol, according to the Official Journal of the European Union[2], is also food compatible and can be used for foodstuffs, but it has a "*Maximum residue limit in the extracted foodstuff or food ingredient*" of 10 mg/Kg.

After maceration, only the grape skin extract obtained from *Thesis 1* was also subjected to a purification protocol based on the use of adsorbent resins, for the removal of sugars and proteins.

1 Official Journal of the European Union L 141/1, 6th June 2009 Annex I, Part I

2 Official Journal of the European Union L 141/1, 6th June 2009 Annex I, Part II

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 GRAPE SAMPLES

As already explained, the aim of the work reported herein was to study the phenolic composition of *Uva di Troia ad acino piccolo* seeds and skins in dependence of the fermentation and vinification process, through the development of appropriate extraction and purification procedures.

In order to achieve this goal, we extracted, purified and characterized the phenolic content of four fractions of grape seeds and skins, collected at four different fermentation stages. We separately analyzed seeds and skins because of the significant differences in the phenolic content and composition between the two tissues.

Uva di Troia ad acino piccolo grapes used for the investigation came from the crop of the Agricultural Research Council (CRA) of Barletta (Apulia, South Italy), a National Research Organization which operates under the supervision of the Ministry of Agriculture, with general scientific competence within the fields of agriculture, agroindustry, food, fishery and forestry.

The harvest took place in October 2009 near Canosa di Puglia (BAT Province, Apulia, South Italy) and the crop obtained, amounting to about 370 Kg, was immediately divided into four equal fractions, called *thesis*, processed independently from each other.

Generally, during the vinification process, grapes are crushed, pressed and poured into open fermentation tanks, leaving the skins in contact with the must throughout the fermentation, that is achieved by maceration. Moreover, for red winemaking, stems of the grapes are usually removed before fermentation since the stems have a relatively high tannin content and could be extracted into wine in addition to grape seed tannins. Natural yeasts present in fresh grape lead to the so-called primary or alcoholic fermentation, during which wild yeast cells feed on the sugars in the must

and multiply, thus producing carbon dioxide gas and alcohol. The carbon dioxide produced pushes grape skins to the surface of the must and this layer, made of skins and other solids, is known as the cap (Fig. 22). On the other side, seeds sink to the bottom of the tank. Because of the unpredictable fermentation process exerted by natural yeast, cultured yeast is often added to the must, so we can distinguish in detail four distinct moments of the winemaking process.

Figure 22: A cap of red grape skins in the fermentation tank (Font: www.wikipedia.org)

Thus, the fermentation stages we chase for the fractionation of the crop are related to these four moments of the vinification process:

- ***1st Thesis***. October 7, 2009. This aliquot of the crop was processed as in white winemaking, minimizing the contact between solid and liquid parts, i.e. the marc (or pomace) and the must. More specifically, after the harvesting, grapes were destemmed to remove the grapes from the rachis, and crushed to break the skins and liberate the contents of the berries. Then, the marc with the skins and seeds was immediately pressed to separate liquid parts (wine) from the solid matter of grapes. Then, all solid parts were collected to constitute our samples, which amounted of about 25 Kg of pomace. *Thesis 1* was then washed and drained before freezing at -20°C and storage.
- ***2nd Thesis***. October 9, 2009. After the harvesting, grapes were crushed, destemmed and pressed into open fermentation tanks. Once obtained a spontaneous separation and layering of the various components (skins, seeds and must) in the container, sampling of skins and seeds was carried out, thus

obtaining about 15 Kg of pomace and 300 g of seeds. By convention, this time is called Tz (time zero of fermentation). Then, both skins and seeds were washed, drained and frozen at -20°C.

- ***3rd Thesis***. October 12, 2009. After the harvesting, grapes were crushed, destemmed, pressed into open fermentation tanks and added of selected cultured yeasts in order to achieve a better fermentation. Once an alcohol content of 5-6% was reached, sampling of skins and seeds was carried out, thus obtaining about 13 Kg of pomace and 940 g of seeds. Then, both skins and seeds were washed, drained and frozen at -20°C.
- ***4th Thesis***. October 14, 2009. After the harvesting, grapes were crushed, destemmed, pressed into open fermentation tanks and added of selected cultured yeasts in order to achieve a better fermentation. Once the fermentation process went to completion, i.e. when sugars were completely converted into alcohol and carbon dioxide, and before wine racking, sampling of skins and seeds was carried out, thus obtaining about 12 Kg of pomace and 1250 g of seeds. Then, both skins and seeds were washed, drained and frozen at -20°C.

Then, in order to let us start the extractive studies, Farmalabor provided the dispatch of the material to be analyzed at our department by courier service, capable of maintaining the cold chain, that was essential for the proper storage of the drugs at -20°C.

Once arrived in our laboratory, the four grape samples showed a different aspect between each other, particularly *Thesis 1*, because it was composed of the entire marc after pressing and so the spontaneous separation of seeds from skins in the tanks did not occur. Therefore, *Thesis 1* seeds needed to be manually separated from skins before starting the extraction operations. Manual separation will be further described in detail.

On the contrary, a variable quantity of seeds and skins coming from the other Theses was already separated, thanks to the spontaneous sedimentation of the seeds in the

fermentative tank (Fig. 23). However, most of the seeds of *Thesis 2*, *3* and *4* also needed to be manually separated from the marc and washed to obtain higher amounts.

Figure 23: *Thesis 1* pomace and *Thesis 4* seeds of *Uva di Troia ad acino piccolo* 2009 harvest

3.2 MANUAL SEPARATION OF SEEDS AND SKINS

An appropriate separation of seeds and skins from the whole grape pomace was necessary to accomplish a better characterization of the phenolic compounds present in the samples of *Uva di Troia ad acino piccolo*.

Therefore, the below steps were followed for each Thesis:

- Small amounts of frozen material were gradually withdrawn, in order to minimize thawing;
- Seeds were carefully separated from skins, taking also care to those inside the whole berries;
- Once separated, seeds were first washed with tap water and then with deionized water for the complete removal of skin residues on the surface;
- Seeds were gently dried with paper before storage;
- Separated skins and seeds were again stored at -20°C.

3.3 INSTRUMENTATION

- Laboratory balance with sensibility ± 0,01 mg (Sartorius, Germany);
- Laboratory oven FN 500 (Nüve, Turkey);

- Desiccators containing $CaCl_2$ anhydrous;
- Magnetic agitator IKA® RCT Classic (Germany), with Thermometer VWR™ VT-5 (France);
- UV-Visible Spectrophotometer Cary 50 Scan (Varian, Italy);
- Analog Vortex Mixer (VWR, France);
- Rotavapor R-114 with B-480 bath (Büchi, Switzerland);
- TLC Silica gel plates 60 F_{254} 20x20 cm (Merck, Germany);
- TLC spotting capillaries 2 µL capacity (Brand, Germany);
- Ultrasonic bath Branson 3200 (Colaver, Italy);
- Syringe Nylon filters 25 mm GD/X 0.45 µm pore size (Whatman, UK);
- Membrane filters Hydrophilic Polypropylene 47 mm 0.45 µm pore size (Pall Life Sciences, NY, USA);
- Carousel rotating agitator, variable rotational speed F205 (Falc Instruments, Italy);
- Benchtop centrifuge EBA 2.0 (Hettich, Germany);
- Blender Chopper 100 (Termozeta, Italy);
- pH meter Cyberscan 1100 RS-232® (Eutech Instruments, The Netherlands);
- Socorex® fixed volume micropipettes with 50 µL and 100 µL capacity (Swiss);
- P200 Transferpette S (20-200 µL) (Brand, Germany);
- P1000 Kartell Pluripet (200-1000 µL) (Italy);
- Graduated glass pipettes (AS class);
- Volumetric glass pipettes (AS class) with 5 ± 0.015 mL, 3 ± 0.01 mL, 2 ± 0.010 mL, 1 ± 0.007 mL capacity, 0.5 ± 0.005 mL;
- Volumetric glass flasks (A class) with 10 ± 0.04 mL and 5 ± 0.04 mL capacity;
- Volumetric glass flask (B class) with 1000 mL capacity;
- Round-bottom glass flasks, various capacities;
- Separating funnel, 100 mL capacity;
- 2 mL glass crimp autosampler vials with rubber/teflon® caps;
- Pasteur glass pipettes;

- 10 mL glass tubes with screw caps and rubber/teflon® under caps;
- Percolator INOX, 2.5 L capacity (Albrigi Luigi, Italy);
- Peristaltic pump (Marcello Cellai, Italy).

3.4 CHEMICALS AND REAGENTS

- (+)-Catechin Hydrate, CAS: 225937-10-0 (Sigma, Italy – Batch no. BCBB1843);
- (-)-Epicatechin, CAS: 490-46-0 (Sigma, Italy – Batch no. 0001423660);
- Procyanidin B1, CAS: 20315-25-7 (Fluka, Italy – Batch no. BCBC2219);
- Procyanidin B2, CAS: 29106-49-8 (Fluka, Italy – Batch no. BCBC5014);
- Gallic Acid, CAS: 149-91-7 (Sigma, Italy – Batch no. 040M0052);
- Leucocyanidins Leucoselect™ (Indena SpA, Italy – Batch no. 646/35);
- Vitis vinifera E.S. 95% (Farmalabor, Italy – Batch no. 0002322002);
- Cyanidin chloride, CAS: 528-58-5 (Sigma, Italy – Batch no. BCBD3242);
- Kuromanin chloride, CAS: 7084-24-4 (Sigma, Italy – Batch no. BCBF9745V);
- Oenin chloride, CAS: 7228-78-6 (Sigma, Italy – Batch no. BCBD2218);
- Myricetin, CAS: 529-44-2 (Sigma, Italy – Batch no. BCBC8837V);
- Quercetin, CAS: 117-39-5 (Quimdis, France – Batch no. 080306-1);
- Quercetin-3-*β*-D-glucoside, CAS: 482-35-9 (Sigma, Italy – Batch no. BCBD3233V);
- *t*-Resveratrol, CAS: 501-36-0 (Sigma, Italy – Batch no. 030M5216V);
- Polydatin, CAS: 65914-17-2 (Sigma, Italy – Batch no. BCBG3260V);
- Revidox™ (Paladin Pharma, Italy – Batch no. L0022);
- Milli-Q purified water with resistivity below 18,2 mΩ/cm obtained with Millipore System (Millipore, Massachusetts, USA);
- Methanol G Chromasolv® (Sigma-Aldrich, Germany);
- Absolut Ethanol (Carlo Erba, Italy);
- Acetone (VWR, Pennsylvania, USA);
- Ethyl Acetate (Sigma-Aldrich, Germany);
- Citric acid anhydrous (Fluka, Italy);

- Butyl Acetate (Sigma-Aldrich, Germany);
- Formic acid 98-100% (Merck, Germany);
- Fast Blue solid B (Merck, Germany);
- Vanillin (Merck, Germany);
- Sulphuric Acid 95-97% (Fluka, Italy);
- Sodium Hydroxide NaOH 1 N (J.T. Baker, New Jersey, USA);
- Sodium chloride (J.T. Baker, New Jersey, USA);
- Acetonitrile (CH_3CN) Chromasolv® Plus for HPLC (Sigma-Aldrich, Germany);
- Orthophosphoric acid 85% (Merck, Germany);
- Synthetic Adsorbent Sepabeads® SP-207 (Resindion, Italy – Batch no. 8D551).

4. GRAPE SEED CHARACTERIZATION

4. GRAPE SEED CHARACTERIZATION

The first purpose of our research was to define the phenolic composition of seeds of *Uva di Troia ad acino piccolo* grapes, coming from the four Theses collected. Hence, we focused the attention on the seed content of Catechins and Procyanidins (PCs), the most abundant polyphenols present in this particular grape tissue.

In order to accomplish our objective, we followed the below steps:

- Optimization of the extraction procedure of polyphenolic from grape seeds;
- Determination of seeds humidity percentage;
- Extraction of seeds polyphenols with two different organic solvents;
- Extraction of dried seeds;
- Thin Layer Chromatography (TLC) analyses of the extracts;
- Liquid Chromatography (LC) characterization of the extracts;
- Purification of the extracts with Ethyl Acetate.

The studies were always performed on intact seeds, that is no grinding was carried out in order to avoid the extraction of lipids present inside the seeds.

4.1 EXTRACTION CONDITIONS

The first step to be undertaken was the optimization of grape seeds extraction conditions. This purpose was achieved by defining the ideal contact time between the seeds and the specific organic solvent during a continuous extraction, achieved by maceration in beaker under magnetic stirring. Then, the ideal contact was determined thanks to spectrophotometric analyses of the extracts collected every thirty minutes from the beginning of the extraction.

Methanol, ethanol and acetone variously mixed with water represent the most common solvents used for grape seed extraction. In this case, considering the final objective of the project related with the development of a nutraceutical product, we decided to use acetone or ethanol as solvents; particularly, for the evaluation of time

contact, acetone does not represent a suitable solvent for the spectrophotometric analyses since it interferes in the UV absorption, consequently a mixture of 70:30 ethanol/water was chosen.

The continuous extraction was performed on *Uva di Troia ad acino piccolo* grape seed samples gently supplied by the Agricultural Research Council (CRA) of Barletta. Their intrinsic water content was evaluated by means of loss on drying experiments.

4.1.1 Loss on drying of "CRA" seeds

About 50 g of grape seeds of the CRA cultivar of *Uva di Troia ad acino piccolo* were exsiccated in oven at 60°C until a constant weight was reached. Particularly, 50.0022 g of frozen seeds were weighted twice a day during 5 days, once cooled in the dry atmosphere of a desiccator. The final weight was: 28.2193 g, thus the weight passed from 50.0 g to 28.2 g.

The humidity percentage of the "CRA" seeds was then calculated as follows:

$$50.0\ g : 100 = 28.2\ g : (100-x)$$

$$x = \mathbf{43.6\%\ H_2O}$$

4.1.2 Continuous extraction

50.0700 g of frozen "CRA" grape seeds were added with Ethanol q.s. to reach a final concentration of 70% (V/V) of ethanol in water, considering the intrinsic humidity percentage of 43.6%. Ethanol (EtOH) was added just to cover the seeds in a 400 mL glass beaker with a stir bar of suitable dimension to allow agitation. The beaker was protected from light and air exposure by aluminium foils in order to avoid polyphenols oxidation and photodegradation as much as possible.

Then, seeds were placed under magnetic agitation and once reached the room temperature (23°C), the continuous extraction started (t_0) and samples of the extraction solvent were collected every thirty minutes. Particularly, 50 µL of solvent were diluted to 5.0 mL of 70% EtOH and immediately analyzed by UV-Vis Spectrophotometry between 245 and 400 nm as wavelengths, because of the

characteristic maximum absorption band of PCs at about 280 nm. The blank control was 70% EtOH.

T_0 was recorded at 10:30 a.m. and samples were taken off until 5 hours (h) of extraction were reached, thus, until 3:30 p.m. Then, the last sample was withdrawn after 24 h of maceration (overnight extraction).

Table 2 describes sampling times and the absorbance of the samples collected during 24 h, while Figures 24 and 25 show the curves of absorbance of the samples between 245 and 400 nm.

Time	Sample	Abs at 280 nm
t_0	0h 0′	0
t_1	0h 30′	0.405
t_2	1h	0.497
t_3	1h 30′	0.602
t_4	2h	0.753
t_5	2h 30′	0.936
t_6	3h	1.033
t_7	3h 30′	1.159
t_8	4h	1.316
t_9	4h 30′	1.423
t_{10}	5h	1.547
t_{11}	o.n. (overnight)	>>3.5

Table 2: Sampling times and relative absorbances

The table and the figures show how the maximum of absorbance of Catechins and Procyanidins increments with the time of extraction; as a consequence, in accordance to the Lambert-Beer law, also the concentration of this class of phenolic compounds progressively grows. At the same time, the values obtained reveal that the saturation of the extraction solvent doesn't occur during 24 h of maceration at room temperature.

Therefore, in order to achieve the best phenolic recovering as possible, it was chosen to perform for each sample two extractions per day with a contact time of 3 hours, and a total number of 6 extractions during 48 hours, taking into account also 2 overnight extractions.

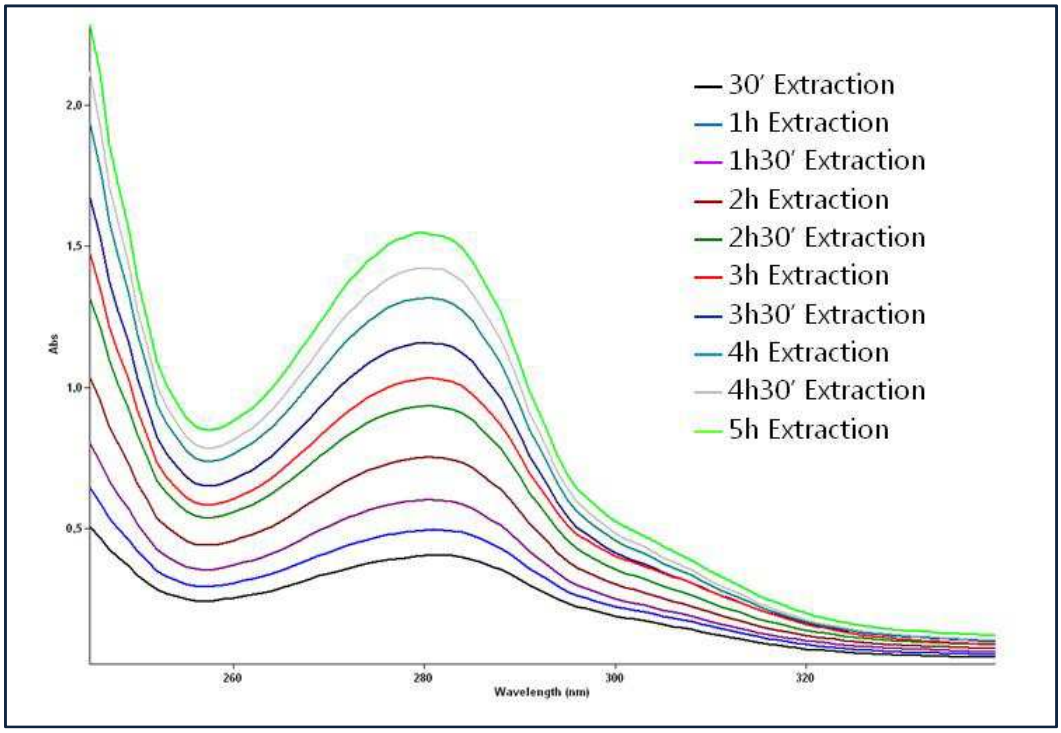


Figure 24: Absorbance between 245 and 400 nm

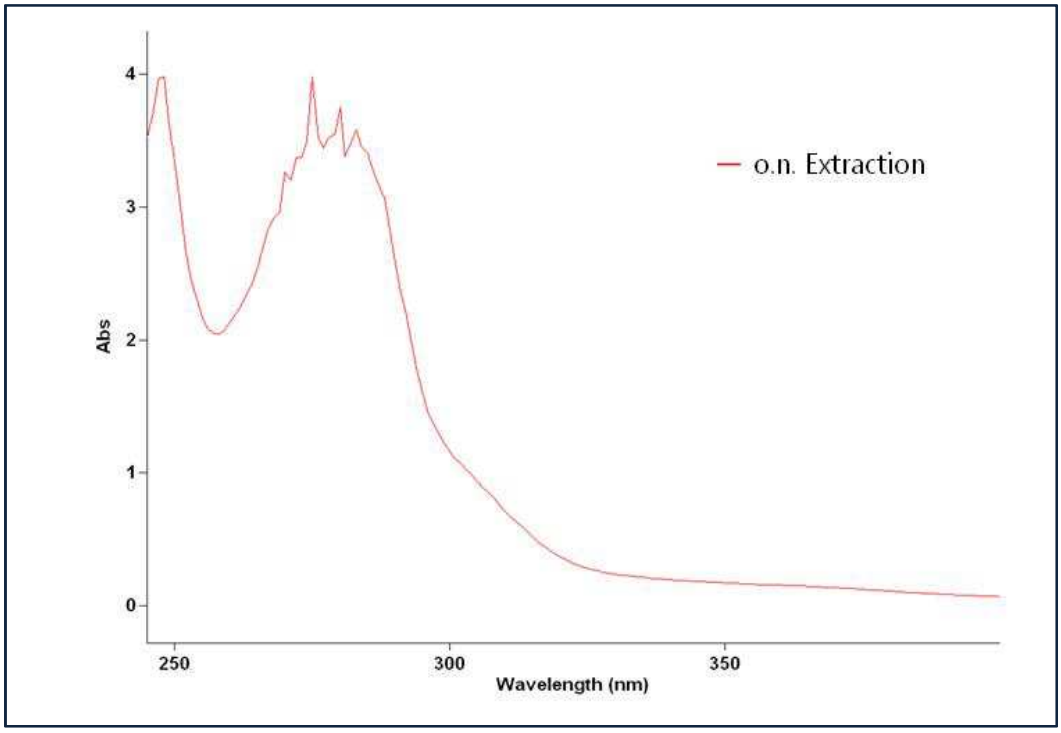


Figure 25: Absorbance of the o.n. sample between 245 and 400 nm

This extraction protocol was subsequently followed for the extraction of the seeds coming from each of the four Theses of *Uva di Troia ad acino piccolo* with two different solvent mixtures: 70:30 ethanol/water and 70:30 acetone/water.

4.2 *UVA DI TROIA* SEED LOSS ON DRYING

In order to evaluate the humidity percentage of each Thesis of *Uva di Troia ad acino piccolo* grape seeds, loss on drying experiments were carried out as for seeds coming from the CRA cultivar. Thus, about 25 or 50 g of grape seeds were desiccated in oven at 60°C and weighted twice a day during 5 days until a constant weight was reached. The humidity percentage of each sample was then calculated as follows:

$$W_i : 100 = W_f : (100\text{-}x)$$

Where W_f is the final weight of the seeds, while W_i is the initial weight of the sample. The results are illustrated in Table 3:

Sample	W_i (g)	W_f (g)	% H_2O
THESIS 1	25.0	16.2	**35.2**
THESIS 2	50.0	32.0	**36.0**
THESIS 3	50.0	28.9	**42.2**
THESIS 4	50.0	28.0	**44.0**

Table 3: Loss on drying of *Uva di Troia ad acino piccolo* grape seeds

Seed water content increases from *Thesis 1* to *Thesis 4*, maybe because of the protracted contact of the seeds with the must.

4.3 GRAPE SEED EXTRACTION

Once chosen the ideal conditions, the extraction of each sample of *Uva di Troia ad acino piccolo* seeds was performed with 70:30 ethanol/water and 70:30 acetone/water following the protocol described below:

- About 25 g or 50 g of frozen seeds were accurately weighted;
- Considering the water percentage of each Thesis, ethanol or acetone were added q.s. to reach a final concentration in the sample of 70% and to cover the seeds;
- Seeds were extracted under magnetic stirring in glass beakers protected from lights and air by aluminum foils;

- The first 3 h extraction started (t_0) when the seeds reached room temperature (R.T.), which was recorded every day and was of about 23°C ± 2 during all the extractions;
- At the end of the 3 h extraction, seeds were filtered under vacuum on a büchner funnel and the extract kept in fridge in an exactly weighted round-bottom flask;
- Once filtered, seeds were subjected to the second 3 h extraction adding fresh solvent (25 mL or 50 mL depending on the initial amount of seeds);
- At the end of the second 3 h extraction, seeds were filtered under vacuum on a büchner funnel and the extract kept in fridge combined with the previous one;
- Seeds were extracted again with fresh solvent (25 mL or 50 mL) during the whole night (1^{st} o.n. extraction);
- Once finished the o.n. extraction, seeds were filtered under vacuum on a büchner funnel and the extract kept in fridge combined with the previous ones;
- Steps from *d.* to *i.* were repeated until the sixth and last extraction, i.e. the 2^{nd} o.n. extraction;
- Then, solvent was completely removed with a rotary evaporator at 40°C and by exsiccation in oven for at least 12 h at 60°C.

For each sample, the dried matter was weighted and the recovery percentage (REC %), which expresses the quality of the extraction procedure, was calculated as follows:

$$REC\,\% = \frac{W_{fin}\ \text{dry extract}}{W_{init}\ \text{dried seeds}}\ X\,100 = \frac{W_{fin}\ \text{dry extract}}{\frac{W_{init}\ x\ (100 - \%\ H_2O)}{100}}\ X\,100$$

Where W_{fin} is the final weight of the extract evaporated to dryness, while W_{init} is the initial weight of the sample, considering the intrinsic content of water.

All dry extracts appeared to be like a purple-red fine powder (Fig. 26) that was easily ground in a mortar before chromatographic analyses.

Figure 26: Aspect of *Thesis 4* seeds dry extract

4.3.1 Extraction of frozen seeds

Frozen seeds of *Thesis 1*, *2*, *3* and *4* were subjected to the extraction protocol described in the previous paragraph, using two different solvent mixtures: 70:30 ethanol/water and 70:30 acetone/water.

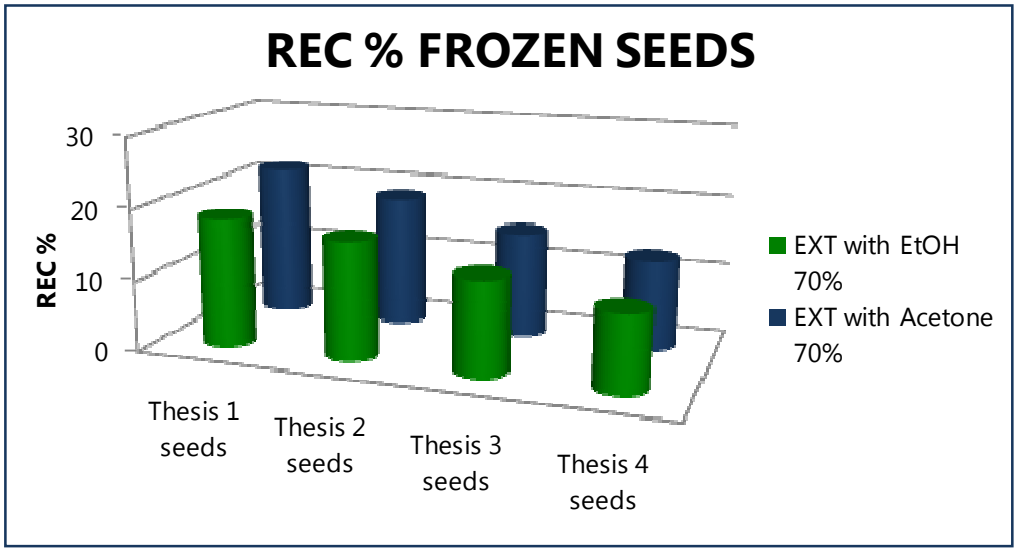
The results related to the recovery percentages are described in Table 4.

Sample	Solvent	W_{init} (g)	W_{fin} (g)	REC % (w/w)
THESIS 1	EtOH 70%	25.0093	2.941	**18.15**
THESIS 1	Acetone 70%	25.0032	3.508	**21.65**
THESIS 2	EtOH 70%	25.0009	2.667	**16.67**
THESIS 2	Acetone 70%	25.0051	2.969	**18.55**
THESIS 3	EtOH 70%	50.0210	3.838	**13.27**
THESIS 3	Acetone 70%	50.0072	4.297	**14.87**
THESIS 4	EtOH 70%	50.0160	2.994	**10.69**
THESIS 4	Acetone 70%	50.0210	3.530	**12.60**

Table 4: REC % of frozen grape seeds extraction

The results show how the recovery % decrements from the extraction of *Thesis 1* seeds to *Thesis 4* seeds (Graph 1). Even if these values are not absolutely related to the phenolic compounds present in our samples, it could be assumed that the recovery percentage represents a valid marker of the phenolic content in the vegetable drug. Moreover, the vinification process leads to the extraction of phenolic compounds from the grapes to the must, thus depleting seed and skin polyphenols.

On the other hand, the mixture acetone/water seems to be the best solvent for seeds extraction. However, the little difference of the recovery values can't demonstrate also a difference in the phenolic extraction between the two solvents. This aspect will be further investigated by chromatographic analyses.



Graph 1: Trend of REC % in *Uva di Troia ad acino piccolo* seed samples

4.3.2 Extraction of dried seeds

About 10 g of *Thesis 1* and *4* dried seeds were also subjected to the same extraction protocol described in the paragraph 4.3, using 70:30 acetone/water. The dried seeds were obtained by exsiccation in oven at 60°C. This step was accomplished in order to evaluate if the drying procedure at 60°C could affect the phenolic content of grape seeds with respect to the frozen vegetable material.

The recovery percentages were calculated as follows:

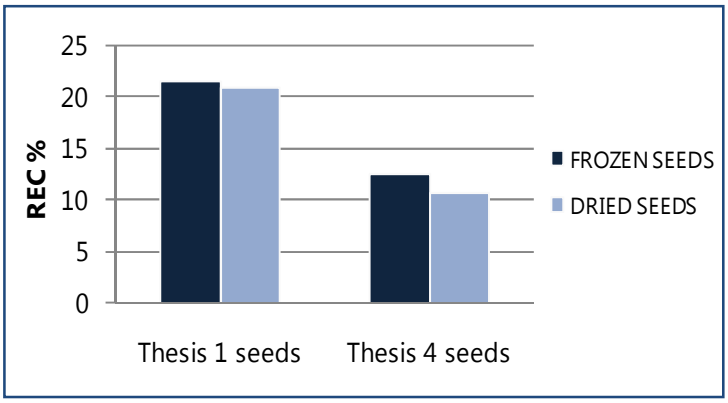
$$\text{REC \%} = \frac{W_{fin}\ \text{dry extract}}{W_{init}\ \text{dried seeds}} \quad X\ 100$$

Results are reported in Table 5.

Sample	Solvent	W_{init} (g)	W_{fin} (g)	REC % (w/w)
THESIS 1	Acetone 70%	10.0025	2.109	**21.08**
THESIS 4	Acetone 70%	10.0058	1.080	**10.79**

Table 5: REC % of dried grape seeds extraction

As we can see by the results illustrated also in Graph 2, the recovery percentages obtained from the extraction of grape seeds exsiccated at 60°C are slightly lower than those obtained by frozen seeds, but the values are not significantly different from each other. By the way, the recovery values don't give us any information about the phenolic content, which will be further investigated by chromatographic analyses.



Graph 2: REC % of frozen seeds *vs* dried seeds extractions

4.4 TLC ANALYSES

The grape seeds extracts obtained as in paragraphs 4.3.1 and 4.3.2 were analyzed by Thin Layer Chromatography (TLC) in order to control the extractive process and to have qualitative information about the phenolic content of the extracts. Samples were compared to reference standards.

However, the composition of the elution solvent and the specific colour reagents used to visualize spots needed to be studied.

4.4.1 Preparation of the standards

For the standards (+)-Catechin Hydrate and (-)-Epicatechin, 0.0100 g of standard were exactly weighted and solved in a 10 mL volumetric flask, thus obtaining a final concentration of 1 mg/mL in methanol. On the other side, samples were also compared to two grape seed standardized extracts: Leucoselect™ and Vitis vinifera E.S. 95%. For their preparation, 0.0500 g of each standard were exactly weighted and solved in methanol in a 5 mL volumetric flask. These solutions have a final concentration of 10 mg/mL, that is 1% (W/V) in methanol.

4.4.2 Preparation of the samples

For each of the ten dry grape seed extracts, 0.0500 g of extract were exactly weighted and solved in methanol in a 5 mL volumetric flask. These solutions have a final concentration of 10 mg/mL, that is 1% (W/V) in methanol.

The same procedure was followed for the preparation of the "CRA" seed extract obtained as in paragraph 4.1.2, that was used for the development of the TLC conditions.

4.4.3 Development of the chromatographic conditions

The first issue to be investigated was related to the elution solvents. Standards of grape seed extracts (e.g. Leucoselect™ and Vitis vinifera E.S. 95%) were compared to the dry extract obtained by the extraction of "CRA" grape seeds, at the concentration of 1% (W/V) in methanol.

The following solvent mixtures were tested:

1) ACETONE/TOLUENE/FORMIC ACID 15:15:5
2) BUTYL ACETATE/ETHANOL/FORMIC ACID/WATER 8:1:1:1
3) ETHYL ACETATE/ETHANOL/WATER 10:2:3
4) CHLOROFORM/METHANOL 2:1
5) *n*-BUTANOL/ETHANOL/WATER 60:15:25
6) ETHYL ACETATE/WATER/FORMIC ACID 10:4:1

Mixtures 4), 5), 6) showed an unsuitable polarity for the separation of catechins and procyanidins, in fact there wasn't any separation of the components present in the extract, neither in the standards.

On the other side, eluents 1), 2) and 3) led to a better separation of the components present in the extracts. Nevertheless, mixture 2) represents the best eluent for seeds phenolics, since it permits also a clear separation of the monomers Catechin and Epicatechin. Therefore, this solvent mixture was chosen for grape seed extract analyses.

4.4.4 TLC stains

In order to visualize spots on TLC plates, two methods were tested after the detection under the UV-lamp at 254 nm. These methods are based on the use of two different specific colour reagents that, sprayed on the plates, stain the spots.

- ***VANILLIN REAGENT***: a 5% solution of Sulphuric acid in EtOH and 1% Vanillin in EtOH are mixed in 1:1 ratio. TLC plates are first sprayed with 20% H_2SO_4 and then with the vanillin reagent freshly prepared before heating carefully with a heat gun, until the spots appear.
- ***FAST BLUE BASIC SOLUTION***: a small amount of Fast Blue solid B is diluted to 10 mL with 1 N NaOH. TLC plates are first sprayed after shaking the solution well.

Both reagents showed a good selectivity for phenolic compounds, but the first one is even better because of the easier detection on plates. Thus the vanillin reagent was chosen as the reagent of choice for TLC spots detection.

4.4.5 TLC results

The ten grape seeds extracts and the standards were then analyzed by TLC as previously described.

Samples were compared to standards of Catechin and Epicatechin and to commercialized products derived from the extraction of different grape seeds biotypes: Leucoselect™ (Indena SpA) and Vitis vinifera 95% extract (Farmalabor Srl).

2 µL of each sample were applied on TLC plates characterized by a stationary phase made of Silica gel added with a fluorescence indicator. Each sample had a concentration of 1% (W/V) in methanol as diluting solvent.

Once the chromatographic separation finished, plates were viewed under the UV-lamp to verify the presence of UV-active substances. Phenolic compounds manifest a characteristic absorption in the UV region, therefore it was possible to confirm their extraction. However, eluted samples were all characterized by an homogenous strip

along the migration distance of the substance and no further separation was detected.

On the contrary, the reaction with Vanillin permitted to visualize not only the strip above described, but also two clear spots above the band. These red-violet spots were identified as Catechin and Epicatechin, thanks to the comparison with the reference standards (Fig. 27).

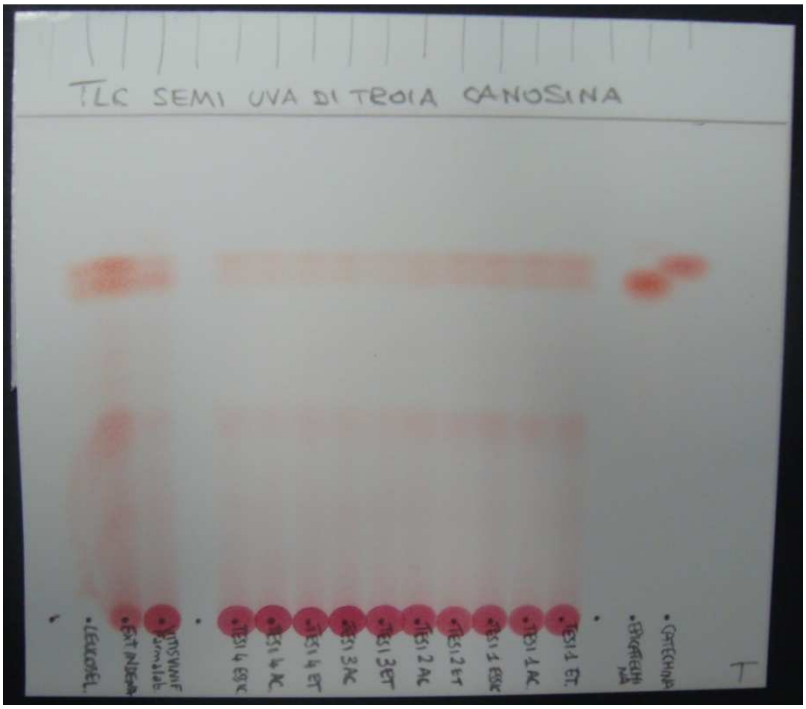


Figure 27: TLC plate of *Uva di Troia canosina* seed extracts

As we can see in Figure 27, grape seed polyphenols, such as Catechins and Procyanidins, are well extracted with the protocol developed. Particularly, we can identify with any certainty the presence of the monomers Catechin and Epicatechin.

Furthermore, the TLC separation gives us an idea of the differences in the phenolic content between the various *Uva di Troia ad acino piccolo* Theses. In fact, it's pretty clear how the colour intensity of the spots increases from *Thesis 4* extract to *Thesis 1* extract.

This result is comparable to that obtained by the recovery values, thus confirming that the polyphenolic content of grape seeds is influenced by the fermentative process, which implies a progressive extraction of the active principles from the seeds to the must and so to the wine.

This trend will be quali-quantitatively confirmed by liquid chromatography analyses.

4.5 LC ANALYSES OF GRAPE SEEDS

The next step for the characterization of grape seed extracts was based on liquid chromatography (LC) analyses, which permitted to separate and identify the phenolic active ingredients present in the extracts, as well as quantify them by comparison with reference standards.

In order to develop a suitable method for grape seed polyphenol characterization, literature data suggest that liquid chromatography coupled to a spectrophotometric detector represents the analytical technique of choice for the direct identification and quantification of the most common and active grape seed components, such as the monomers Catechin and Epicatechin and the dimers Procyanidin B1 and Procyanidin B2 (PC B1 and PC B2). Moreover, a reversed phase (RP) stationary phase is the most suitable one for the separation of this class of compounds, especially for these molecules with a low degree of polymerization.

In our research, we compared our crude grape seed extracts with a patented highly standardized grape seed extract, i.e. Leucoselect™, supplied by Indena SpA. Therefore, we characterized our extracts using the LC method developed by Gabetta *et al.* (2000) for the complete characterization of the proanthocyanidin constituents of Leucoselect™. However, some modifications of the method were carried out in order to improve the resolution.

4.5.1 Apparatus

HPLC analyses were performed on a Varian™ Pro Star equipped with two chromatographic pumps mod. 210, a Varian™ 410 autosampler and diode array detector mod. 335. The instrument is controlled by Software Galaxie which permits data management.

4.5.2 Chromatographic conditions

The grape seeds extracts obtained were studied and analyzed by HPLC-DAD technique, using a Reversed Phase C18 column and a binary solvent gradient with the following chromatographic conditions:

- Chromatographic column: Zorbax SB C18 250 x 4.6 mm i.d. particle size 5 µm (Agilent Technologies™);
- Pre-column: SecurityGuard Cartridges C18 4 x 2.0 mm (Phenomenex™);
- Column temperature: R.T.;
- Detection wavelength: 278 nm;
- Flow rate: 1.0 mL/min;
- Injection volume: 10 µL;
- Syringe washing solvent: methanol/water 1:1
- Solvent A: 0.3% H_3PO_4 in water;
- Solvent B: Acetonitrile;
- Mobile phase: solvents were filtered under vacuum on 0.45 µm membrane filters and degassed by immersion in ultrasonic bath for 15 minutes before column conditioning;
- Gradient:

Time (min)	Solvent A %	Solvent B %
0	90	10
45	80	20
65	40	60
66	90	10
85	90	10

Table 6: Gradient for seed extracts LC method

- Riequilibration time: 5 minutes.

4.5.3 Preparation of the standard solutions

10.0 mg of each of the five investigated grape seed characteristic polyphenols ((+)-Catechin Hydrate, (-)-Epicatechin, Gallic acid, Procyanidin B1 and Procyanidin B2)

were exactly weighted and solved in a 1:1 mixture of Solvent A/Solvent B in a 10 mL volumetric flask, thus obtaining 1 mg/mL solutions. These solutions were then diluted 1:10 (using 10 mL volumetric flasks) with the same solvent mixture in order to obtain solutions with a concentration of 0.1 mg/mL.

4.5.4 Preparation of Leucoselect™

10.0 mg of Leucoselect™ were exactly weighted and solved in a 1:1 mixture of Solvent A/Solvent B in a 10 mL volumetric flask, thus obtaining a 1 mg/mL solution. This solution was filtered on 0.45 µm nylon filters before injection.

4.5.5 Preparation of the samples

100.0 mg of each of the ten crude dry extracts were exactly weighted and solved in a 1:1 mixture of Solvent A/Solvent B in a 10 mL volumetric flask, thus obtaining a 10 mg/mL solution. These solutions were then filtered on 0.45 µm nylon filters before injection. Each solution was named depending on the Thesis used, the typology of seed (frozen or dried) and the extraction solvent (EtOH or Acet 70%) used, for example *Thesis 1 dried seeds Acet 70%*.

4.5.6 Calibration curve

In order to quantify the grape seed polyphenols present in our crude extracts, a calibration curve of the standard (-)-Epicatechin was built analyzing a series of standards across a range of concentrations near the expected concentration of the analytes in the samples.

10.0 mg of (-)-Epicatechin were exactly weighted and solved in a 1:1 mixture of Solvent A/Solvent B in a 10 mL volumetric flask, thus obtaining a 1 mg/mL solution. This solution was then diluted 1:2, 1:5 and 1:10 in volumetric flasks with the same solvent mixture in order to obtain 0.5, 0.2 and 0.1 mg/mL solutions, respectively.

The 0.1 mg/mL solution was further diluted 1:2, 1:5 and 1:10 in volumetric flasks with the same solvent mixture in order to obtain 0.05, 0.02 and 0.01 mg/mL solutions, respectively.

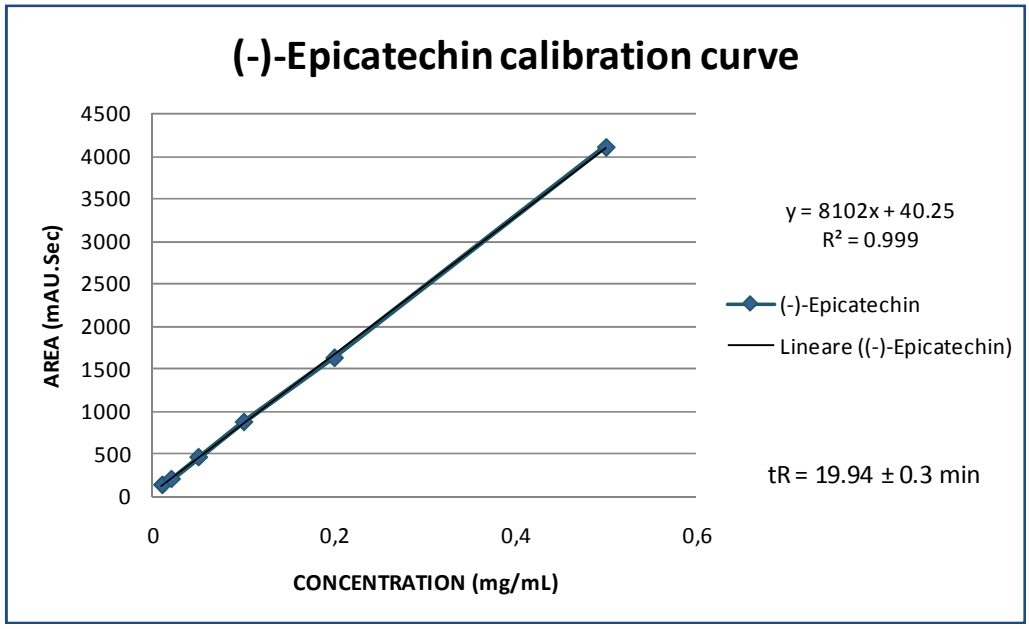
Consequently, the range of concentrations was: 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mg/mL of standard (-)-Epicatechin. These solutions were analyzed by LC-DAD with the method above described (Appendix 1).

Retention times (t_R) and the peak area for each standard are reported in Table 7.

t_R (min)	C (mg/mL)	A (mAU.Sec)
20.34	0.01	127.6
19.97	0.02	197.0
19.83	0.05	454.0
19.85	0.10	867.5
19.98	0.20	1623.2
19.69	0.50	4102.2

Table 7: t_R, concentrations and areas of Epicatechin in the calibration curve

The plot of the instrument response, based on the peak area under the curve (AUC) of the standard *versus* its concentration, showed a linear relationship and a very good correlation of determination R^2, as shown in Graph 3.



Graph 3: Calibration curve of std (-)-Epicatechin

4.5.7 LC analysis of a blank sample

The specificity of a method is defined as its ability to unequivocally assess the analytes of interest in the matrix analyzed. It represents a common parameter used for the validation of a method, even if this is not a purpose of our investigation.

As regarding the optimized LC method, the presence of any interferent peak during the whole analysis time was evaluated by injection of solvent blank samples, because of the impossibility to get a blank vegetable matrix. Blank samples were prepared as a 1:1 mixture of Solvent A/Solvent B (Appendix 2).

4.5.8 LC analysis of Leucoselect™

The LC analysis of Leucoselect™ was used as a controller of the chromatographic separation and efficiency, due to its standardized polyphenolic profile. In fact, its content of monomeric procyanidins is well known and it is attested on about the 15-16% of the extract. Particularly, HPLC-UV analysis of Leucoselect™ by Gabetta *et al.* (Fig. 28), verified the presence of 9.2% of Catechin and 6.8% of Epicatechin and a total concentration of both monomers equal to 16%.

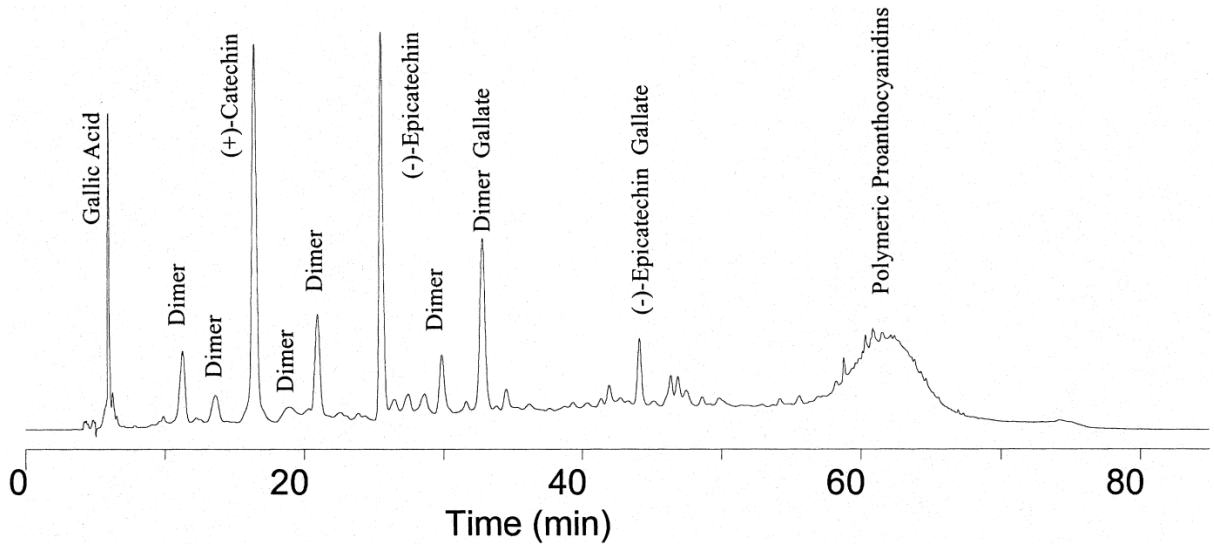


Figure 28: HPLC-UV profile of Leucoselect™ (Font: Gabetta *et al.*, 2000)

In the LC-UV profile of Leucoselect™ we can notice how low molecular weight constituents, such as monomers and dimers, are well-separated, while polymeric proanthocyanidins co-elute as a hump at approximately 60 min. This relief on the chromatographic profile is due to the retention of the polymeric proanthocyanidins in the column at the beginning of the analysis at lower concentrations of Solvent B.

Our LC-DAD analyses of Leucoselect™ 1 mg/mL (Appendices 3-4) show a very similar chromatographic profile. The most important analytes of interest were identified by

comparison with the retention times of the standards and with their spectrophotometric characteristics (Appendix 5).

For the quantification of Leucoselect™ polyphenols, the 1 mg/mL standard was injected twice and the peak areas of the analytes of interest (Gallic acid, Catechin, Epicatechin, PC B1 and PC B2) were interpolated to the (-)-Epicatechin calibration curve to find their concentrations.

The results confirmed us the efficiency of the method towards the characterization of grape seed extracts, in fact the concentrations of Catechin and Epicatechin were consistent with those expected. Results are shown in Table 8.

Analyte	t_R (min)	AREA (mAU.sec)	AREA AVERAGE	CONC. (mg/mL)	CONC %
(+)-CATECHIN	12.43	838.6	849.4	0.0999	**9.99**
(+)-CATECHIN	16.90	860.2			
(-)-EPICATECHIN	18.92	554.0	546.6	0.0625	**6.25**
(-)-EPICATECHIN	24.36	539.1			
GALLIC Acid	4.17	99.8	107.8	0.0083	**0.83**
GALLIC Acid	5.88	115.8			
PROCYANIDIN B1	9.02	225.7	237.1	0.0243	**2.43**
PROCYANIDIN B1	12.41	248.4			
PROCYANIDIN B2	16.20	410.4	392.5	0.0435	**4.35**
PROCYANIDIN B2	21.18	374.6			

Table 8: Quantification of Leucoselect polyphenols™

As we can see in Table 8, the sum of the concentrations % of the monomers Catechin and Epicatechin is equal to 16.24%, a value that is comparable to that obtained by Gabetta *et al.*

Data reveal another aspect of interest: the high variability in analytes retention times between the two injections. Leucoselect™ samples were injected at different moments of the day because of the duration of the chromatographic analysis (85 min) and, moreover, it was not possible to thermostate the column because of its length. Therefore, this problem could be associated to the high variation of the laboratory temperature during the whole day, which strongly influences mobile phase viscosity and, consequently, analytes retention times.

4.5.9 LC analysis of the samples

The ten crude extracts, obtained by the extraction of *Uva di Troia ad acino piccolo* grape seeds, were analyzed by LC-DAD and the chromatographic profile was quite comparable with that obtained from the analysis of the std Leucoselect™ (Appendices 6-15). A high variability of analytes retention times was again found.

In Table 9, the concentrations of the main phenolic compounds present in our extracts are quantified by interpolation of the averaged peak area (Appendices 16-17) of each analyte of interest (Gallic acid, Catechin, Epicatechin, PC B1 and PC B2) to the (-)-Epicatechin calibration curve. Samples were analyzed in duplicate.

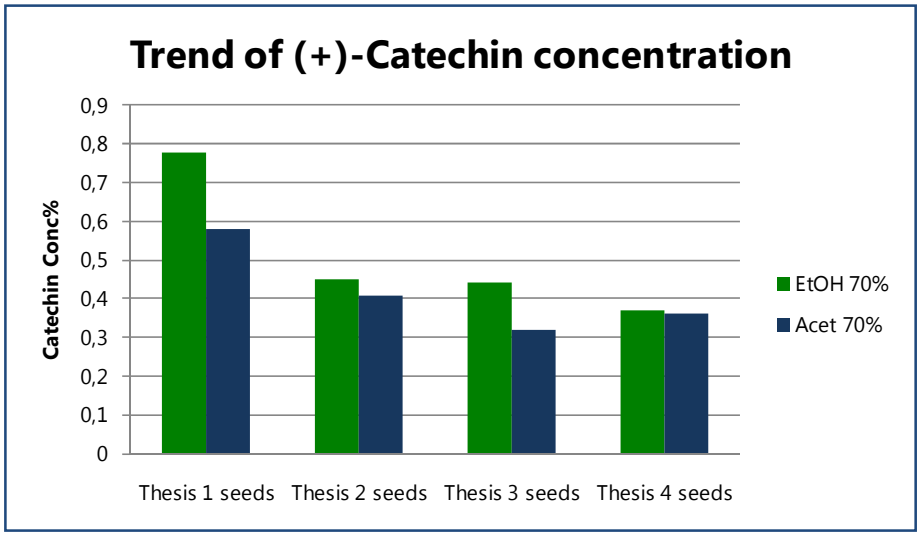
Sample and extraction solvent	Conc % (+)-Catechin	Conc % (-)-Epicatechin	Conc % Gallic Ac.	Conc % PC B1	Conc % PC B2
Thesis 1 frozen seeds EtOH 70%	0.78	0.37	0.16	0.28	0.31
Thesis 2 frozen seeds EtOH 70%	0.45	0.18	0.20	0.22	0.20
Thesis 3 frozen seeds EtOH 70%	0.44	0.18	0.29	0.15	0.25
Thesis 4 frozen seeds EtOH 70%	0.37	0.18	0.18	0.16	0.20
Thesis 1 frozen seeds Acet 70%	0.58	0.42	0.24	0.24	0.26
Thesis 2 frozen seeds Acet 70%	0.41	0.19	0.22	0.16	0.25
Thesis 3 frozen seeds Acet 70%	0.32	0.14	0.30	0.15	0.13
Thesis 4 frozen seeds Acet 70%	0.36	0.13	0.11	0.12	0.20
Thesis 1 dried seeds Acet 70%	0.58	0.43	0.29	0.12	0.23
Thesis 4 dried seeds Acet 70%	0.25	0.17	0.34	0.10	0.20

Table 9: Quantification of grape seed extracts polyphenols

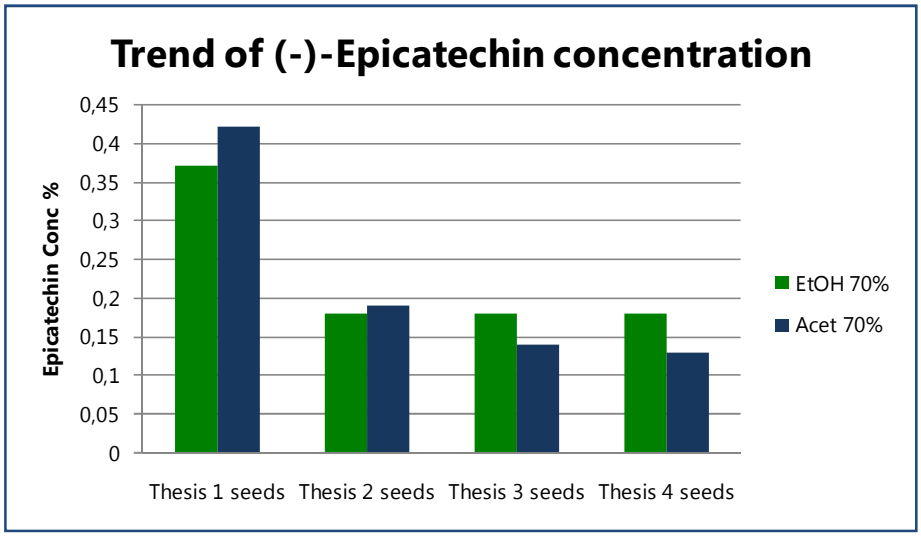
As we can assume from the above table, the phenolic content of the samples is much lower from that of Leucoselect™ and is influenced by the fermentative process, as well by the solvent used for the extraction. In fact, it decreases from *Thesis 1* frozen seeds to *Thesis 4* frozen seeds, thus confirming the previous data obtained by TLC analyses. This trend is consistent with the fact that the polyphenolic content of grape

seeds is influenced by the vinification process, which implies a progressive extraction of the active ingredients from the seeds to the must, thus exhausting seed phenolic content.

Furthermore, the recovery values indicated acetone/water 70% (V/V) as the best solvent mixture for seeds extraction. The differences in the recovery values can't demonstrate also a difference in the extraction of phenolics between the two solvents, whereas LC analyses can. In fact, Graphs 4 and 5 point up that Ethanol 70% better extracts (+)-Catechin than Acetone 70% in all the four Theses, and the same trend appears for the (-)-Epicatechin extraction, except for *Thesis 1* seeds.



Graph 4: Catechin concentration in frozen seeds

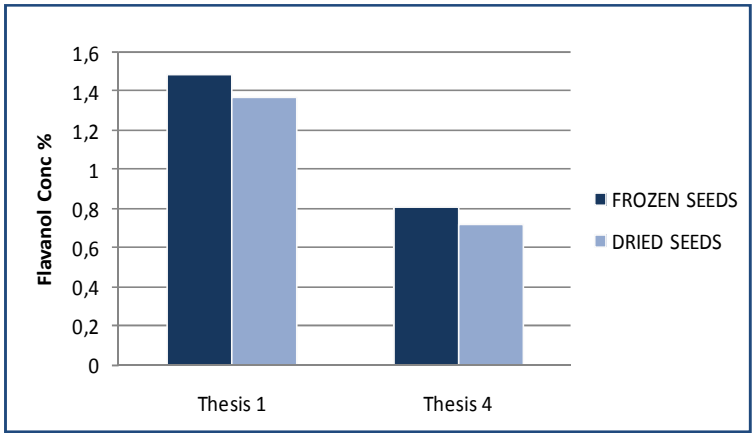


Graph 5: Epicatechin concentration in frozen seeds

Therefore, we can deduce that Acetone 70% represents a less selective solvent for polyphenol extraction from grape seeds.

For what concerns the extraction of Procyanidin B1 and Procyanidin B2, the same tendency is observed, while Gallic acid concentration seems to be more unpredictable among the samples.

The phenolic content of dried seeds, instead, doesn't seem to be influenced by the drying procedure at 60°C. Graph 6 illustrates that the differences in Flavanol (Catechins plus Procyanidins) concentrations between frozen and dried seeds of *Thesis 1* and *Thesis 4* extracted with Acetone 70% are insignificant. This trend is important because of the easier storage of dried seeds and for the higher analytical reproducibility given by them, with respect to frozen seeds.



Graph 6: Flavanol concentration trend between *Thesis 1* and *Thesis 4* frozen and dried seeds

From the analytical point of view, it was necessary to inject higher concentrations of the samples than that of Leucoselect™, i.e. 10 mg/mL for crude extracts *vs* 1 mg/mL for the std, to clearly identify the peaks of interest and quantify them.

In addition, chromatograms show that the broad peak appearing at about 60 minutes of the analysis, relative to the elution of polymeric proanthocyanidins, is definitely bigger than that of Leucoselect™. This huge amount of high polymeric components strongly interferes with the elution of the low molecular weight constituents, as it can be deduced observing the chromatogram baseline (Appendices 6-15).

The high sample concentration necessary for the chromatographic analysis and the abundance in polymeric proanthocyanidins are two negative aspects of our analyses

and are due to the lack of purification steps in the preparation of the extracts. Indeed, the industrial production of Leucoselect™ includes also a patented purification process, that eliminates highly polymerized compounds poorly active on human health and, consequently, enriches the antioxidant phenolic content of the extract.

4.6 PURIFICATION OF GRAPE SEED EXTRACTS

Different purification methods were studied in order to improve and standardize the phenolic composition of grape seed extracts of *Uva di Troia ad acino piccolo*, thus obtaining enriched extracts for which the pattern of characteristic constituents can be determined. Refining extracts, indeed, is acted to reduce analytical problems and to increase the content of active constituents.

4.6.1 Development of the purification procedure

In order to purify grape seed extracts, two methods were studied in a preliminary test performed on one dry extract, i.e. *Thesis 4 frozen seeds EtOH 70%*. Both methods involve a liquid-liquid extraction (LLE) using Ethyl acetate as organic solvent for the removal of interferents, such as lipids, proteins, sugars and highly polymerized compounds.

Particularly, one method is based on the use of the pure organic solvent, while the second involves the concomitant use of an inorganic salt, e.g. NaCl, that should lead to the precipitation of the high weight interferents above cited.

In any case, because of the partial miscibility of Ethyl acetate in water, a previous step of solvent saturation was performed. Therefore, water and Ethyl acetate were placed in a 1:1 ratio in a separating funnel and well mixed. Saturated water was then used to solubilise dry extracts, while saturated Ethyl acetate to purify them.

In the case of the purification with salt, a precise amount of NaCl was added to saturated water to obtain a final concentration of 15% of salt, before LLE.

Both methods were then performed following the below steps:

- 0.50000 g of *Thesis 4 frozen seeds EtOH 70%* dry extract were exactly weighted and transferred in a 10 mL glass tube with screw caps;
- The dry extract was solubilised in 2.5 mL of warm water, thus obtaining a 20% (W/V) solution, and placed for 30' in an ultrasonic bath to better achieve solubilisation of the components;
- 2.5 mL of Ethyl acetate were added and LLE performed on a carousel rotating agitator for 15';
- After centrifugation for 3' at 5000 rpm, the organic phase was collected in a calibrated round-bottom flask;
- Steps from *c.* to *d.* were repeated five times in total.

The collected organic phases were evaporated to dryness under vacuum on a rotary evaporator set at 40°C and by exsiccation in oven for 12 h at 60°C.

When salted water was added to the dry extract, there was the formation of a precipitate that made the solubilisation difficult, so it was necessary to decant the solution leaving it for 12 h in the fridge.

Afterwards, the dried matter, appearing as a red fine powder, was weighted and the recovery percentage (REC %) calculated.

- ***ETHYL ACETATE***: the final weight was W_f = 0.04470 g, so REC % was:

$$\text{REC \%} = \frac{W_{fin}\ \text{dry extract}}{W_{init}\ \text{dry extract}} \text{ X 100} = \frac{0.04470}{0.50000} \text{ X 100} = \mathbf{8.94\ \%}$$

- ***ETHYL ACETATE with NaCl***: the final weight was W_f = 0.03410 g, so REC %:

$$\text{REC \%} = \frac{0.03410}{0.50000} \text{ X 100} = \mathbf{6.82\ \%}$$

Therefore, purification with Ethyl acetate plus salt led to a lower REC % (w/w) value than the pure solvent.

Finally, the two purified extracts were ground in mortar and the corresponding 4 mg/mL (1:1 in Solvent A/Solvent B) solutions were filtered and analyzed by LC-DAD

with the method described in paragraph 4.5.2, in order to control the efficiency of the purification process (Appendices 18-19).

As we can see by the relative chromatograms, both methods strongly reduce the content of polymeric proanthocyanidins in our extracts, but the method chosen for further analyses is the one involving the use of the pure Ethyl acetate. Actually, it gives higher REC% values, a better chromatographic profile and, moreover, it is less time-consuming.

4.6.2 Purification of the extracts

The four dry extracts obtained by the extraction of *Thesis 1* to *Thesis 4* frozen seeds with Ethanol 70%, were subjected to the purification protocol explained in the preceding paragraph. Hence, samples were solubilised in water at 20% (W/V) and the LLE was performed with Ethyl acetate following the described steps.

For each Thesis the REC % was calculated as shown in paragraph 4.6.1 and the final yield of extraction (Table 10) as follows:

$$\text{YIELD \%} = \frac{\text{REC \% crude extracts X REC \% purified extracts}}{100}$$

Sample	W_{init} (g)	W_{fin} (g)	REC %	YIELD %
THESIS 1	0.5000	0.0567	11.34	**2.06**
THESIS 2	0.5000	0.0488	9.76	**1.63**
THESIS 3	0.5000	0.0459	9.18	**1.22**
THESIS 4	0.5000	0.0482	9.64	**1.03**

Table 10: Final yield of grape seed extraction

As expected, the final yield of the whole extractive process is very low.

4.6.3 Preparation of the samples for LC analyses

20.0 mg of each purified dry extract were exactly weighted and solved in a 1:1 mixture of Solvent A/Solvent B in a 5 mL volumetric flask, thus obtaining a 4 mg/mL solution. These solutions were then filtered on 0.45 µm nylon filters before injection.

4.6.4 LC analysis of purified extracts

The four purified extracts were finally analyzed by LC-DAD with the method previously described. As we can see by relative chromatograms, the profiles of the purified extracts are more comparable with that of the Leucoselect™ than those obtained by the analysis of the crude extracts (Appendices 20-23). As a matter of fact, the content of polymeric proanthocyanidins, eluting at approximately 60 minutes, is drastically reduced.

In Table 11, the concentrations of the main phenolic compounds present in our extracts are found by interpolation of the averaged peak area (Appendix 24) of each analyte of interest (Gallic acid, Catechin, Epicatechin, PC B1 and PC B2) to the (-)-Epicatechin calibration curve. Samples were analyzed in duplicate.

Sample	Conc % (+)-Catechin	Conc % (-)-Epicatechin	Conc % Gallic Ac.	Conc % PC B1	Conc % PC B2
Thesis 1 purified seeds	6.35	4.60	0.29	0.73	1.98
Thesis 2 purified seeds	3.64	3.16	0.24	0.80	1.76
Thesis 3 purified seeds	2.55	1.80	0.61	0.63	1.11
Thesis 4 purified seeds	2.73	2.05	0.61	0.56	1.40

Table 21: Characterization of purified grape seed extracts

The table shows how the concentration % of the analytes in the purified extracts is significantly higher than that of non purified hydro-alcoholic extracts. Specifically, the flavanol content of Catechin, Epicatechin, PC B1 and PC B2 is strongly enriched in the refined extracts, while Gallic acid content is not particularly influenced by the purification process, especially in *Thesis 1* and *Thesis 2* extracts.

As a matter of fact, the absolute increment is more evident for *Thesis 1* seeds, where the sum of monomer concentration is 1.15% in the crude extract (see Table 9) *vs* 10.95% in the purified extract, a value that is closer to that of Leucoselect™.

Concluding, the method studied is extremely simple and provides a commercial feasible procedure for the production of purified grape seed extracts.

4.7 EXTRACTION BY PERCOLATION

The final objective of this research, as explained in the previous chapters, is to understand the best extraction and analytical conditions to develop a new nutraceutical product based on the benefits of polyphenols from *Uva di Troia ad acino piccolo*.

Therefore, due to the low yield of the extraction procedure and to the amount of seeds extracted, the small quantity of grape seed extracts obtained for each Thesis is not adequate for the industrial development of the food supplement.

For this reason, in order to simulate an industrial extraction process, we decided to test an extraction in larger scale with a percolator connected to a peristaltic pump, which ensures the continuous agitation of the seeds covered by the extraction medium (Fig. 29).

Figure 29: Percolator connected to a peristaltic pump

Thus, 500.00 g of *Thesis 1* grape seeds were subjected to an extraction by percolation with 70% Ethanol as organic solvent, following the same extraction conditions described in paragraph 4.3. Obviously, considering the capacity of 2.5 L of the percolator, the fresh solvent added to each extraction amounted to about 900 mL.

The seeds herein investigated came from the same crop and harvest of the *Thesis 1* seeds previously analyzed, but they were subsequently sent to our laboratory. For this

reason, another loss on drying experiment was applied to this fraction, thus obtaining a water content of 38.6%.

Once performed all the six extractions, the whole crude extract was evaporated to dryness under vacuum with a rotary evaporator set at 40°C and completely exsiccated in oven at 60°C for at least 12 h. Contrary to the extraction of 50 g of seeds in beaker, the extract thus obtained didn't appear as a perfectly dry powder, but it had some soft consistency that made difficult weighting and sampling procedures. This may be due to the huge amount of extract to evaporate with laboratory instruments.

Anyway, in order to evaluate the quality of this extraction procedure, the dry extract was weighted (W_{fin} = 52.313 g) and the recovery percentage (REC %) then calculated was of 17.04% (w/w). The value obtained is lower than the corresponding extraction by maceration in beaker (18.15% (w/w)), but this could be caused by the higher loss of solvent during percolation. In fact, the connecting tubes couldn't be completely emptied from the extraction solvent and, moreover, the seeds extracted by percolation were more impregnated of solvent, since filtration under vacuum of 0.5 Kg of seeds could not be achieved after each extraction.

5. GRAPE SKIN CHARACTERIZATION

5. GRAPE SKIN CHARACTERIZATION

After grape seed characterization, the second purpose of our research was to define the phenolic composition of the skins of *Uva di Troia ad acino piccolo* grapes. In order to achieve this goal, we extracted, purified and analyzed by liquid chromatography grape skins coming from the four Theses collected. Hence, we focused our attention on the characteristic polyphenols of grape skins, such as Anthocyans (Anthocyanidins and their glycosides), Flavonols and, especially, Resveratrol.

Studies were performed on separated skins, obtained as described in paragraph 3.2. Furthermore, skin polyphenols are more sensitive to high temperatures, thus working with skins always means working with the frozen drug.

5.1 LOSS ON DRYING OF *UVA DI TROIA* SKINS

First of all, the humidity percentage of each Thesis of *Uva di Troia ad acino piccolo* grape skins was evaluated by loss on drying experiments. In order to realize this aim, about 5 g of grape skins were desiccated in oven at 100°C and weighted, once cooled in the dry atmosphere of a desiccator, twice a day during 5 days until a constant weight was reached. The humidity percentage of each sample was then calculated as follows:

$$W_i : 100 = W_f : (100\text{-}x)$$

Where W_f is the final weight of the skins, while W_i is the initial weight of the sample. Results are illustrated in Table 12.

Sample	W_i (g)	W_f (g)	% H_2O
THESIS 1	5.367	1.492	**72.20**
THESIS 2	5.715	1.536	**73.12**
THESIS 3	5.068	1.504	**70.32**
THESIS 4	5.019	1.413	**71.85**

Table 12: Loss on drying of *Uva di Troia ad acino piccolo* grape skins

The content of water resulted quite constant among the samples.

5.2 GRAPE SKIN EXTRACTION

Grape skins extraction was achieved with a one step extraction with methanol, and particularly by maceration of the skins in a beaker under magnetic stirring. Methanol was chosen as the organic solvent for the extraction in order to maximize the recovery of polyphenols, thanks to its high extractive potential. Furthermore, as already explained, methanol is food compatible, even if its residue can't exceed the limit of 10 mg/Kg in the final food supplement (chapter 2).

The extraction method was developed considering that the degradation rate of Anthocyanins is time and temperature dependent, so drying by heating at temperatures higher than 70°C must be avoided. For this reason, we performed grape skin extraction at room temperature (R.T.= 22°C ± 2) to minimize degradation.

Hence, frozen skins of *Thesis 1*, *2*, *3* and *4* were independently subjected to the following extraction protocol:

a. Separated frozen skins were milled with an electronic grinder;
b. 100.0 g of the drug were accurately weighted and suspended in a 500 mL beaker, protected from light and air exposure by aluminium foils, with about 250 mL of methanol (MeOH);
c. Once reached the R.T., extraction was carried out for 45 minutes;
d. The homogenized material was then transferred to a 1000 mL volumetric flask and recipients abundantly washed with MeOH before making up the solution;
e. The extract was filtered under vacuum on a büchner funnel before further analyses.

The weight of each Thesis is reported in Table 13.

Sample	W_i (g)
THESIS 1	100.33
THESIS 2	100.33
THESIS 3	100.33
THESIS 4	100.10

Table 13: Weight of each skin sample

Each crude methanolic extract was then subjected to three different characterizations: evaluation of the extraction procedure, UV-Vis analysis and LC analysis.

5.3 SKIN EXTRACTION RECOVERY

For each grape skin extract, the quality of the extraction procedure was measured calculating the recovery percentage (REC %).

For this purpose, exactly 100.0 mL of each extract were completely evaporated to dryness under vacuum on a rotary evaporator set at 40°C, the dried matter was then weighted and the recovery percentage (REC %) was calculated as follows:

$$\text{REC \%} = \frac{W_{fin}\ \text{dry extract}}{W_{init}\ \text{frozen skins}} \quad X\,100$$

Where W_{fin} is the final weight of the extract evaporated to dryness, while W_{init} is the initial weight of the sample, without considering its intrinsic content of water. W_{init} considers the volume exactly measured, i.e. 100 mL, that is 1/10 of the whole extract (1000 mL); consequently, W_{init} is 1/10 of the initial weight of frozen skins (e.g. for *Thesis 1* W_{init} is 10.033 g).

In addition, contrary to grape seed extracts, skin dried extracts appeared as dark violet soft extracts, that are semi-solid preparations of an intermediate consistency between liquid and dry extracts (Fig. 30).

Figure 30: Aspect of *Thesis 2* skin dry extract

This consistency makes difficult (if not impossible) grounding, weighting, sampling and the storage of the extracts and is presumably related to the extraction of the sugars present in grape skins.

Anyway, the recovery percentages of such soft extracts are described in Table 14.

Sample	Solvent	W_{init} (g)	W_{fin} (g)	REC % (w/w)
THESIS 1	MeOH	10.033	2.221	**22.14**
THESIS 2	MeOH	10.033	1.586	**15.81**
THESIS 3	MeOH	10.033	0.579	**5.77**
THESIS 4	MeOH	10.010	0.4904	**4.90**

Table 14: REC % of frozen grape seeds extraction

The above table clearly shows that the recovery % decrements from the extraction of *Thesis 1* skins to *Thesis 4* skins. Thus, also in grape skin extractions, it could be assumed that the vinification process leads to the depletion of skin phenolic compounds, which are extracted into the must. This aspect will be further investigated by UV-Vis and chromatographic analyses.

Moreover, the low yield is presumably caused by the high level of moisture of the grape skin extract, which restricts the extraction efficiency with respect to a dry material.

5.4 DETERMINATION OF TOTAL ANTHOCYANS

The total amount of anthocyans in *Uva di Troia ad acino piccolo* skin extracts was determined by measuring the absorbance between 200 and 700 nm, and particularly around 535 nm, against a blank of 2% HCl in methanol.

UV-Vis spectra show two characteristic absorption bands for each extract, one at 281-282 nm and the other at approximately 535 nm (Fig. 31). The former is due to Proanthocyanidins absorption, thus confirming their presence also in grape skins, while the latter is relative to Anthocyans.

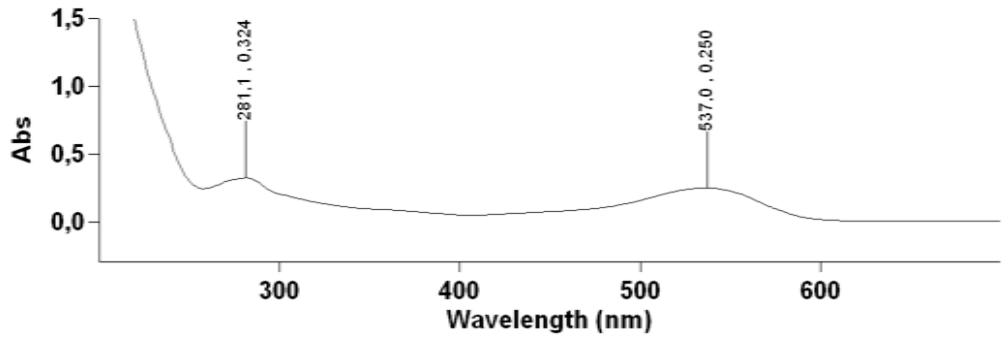


Figure 31: UV-Vis detection of *Thesis 1* grape skins between 200 and 700 nm

In order to quantify total anthocyans, the maximum of absorbance red at ≈535 nm was compared to Malvidin specific absorbance [1%, 1 cm], i.e. the absorbance of a solution containing 1 g of substance in 100 mL of solvent in a 1 cm shell. Malvidin specific absorbance at 537 nm was given by Schou (1927) and it is equal to 1010.

All the extracts were diluted with 2% HCl in methanol until the absorbance was within acceptable limits of absorbance. The most suitable dilution resulted 1:100 in 2% HCl in methanol; analyses were conducted in duplicate.

The amount of total anthocyans in the skin extracts was calculated as follows:

$$A_{537} : x = 1010 : 1\ \%$$

Where A_{537} is the average absorbance at the specific wavelength (WL) obtained by the two analyses of the extract considered.

The concentration of anthocyans thus obtained, related to 1 mL of grape skin extract, was multiplied by the dilution factors of the extract volume and divided by the weight (W_{init}) of skins employed. This value was then multiplied by 100 to express results as % of anthocyans equivalent to Malvidin. Results are shown in Table 15.

Sample	WL	Abs_1	Abs_2	Abs_{med}	W_{init} (g)	CONC %
THESIS 1	537	0.250	0.250	0.250	100.33	**0.247**
THESIS 2	533	0.117	0.117	0.117	100.33	**0.115**
THESIS 3	533	0.109	0.109	0.109	100.33	**0.108**
THESIS 4	535	0.086	0.087	0.087	100.10	**0.086**

Table 15: Determination of total anthocyans in grape skin extracts

As we can see, the total amount of anthocyans decreases from *Thesis 1* to *Thesis 4* grape skin extracts, thus confirming the progressive extraction of skin phenolic compounds in the must during winemaking processing.

5.5 LC ANALYSIS OF GRAPE SKINS

5.5.1 Method development

Numerous methods have been developed for anthocyans and flavonols characterization, being reversed phase liquid chromatography (RP-LC) the most employed one. The chromatographic separation is, therefore, a function of the polarity of the analytes and depends on their chemical status. For this reason, the addition of an acid to the mobile phase is necessary to maintain carboxyl and hydroxyl groups in the protonated form and to improve the resolution.

In this work, the individual separation of the analytes of interest in the grape skin extracts was performed on a C18 reversed stationary phase, using a LC instrument coupled to a spectrophotometric detector. The influence of different organic phases and gradients was studied in order to optimize the chromatographic conditions that allow the separation of grape skin polyphenols, focusing the attention on *t*-Resveratrol.

First of all, due to the high variability on the UV absorbance of each class of phenolics present in grape skins, we decided to set the detector at a wavelength suitable for all the components, i.e. 254 nm.

Then, the mobile phases and the gradient were investigated, as well as the acid to use; the method behaviour was evaluated by injection of a mix of selected polyphenols, representative of those present in grape skins (see paragraph 5.5.4). Preliminary experiments carried out with methanol and phosphate buffer pH 3.3 as mobile phases B and A, respectively, resulted in unsatisfactory separations of the polyphenols, both under isocratic either gradient elution mode. The negative aspects of these tests involve the long duration of the analysis and evident peak broadenings.

Hence, acetonitrile as organic phase was preferred over methanol, due to its lower viscosity, and also a lower pH was tested. Particularly, those solvents already used for grape seed characterization were chosen as mobile phases also for grape skin characterization, i.e. H_2O added with 0.3% of H_3PO_4 (pH ≈ 1.7) as solvent B and Acetonitrile as solvent A. Also acetonitrile as solvent A organic modifier was tested, but unsuccessfully.

The separation of the grape skin polyphenols present in the mix was at last achieved under a 65 minutes multisegment gradient elution mode with increasing concentration of acetonitrile. This gradient will be ahead described in detail.

Finally, considering the impossibility to obtain perfectly dry extracts from grape skins, the preparation of the samples for LC analyses was investigated. Skin methanolic extracts were injected as such or diluted 1:10 in methanol and in mobile phase 70:30 A/B, the latter being the best among these. To further improve peak separation and detection, another method was tested: 1 mL of the crude methanolic extract, previously filtered on 0.45 µm filters, was evaporated to dryness under a stream of nitrogen at 40°C and recovered with 1 mL of a mixture 70:30 of Solvent A/Solvent B. This method resulted the best one.

Analytes were then identified by comparison of their retention times to those of the corresponding standards.

5.5.2 Apparatus

HPLC analyses of grape skin extracts were performed on a 1220 Infinity LC (Agilent Technologies™) equipped with two chromatographic pumps, a manual injector with a 20 µL loop and a UV-Vis detector. The instrument is controlled by Software ChemStation which permits data management.

5.5.3 Chromatographic conditions

The four grape skin extracts obtained were studied and analyzed by HPLC-UV, following the chromatographic conditions reported below:

- Chromatographic column: Zorbax SB C18 250 x 4.6 mm i.d. particle size 5 µm (Agilent Technologies™);
- Pre-column: SecurityGuard Cartridges C18 4 x 2.0 mm (Phenomenex™);
- Column temperature: R.T.;
- Detection wavelength: 254 nm;
- Flow rate: 0.7 mL/min;
- Injection volume: 20 µL;
- Syringe washing solvent: methanol;
- Solvent A: 0.3% H_3PO_4 in water;
- Solvent B: Acetonitrile;
- Mobile phase: solvents were filtered under vacuum on 0.45 µm membrane filters and degassed by immersion in ultrasonic bath for 15 minutes before column conditioning;
- Gradient:

Time (min)	Solvent A %	Solvent B %
0	98	2
5	98	2
10	80	20
15	80	20
30	70	30
35	70	30
50	55	45
55	40	60
58	40	60
60	98	2
65	98	2

Table 36: Gradient for skin extracts LC method

5.5.4 Preparation of the standard solutions

5.0 mg of Quercetin-3-*β*-D-glucoside, Myricetin and *t*-Resveratrol were exactly weighted and solved in methanol in a 5 mL volumetric flask, thus obtaining 1 mg/mL solutions. These solutions were then diluted 1:10 (using 10 mL volumetric flasks) with

a 70:30 mixture of Solvent A/Solvent B in order to obtain solutions with a concentration of 0.1 mg/mL.

5.0 mg of Quercetin were exactly weighted and solved in methanol in a 5 mL volumetric flask, thus obtaining a 1 mg/mL solution, that was then diluted 1:40 with a 70:30 mixture of Solvent A/Solvent B in order to obtain a final concentration of 0.025 mg/mL.

1.0 mg of Cyanidin chloride, Kuromanin chloride and Oenin chloride were solved in methanol to obtain 1 mg/mL solutions.

Then, 500 µL of Cyanidin and Kuromanin solutions were diluted to 5 mL of methanol to obtain 0.1 mg/mL solutions; for the solution of Oenin chloride, 125 µL were diluted to 5 mL of methanol to obtain a 0.025 mg/mL solution.

The mix of grape skin polyphenols, cited in the method development, was prepared with the solutions above described, as follows: 100 µL Kuromanin-Cl 0.1 mg/mL + 250 µL Oenin-Cl 0.025 mg/mL + 150 µL Cianidin-Cl 0.1 mg/mL + 150 µL Quercetin 0.025 mg/mL + 100 µL Quercetin-3-*β*-D-glucoside 0.1 mg/mL + 100 µL Myricetin 0.1 mg/mL + 100 µL *t*-Resveratrol 0.1 mg/mL.

5.5.5 Preparation of the samples

1 mL of each crude methanolic grape skin extract, previously filtered on 0.45 µm nylon filters, was evaporated to dryness under a stream of nitrogen at 40°C and recovered with 1 mL of a 70:30 mixture of mobile phases A/B. The solutions thus obtained were directly injected in the LC instrument.

5.5.6 Identification of the analytes

The comparison of peak retention times to those of the standards, as well as the evaluation of the UV spectra, generally permit to identify with almost any certainties the analytes of interest in the samples analyzed by LC-DAD.

However, the use of a UV detector set at one single wavelength and the complexity of our matrix didn't ensure us the absolute identification of grape skin polyphenols in our extracts just by the comparison of the retention times.

Therefore, sum tests were performed to guarantee the exact nature of the peaks eluted. In order to carry out these tests, retention times were first checked out in the samples and those similar to the standards were chosen as putative analytes of interest. Then, the area of the related standard was divided by the area of the unknown peak in order to obtain a dilution factor that was used to dilute the standard and make its concentration comparable to that of the analyte to identify. The standard diluted and the sample were subsequently mixed in a 1:1 ratio and the mixture was injected in the LC column. In the obtained chromatogram, sample peak areas and heights were half of the original ones, except to that of the standard if it corresponded to the putative peak. If the unknown peak didn't correspond to the standard, even if the retention times were similar, the chromatogram showed two different peaks close together with comparable area and height. An example of sum test is shown in Appendix 25.

5.5.7 Quantification of the analytes

The quantification of the analytes was achieved by comparison of the peak area of each analyte identified to that of the standard of the same phenolic class, through the measurement of the Response Factor (RF), taking into account the amount of skins extracted. RF was calculated as follows:

$$RF = \frac{\text{STD AREA}}{\text{CONC (mg/mL)}}$$

Quercetin 0.025 mg/mL and Oenin-Cl 0.025 mg/mL were used as reference STDs and analyzed at least in triplicate by LC-UV with the conditions above described, in order to control the linearity of the method. Then, the average of the response factors was used to calculate analyte concentration in our samples, thus:

$$\text{CONC \%} = \frac{\text{AREA}}{RF_{med} \times W_{init}} \quad X\,100$$

Where W_{init} is the initial amount of skins extracted, RF_{med} is referred to as the average of the response factors and $AREA_{med}$ is the average of analytes peak areas.

The results obtained by the analyses of the STDs are illustrated in Table 17.

STD	C (mg/mL)	Area (mAU.sec)	RF	RF med	STD DEV	CV %
Quercetin	0.025	2347.45898	93898.3592	91692.25253	2124.49	2.32
	0.025	2241.50195	89660.0780			
	0.025	2287.95801	91518.3204			
Oenin-Cl	0.025	583.37781	23335.1124	23960.4846	445.71	1.86
	0.025	599.77325	23990.9300			
	0.025	603.54437	24141.7748			
	0.025	609.35303	24374.1212			

Table 17: Response factors and coefficients of variation (CV) of Quercetin and Oenin-Cl

5.5.8 LC analysis of the standards

Each one of the standard solution described as in paragraph 5.5.4 was separately analyzed with the developed chromatographic method, in order to verify the specific retention time. Moreover, as already explained, a mix of the selected standards was injected to ensure the chromatographic separation of each standard (Appendix 26).
The chromatogram relative to the mix standard clearly shows the excellent separation of the analytes, whose retention times (t_R) are listed in Table 18.

STD	Phenolic class	t_R (min)
Cyanidin chloride	Anthocyanidin	17.40
Kuromanin chloride	Anthocyanin	14.43
Oenin chloride	Anthocyanin	15.11
Quercetin	Flavonol	37.49
Quercetin 3-*β*-D-glucoside	Flavonol	21.15
Myricetin	Flavonol	29.31
***t*-Resveratrol**	Stilbene	33.16

Table 18: STDs and their retention times

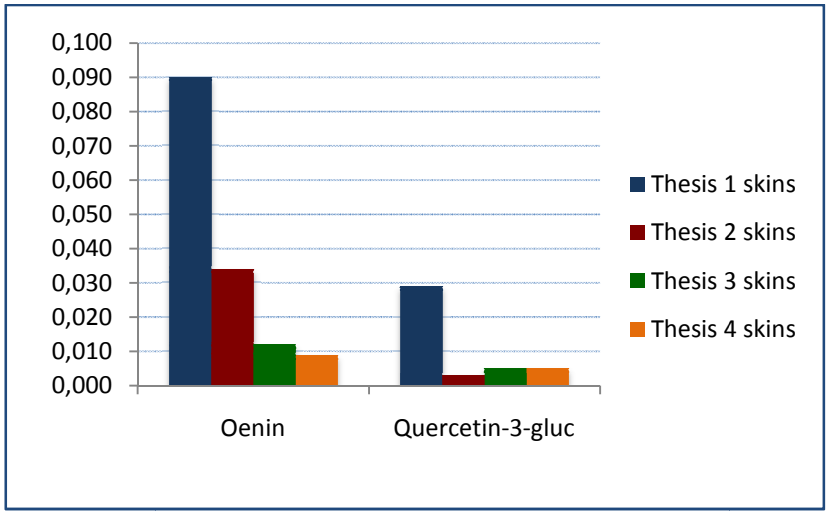
5.5.9 LC analysis of the samples

The four crude grape skin extracts were analyzed by LC-UV using the method developed, and peaks were identified by comparison with standards retention times

and by sum tests (Appendices 27-30). Grape skin extracts proved to be very complex matrices, because of the difficulties encountered in the separation of the analytes and in their identification. The phenolic compounds certainly identified in our extracts, depending on the Thesis explored, were: Oenin, Quercetin-3-*β*-D-glucoside and Quercetin. On the other hand, Kuromanin, Cyanidin and Myricetin were also found, but in trace; conversely, *t*-Resveratrol wasn't found at all. In Table 19, the areas and the relative concentration of the polyphenols present in our extracts are quantified as previously explained. Samples were analyzed in duplicate.

SAMPLE	ANALYTE	AREA 1 (mAU.sec)	AREA 2 (mAU.sec)	AREA $_{med}$	CONC %
THESIS 1	Oenin	2188.75000	2121.88208	2155.31604	0.090
	Quercetin-3-gluc	2402.09570	2872.67041	2637.38306	0.029
THESIS 2	Oenin	792.82379	839.61322	816.21851	0.034
	Quercetin-3-gluc	346.96301	271.95877	309.46089	0.003
	Quercetin	495.78073	465.76526	480.77300	0.005
THESIS 3	Oenin	301.95581	296.48740	299.22161	0.012
	Quercetin-3-gluc	466.19031	483.40906	474.79969	0.005
	Quercetin	445.91943	412.43497	429.17720	0.005
THESIS 4	Oenin	212.77513	238.62590	225.70052	0.009
	Quercetin-3-gluc	407.91098	423.44232	415.67665	0.005
	Quercetin	596.47479	585.32288	590.89884	0.006

Table 19: Concentration % of polyphenols in grape skin extracts



Graph 7: Trend of Oenin and Quercetin-3-glucoside concentrations between the four Theses

Data show how the phenolic concentration of the analytes of interest decreases from *Thesis 1* to *Thesis 4* grape skin extracts, even if these values are quite low (Graph 7). Anyway, this trend confirms previous considerations about the progressive extraction of skin active ingredients in the must during winemaking.

5.6 PURIFICATION OF GRAPE SKIN EXTRACTS

As already explained in the previous chapter, the purification of crude extracts represents the best way to reduce analytical problems due to the complexity of the matrix and to increase the content of the active constituents. For instance, the extracts we obtained by the extraction of grape skins of *Uva di Troia ad acino piccolo* had a soft consistency, probably due to the extraction of interferents together with polyphenols, that makes difficult all the analytical procedures. Thus, in order to eliminate such interferents, i.e. sugars and proteins that are present in our crude extracts, a purification method based on the use of adsorbent resins was investigated.

5.6.1 Development of the purification procedure

In order to purify grape skin extracts a selected adsorbent resin was tested, i.e. Sepabeads® SP-207 (Resindion, Italy). This particular resin is a highly porous synthetic adsorbent based on a styrene e DVB (Divinylbenzene) copolymer with a calibrated pore structure and a high degree of hydrophobicity; it can be used as solid extractant for the removal of interfering organic substances.

In general, synthetic adsorbents have a large surface area made of spherical particles and also fine pore structures, hence, they can effectively adsorb organic compounds from aqueous solutions. The porous structure allows the diffusion of smaller solutes, whereas molecules that are larger than pore size can't penetrate inside the particles and so are excluded and, consequently, can't be adsorbed on the resin.

Different types of chemical structures for synthetic adsorbents can exist, such as aromatic, modified aromatic and methacrylic series. The chemical structure influences the degree of hydrophobicity of the resin, a characteristic that is important in

selecting a suitable type of adsorbent according to the chemical nature of the target compounds.

Sepabeads® SP-207 is a modified aromatic synthetic adsorbent characterized by a brominated aromatic matrix (Fig. 32). This attribute represents confers to the resin an enhanced hydrophobicity that provides a great selectivity for non-polar molecules and permits the adsorption of highly hydrophilic substances. For instance, according to its technical sheet, Sepabeads® SP-207 represents a suitable resin for the recovery of aromatic compounds.

Specifically, the resin has a specific surface area of 630 m^2/g, particle size of 250 µm, a pore volume of 1.3 mL/g and an average pore diameter of 100-150 Å.

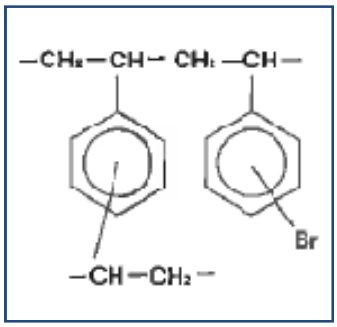


Figure 32: Sepabeads® SP-207 chemical structure

Before use, the resin was activated by keeping it in absolute ethanol overnight. Then, it was filtered under vacuum on a büchner funnel and washed with plenty of distilled water. Once measured the volume of resin to use and filled in the column, the resin was again repetitively washed and backwashed with water, in order to pack it in the column.

5.6.2 Purification of the extracts

The purification procedure with Sepabeads® SP-207 was tested on *Thesis 1* grape skin extract.

16.016 g of *Thesis 1* extract were solved in 50 mL of distilled water and filtered on cotton before loading it into the column containing 100 mL of the activated adsorbent resin. The loading of the sample was then accomplished by a silicon dripper, thus preventing the movement of the resin. After that, the resin was

abundantly washed with water in order to eliminate interfering substances and to control any loss of phenolic compounds. In fact, the washing water was collected in four different fractions and analyzed by UV-Vis spectrophotometry between 200 and 700 nm; no absorption was detected in the selected wavelengths.

Finally, the analytes of interest, i.e. Anthocyanins, were desorbed from the resin by dripping into the column about 500 mL of EtOH 95% with 0.01% of citric acid, being the latter necessary to re-establish the acidic pH of the extract that was altered by the addition of water (Fig. 33).

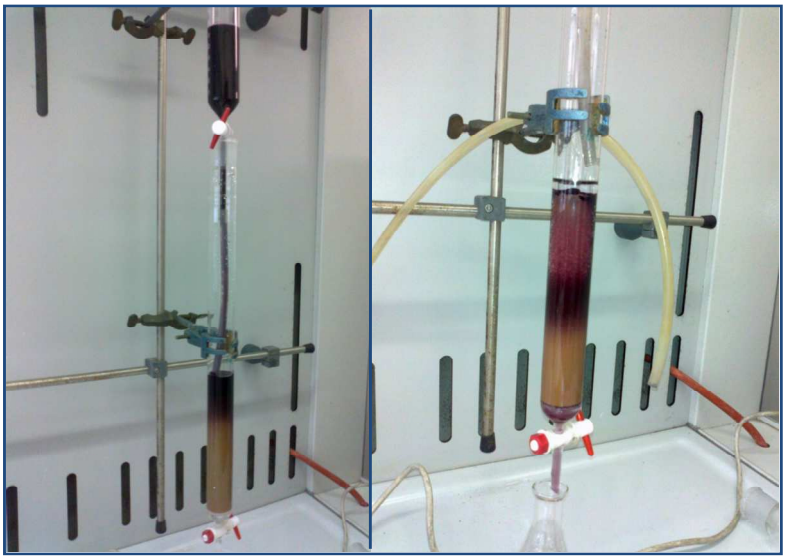


Figure 33: Loading and elution of the sample during the purification process

The elution solvent was fractionated into two parts and analyzed by spectrophotometry to control the complete elution of the analytes from the column. Also the pH of each fraction was measured and it didn't exceed the value of 4.9 for the last aqueous fraction.

5.6.3 Total Anthocyanins in the purified extract

How already explained, the ethanolic fractions obtained by the on column purification were analyzed by spectrophotometry to control the complete desorption of the analytes (Fig. 34). Particularly, the two fractions were diluted 1:100 in a 98:2 mixture of methanol/HCl 37% (50 µL diluted to 5 mL) and analyzed between 200 and 700 nm against 98:2 methanol/HCl 37% as blank.

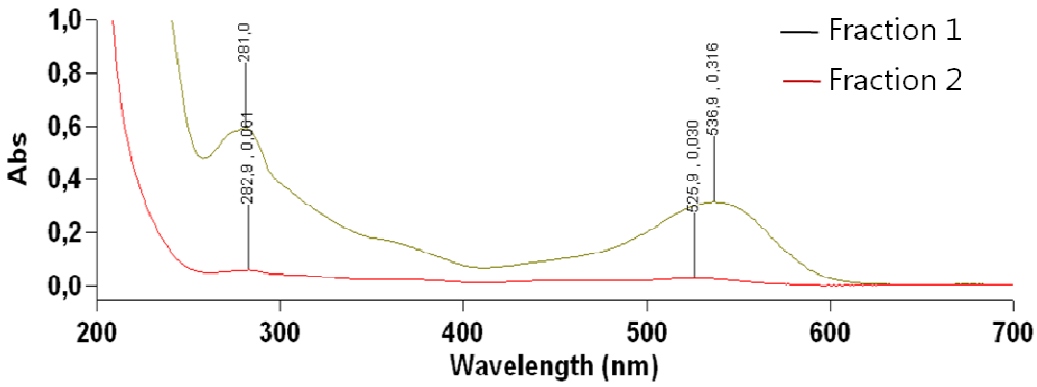


Figure 34: UV-Vis spectra of the ethanolic fractions of *Thesis 1* purified extract

The amount of total anthocyans in the skin extracts was calculated as follows:

$$A_{537} : x = 1010 : 1\ \%$$

Where A_{537} is the absorbance at the specific wavelength (WL) and 1010 is Malvidin specific absorbance [1%, 1 cm].

The concentration of anthocyans thus obtained was multiplied by the dilution factor of the extract volume (V) and divided by the weight (W_{init}) of the crude extract employed. This value was then multiplied by 100 to express results as % of anthocyans equivalent to Malvidin. Results are shown in Table 20.

Sample	WL	Abs	V (mL)	W_{init} (g)	CONC %
FRACTION 1	537	0.316	330	16.010	**0.651**
FRACTION 2	526	0.030	100	16.010	**0.019**

Table 20: Determination of total anthocyans in *Thesis 1* purified extract

Fraction 2 Anthocyanin concentration represents only the 2.92% of Fraction 1, so that the desorption of the analytes of interest can be considered complete.

5.6.4 Recovery % of purified extracts

The quality of the purification procedure was measured calculating the recovery percentage (REC %). For this purpose, the two ethanolic fractions of purified grape skins were combined and evaporated to dryness under vacuum on a rotary

evaporator set at 40°C. The dry matter thus obtained was weighted and the recovery percentage (REC %) was calculated as follows:

$$REC\,\% = \frac{W_{fin}\ \text{purified extract}}{W_{init}\ \text{crude extract}}\ X\,100$$

Where W_{fin} is the weight of the dry purified extract, i.e. **1.238 g**, while W_{init} is relative to the initial weight of the crude extract, i.e. 16.016 g. Therefore, the REC % of the purified extract was equal to **7.73 %** (w/w).

Considering that the crude extracts was obtained by the extraction of 100.33 g of frozen skins with a REC % = 22.14 % (w/w), the final yield of the whole extractive process was:

$$YIELD\,\% = \frac{REC\,\%\ \text{crude extracts}\ X\ REC\,\%\ \text{purified extracts}}{100} = \frac{22.14 \times 7.73}{100} = \mathbf{1.71\ \%}$$

As already seen with grape seeds, the final yield is very low.

However, what most attracts our attention is the consistency of the purified extract, contrary to the crude extract. In fact, as shown in Figure 35, the grape skin purified extract appears as a fine violet powder, i.e. a dry extract that confirms the efficiency of the purification process tested.

The dry extract was ground in a mortar and then analyzed by LC-UV with the chromatographic method developed for crude extracts (Paragraph 5.5.3).

Figure 35: Aspect of *Thesis 1* grape skin purified dry extract

5.6.5 Preparation of the sample for LC analysis

10.0 mg of the purified grape skin dry extract were exactly weighted and solved in a 70:30 mixture of Solvent A/Solvent B in a 10 mL volumetric flask, thus obtaining a 1 mg/mL solution. The solution was filtered on a 0.45 µm nylon filter before injection.

5.6.6 LC analysis of the purified extract

The purified extract obtained by the purification of *Uva di Troia ad acino piccolo Thesis 1* grape skin crude extract, was analyzed by LC-UV with the method previously described for grape skin characterization. Peaks were identified by comparison with the retention times of the standards and by sum tests; particularly the presence of Oenin and Quercetin-3-*β*-D-glucoside was confirmed in the purified extracts. The other compounds that were supposed to be in traces weren't detected, as well as *t*-Resveratrol.

The relative chromatogram (Appendix 31) shows that the chromatographic separation of the analytes of the purified extract doesn't ameliorate, with respect to that of the crude extract, and indeed is even worse. This effect could be due to the higher concentration of the analytes in the sample.

In Table 21, the concentration of the main phenolic compounds present in our purified extract was found as indicated in paragraph 5.5.7, considering the concentration of the sample. The extract was analyzed in duplicate.

ANALYTE	tR (min)	Area (mAU.sec)	Area $_{med}$	CONC %
Oenin	15.20	917.03979	924.50882	**3.858**
Oenin	15.09	931.97784		
Quercetin-3-gluc	20.74	1203.36438	1181.46857	**1.289**
Quercetin-3-gluc	21.12	1159.57275		

Table 21: Characterization of the purified *Thesis 1* grape skin extract

The table shows how analytes concentration % in the purified extract is significantly higher than that of the non purified extract (Table 19). For example, the concentration

of Oenin passes from 0.090% to 3.858%, thus confirming a considerable enrichment of the polyphenolic content in the refined extract.

Therefore, the purification method tested provides a feasible procedure for the production of purified grape skin extracts with better handling characteristics, but from the analytical point of view, it doesn't grant any improvement to the chromatographic resolution.

6. IN SEARCH OF *t*-RESVERATROL

6. IN SEARCH OF *t*-RESVERATROL

Due to its strong antioxidant properties, which exert beneficial effects on human health, and to its previous detection in *Uva di Troia* berries (Nicoletti *et al.*, 2008), the absence of *t*-Resveratrol in our grape skin samples was immediately noticed. Consequently, we decided to further investigate on this important phenolic compound.

First of all, as already explored in Chapter 1, Resveratrol can also be encountered in the 3-glycosylated form, which is referred to as Polydatin or Piceid and should be present in quantities comparable to free Resveratrol in grape berries and wines, or even higher. Thus, we imagined that in our samples of *Uva di Troia ad acino piccolo* skins, *t*-Resveratrol could be exclusively found as the glycosylated form of *t*-Piceid. As a result, a hydrolytic process was studied, as well as the chromatographic behavior of *t*-Piceid itself.

6.1 HYDROLYSIS OF GRAPE SKIN EXTRACTS

Hydrolyses are frequently used to remove sugar moieties from glycosylated compounds and can be based on the use of a strong acid or base, otherwise on an enzymatic reaction that selectively cut the link between the sugar and the compound. Hydrolysis of anthocyanins is traditionally done in refluxing HCl solutions (Merken and Beecher, 2000), but for the hydrolysis of *t*-Piceid we had a few reference data. For example, *t*-Piceid hydrolysis with H_2SO_4 and under enzymatic conditions was investigated by Wang *et al.* (2007).

Anyway, since Resveratrol is unstable in alkaline solution, the acidic hydrolysis was performed.

6.1.1 Hydrolytic conditions

In order to obtain the aglycon form of *t*-Resveratrol, an acidic hydrolysis was performed on *Thesis 1* grape skin crude extract, following the procedure below:

- About 1 g of *Thesis 1* grape skin crude extract was weighted in a round-bottom flask pre-calibrated and solved in a 85:15:5 mixture of CH_3OH/HCl 37%/H_2O;
- The acid aqueous solution was then heated under reflux for 3 h (Fig. 36);
- After cooling, the extract was concentrated under vacuum on a rotary evaporator set at 70°C and recovered with about 15 mL of the pure methanol;
- The hydrolyzed extract was then completely evaporated to dryness in the same conditions described above.

Figure 36: Acidic hydrolysis of *Thesis 1* grape skin extract

6.1.2 LC analysis of the hydrolyzed extract

The hydrolyzed extract of *Thesis 1* grape skins was subsequently analyzed by LC-UV in the conditions described in paragraph 5.5.3 (Appendix 32).

Particularly, 10.0 mg of the hydrolyzed extract were weighted and solved in a 70:30 mixture of Solvent A/Solvent B in a 10 mL volumetric flask, thus obtaining a final concentration of 1 mg/mL; the solution was then filtered on 0.45 µm nylon filters before the LC analysis.

The chromatographic profile that resulted after the hydrolytic reaction is very different from that of the crude extract of *Thesis 1* grape skins. Moreover, *t*-Resveratrol wasn't detected at all.

Therefore, we can deduce that *t*-Piceid is not present as well in our extracts, otherwise the strong hydrolytic conditions do not represent a feasible method to obtain *t*-Resveratrol, because of the presumable and consequent decomposition of the target compound during hydrolysis.

6.2 ANALYSIS OF POLYDATIN

The hydrolytic reaction performed on grape skin crude extracts represented a rapid procedure that could confirm us the presence of Resveratrol, whenever it had been in the glycosylated form. However, the absence of the target molecule didn't ensure us its presence, nor its absence, due to the impossibility to know if our hydrolytic reaction was effective.

Therefore, a standard of *t*-Piceid, also referred to as Polydatin, was bought and analyzed by LC.

6.2.1 Preparation of the standard solution

5.0 mg of Polydatin were exactly weighted and solved in methanol in a 5 mL volumetric flask, thus obtaining a 1 mg/mL solution. This solution was then diluted 1:10 with a 70:30 mixture of Solvent A/Solvent B in order to obtain a final concentration of 0.1 mg/mL.

6.2.2 LC-UV analyses

The 0.1 mg/mL STD of Polydatin was analyzed by LC-UV applying the chromatographic conditions previously described, thus obtaining a peak with a retention time of 20.94 minutes (Appendix 33).

With reference to the LC-UV profile of *Thesis 2* grape skin extract (Appendix 28), we observe that at this elution time there are co-eluting peaks, including that belonging to Quercetin-3-*β*-D-glucoside at 21.28 min.

For this reason, a sum test was carried out between the standard of Polydatin conveniently diluted and *Thesis 2* grape skin extract, following the procedure described in paragraph 5.5.6 (Appendix 34). The peak that was thought to be the target analyte was at 21.09 min in the crude extract.

Figure 37 compares the chromatograms relative to the sum test and to the pure extract, zoomed between 19 and 24 minutes of analysis.

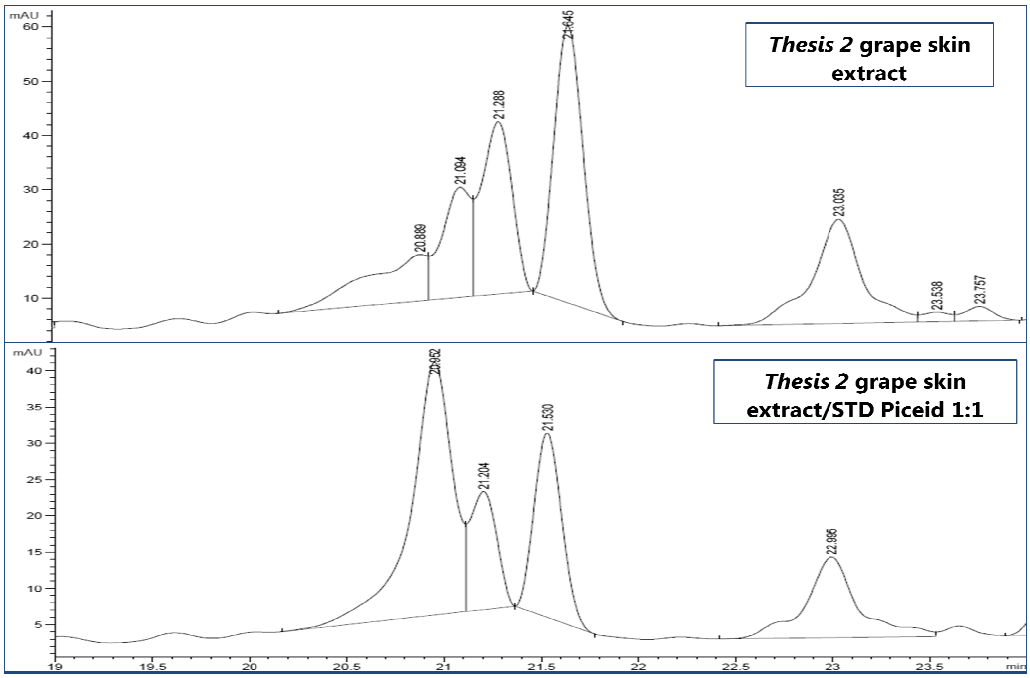


Figure 37: Zoom of *Thesis 2* and sum test chromatograms

As shown by the figure above, the peak at 21.09 min of *Thesis 2* grape skin extract (upper chromatogram) is attributable to Polydatin, however we can't be sure of this just comparing the chromatograms.

6.2.3 LC-DAD analyses

In order to confirm the identity of Polydatin in our extracts, since Stilbenes show a characteristic absorbance at about 300 nm of the UV region, samples were also

analyzed by LC-DAD using the same chromatographic conditions developed for LC-UV analyses.

LC-DAD analyses were performed on a Varian™ Pro Star equipped with two chromatographic pumps mod. 210, a Varian™ 410 autosampler and diode array detector mod. 335, and controlled by Software Galaxie.

Standards 0.1 mg/mL of *t*-Resveratrol and *t*-Piceid were thus analyzed in order to compare their retention times and characteristic absorbances to those of our extracts of *Uva di Troia ad acino piccolo* skins. The two standards eluted at about 35.78 min and 23.89 min, respectively.

As we can see in Appendix 35, *Thesis 2* grape skin chromatographic profile is comparable to that obtained by LC-UV analysis, especially for what concern the co-eluting peaks at about 24 minutes of the analysis. Thus, the absence of *t*-Resveratrol was confirmed, but that of *t*-Piceid had to be further investigated.

In order to achieve this goal, the absorbance of the standard *t*-Piceid was compared to that of the presumable corresponding peak in *Thesis 2* grape skin extract at 24.05 min (Fig. 38).

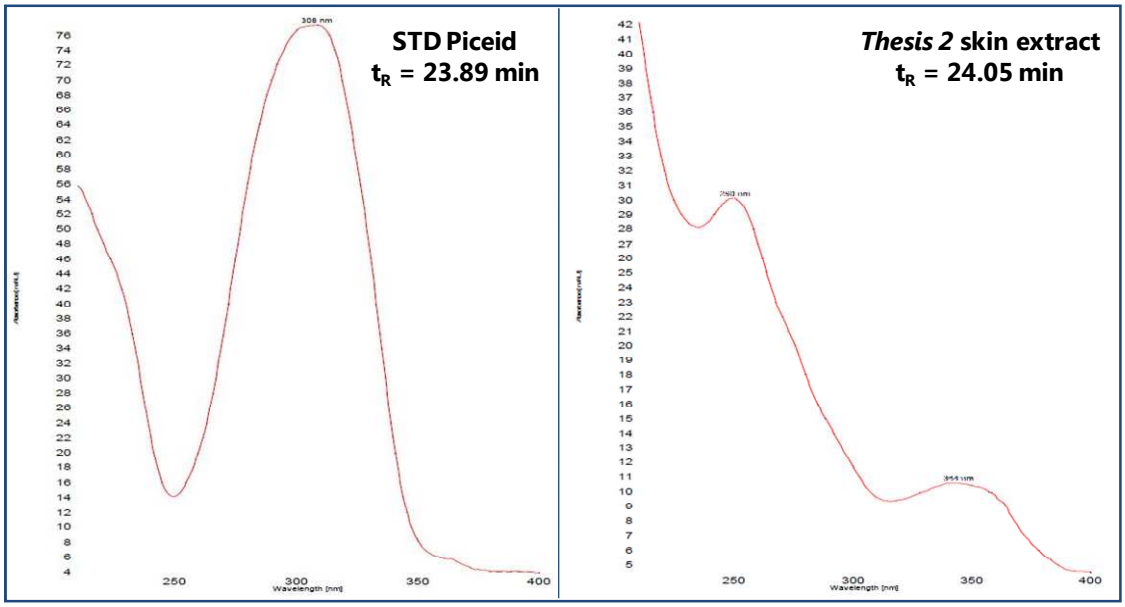


Figure 38: UV absorbance of STD Piceid and *Thesis 2* skin extract at ≈24 min

Fig. 35 shows that the peak eluted at 24.05 min of our skin extract has a UV absorbance completely different from Polydatin characteristic absorbance (≈ 300

nm), thus clearing any doubt about its presence in *Uva di Troia ad acino piccolo* grape skin extracts.

6.3 ANALYSIS OF REVIDOX™

Resveratrol is a strong antioxidant phenolic compound that has been found in many grape cultivars and, especially, in wine. For its important beneficial effects on human health, that include anticancer and anti-inflammatory properties, this natural compound has been of great interest in the area of nutritional supplements during recent years. As a result, a dietary supplement made of Resveratrol and other phenolic compounds has been developed by a group of researchers of the Spanish National Research Council (CSIC), i.e. Revidox™ with Stilvid®. The latter represents the standardized extract derived from *Vitis vinifera* grape skins and seeds; it contains a high concentration of Resveratrol, i.e. about 8 mg of Resveratrol per capsule of Revidox™. Thus, we decided to analyze it by liquid chromatography.

6.3.1 Preparation of Revidox™

100.0 mg of Revidox™ (Paladin Pharma, Italy) were exactly weighted and solved in a 10 mL volumetric flask with a 70:30 mixture of Solvent A/Solvent B, thus obtaining 10 mg/mL solution. This solution was filtered on 0.45 µm nylon filters before injection.

6.3.2 LC analysis of Revidox™

As explained above, we decided to analyze the nutritional supplement Revidox™ by LC-UV with the chromatographic method widely described in the previous chapter, in order to get a qualitative comparison with our extracts (Appendix 35).

Revidox™ relative chromatogram clearly shows the presence of the analyte of interest, i.e. *t*-Resveratrol, together with other phenolic compounds.

As enlightened in Chapter 1, Resveratrol concentration in plants strictly depends on the elicitation by emergency situations, in fact it is biosynthesized in response to biotic and abiotic stress, such as fungal attack or ultraviolet radiations. For this

reason, the patented process that leads to Stilvid® is probably based on the UV exposure of grapes, thus enhancing Resveratrol biosynthesis.

Therefore, the absence of such molecule in our extracts could be caused by the lack of environmental elicitors in the investigated cultivar during the maturation of the *Uva di Troia ad acino piccolo* grapes collected in harvest 2009.

Anyway, Revidox™ information leaflet, about the medium content of active ingredients in the product, is illustrated in Table 22.

ANALYTE	in 100 g of product	per capsule
STILVID® (Grape dry extract)	28.60 g	133.00 mg
Resveratrol	1.72 g	8.00 mg
Antocyanosides	0.14 g	0.67 mg
Procyanidins	3.15 g	14.63 mg
Flavonoids	0.86 g	0.40 mg
POMEGRANATE dry extract	26.88 g	125.00 mg
Ellagic acid	1.88 g	8.75 mg
Procyanidins	0.80 g	3.75 mg
SELENIUM	10.75 mg	50.0 µg (90% RDA)

Table 22: Revidox™ medium content (RDA = Recommended Daily Allowance)

7. LC-MS/MS EXPERIMENTS

7. LC-MS/MS EXPERIMENTS

HPLC methods combined with UV and fluorescent detectors have been widely used in the past decades for the study of food polyphenols. However, these detection methods often lead to misattribution of peaks and do not give many information about the chemical structure of the compounds investigated.

For this reason, LC-MS is a chromatographic technique that is gradually replacing the other ones in the investigation of phenolic compounds, since it gives the possibility to improve the identification and structural characterization, as well as the quantification, of such compounds. The interface and the mass analyzer provided by the equipment represent very important tools in mass spectrometry, being the Electrospray ionization (ESI) one of the most common interfaces and triple quadrupole (QqQ) a mass spectrometer extensively used for the identification and quantification of polyphenolic compounds in tandem mass (MS/MS) studies. ESI, at the same time, has been proven to be suitable for the analysis of polar compounds in aqueous solutions and is typically used in the positive-ion mode for the analysis of Anthocyanins (Flamini, 2003).

Thus, we decided to analyze by LC-MS the grape skin extracts obtained by the extraction of *Uva di Troia ad acino piccolo* grapes.

Particularly, using an ESI ion source coupled to a triple quadrupole mass spectrometer, we preliminary characterized the fragmentation pattern of selected polyphenol standards, representative of those present in grape skins.

The analytical procedure used in our research is described in the next paragraphs.

7.1 APPARATUS

The analyses were performed on a Varian LC-320 with ESI source and a 320-MS triple quadrupole mass spectrometer, equipped with two Varian 212 LC chromatographic

pumps and a Varian 410 tray cooled autosampler. The system is managed by Varian MS Workstation software (Version 6.9.1).

7.2 PREPARATION OF THE STDs

1.0 mg of Cyanidin chloride, Kuromanin chloride and Oenin chloride were solved in methanol to obtain 1 mg/mL solutions. Kuromanin chloride and Oenin chloride solutions were then diluted 1:100 (using 10 mL volumetric flasks) with 0.1% aqueous solution of formic acid, thus obtaining a final concentration of 10 µg/mL; Cyanidin chloride solution, instead, was diluted 1:10 in methanol in order to obtain a final concentration of 0.1 mg/mL.

10.0 mg of *t*-Resveratrol, Quercetin and Myricetin were exactly weighted and solved in methanol in a 10 mL volumetric flask, separately, thus obtaining 1 mg/mL solutions. Myricetin and *t*-Resveratrol solutions were then diluted 1:100 (using 10 mL volumetric flasks) with methanol, in order to obtain a final concentration of 10 µg/mL; Quercetin solution, instead, was diluted 1:10 in methanol in order to obtain a final concentration of 0.1 mg/mL.

7.3 PREPARATION OF THE SAMPLES

1 mL of *Thesis 2* grape skin methanolic extract, previously filtered on 0.45 µm filters, was diluted with methanol in a 10 mL volumetric flask, thus obtaining a final concentration of 0.1 mg/mL.

10.0 mg of *Thesis 1* grape skin purified extract were exactly weighted and solved in methanol in a 10 mL volumetric flask. 0.250 mL of this solution were then diluted with methanol in a 5 mL volumetric flask, in order to obtain a final concentration of 50 µg/mL.

7.4 MASS SPECTROMETER SETUP

The ESI-triple quadrupole mass spectrometer (QqQ) was set to perform collision-induced dissociation experiments in positive ionization mode, using Argon as collision gas.

Particularly, a Multiple Reaction Monitoring (MRM) mode, also referred to as Selected Reaction Monitoring (SRM), was used. Such experiments are widely used for quali-quantitative purposes on QqQ because of the great sensitivity, in order to confirm the presence of an unknown compound in a matrix.

MRM experiments are based on the filtration of the molecular (precursor) ion of the investigated compound in the first quadrupole, which is fragmentized in an Argon enriched atmosphere at different collision energies in the second quadrupole (collision chamber) to produce its one or more product ions. Finally, the third quadrupole filters only one of the fragment ions, which is subsequently sent to the detector (Fig. 39).

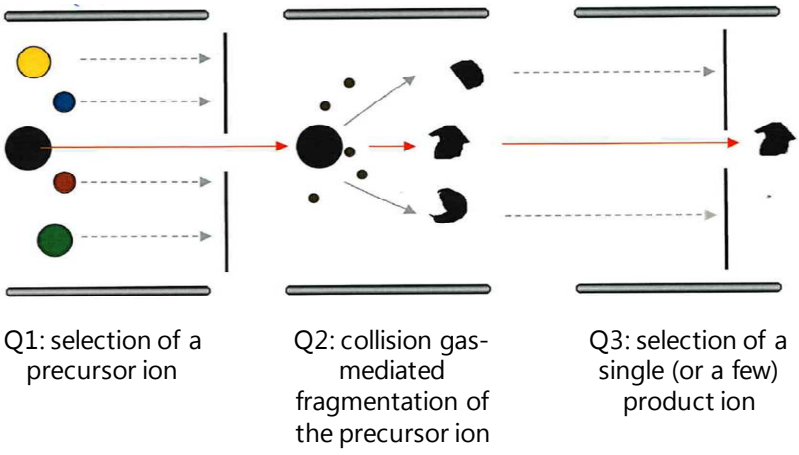


Figure 39: Schematic representation of a MRM experiment

Then, the quantitation of the analytes of interest is achieved by the comparison between the product ion abundance in the sample and in the standard solution. Nevertheless, up to now, MRM experiments on grape extracts have been realized with a qualitative purpose only.

Consequently, analyses were conducted by the continuous injection - at a rate of 20 µL/min - of the standards of interest into the mass spectrometer set in positive

ionization mode, in either aqueous or methanolic solutions. In order to enhance ion formation and to improve conductivity, the solvent for electrospray ionization was prepared by mixing formate buffer 3 mM pH 3 with 0.1% HCOOH acetonitrile. The method was optimized for the research of the ions of interest, e.g. the specific MRM transitions of each standard. In detail, once recognized the molecular ion of each standard ($M+H^+$), MRM experiments were performed to study the characteristic fragmentation pattern of the analytes Kuromanin chloride, Cyanidin chloride, Oenin chloride, Quercetin, Myricetin and *t*-Resveratrol.

Table 23 shows the specific MRM transitions of each analyte, i.e. its molecular ion and fragment ions chosen for the identification, as well as the capillary voltage and the collision energy at which the fragment ions are produced in the second quadrupole.

ANALYTE	Q1FM	Q3FM	Capillary (V)	Coll. Energy (V)
Kuromanin-Cl	449.0	449.0	28.5	4.5
	449.0	287.0	28.5	18.0
Oenin-Cl	493.4	493.4	71.4	4.0
	493.4	331.0	71.4	17.5
	493.4	315.1	71.4	43.5
	493.4	287.0	71.4	45.0
	493.4	270.1	71.4	39.0
Cyanidin-Cl	287.0	287.0	50.0	4.5
	287.0	213.1	50.0	28.5
	287.0	137.0	50.0	30.5
Myricetin	341.1	341.1	45.0	4.0
	341.1	285.1	45.0	7.5
	341.1	243.0	45.0	12.5
***t*-Resveratrol**	229.1	229.1	52.9	5.0
	229.1	135.1	52.9	12.0
	229.0	118.9	52.9	10.5
	229.1	107.1	52.9	19.0
Quercetin	303.0	303.0	49.2	6.5
	303.0	257.0	49.2	21.5
	303.0	153.2	49.2	30.5
	303.0	127.2	49.2	46.5

Table 23: Fragmentation pattern of the standards analyzed by LC-MS/MS

ESI source settings and mass spectrometer parameters used for compound identification are described below:

- ***POSITIVE IONIZATION MODE:***

  Needle Voltage: + 5000 V

  Shield Voltage: + 600 V

  Nebulizing Gas (N_2) Pressure: 40.00 psi

  Drying Gas (N_2) Pressure: 15.00 psi

  Drying Gas (N_2) Temperature: 400°C

  Q0 Offset: + 3.261 V

  L4 Offset: + 2.000 V

- ***MS/MS PARAMETERS***

  Housing Temperature: 50°C

  CID Gas (Ar) Pressure: 2.00 mTorr

  Electron multiplier: 1650.0 V

  Scan time: 4.0 sec

  Dwell time: 0.1 sec

7.5 LC-MS/MS ANALYSIS OF THE SAMPLES

Finally, in order to gain information on *Uva di Troia ad acino piccolo* grape phenolic content, grape skin crude and purified extracts were directly injected in the ESI source with a syringe pump flow rate of 1.0 µL/min, without performing any chromatographic separation. The identification of each analyte in the extract is based on the presence of all its specific MRM transitions.

Appendix 37 illustrates the positive matches between standard characteristic MRM transitions and *Thesis 2* grape skin crude extract., i.e. Oenin chloride and Quercetin. On the other hand, Appendix 38 shows the detection of the characteristic MRM transitions of Kuromanin and Resveratrol in our *Thesis 2* extract, but the scarce resolution, the low intensity of the peaks and, especially for Resveratrol, the mismatch

among some MRM retention times, can't allow an unequivocal identification of these two compounds.

At the same time, *Thesis 1* grape skin purified extract was analyzed by LC-MS/MS with the method developed. The Oenin represents the only analyte whose presence in the purified extract is confirmed without any doubt, while Kuromanin is maybe present in traces (Appendix 39).

8. CONCLUSIONS

8. CONCLUSIONS

The present Ph.D. project took its origin form the partnership between the University of Milan and the pharmaceutical society Farmalabor Srl with the aim of studying the polyphenolic content of a particular Apulian grapevine, i.e. *Uva di Troia ad acino piccolo* (*Uva di Troia* with small berry), in dependence of the vinification process.

The characteristic small berry confers to *Uva di Troia* a smaller pulp/skins ratio than the other grape varieties having a big berry, thus, this biotype is considered unproductive from the oenological point of view. However, the small berry is also linked to a major content of polyphenols extracted during fermentation processes, with respect to big size berries, and previous studies confirmed high levels of polyphenols in this grape biotype and a great wine aging potential (Suriano *et al.*, 2005).

Thus, the best extraction and analytical conditions were investigated in order to valorise the cultivation of this autochthonous grape biotype, both for the production of wine either to create a new nutritional supplement based on the beneficial antioxidant activity of such phenolic compounds.

For this purpose, we extracted, purified and characterized the phenolic content of four fractions of grape seeds and skins, called *thesis*, collected at four different fermentation stages (from no fermentation to complete fermentation). As already explained, we separately investigated on grape seeds and skins because of the significant differences in the phenolic content and composition between the two tissues. An important issue was represented by the choice of the extracting solvents, i.e. methanol, acetone and ethanol. These solvents are food compatible according to the Official Journal of the European Union[3], as they can be used for the production of nutritional supplements.

[3] Official Journal of the European Union L 141/1, 6th June 2009 Annex I

First of all, the research was focused on the extraction of flavanols from grape seeds and on their characterization. Once chosen the best extractive protocol, we performed seeds extraction by maceration with two solvent mixtures, 70:30 ethanol/water and 70:30 acetone/water, being the former the best one. In fact, the analysis of the extracts by reversed phase LC-DAD confirmed higher levels in polyphenols in the ethanolic extracts, than in the acetonic ones. Our extracts were also compared to a highly standardized grape seed extract, i.e. Leucoselect™ (Indena, Italy). As expected, the crude extracts showed a significant lower content of Flavanols (monomeric Catechins, Procyanidins B1 and B2) than Leucoselect™, that is 1.74% for *Thesis 1* grape seed ethanolic extract and 23.02% for Leucoselect™. However, the two chromatographic profiles were quite comparable. The only remarkable difference was represented by a considerable broader peak at approximately 60 minutes of the analysis, relative to the elution of highly polymeric Procyanidins (PC), which were very abundant in our extracts. Thus, in order to enrich our crude grape seed extracts and at the same time reduce the content of polymeric PCs, that are less active on human health, a purification method was optimized. The best purification conditions were represented by a L-L extraction of the crude extracts with pure Ethyl acetate, because it gave a good yield of extraction and a better chromatographic profile (more comparable to that of Leucoselect™) due to the drastic reduction of polymeric PCs. In addition, the flavanol content of Catechin, Epicatechin, PC B1 and PC B2 was strongly enriched in the refined extracts with respect to the non purified hydro-alcoholic extracts, being for example 13.66% for *Thesis 1* seeds.

Finally, in order to simulate an industrial extraction process, we decided to test a larger scale extraction by percolation on about 0.5 Kg of *Thesis 1* seeds.

The second purpose of our research was then to define the phenolic composition of *Uva di Troia ad acino piccolo* grape skins. In order to achieve this goal, we extracted, purified and analyzed by liquid chromatography grape skins coming from the four Theses collected. Hence, we focused our attention on the skin content of Anthocyanidins and Flavonols and, especially of Resveratrol.

Skin extraction was achieved by a single step extraction by maceration in methanol as organic solvent, then, we turned our attention on the development of the LC conditions and on the purification of the extracts. In fact, purification of the extracts was mandatory, considering the low content of polyphenols in the crude extracts and, especially, the soft consistency of skin dried extracts that was due to the concomitant extraction of sugars and proteins, and limited sampling and analytical procedures.

The best LC conditions were accomplished on a reversed phase liquid chromatography column coupled to a spectrophotometer detector; separation of the analytes of interest was achieved with a binary multisegment gradient elution mode. The identification of the analytes was then realized by comparison with standard retention times and by specific sum tests, which confirmed the presence of Oenin chloride and Quercetin-3-*β*-D-glucoside in each Thesis and of Quercetin in *Thesis 2*, *3* and *4*, even if the concentration attested was very low.

On the other hand, the purification of *Thesis 1* grape skin extract was performed on a bromurated synthetic adsorbent resin, i.e. Sepabeads® SP-207 (Resindion, Italy). The purified extract thus obtained showed better handling characteristics and an enriched polyphenolic content; for instance, the concentration % of Oenin chloride passed from 0.09% to 3.86% in the purified extract. However, from the analytical point of view this extraction method didn't give any improvement to the chromatographic resolution.

Concluding, we extracted, purified and analyzed by LC skins and seeds derived from the berries of *Uva di Troia ad acino piccolo*, collected at four different stages of the vinification process. From the data obtained, we observe that the phenolic content of both grape seeds and skins decreases from *Thesis 1* to *Thesis 4*. This trend is consistent with the fact that the polyphenolic content of grapes is influenced by the winemaking process, which implies a progressive extraction of the active ingredients from the vegetable matter to the must and so to the wine, thus exhausting its phenolic content.

In addition, grape skins are characterized by a very low content of phenolics and the extraction and characterization of this matrix involve many analytical problems, such as a complicated storage of frozen samples, a difficult chromatographic separation of the analytes of interest and also the necessity of performing a purification step with adsorbent resins to remove sugars and proteins from the crude extract.

For this reasons, in order to develop a new nutritional supplement based on the beneficial properties of grape active ingredients, we decided to chose the seeds as the vegetable drug to extract. Indeed, grape seeds represent a rich font of flavanols that, as explored in Chapter 1, exert strong antioxidant, cardioprotective, anti-inflammatory and anticancer actions.

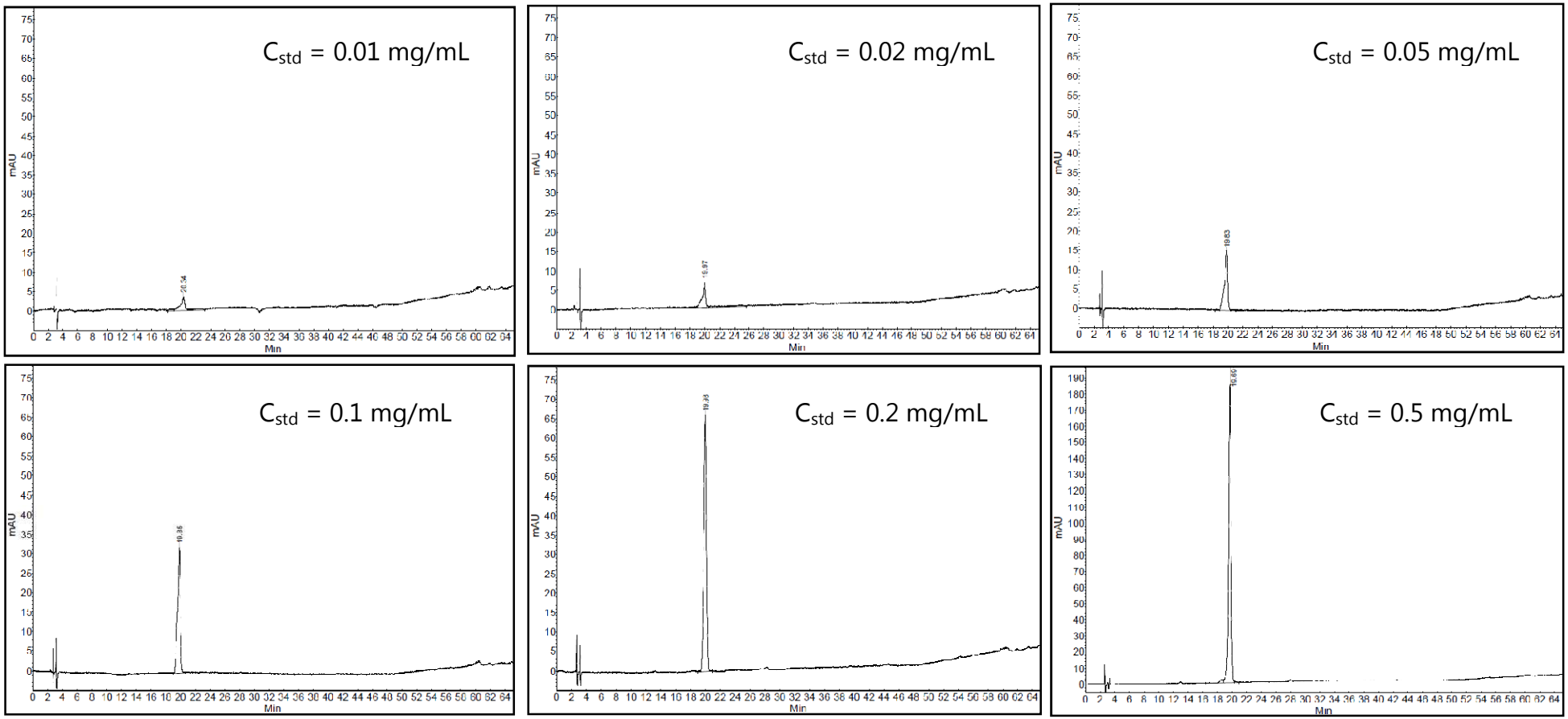
Furthermore, the *Uva di Troia ad acino piccolo* grape sample chosen for the development of the nutraceutical should preserve at the same time the production of wine, thus leading to the total exploitation of the cultivar and limiting the remaining solid waste.

Hence, among the Theses investigated, *Thesis 2* represents the most suitable fraction to achieve this purpose, considering the possibility to use the corresponding seeds for the extraction of the flavanols that will constitute the phytocomplex, while the remaining marc in the must continues the vinification process.

Obviously, this research will continue in the future, taking into account the industrial outcome. In fact, the seed extraction by percolation will be further developed and, in particular, the purification procedure will be further investigated. Moreover, seeds coming from the 2011 harvest of Farmalabor experimental vineyard will be extracted, purified, analyzed by LC-DAD and compared with those thoroughly studied in this Ph.D. project.

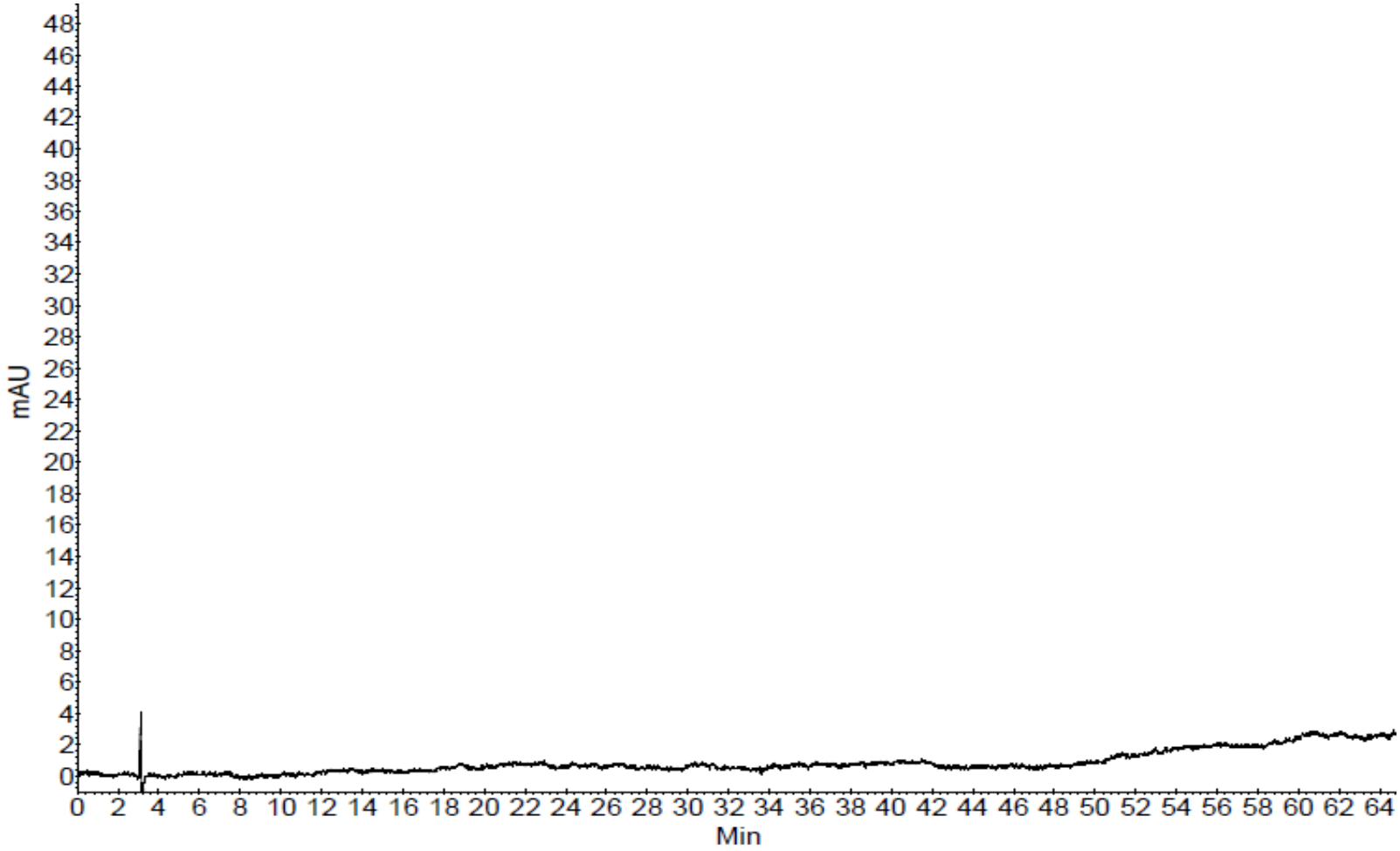
APPENDICES

Appendix 1



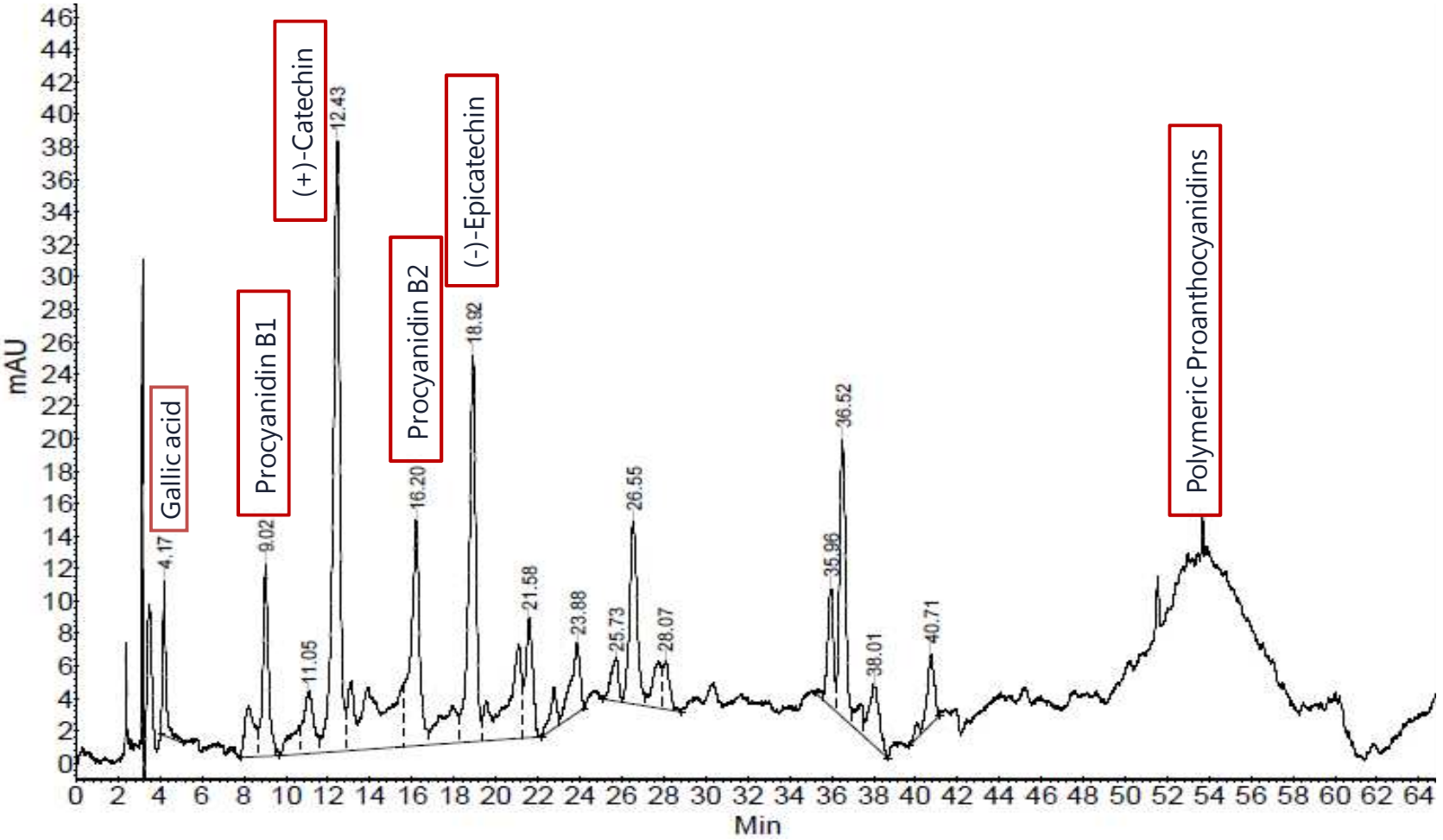
Analyses of six concentrations of (-)-Epicatechin used for the building of a calibration curve

Appendix 2



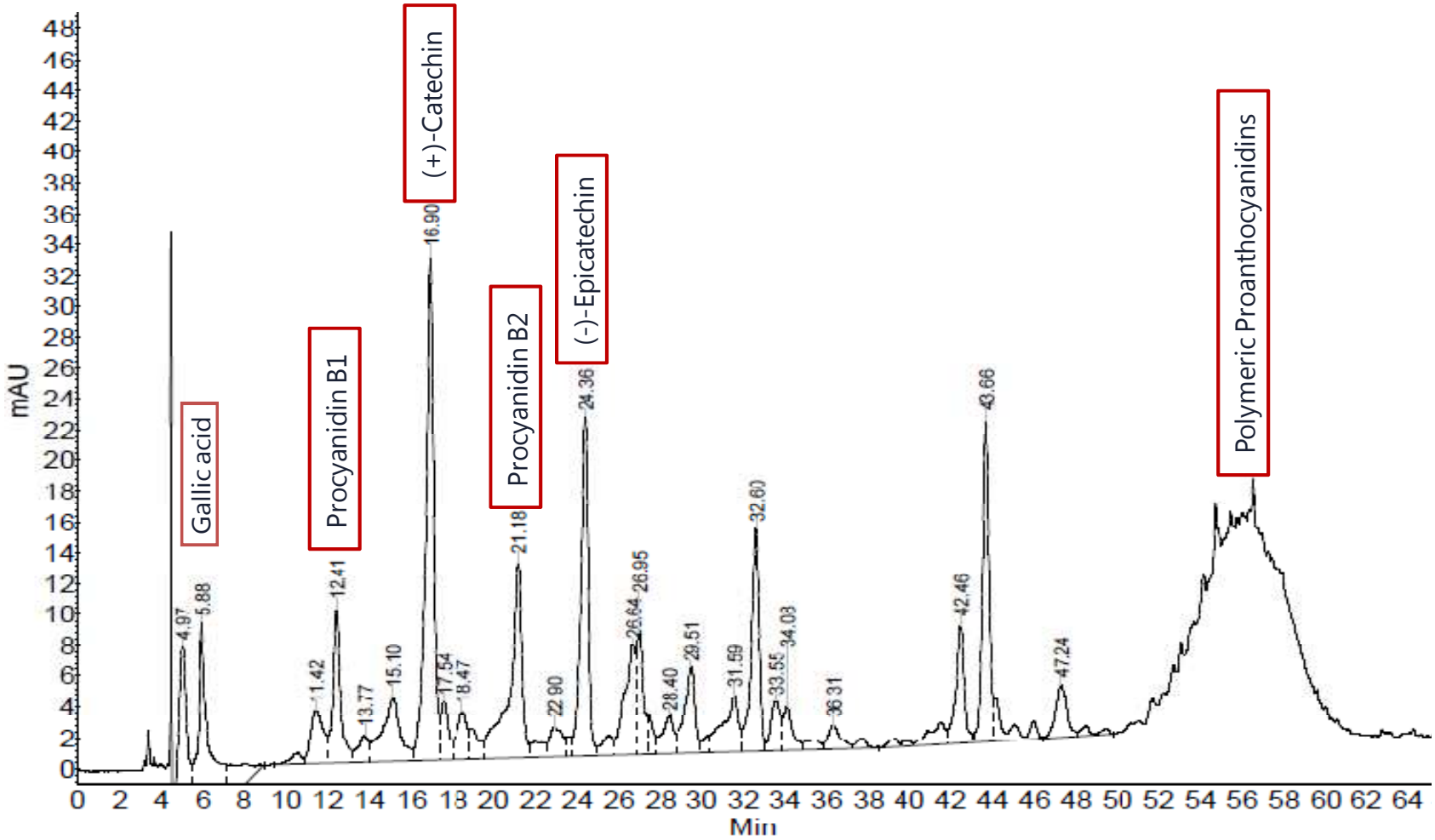
LC-DAD profile of a blank sample

Appendix 3



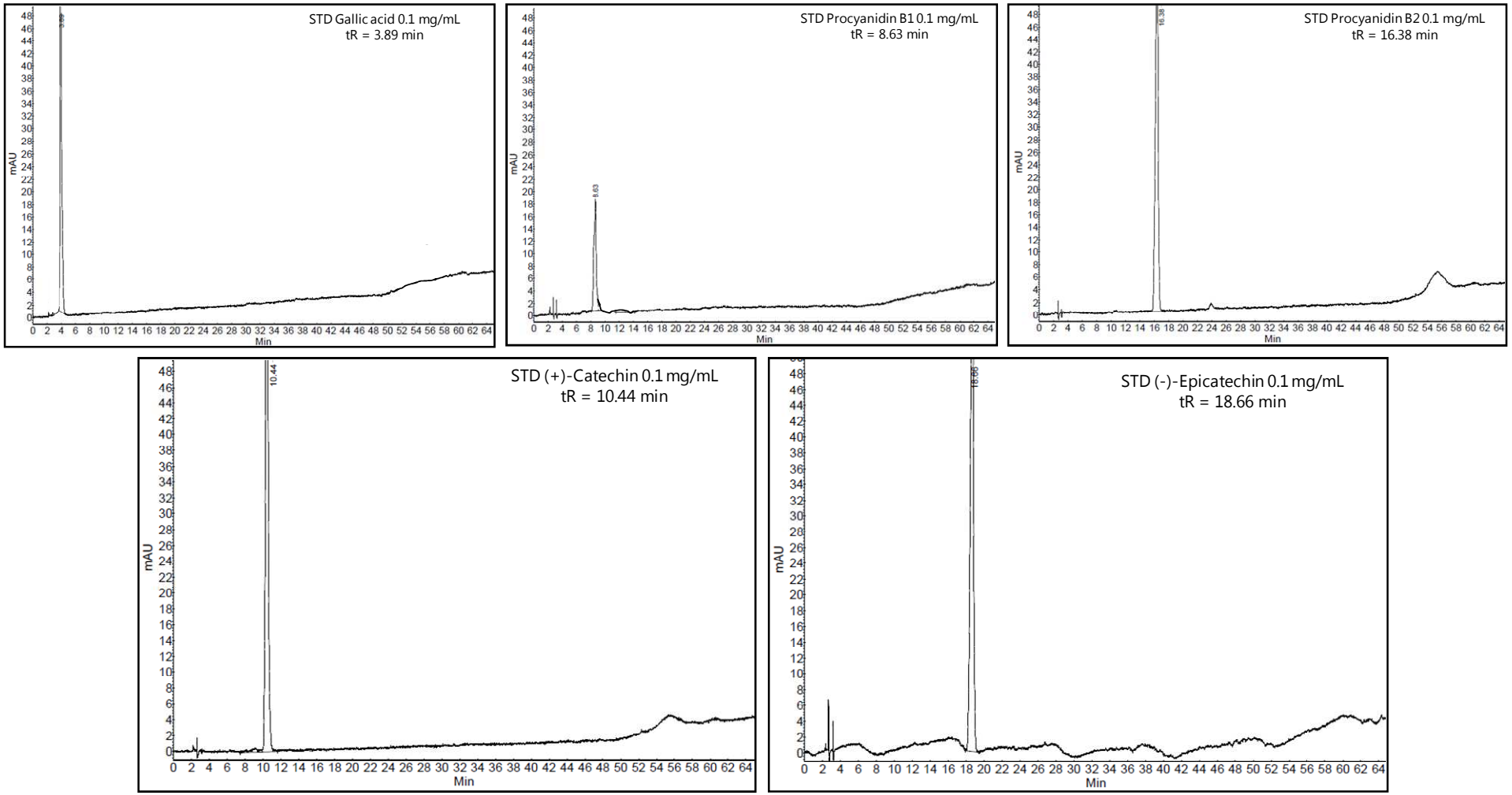
LC-DAD profile of Leucoselect™ 1 mg/mL (1)

Appendix 4



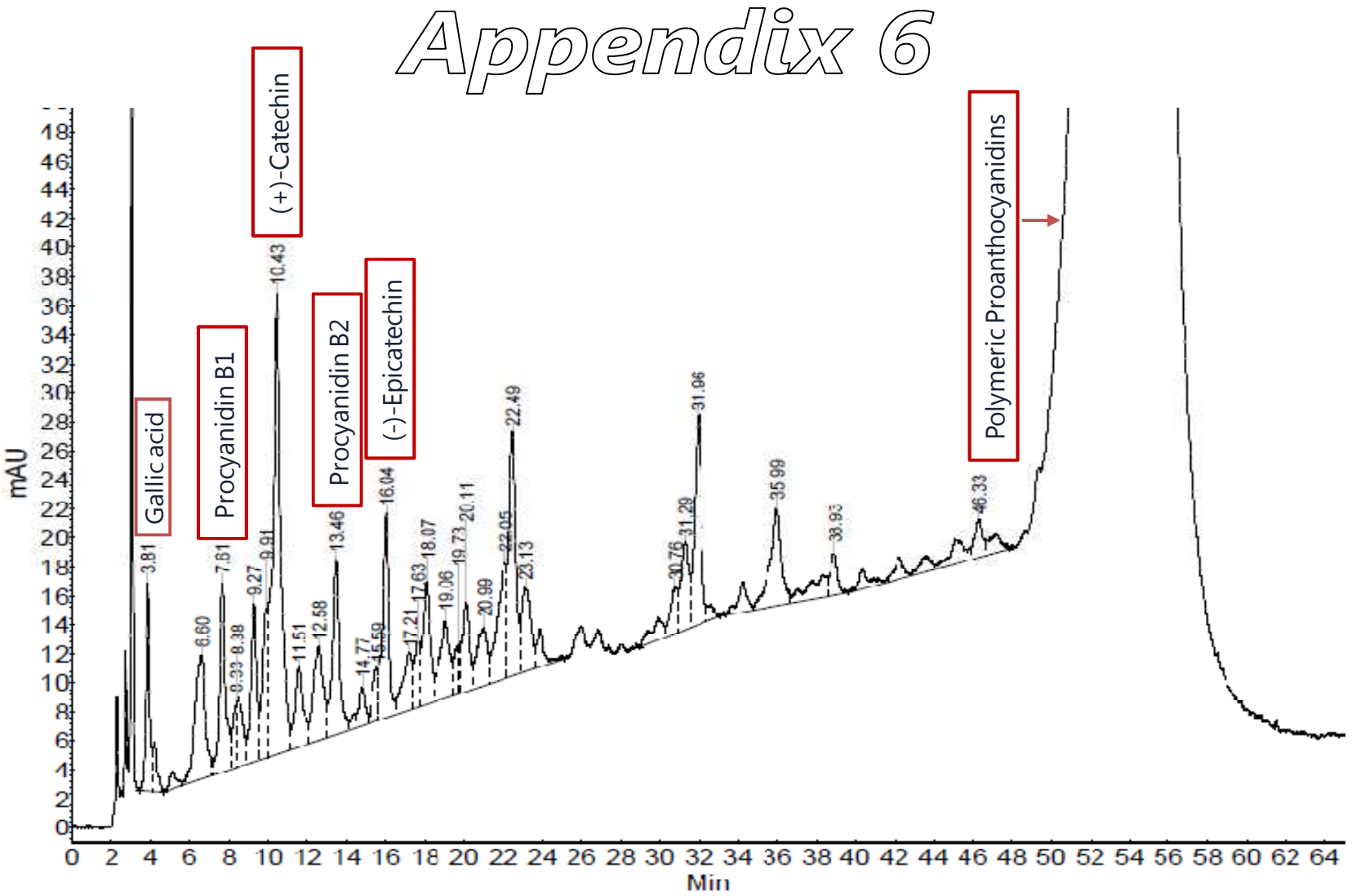
LC-DAD profile of Leucoselect™ 1 mg/mL (2)

Appendix 5



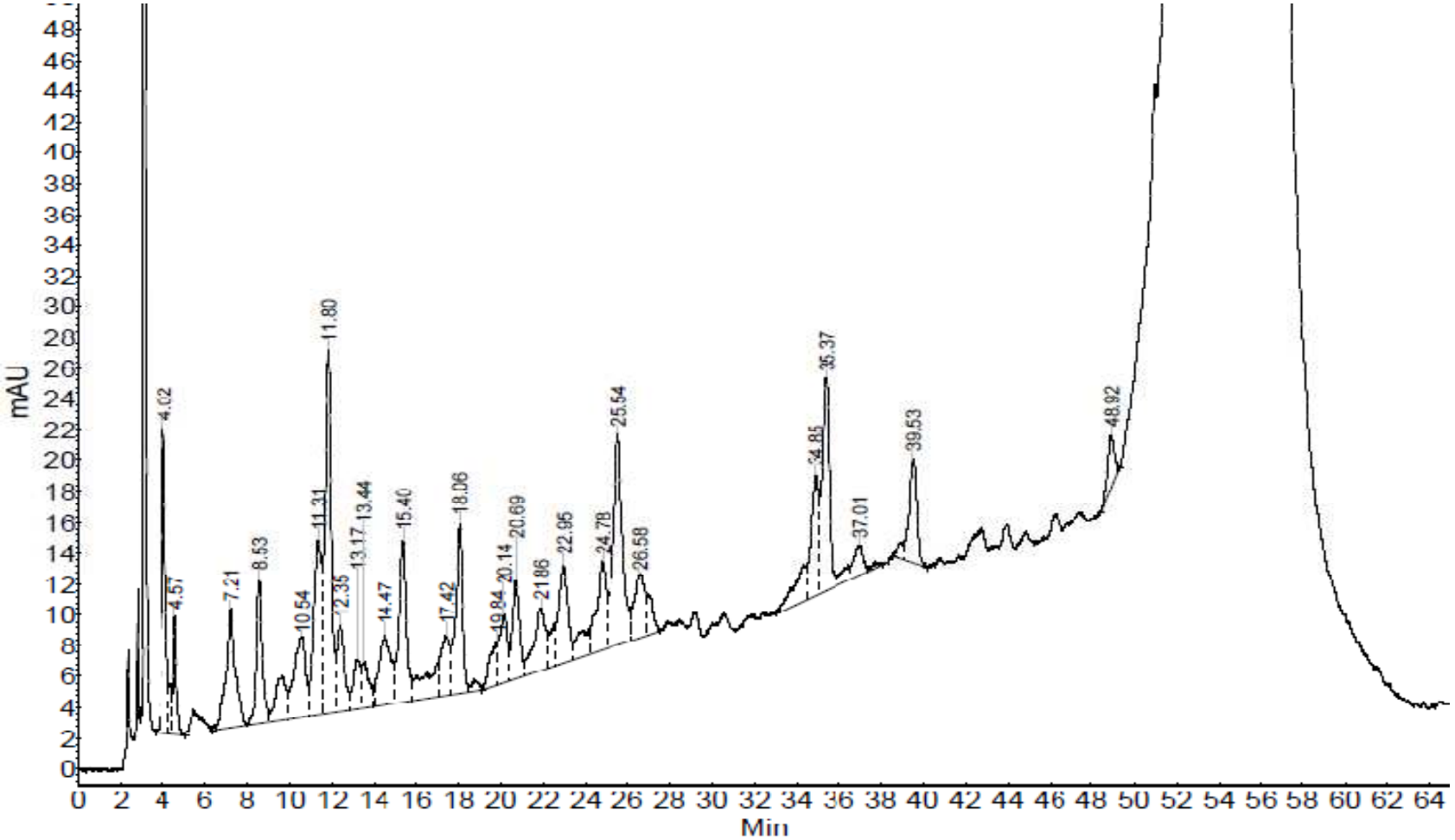
LC-DAD profile of reference standards

Appendix 6



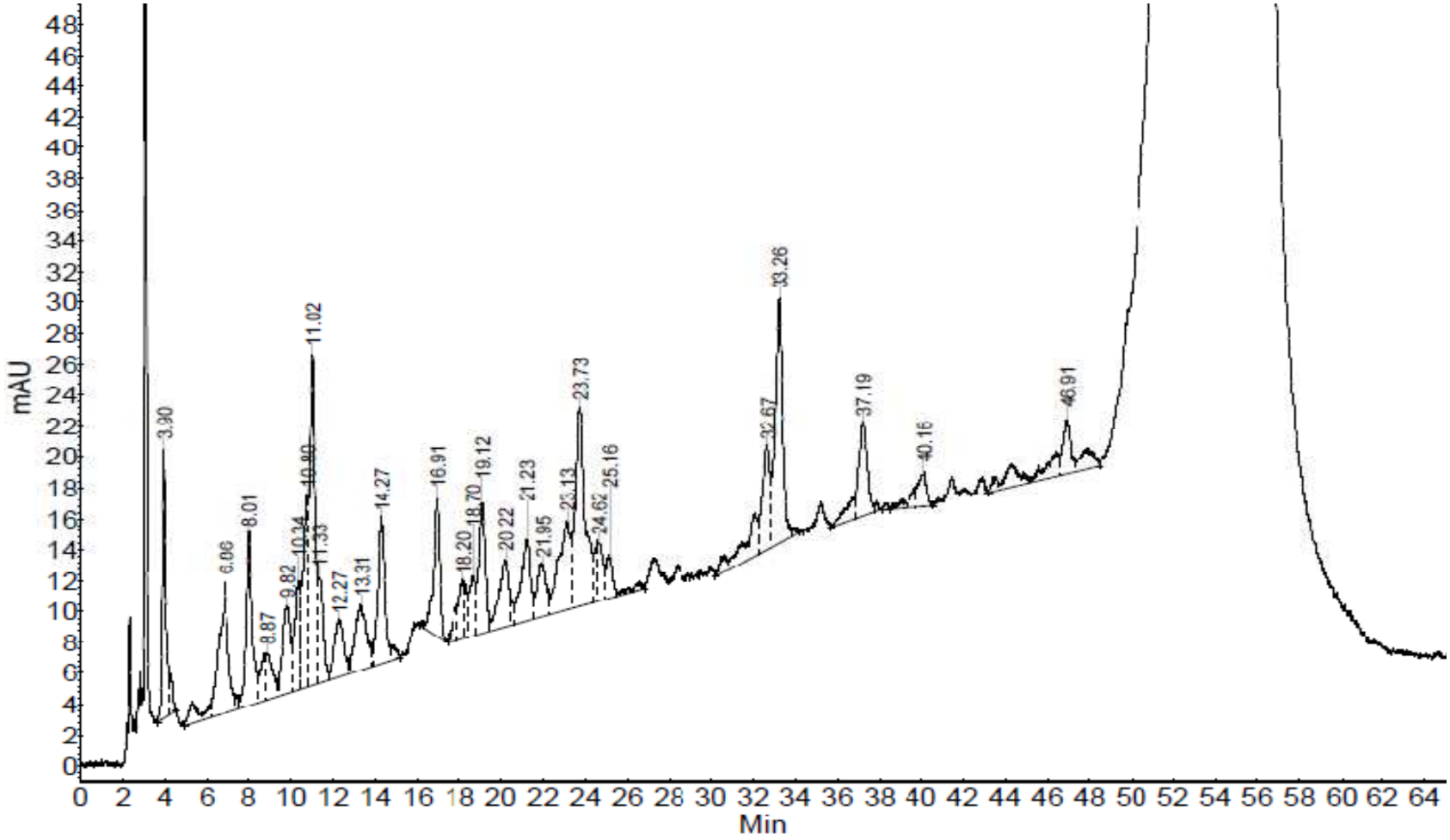
LC-DAD profile of Thesis 1 frozen seeds EtOH 70%

Appendix 7



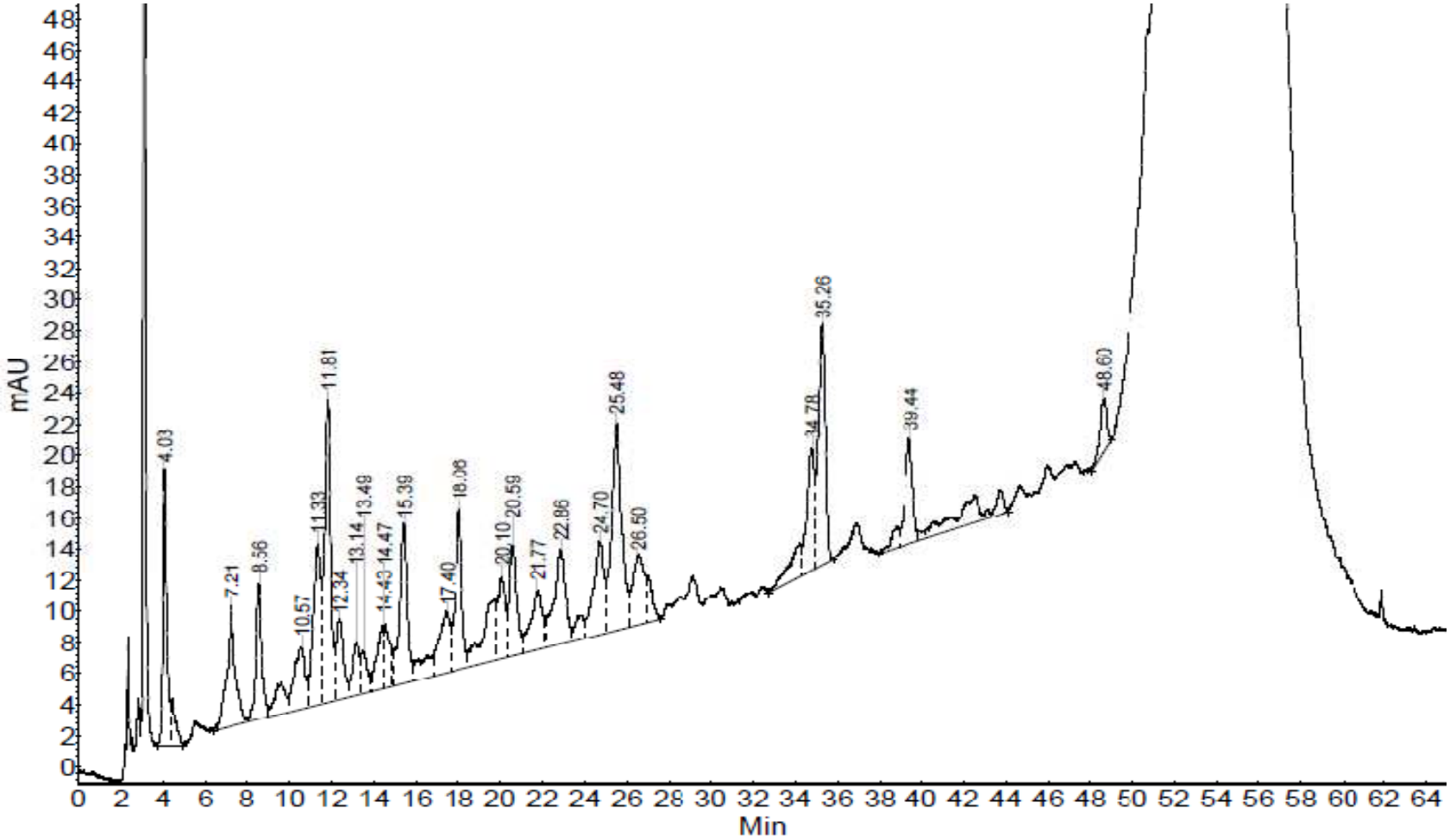
LC-DAD profile of Thesis 1 frozen seeds Acet 70%

Appendix 8



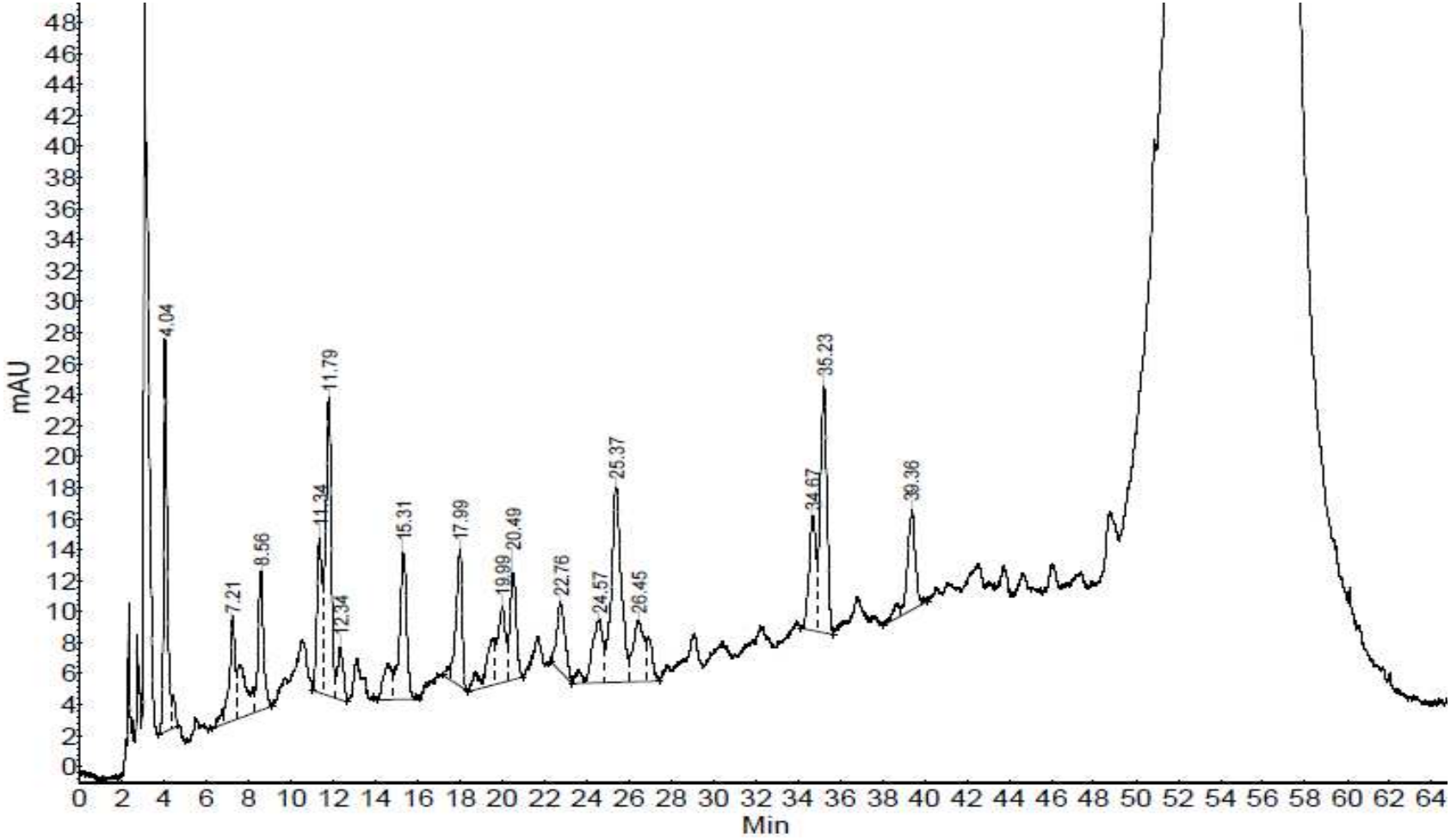
LC-DAD profile of Thesis 2 frozen seeds EtOH 70%

Appendix 9



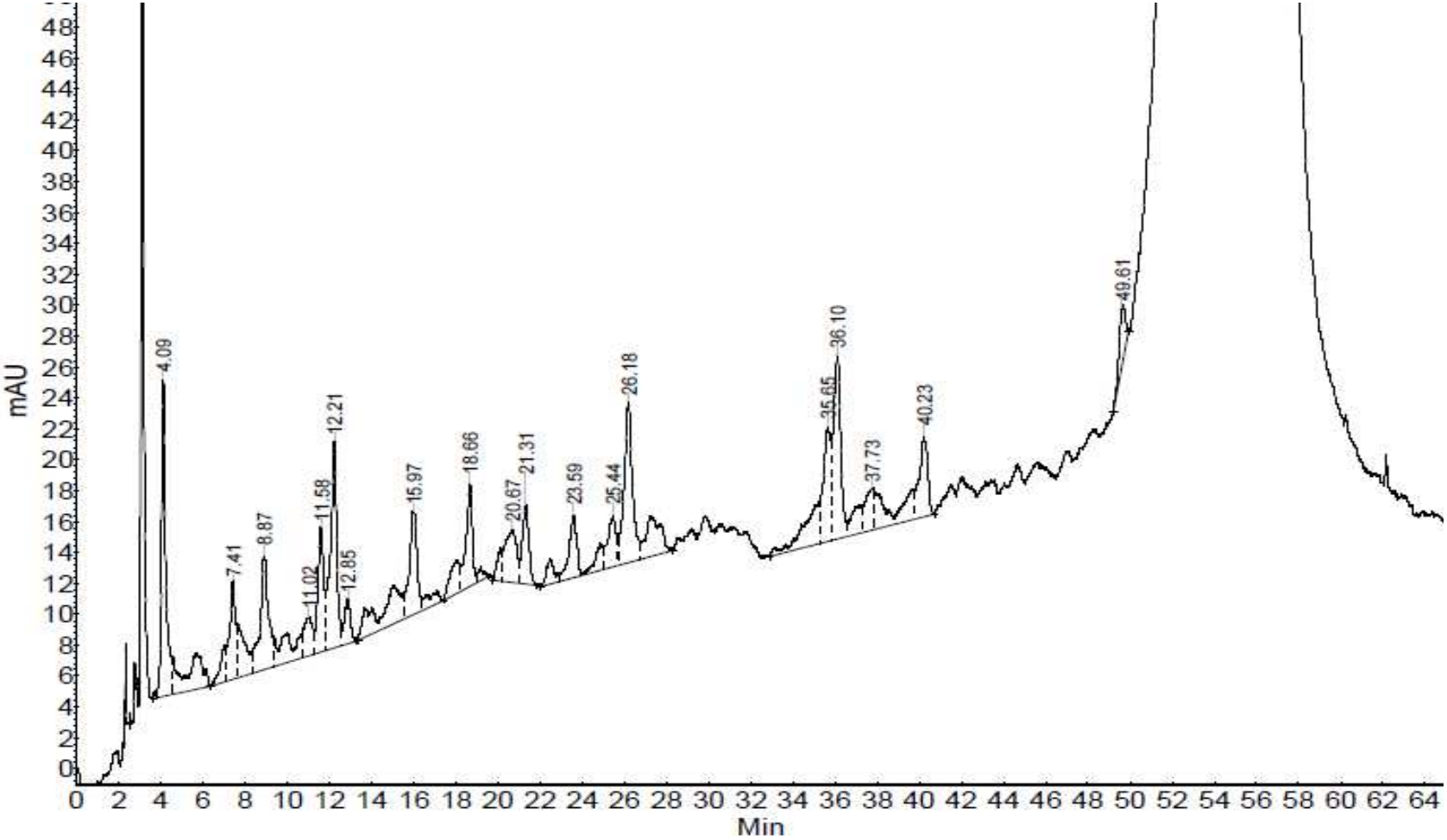
LC-DAD profile of Thesis 2 frozen seeds Acet 70%

Appendix 10



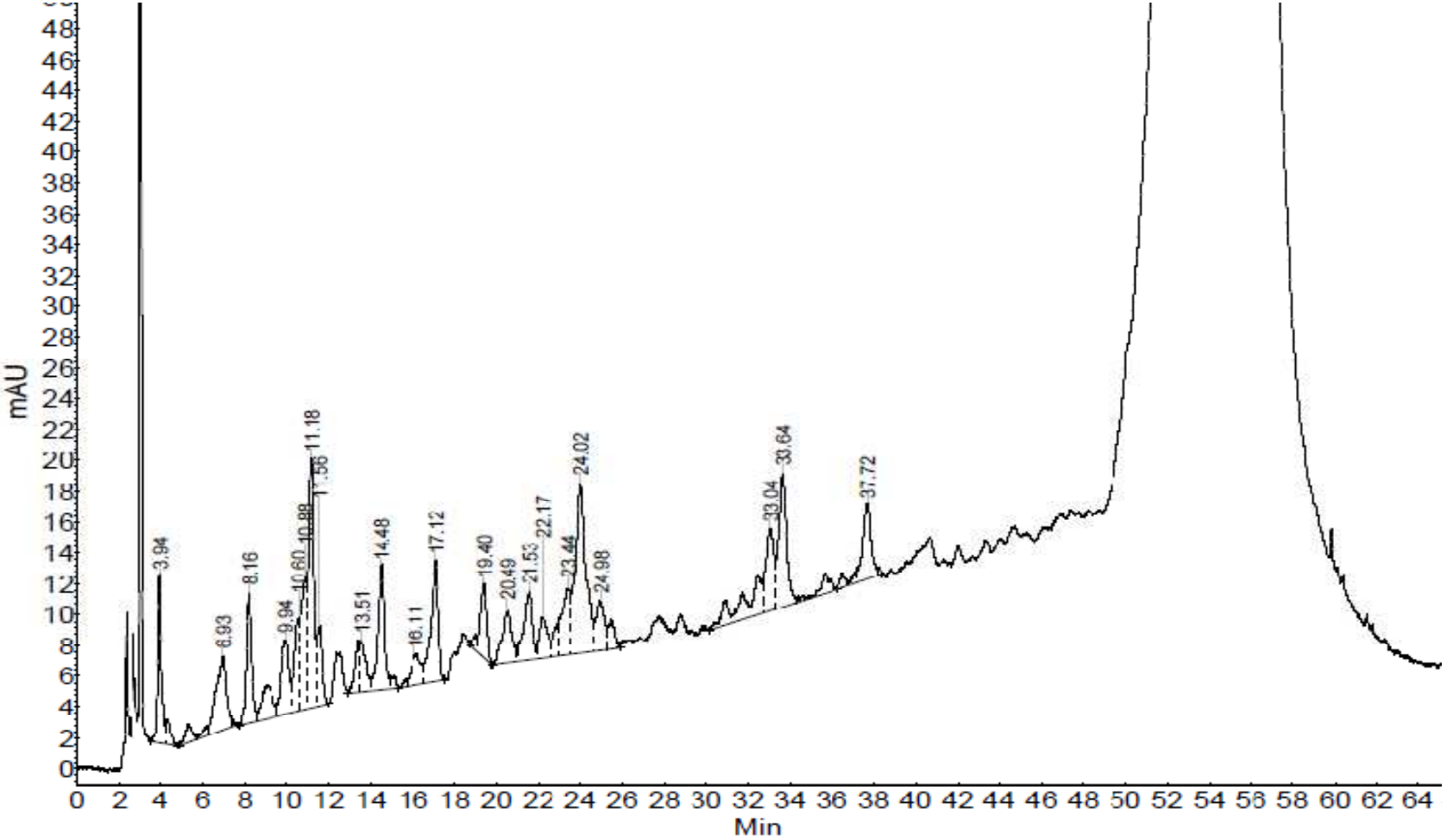
LC-DAD profile of Thesis 3 frozen seeds EtOH 70%

Appendix 11



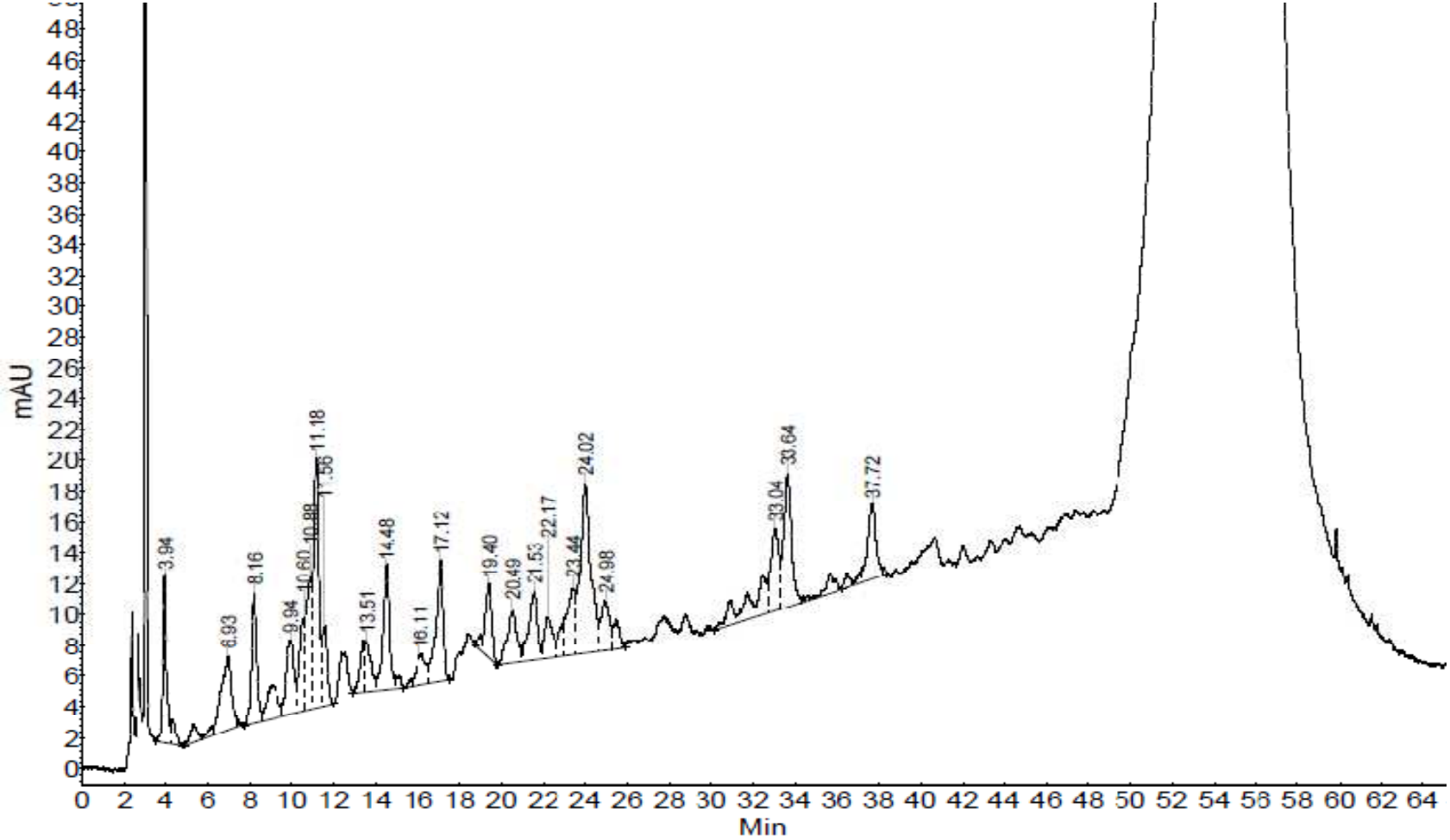
LC-DAD profile of Thesis 3 frozen seeds Acet 70%

Appendix 12



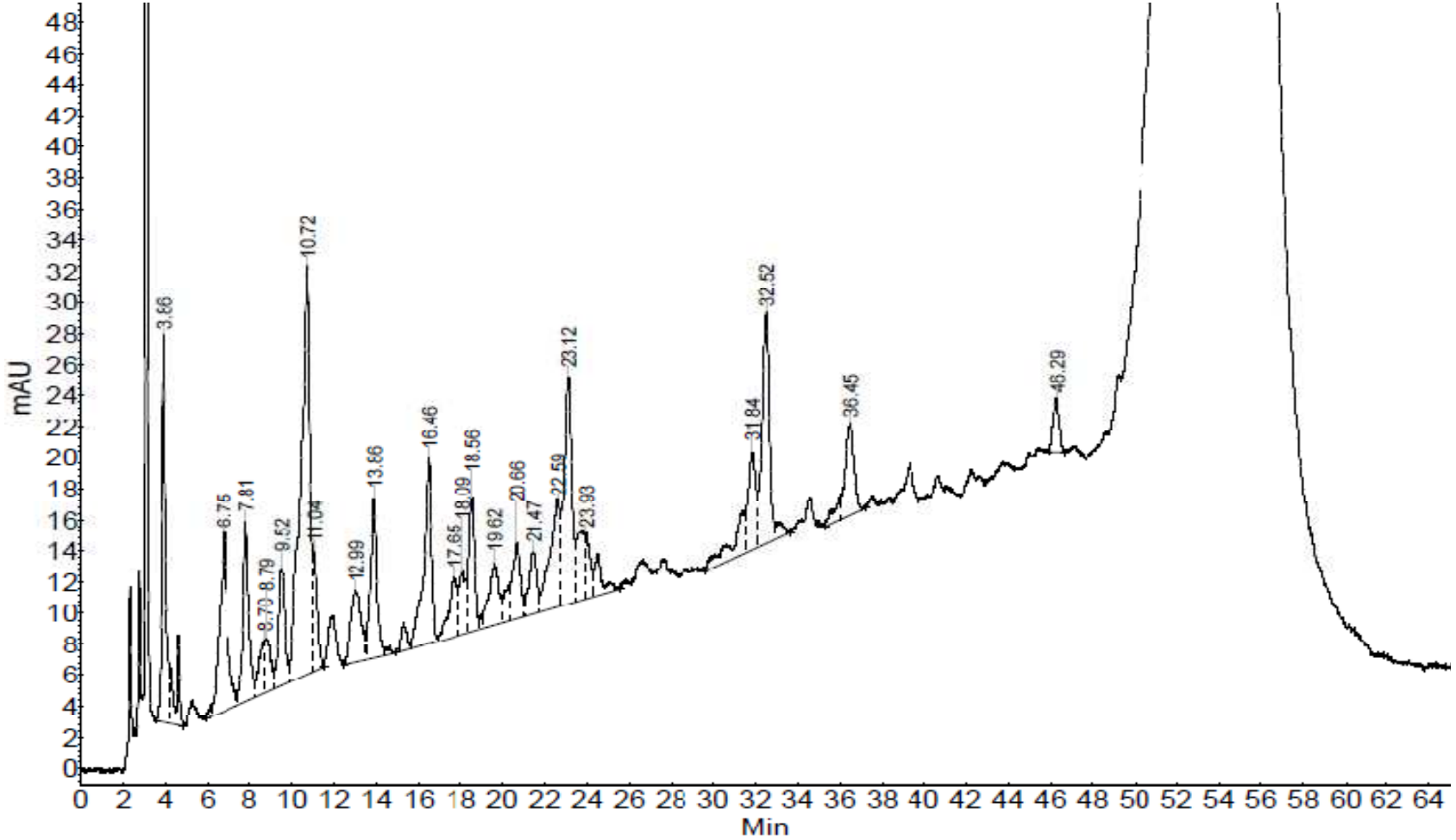
LC-DAD profile of Thesis 4 frozen seeds EtOH 70%

Appendix 13



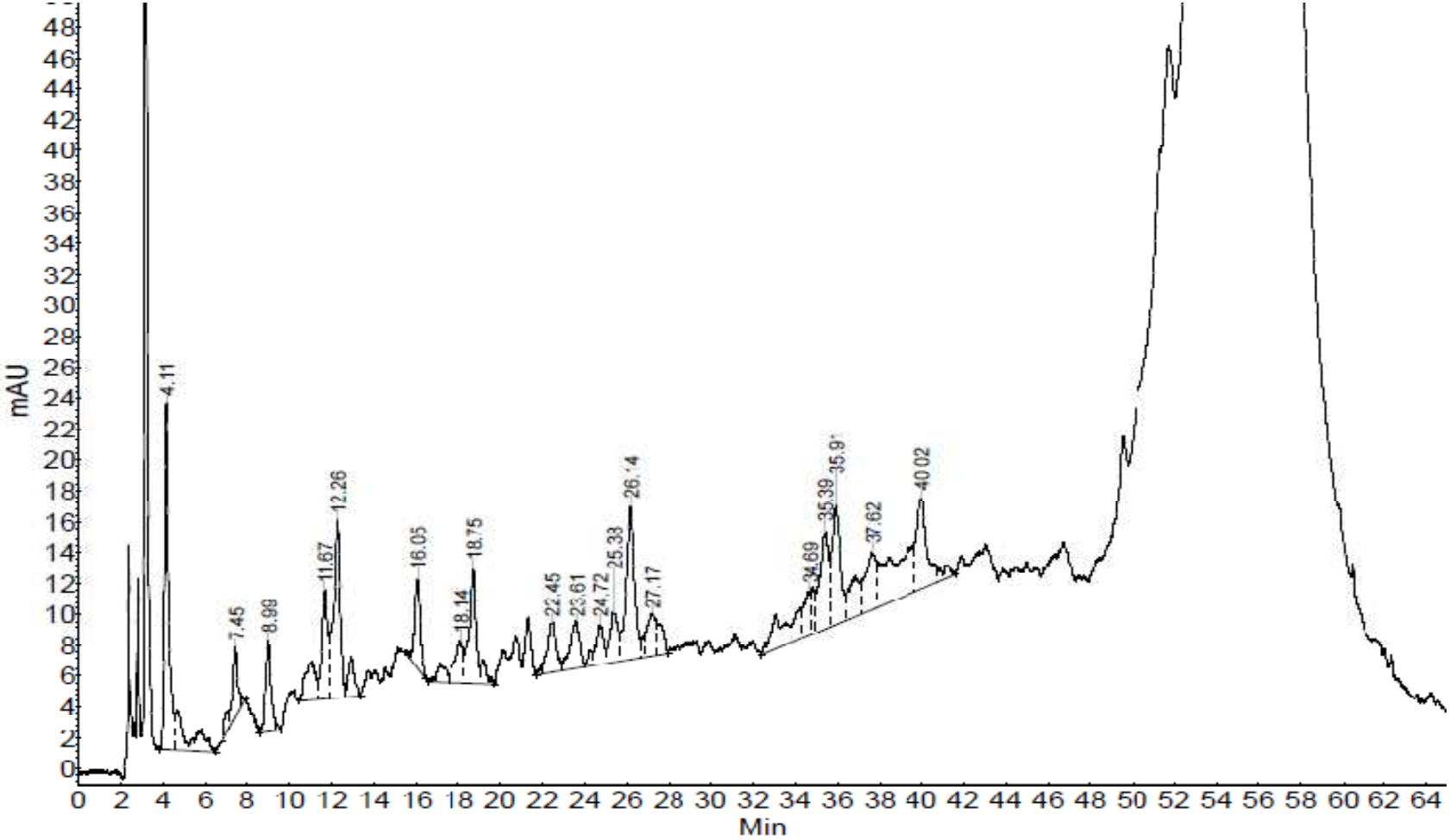
LC-DAD profile of Thesis 4 frozen seeds Acet 70%

Appendix 14



LC-DAD profile of Thesis 1 dried seeds Acet 70%

Appendix 15



LC-DAD profile of Thesis 4 dried seeds Acet 70%

Appendix 16

ANALYTE	AREA (mAU.sec)
CATECHIN	676.0
CATECHIN	669.6
EPICATECHIN	357.5
EPICATECHIN	329.2
GALLIC AC.	167.4
GALLIC AC.	174.1
PC B1	285.9
PC B1	252.9
PC B2	295.2
PC B2	288.6

THESIS 1 FROZEN SEEDS EtOH 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	500.5
CATECHIN	512.8
EPICATECHIN	396.3
EPICATECHIN	360.7
GALLIC AC.	240.0
GALLIC AC.	232.3
PC B1	236.9
PC B1	230.5
PC B2	257.3
PC B2	241.0

THESIS 1 FROZEN SEEDS Acet 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	521.9
CATECHIN	500.0
EPICATECHIN	402.0
EPICATECHIN	380.6
GALLIC AC.	263.4
GALLIC AC.	293.3
PC B1	144.8
PC B1	134.4
PC B2	229.3
PC B2	222.3

THESIS 1 DRIED SEEDS Acet 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	414.2
CATECHIN	395.7
EPICATECHIN	188.0
EPICATECHIN	183.0
GALLIC AC.	197.4
GALLIC AC.	201.3
PC B1	209.9
PC B1	222.5
PC B2	198.6
PC B2	212.4

THESIS 2 FROZEN SEEDS EtOH 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	356.1
CATECHIN	395.6
EPICATECHIN	192.4
EPICATECHIN	196.1
GALLIC AC.	217.8
GALLIC AC.	211.3
PC B1	172.9
PC B1	166.3
PC B2	221.2
PC B2	258.9

THESIS 2 FROZEN SEEDS Acet 70%

Analytes peak area for double LC injections of Thesis 1 and Thesis 2 extracts

Appendix 17

ANALYTE	AREA (mAU.sec)
CATECHIN	416.3
CATECHIN	370.6
EPICATECHIN	193.7
EPICATECHIN	175.7
GALLIC AC.	272.9
GALLIC AC.	277.4
PC B1	158.0
PC B1	158.7
PC B2	267.6
PC B2	225.2

THESIS 3 FROZEN SEEDS EtOH 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	284.6
CATECHIN	307.7
EPICATECHIN	142.6
EPICATECHIN	163.9
GALLIC AC.	270.2
GALLIC AC.	294.9
PC B1	164.1
PC B1	155.7
PC B2	167.4
PC B2	128.5

THESIS 3 FROZEN SEEDS Acet 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	365.0
CATECHIN	313.3
EPICATECHIN	176.9
EPICATECHIN	189.4
GALLIC AC.	206.5
GALLIC AC.	169.1
PC B1	175.7
PC B1	166.1
PC B2	209.9
PC B2	198.0

THESIS 4 FROZEN SEEDS EtOH 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	315.3
CATECHIN	345.5
EPICATECHIN	124.0
EPICATECHIN	170.5
GALLIC AC.	133.6
GALLIC AC.	118.3
PC B1	148.1
PC B1	130.9
PC B2	213.8
PC B2	190.3

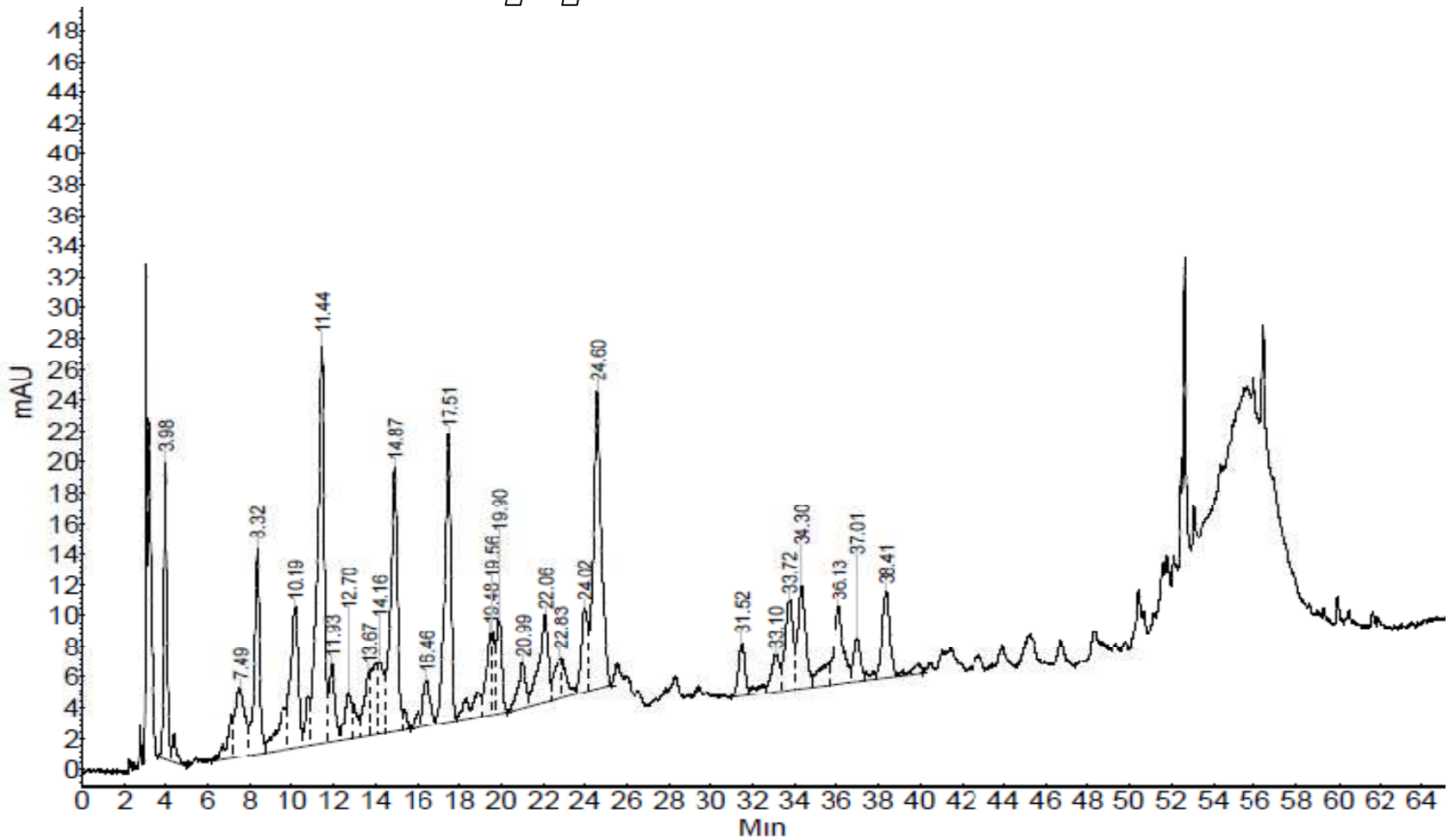
THESIS 4 FROZEN SEEDS Acet 70%

ANALYTE	AREA (mAU.sec)
CATECHIN	245.5
CATECHIN	233.9
EPICATECHIN	168.4
EPICATECHIN	182.3
GALLIC AC.	300.1
GALLIC AC.	329.4
PC B1	108.2
PC B1	142.1
PC B2	197.5
PC B2	205.1

THESIS 4 DRIED SEEDS Acet 70%

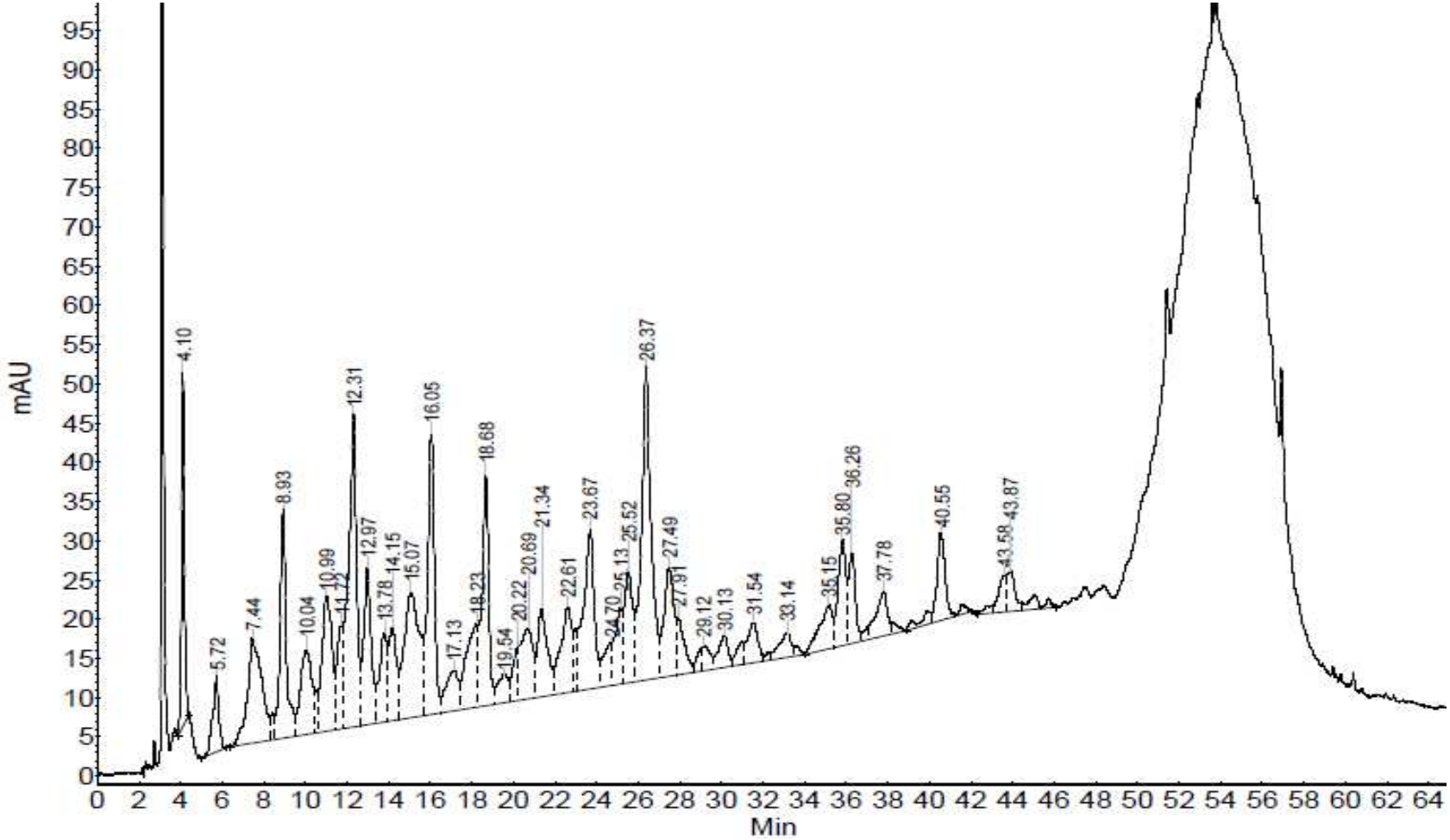
Analytes peak area for double LC injections of Thesis 3 and Thesis 4 extracts

Appendix 18



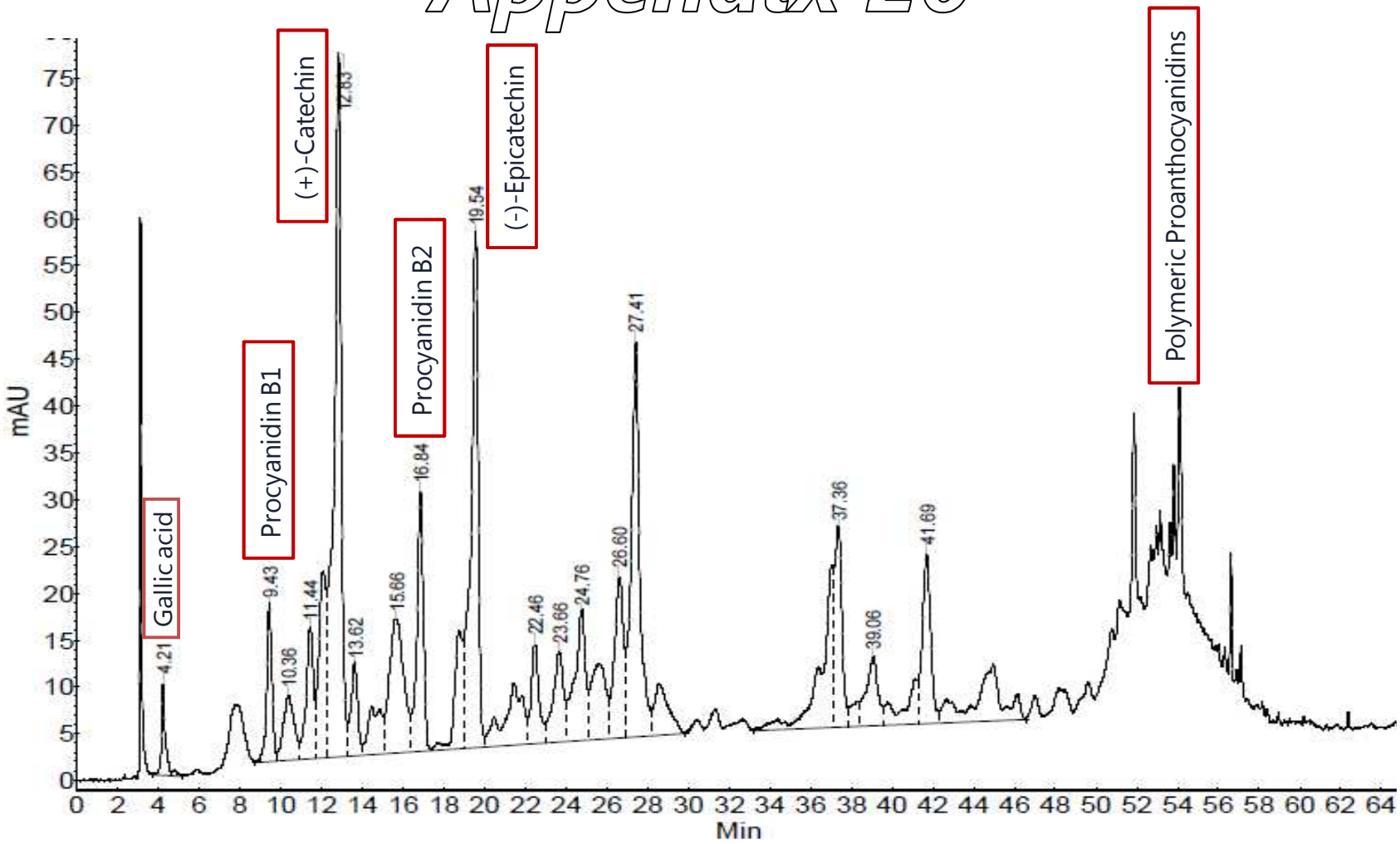
LC-DAD profile of Thesis 4 frozen seeds EtOH 70% purified with Ethyl Acetate (preliminary test)

Appendix 19



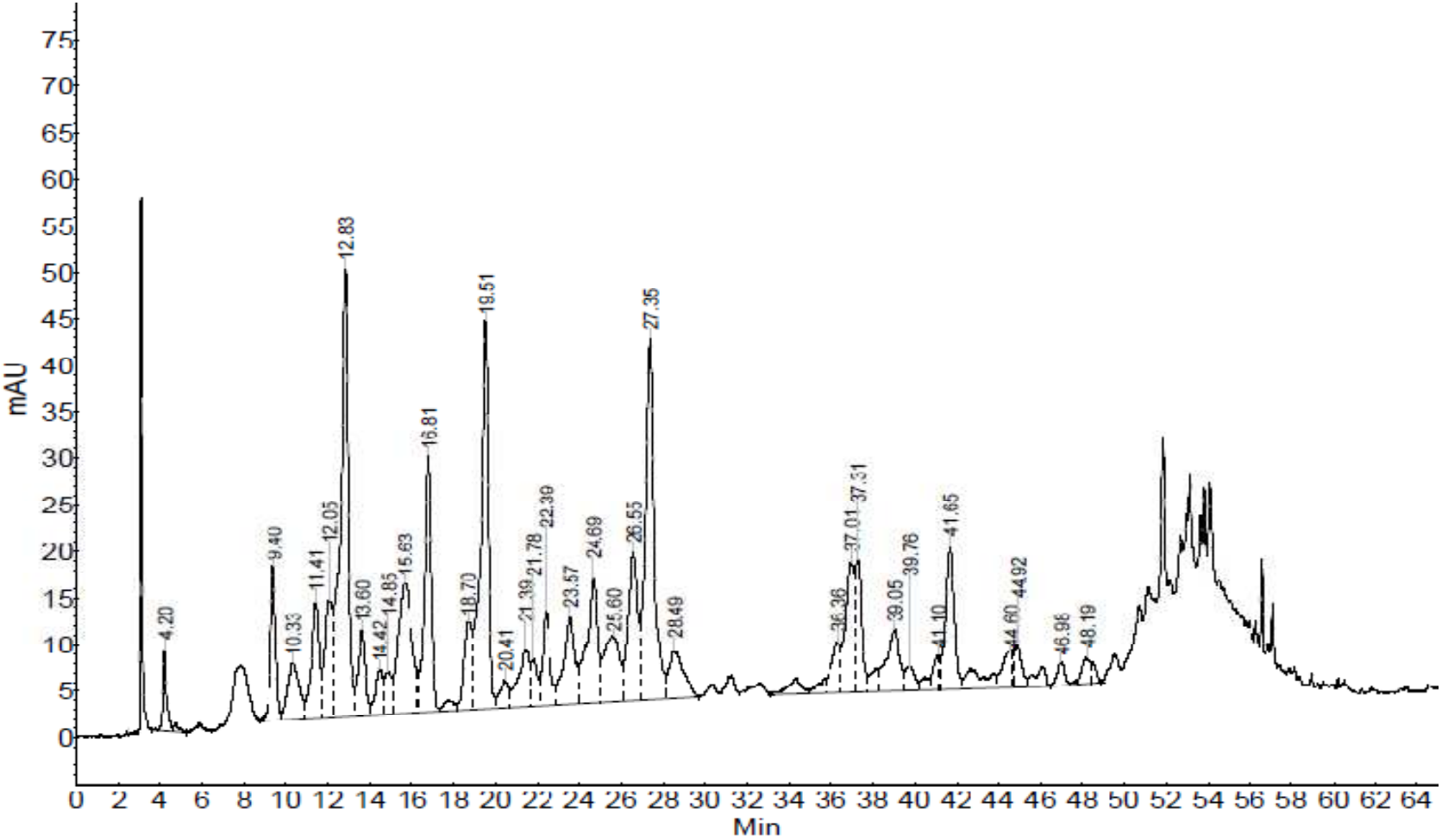
LC-DAD profile of Thesis 4 frozen seeds EtOH 70% purified with Ethyl Acetate and NaCl (preliminary test)

Appendix 20



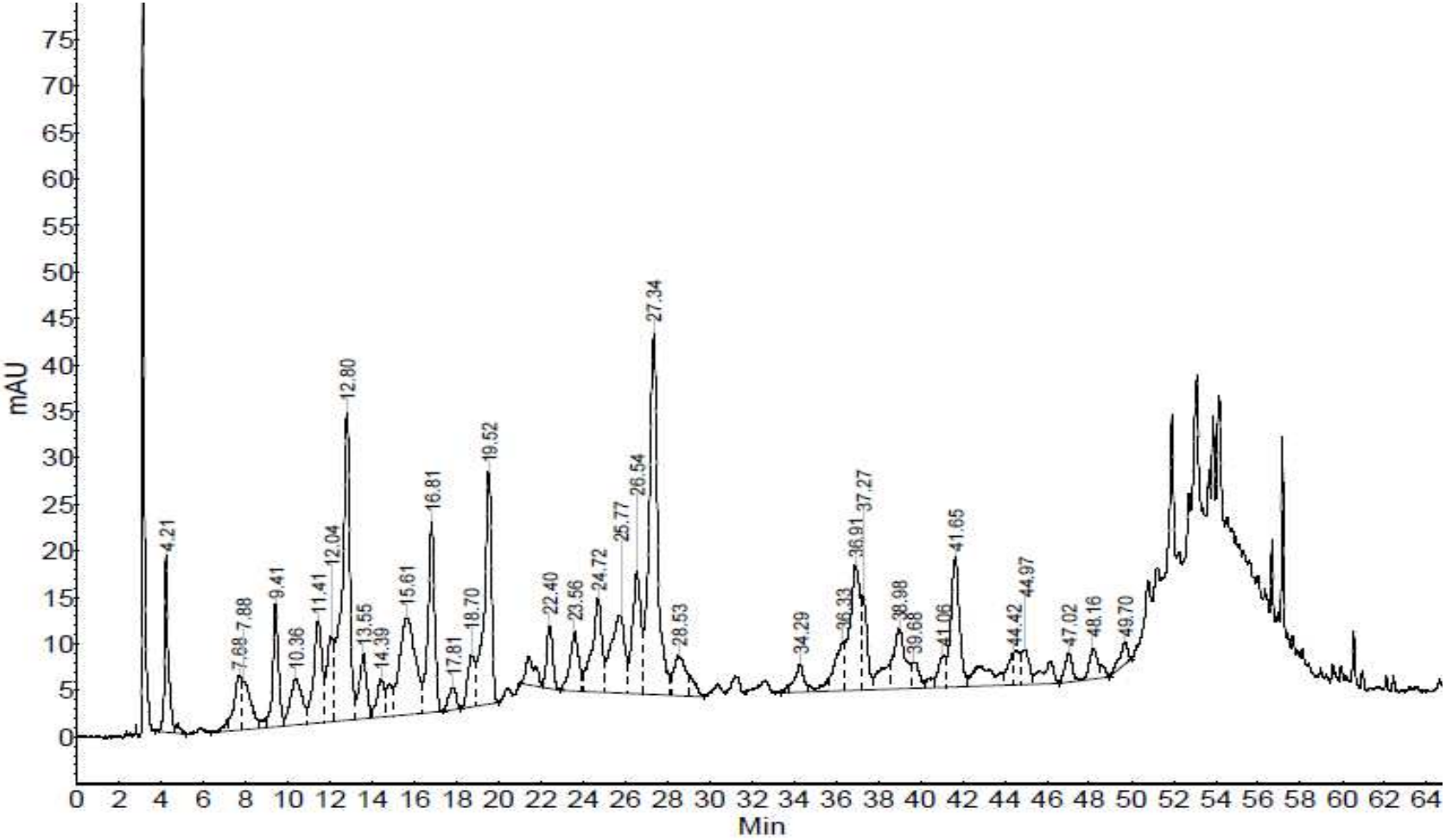
LC-DAD profile of Thesis 1 frozen seeds EtOH 70% purified with Ethyl Acetate

Appendix 21



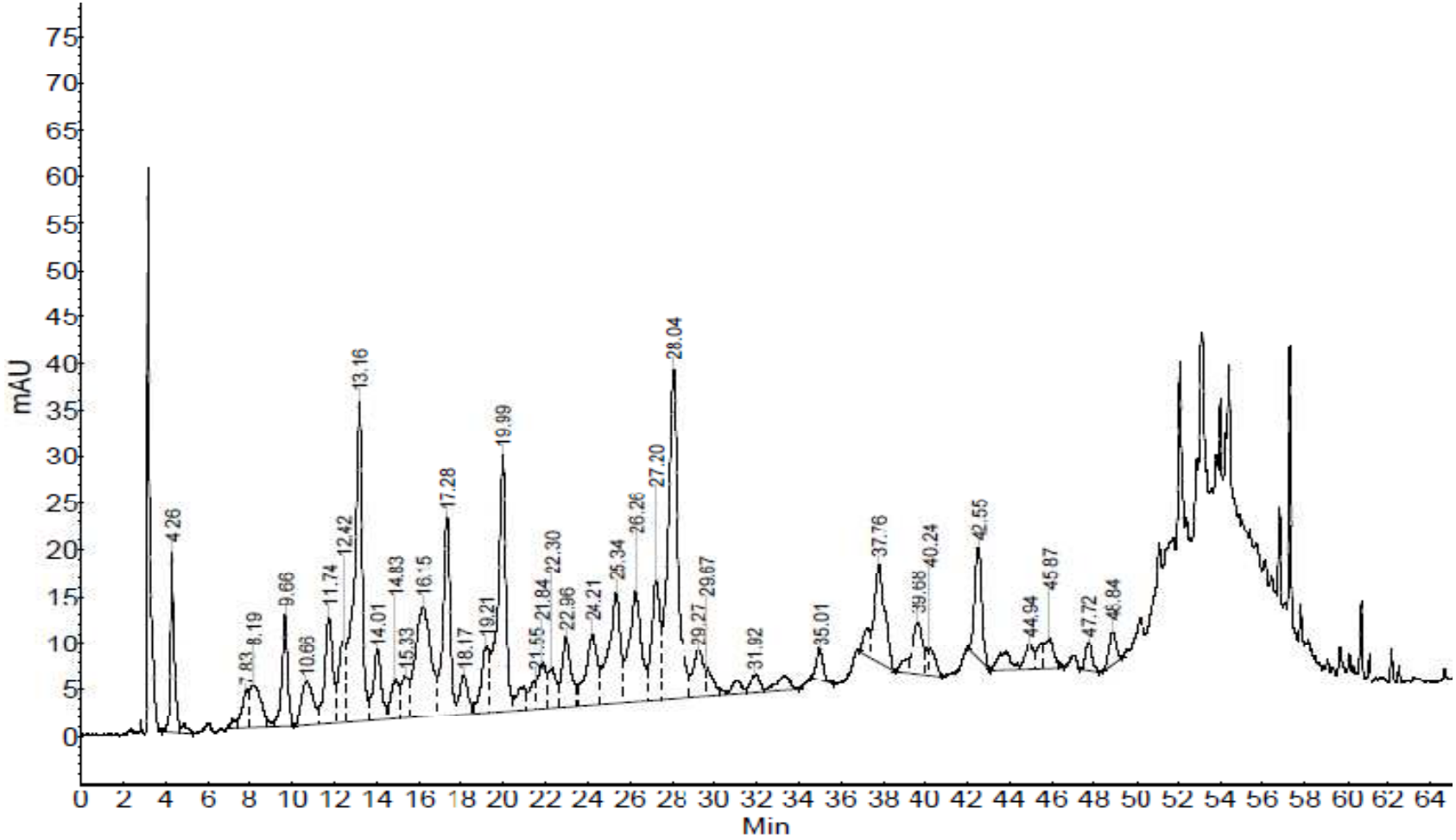
LC-DAD profile of Thesis 2 frozen seeds EtOH 70% purified with Ethyl Acetate

Appendix 22



LC-DAD profile of Thesis 3 frozen seeds EtOH 70% purified with Ethyl Acetate

Appendix 23



LC-DAD profile of Thesis 4 frozen seeds EtOH 70% purified with Ethyl Acetate

Appendix 24

ANALYTE	AREA (mAU.sec)
CATECHIN	1872.3
CATECHIN	2321.4
EPICATECHIN	1366.4
EPICATECHIN	1698.4
GALLIC AC.	124.2
GALLIC AC.	142.4
PC B1	302.3
PC B1	254.5
PC B2	616.6
PC B2	748.3

THESIS 1 PURIFIED SEEDS

ANALYTE	AREA (mAU.sec)
CATECHIN	1198.3
CATECHIN	1242.6
EPICATECHIN	1066.5
EPICATECHIN	1065.1
GALLIC AC.	124.8
GALLIC AC.	109.6
PC B1	294.7
PC B1	303.1
PC B2	614.4
PC B2	605.7

THESIS 2 PURIFIED SEEDS

ANALYTE	AREA (mAU.sec)
CATECHIN	851.0
CATECHIN	884.6
EPICATECHIN	642.0
EPICATECHIN	608.4
GALLIC AC.	243.0
GALLIC AC.	234.0
PC B1	236.0
PC B1	250.4
PC B2	345.0
PC B2	456.5

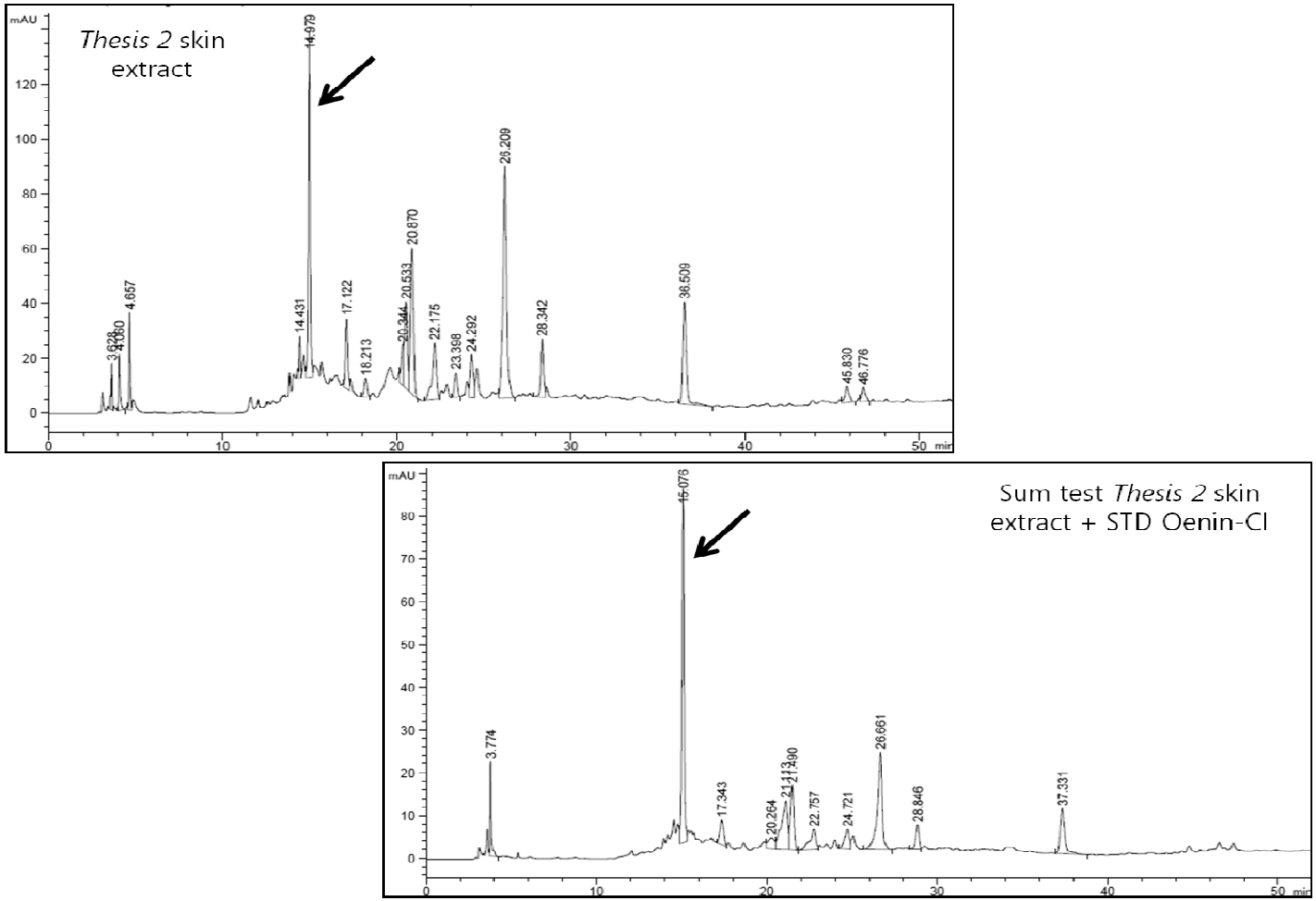
THESIS 3 PURIFIED SEEDS

ANALYTE	AREA (mAU.sec)
CATECHIN	920.1
CATECHIN	926.9
EPICATECHIN	715.4
EPICATECHIN	692.2
GALLIC AC.	235.9
GALLIC AC.	240.1
PC B1	225.7
PC B1	220.4
PC B2	515.7
PC B2	473.2

THESIS 4 PURIFIED SEEDS

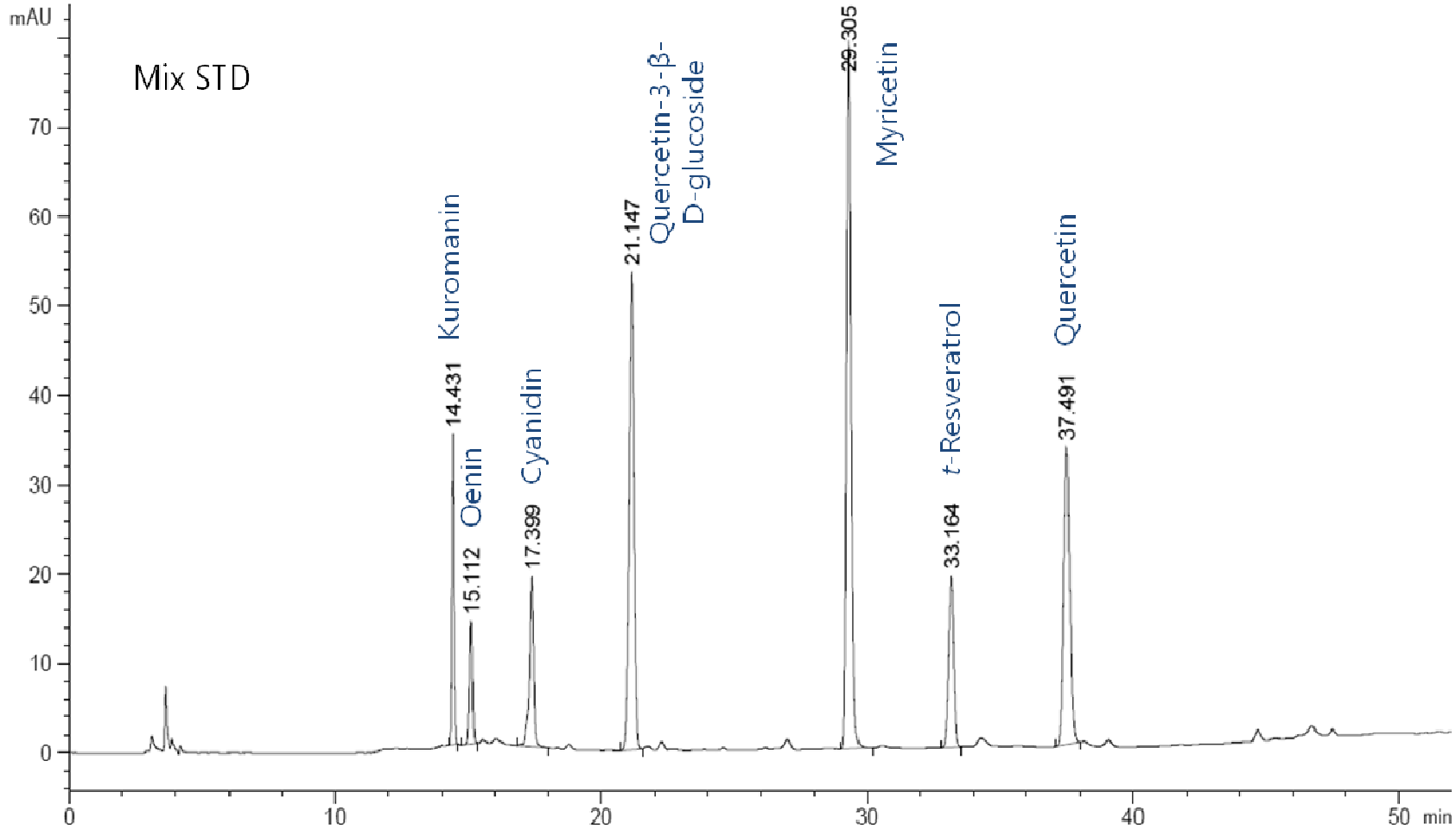
Analytes peak area for double LC injections of purified extracts

Appendix 25



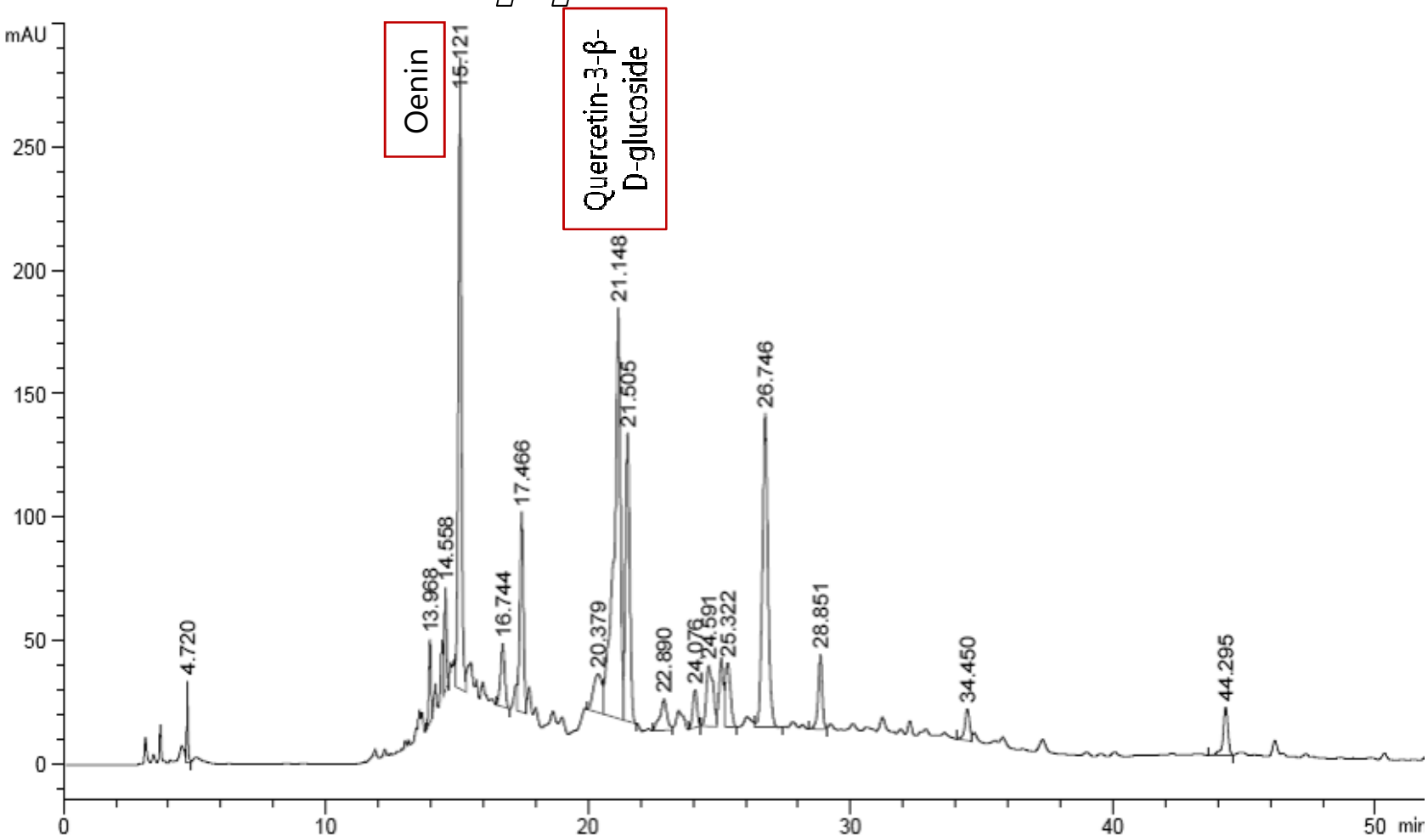
Sum test of Oenin Chloride and Thesis 2 skin extract

Appendix 26



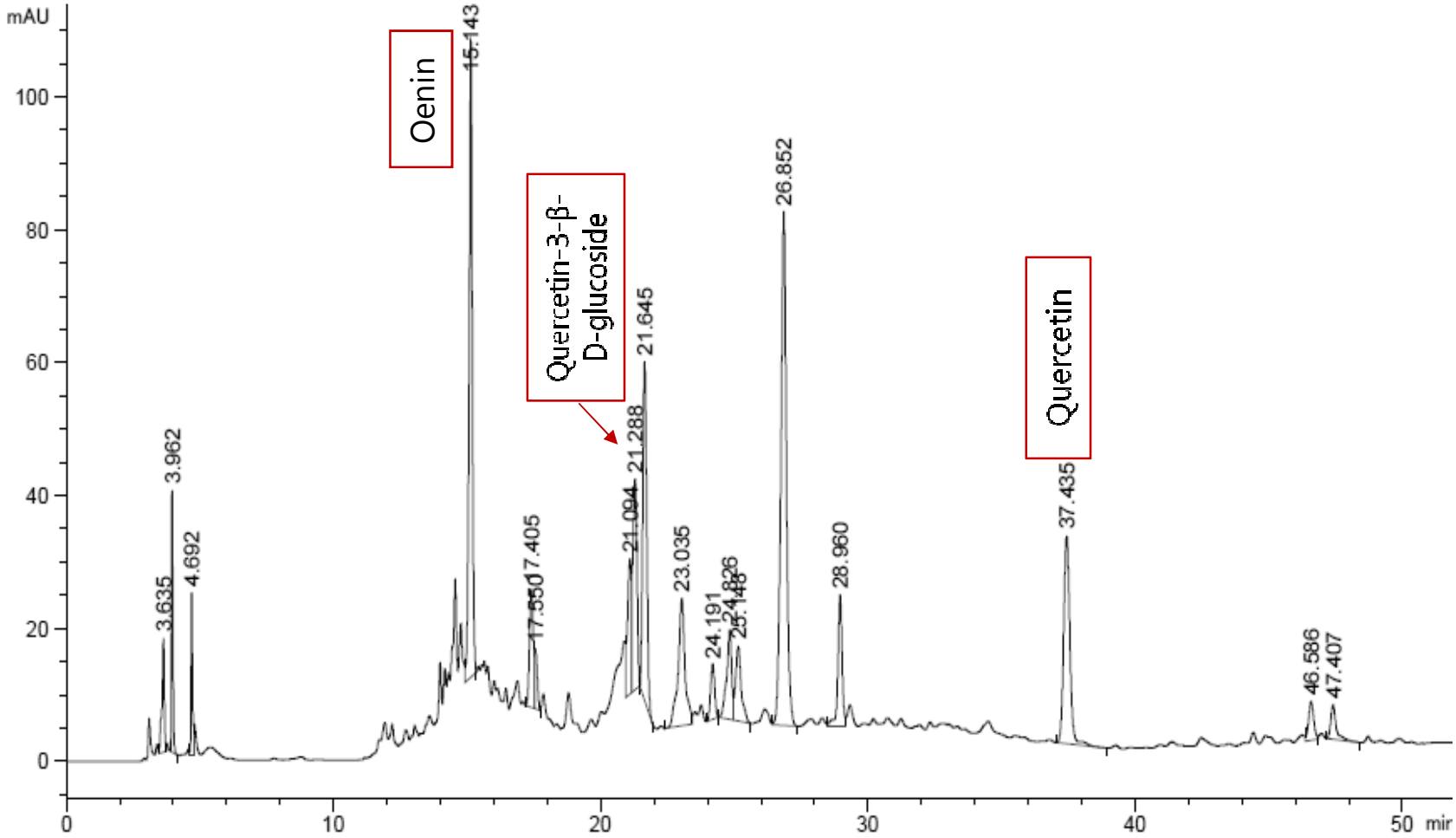
LC-UV profile of MIX STD for grape skins characterization

Appendix 27



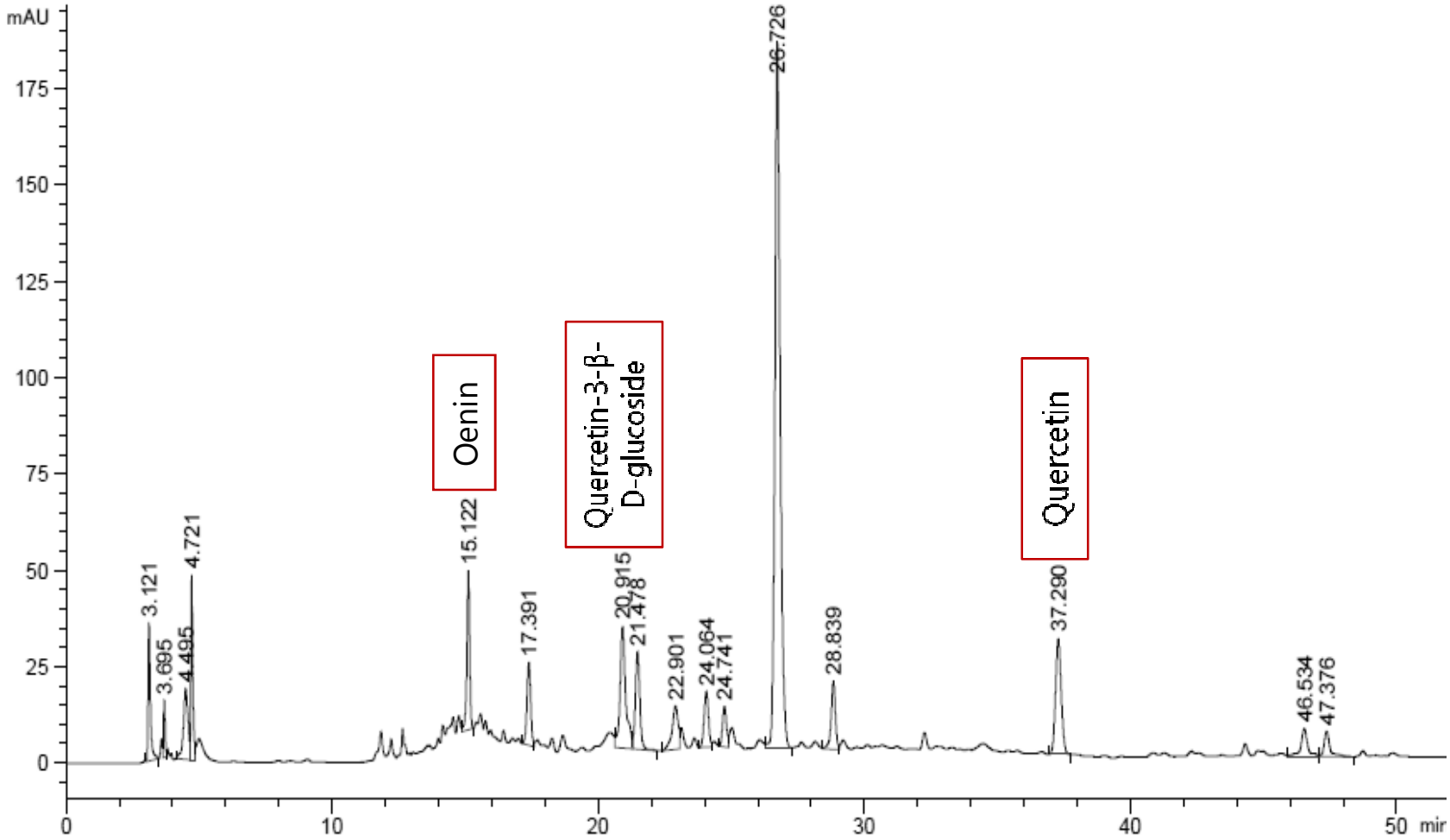
LC-UV profile of Thesis 1 skins

Appendix 28



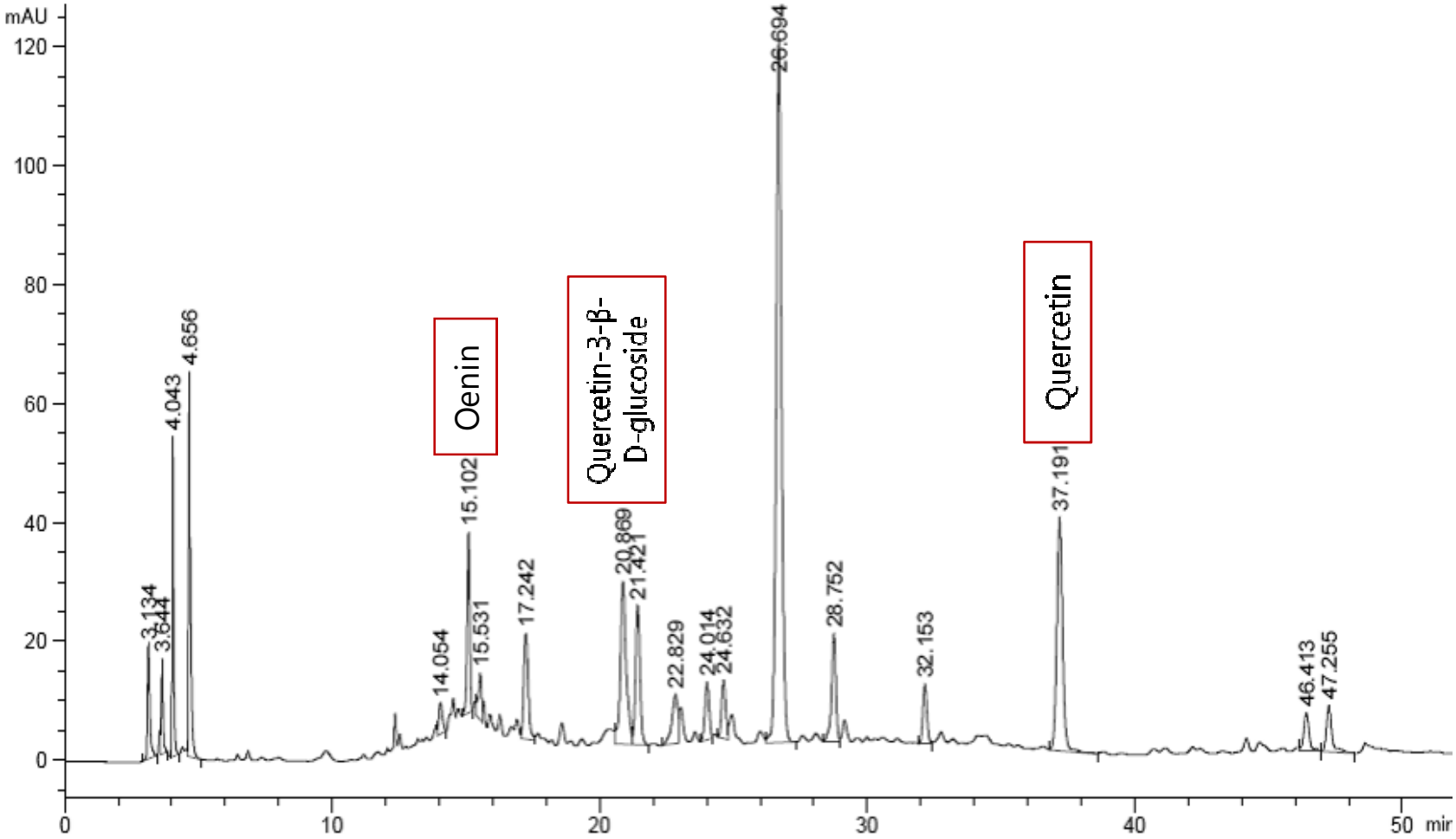
LC-UV profile of Thesis 2 skins

Appendix 29



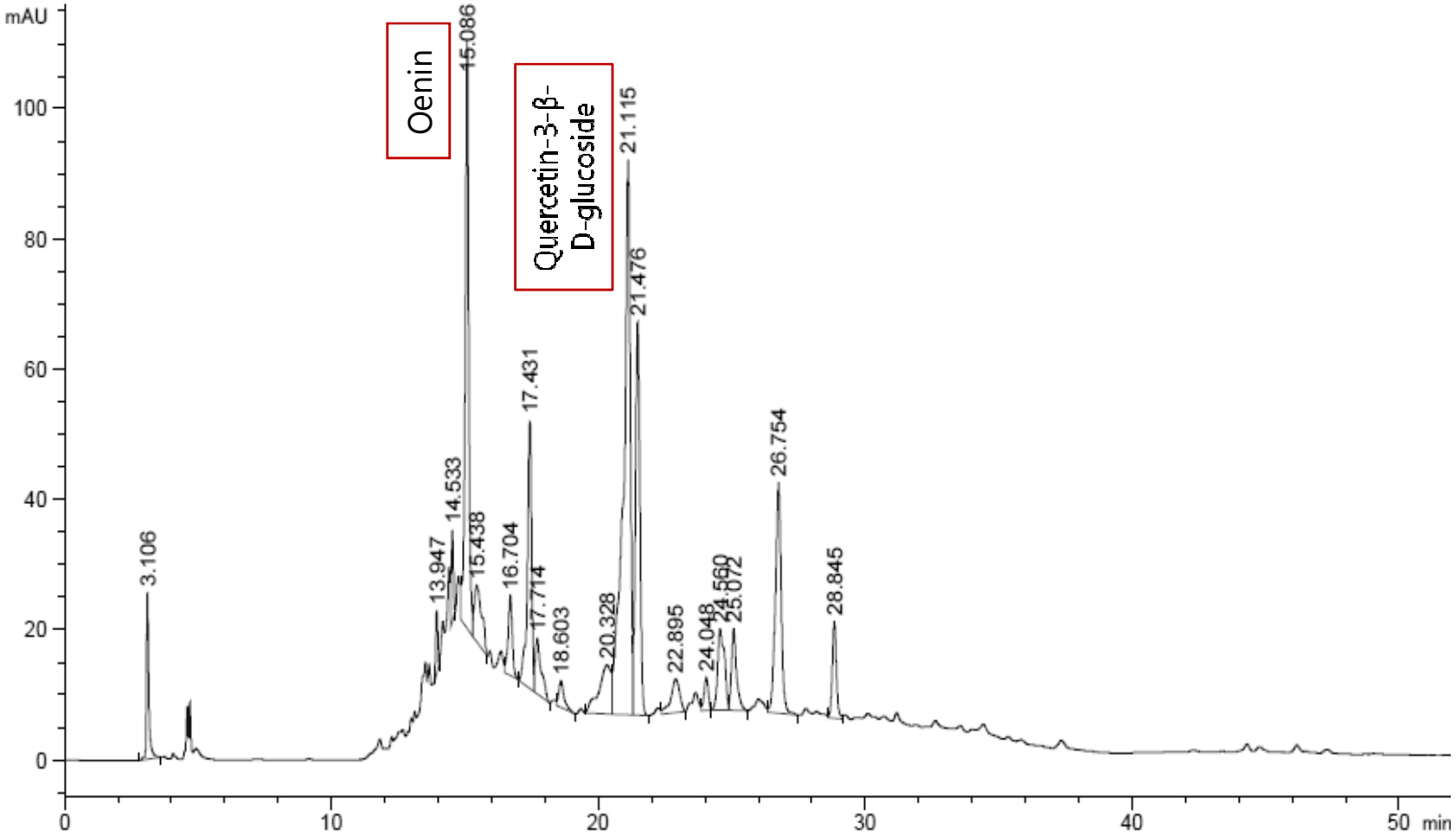
LC-UV profile of Thesis 3 skins

Appendix 30



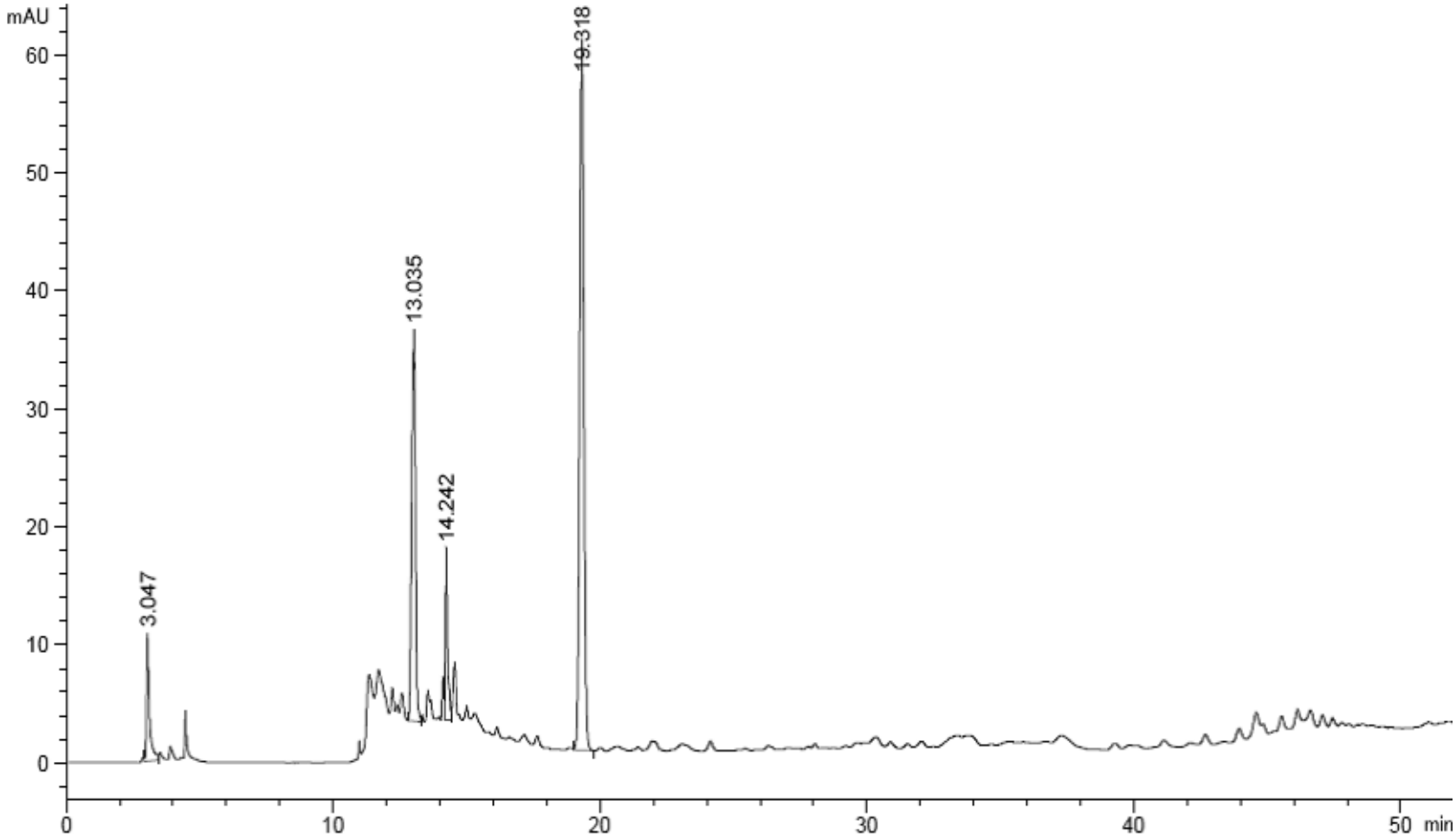
LC-UV profile of Thesis 4 skins

Appendix 31



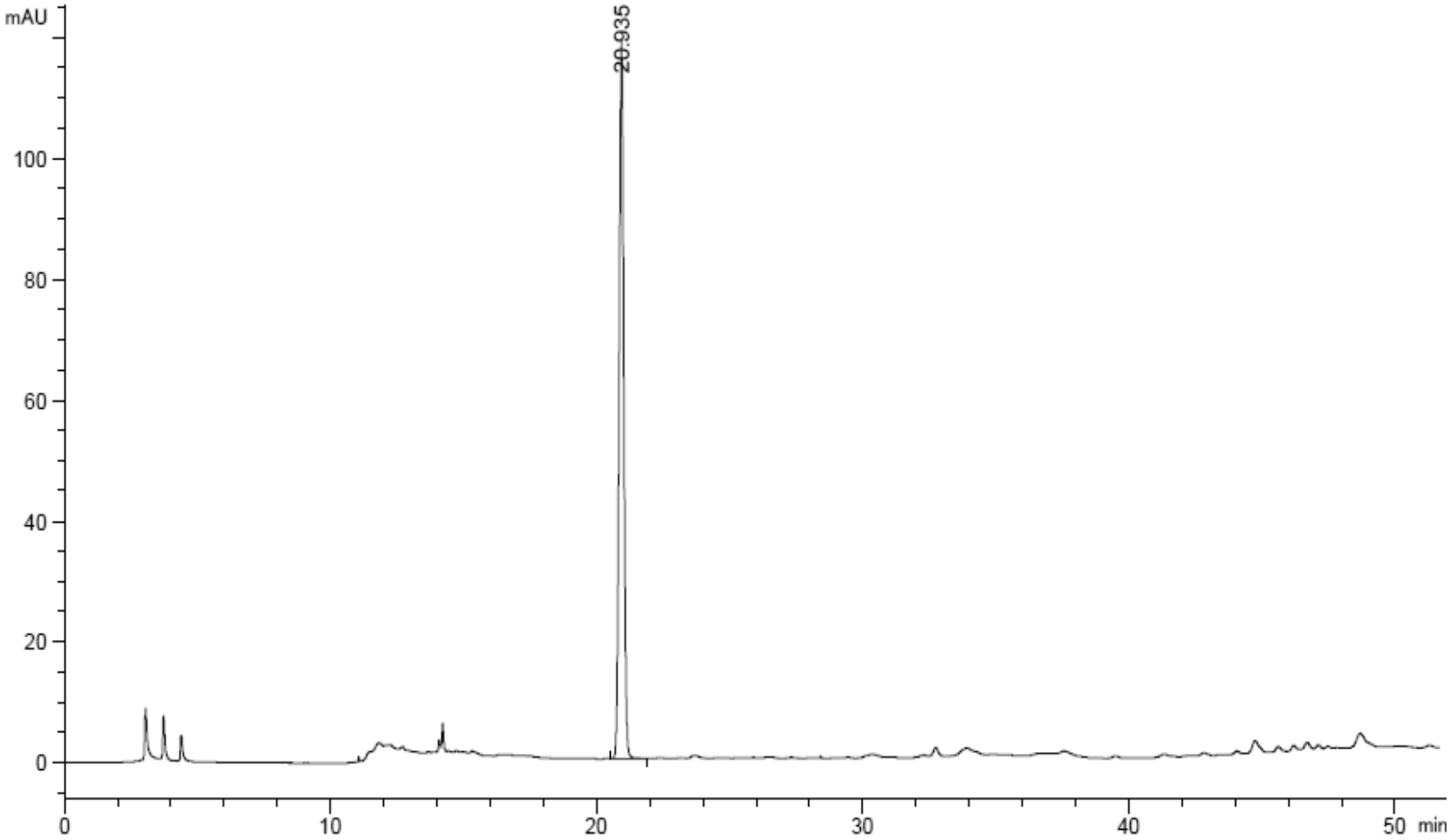
LC-UV profile of Thesis 1 purified skins

Appendix 32



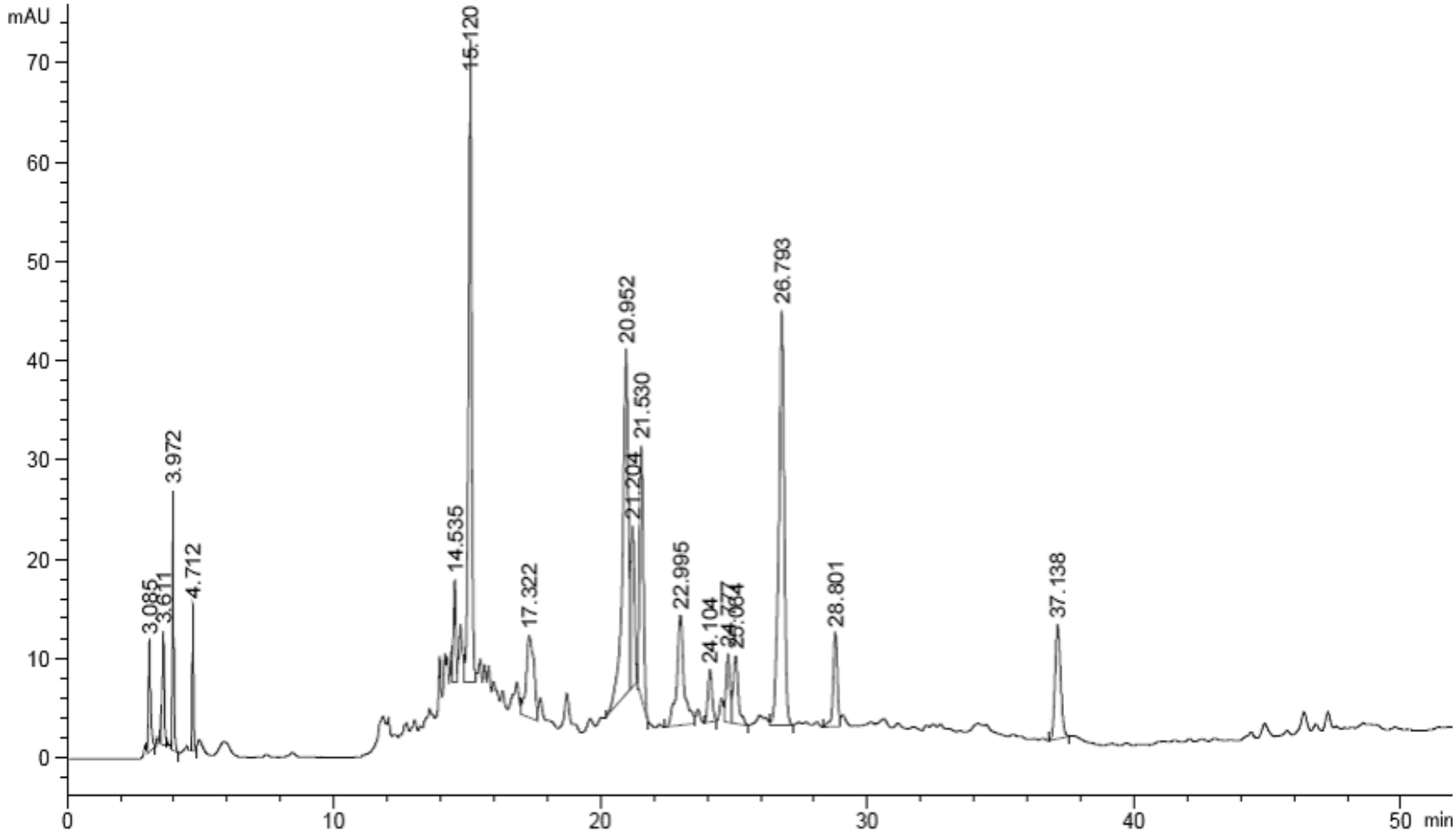
LC-UV profile of Thesis 1 hydrolyzed grape skin extract

Appendix 33



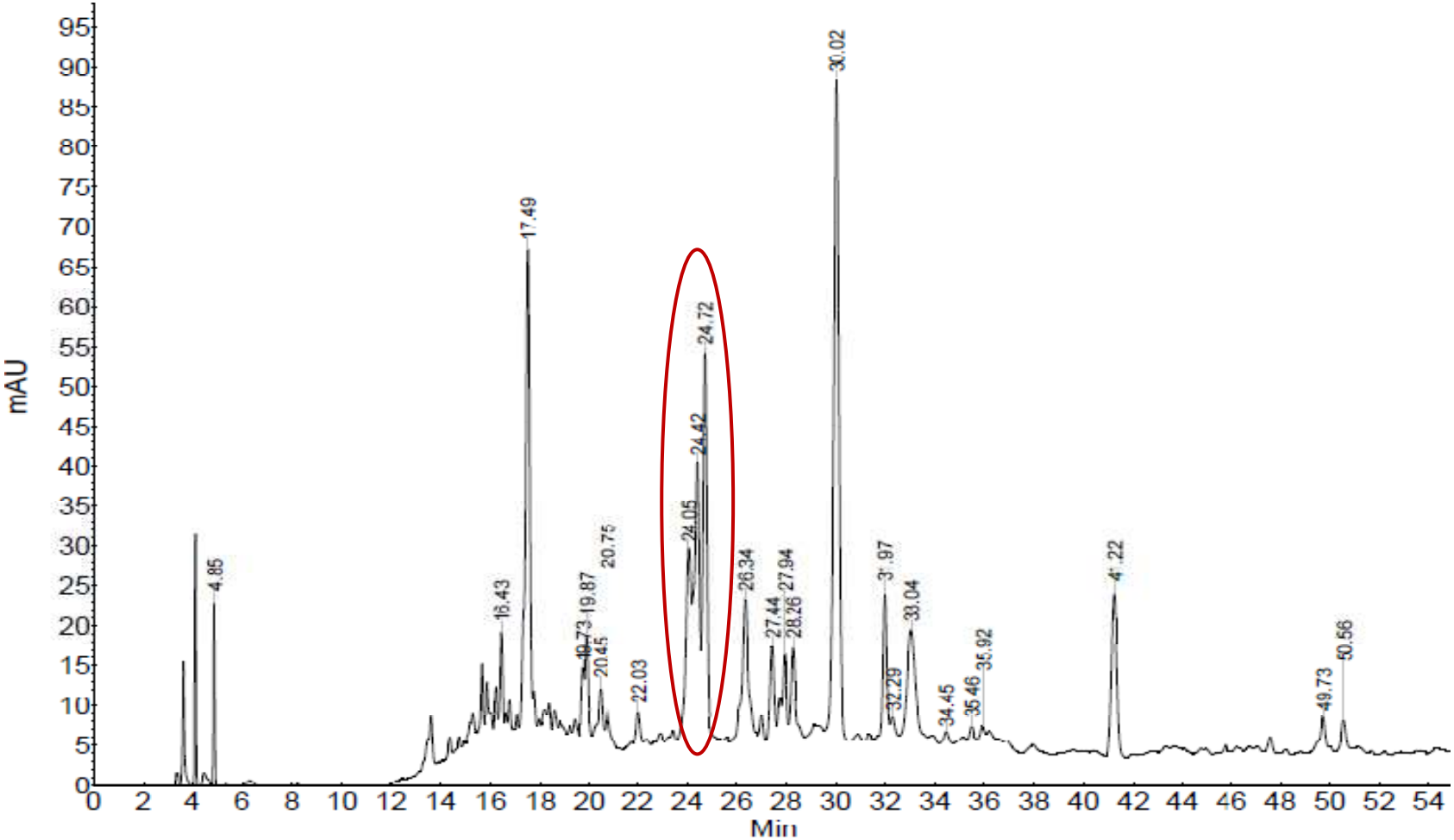
LC-UV profile of STD Polydatin 0.1 mg/mL

Appendix 34



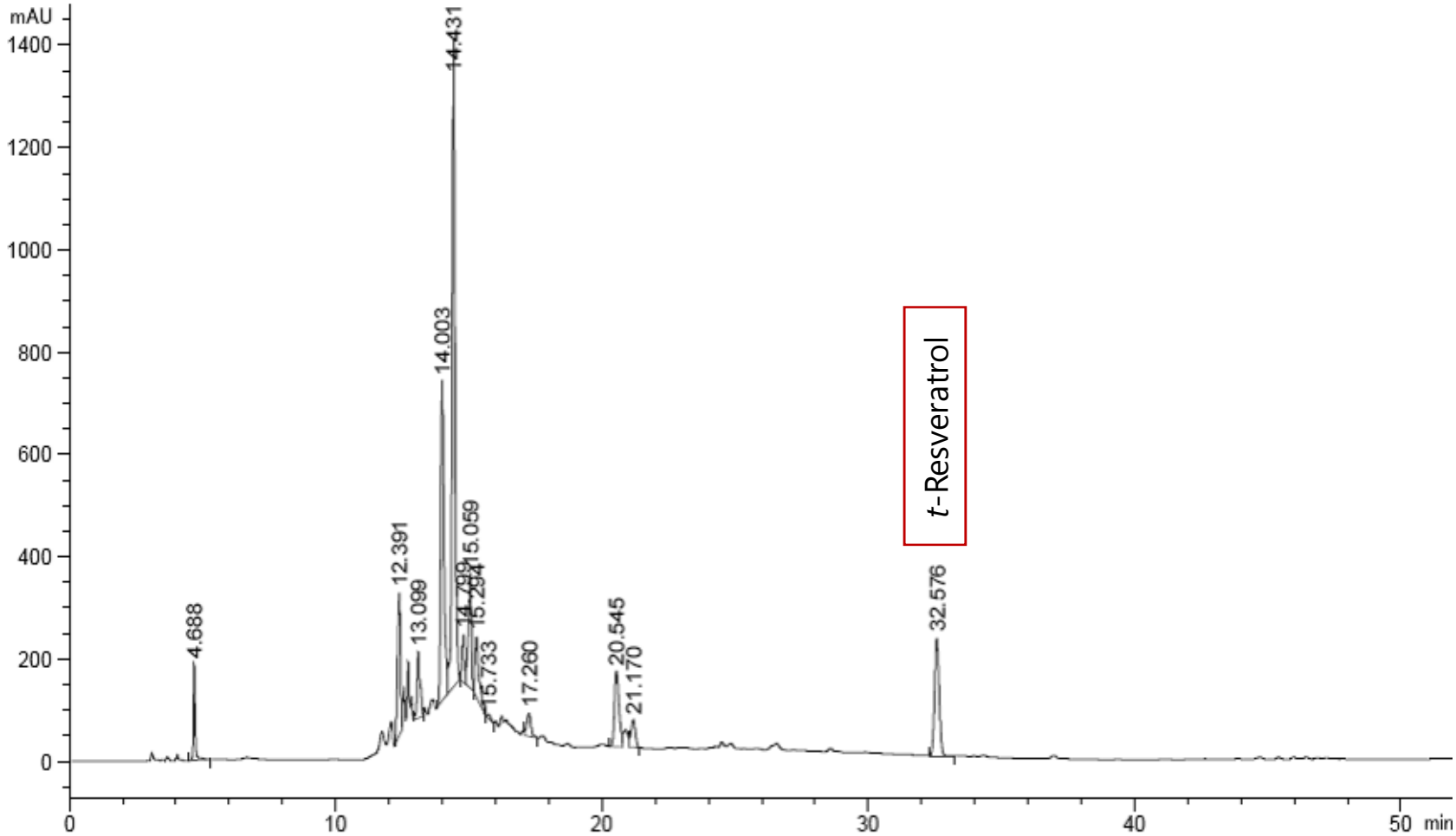
LC-UV profile of the sum test between std Polydatin and Thesis 2 skin extract

Appendix 35



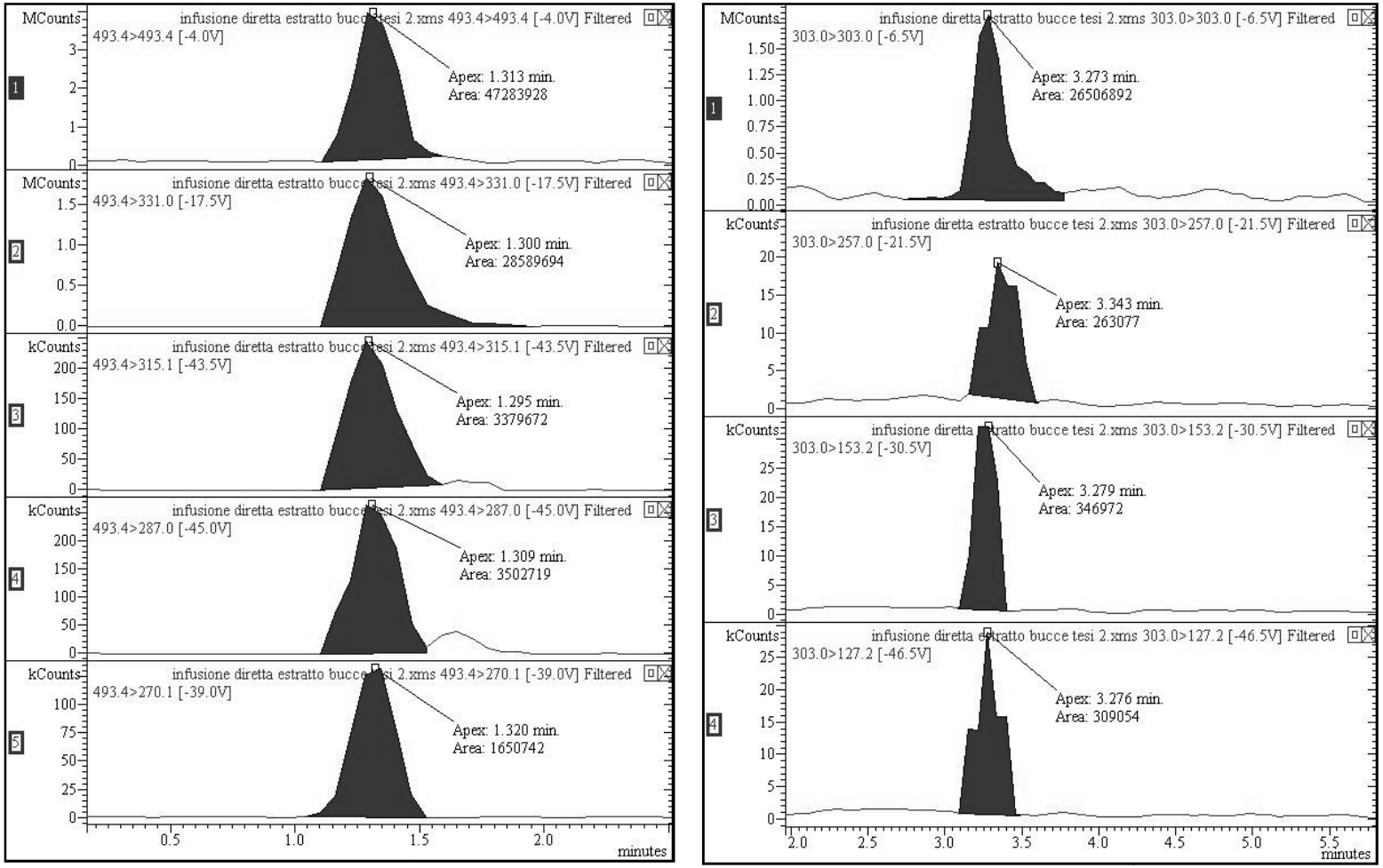
LC-DAD profile of Thesis 2 grape skin extract

Appendix 36



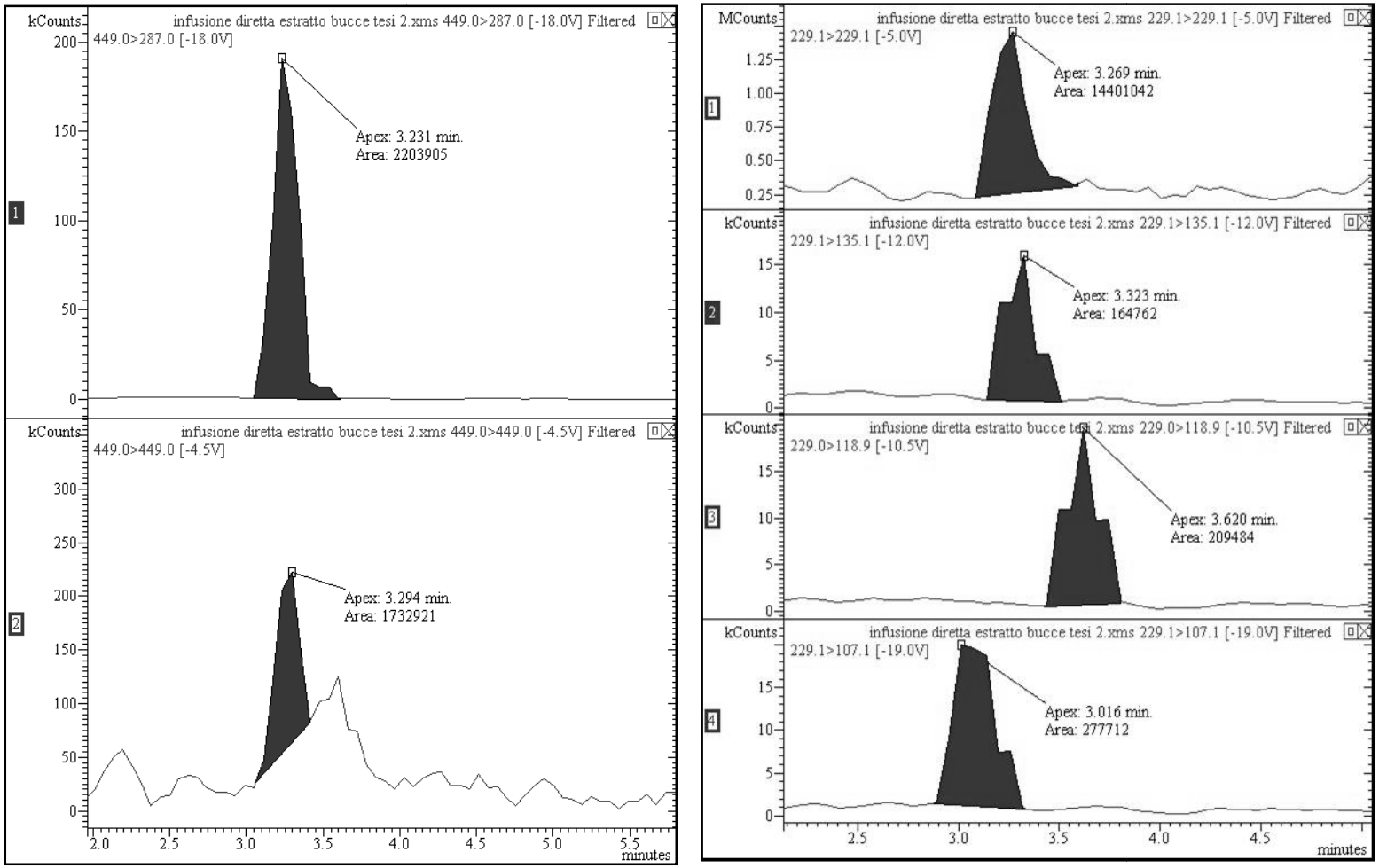
LC-UV profile of Revidox™

Appendix 37



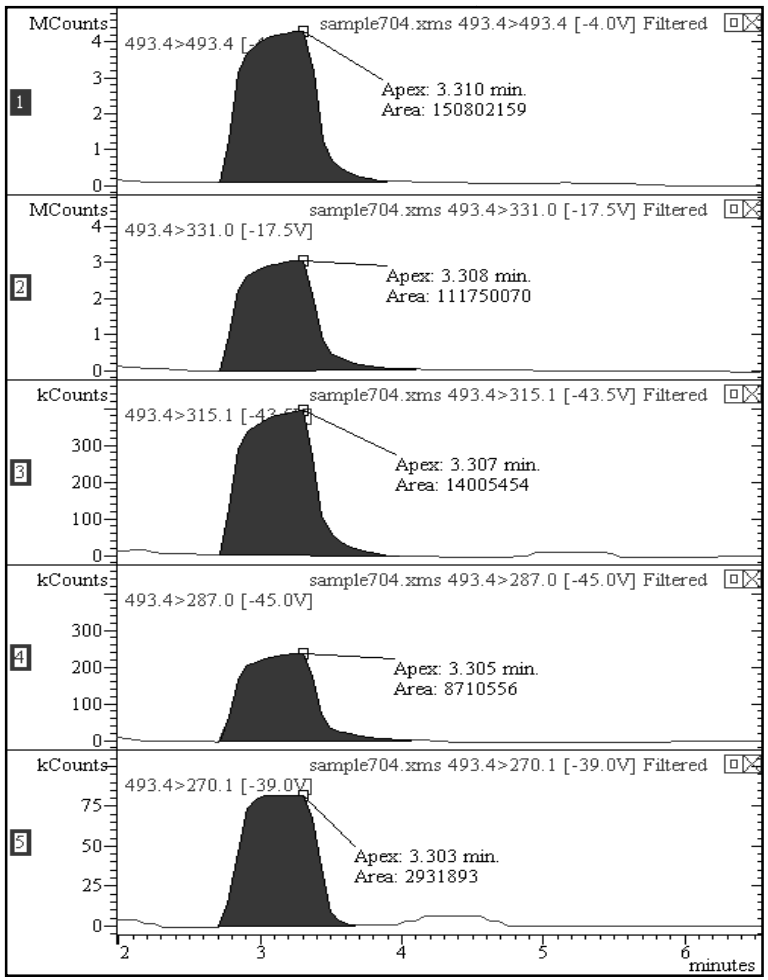
LC-MS/MS analysis of Thesis 2 grape skin extract: positive match with Oenin (left) and Quercetin (right) fragmentation patterns

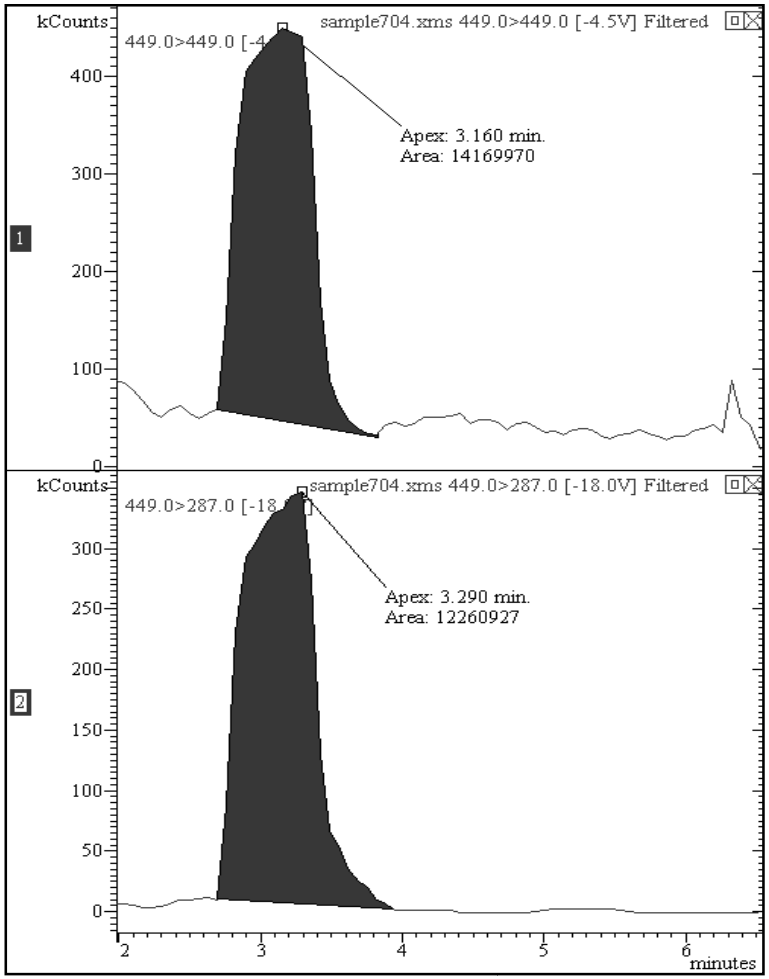
Appendix 38



Match between Thesis 2 extract and Kuromanin (left) or Resveratrol (right) fragmentation patterns

Appendix 39





LC-MS/MS analysis of Thesis 1 grape skin purified extract: match with Oenin (left) and Kuromanin (right) fragmentation patterns

REFERENCES

Alarcón de la Lastra C. and Villegas I.
Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications
Biochemical Society Transactions Vol. 35, part 5, 2007.

Aldini G., Carini M., Piccoli A., Rossoni G., Maffei Facino R.
Procyanidins from grape seeds protect endothelial cells from peroxynitrite damage and enhance endothelium-dependent relaxation in human artery: new evidences for cardio-protection
Life Sciences 73: 2883-2898, 2003.

Amico V., Chillemi R., Mangiafico S., Spatafora C., Tringali C.
Polyphenol-enriched fractions from Sicilian grape pomace: HPLC-DAD analysis and antioxidant activity
Bioresource Technology 99: 5960-5966, 2008.

Aron P.M., Kennedy J.A.
Flavan-3-ols: Nature, occurrence and biological activity
Mol. Nutr. Food Res. 52: 79-104, 2008.

Austin M.B., Bowman M.E., Ferrer J-L, Schröder J., Noel J.P.
An Aldol Switch Discovered in Stilbene Synthases Mediates Cyclization Specificity of Type III Polyketide Synthases
Chemistry & Biology 11: 1179-1194, 2004.

Bartolomé B., Hernández T., Bengoechea M.L., Quesada C., Gómez-Cordovés C., Estrella I.
Determination of some structural features of procyanidins and related compounds by photodiode-array detection.
Journal of Chromatography A, 723: 19-26, 1996.

Bucić-Kojić A., Planinić M., Tomas S., Bilić M., Velić D.
Study of solid-liquid extraction kinetics of total polyphenols from grape seeds
J. of Food Engineering 81: 236-242, 2007.

Careri M., Corradini C., Elviri L., Nicoletti I., Zagnoni I.
Direct HPLC Analysis of Quercetin and trans-Resveratrol in Red Wine, Grape, and Winemaking Byproducts
J. Agric. Food Chemistry 51: 5226:5231, 2003.

Carini M., Maffei Facino R ., Aldini G., Calloni M.T., Bombardelli E., Morazzoni P.
The protection of polyunsaturated fatty acids in micellar systems against UVB-induced photooxidation by procyanidins from *Vitis vinifera* L., and the protective synergy with vitamin E
International Journal of Cosmetic Science 2:, 203-215, 1998.

Carini M., Aldini G., Bombardelli E., Morazzoni P., Maffei Facino R.
UVB-induced hemolysis of rat erythrocytes: Protective effect of procyanidins from grape seeds
Life Sciences 67: 1799-1814, 2000.

Carini M., Stefani R., Aldini G., Orioli M., Maffei Facino R.
Procyanidins from *Vitis vinifera* Seeds Inhibit the Respiratory Burst of Activated Human Neutrophils and Lysosomal Enzyme Release
Planta Medica 67: 714-717, 2001.

Cavaliere C., Foglia P., Gubbiotti R., Sacchetti P., Samperi R., Laganà A.
Rapid-resolution liquid chromatography/mass spectrometry for determination and quantitation of polyphenols in grape berries
Rapid. Commun. Mass Spectrom. 22: 3089-3099, 2008.

Cheynier V.
Polyphenols in foods are more complex than often thought
Am J Clin Nutr 81 (suppl): 223S-9S, 2005.

Dávalos A., de la Peña G., Sánchez-Martín C.C., Guerra M.T., Bartolomé B., Lasunción M.A.
Effects of red grape juice polyphenols in NADPH oxidase subunit expression in human neutrophils and mononuclear blood cells
British Journal of Nutrition 102: 1125-1135, 2009.

De Freitas V.A.P., Glories Y., Bourgeois G., Vitry C.
Characterisation of oligomeric and polymeric Procyanidins from grape seeds by Liquid Secondary Ion Mass Spectrometry
Phytochemistry, Vol. 38, No. 5, 1435-1441, 1998.

Dell'Agli M., Galli G.V., Vrhovsek U., Mattivi F., Bosisio E.
In Vitro Inhibition of Human cGMP-Specific Phosphodiesterase-5 by Polyphenols from Red Grapes
J. Agric. Food Chemistry 53: 1960-1965, 2005.

Delmas D., Aires V., Limagne E., Dutartre P., Mazué F., Ghiringhelli F., Latruffe N.
Transport, stability, and biological activity of resveratrol
Ann. N.Y. Acad. Sciences 1215: 48-59, 2011.

Dixon R.A., Xie D-Y, Sharma S.B.
Proanthocyanidins – a final frontier in flavonoid research?
New Phytologist, Tansley Review, 2004.

Downey M.O., Rochfort S.
Simultaneous separation by reversed-phase high-performance liquid chromatography and mass spectral identification of anthocyanins and flavonols in Shiraz grape skin
Journal of Chromatography A 1201: 43-47, 2008.

Eng E.T., Ye J., Williams D., Phung S., Moore R.E., Young M.K., Gruntmanis U., Braunstein G., Chen S.
Suppression of Estrogen Biosynthesis by Procyanidin Dimers in Red Wine and Grape Seeds
Cancer Research 63: 8516-8522, 2003.

Flamini R.
Mass Spectrometry in grape and wine chemistry. Part I: Polyphenols
Mass Spectrometry Reviews 22: 218-250, 2003.

Fuleki T., Ricardo da Silva J.M.
Catechin and Procyanidin Composition of Seeds from Grape Cultivars Grown in Ontario
J. Agric. Food Chemistry 45: 1156-1160, 1997.

Fuleki T., Ricardo da Silva J.M.
Effects of Cultivar and Processing Method on the Contents of Catechins and Procyanidins in Grape Juice
J. Agric. Food Chemistry 51: 640-646, 2003.

Gabetta B., Fuzzati N., Griffini A., Lolla E., Pace R., Ruffilli T., Peterlongo F.
Characterization of proanthocyanidins from grape seeds
Fitoterapia 71: 162-175, 2000.

Gagliano N., Aldini G., Colombo G., Rossi R., Colombo R., Gioia M., Milzani A., Dalle-Donne I.
The potential of resveratrol against human gliomas
Anti-Cancer drugs 21: 140-150, 2010.

Geny L., Saucier C., Bracco S., Daviaud F., Glories Y.
Composition and Cellular Localization of Tannins in Grape Seeds during Maturation
J. Agric. Food Chemistry 51: 8051-8054, 2003.

Ghiselli A., Nardini M., Baldi A., Scaccini C.
Antioxidant Activity of Different Phenolic Fractions Separated from an Italian Red Wine
J. Agric. Food Chem, Vol. 46, No. 2: 361-367, 1998.

Gómez-Alonso S., García-Romero E., Hermosín-Gutiérrez I.
HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence
J. of Food Composition and Analysis 20: 618-626, 2007.

González R., Ballester I., López-Posadas R., Suárez M.D., Zarzuelo A., Martínez-Augustin O., Sánchez de Medina F.
Effects of Flavonoids and other Polyphenols on Inflammation
Critical Reviews in Food Science and Nutrition 51: 331-362, 2011.

Grønbæk M., Becker U., Johansen D., Gottschau A., Schnohr P., Hein H.O., Jensen G., Sørensen T.I.A.
Type of Alcohol Consumed and Mortality from All Causes, Coronary Heart Disease, and Cancer

Ann Intern Med. 133: 411-419, 2000.

Hammerbacher A., Ralph S.G., Bohlmann J., Fenning T.M., Gershenzon J., Schmidt A.
Biosynthesis of the Major Tetrahydroxystilbenes in Spruce, Astringin and Isorhapontin, Proceeds via Resveratrol and Is Enhanced by Fungal Infection
Plant Physiology 157: 876-890, 2011.

He F., Pan Q-H, Shi Y., Duan C-Q
Biosynthesis and Genetic Regulation of Proanthocyanidins in Plants
Molecules 13: 2674-2703, 2008.

Herrmann K.M.
The Shikimate Pathway: Early Steps in the Biosynthesis of Aromatic Compounds
The Plant Cell 7: 907-919, 1995.

Hogan S., Canning C., Sun S., Sun X., Zhou K.
Effects of Grape Pomace Antioxidant Extract on Oxidative Stress and Inflammation in Diet Induced Obese Mice
J. Agric. Food Chemistry 58: 11250-11256, 2010.

Hollecker L., Pinna M., Filippino G., Scrugli S., Pinna B.
Simultaneous determination of polyphenolic compounds in red and white grape grown in Sardinia by high performance liquid chromatography-electron spray ionisation-mass spectrometry
Journal of Chromatography A, 1216: 3402-3408, 2009.

Kaneko M., Kanesaka M., Yoneyama M., Tominaga T., Jirillo E., Kumazawa Y.
Inhibitory effects of fermented grape marc from *Vitis vinifera* Negroamaro on antigen-induced degranulation
Immunopharmacology and Immunotoxicology 32 (3): 454-461, 2010.

Karvela E., Makrisb D.P., Kalogeropoulosa N., Karathanosa V.T.
Deployment of response surface methodology to optimise recovery of grape (*Vitis vinifera*) stem polyphenols
Talanta 79: 1311-1321, 2009.

Kong J-M, Chia L-S, Goh N-K, Chia T-F, Brouillard R.
Analysis and biological activities of anthocyanins
Phytochemistry 64: 923–933, 2003.

Kurek A., Grudniak A.M., Kraczkiewicz-Dowjat A., Wolska K.I.
New Antibacterial Therapeutics and Strategies
Polish Journal of Microbiology Vol. 60, Nr 1: 3-12, 2011.

Leifert W.R., Abeywardena M.Y.
Cardioprotective actions of grape polyphenols

Nutrition Research 28: 729-737, 2008(a).

Leifert W.R., Abeywardena M.Y.
Grape seed and red wine polyphenol extracts inhibit cellular cholesterol uptake, cell proliferation, and 5-lipoxygenase activity
Nutrition Research 28: 842-850, 2008(b).

Luque-Rodríguez J.M., Luque de Castro M.D., Pérez-Juan P.
Dynamic superheated liquid extraction of anthocyanins and other phenolics from red grape skins of winemaking residues
Bioresource Technology 98: 2705-2713, 2007.

Maffei Facino R., Carini M., Aldini G., Bombardelli E., Morazzoni P., Morelli R.
Free Radicals Scavenging Action and Anti-enzyme Activities of Procyanidines from *Vitis vinifera*
Arzneim.-Forsch./Drug Res. 44 (I),5: 592-601, 1994.

Maffei Facino R., Carini M., Aldini G., Berti F., Rossoni G., Bombardelli E., Morazzoni P.
Procyanidins from *Vitis vinifera* Seeds Protect Rabbit Heart from Ischemia/Reperfusion Injury: Antioxidant Intervention and/or Iron and Copper Sequestering Ability
Planta Medica 64: 495-502, 1996.

Maffei Facino R., Carini M., Aldini G., Calloni M.T., Bombardelli E., Morazzoni P.
Sparing Effect of Procyanidins from *Vitis vinifera* on Vitamin E: *In vitro* Studies
Planta Medica 62: 343-347, 1998.

Maffei Facino R., Carini M., Aldini G., Berti F., Rossoni G., Bombardelli E., Morazzoni P.
Diet enriched with Procyanidins enhances antioxidant activity and reduces myocardial post-ischaemic damage in rats
Life Sciences Vol. 64, Nr 8: 627-642, 1999.

Mané C., Souquet J. M., Ollé D., Verriés C., Véran F., Mazerolles G., Cheynier V., Fulcrand H.
Optimization of Simultaneous Flavanol, Phenolic Acid, and Anthocyanin Extraction from Grapes Using an Experimental Design: Application to the Characterization of Champagne Grape Varieties
J. Agric. Food Chemistry 55: 7224-7233, 2007.

Marles M.H.S., Ray H., Gruber M.Y.
New perspectives on proanthocyanidin biochemistry and molecular regulation
Phytochemistry 64: 367-383, 2003.

Merken H.M., Beecher G.R.
Measurement of Food Flavonoids by High-Performance Liquid Chromatography: A Review
J. Agric. Food Chemistry Vol.48 No.3, 2000.

Morel-Salmi C., Souquet J.M., Bes M., Cheynier V.
Effect of Flash Release Treatment on Phenolic Extraction and Wine Composition

J. Agric. Food Chemistry 54: 4270-4276, 2006.

Muñoz S., Mestres M., Busto O., Guasch J.
Determination of some flavan-3-ols and anthocyanins in red grape seed and skin extracts by HPLC-DAD: Validation study and response comparison of different standards
Analytica Chimica Acta 628: 104–110, 2008.

Mullen W., Marks S.C., Crozier A.
Evaluation of Phenolic Compounds in Commercial Fruit Juices and Fruit Drinks
J. Agric. Food Chemistry 55: 3148-3157, 2007.

Nassiri-Asl M., Hosseinzadeh H.
Review of the Pharmacological Effects of *Vitis vinifera* (Grape) and its Bioactive Compounds
Phytotherapy Research 23: 1197-1204, 2009.

Nawaz H., Shi J., Mittal G.S., Kakuda Y.
Extraction of polyphenols from grape seeds and concentration by ultrafiltration
Separation and Purification Technology 48: 176-181, 2006.

Nichols J.A., Katiyar S.K.
Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms
Arch. Dermatol. Res. 302: 71-83, 2010.

Nicoletti I., Bello C., De Rossi A., Corradini D.
Identification and Quantification of Phenolic Compounds in Grapes by HPLC-PDA-ESI-MS on a Semimicro Separation Scale
J. Agric. Food Chemistry 56: 8801-8808, 2008.

Nuttall S.L., Kendall M.J., Bombardelli E., Morazzoni P.
An evaluation of the antioxidant activity of a standardized grape seed extract, Leucoselect®
Journal of Clinical Pharmacy and Therapeutics 23, 385-389, 1998.

Oak M-H, El Bedoui J., Schini-Kerth V.B.
Antiangiogenic properties of natural polyphenols from red wine and green tea
J. of Nutritional Biochemistry 16: 1-8, 2005.

Official Journal of the European Union
English edition, L 141, Vol. 52, 6 June 2009.

Parris M.K.
Bioavailability and Activity of Phytosome Complexes from Botanical Polyphenols: The Silymarin, Curcumin, Green Tea, and Grape Seed Extracts
Alternative Medicine Review Vol. 14, Nr 3, 2009.

Pereira G.E., Gaudillere J.P., Pieri P., Hilbert G., Maucourt M., Deborde C., Moing A., Rolin D.

Microclimate Influence on Mineral and Metabolic Profiles of Grape Berries
J. Agric. Food Chemistry 54: 6765-6775, 2006.

Pinelo M., Arnous A., Meyer A.S.
Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release
Trends in Food Science & Technology 17: 579-590, 2006.

Priego Capote F., Rodríguez J.M.L., Luque de Castro M.D.
Determination of phenolic compounds in grape skin by capillary electrophoresis with simultaneous dual fluorescence and diode array absorption detection after dynamic superheated liquid leaching
Journal of Chromatography A 1139: 301-307, 2007.

Rogerio A.P., Kanashiro A., Fontanari C., da Silva E.V.G., Lucisano-Valim Y.M., Soares E.G., L.H. Faccioli
Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma
Inflamm. research 56: 402-408, 2007.

Rubilar M., Pinelo M., Shene C., Sineiro J., Nuñez M.J.
Separation and HPLC-MS identification of phenolic antioxidants from agricultural residues: almond hulls and grape pomace
J. Agric. Food Chemistry 55: 10101-10109, 2007.

Santamaria A.R., Mulinacci N., Valletta A., Innocenti M., Pasqua G.
Effects of Elicitors on the Production of Resveratrol and Viniferins in Cell Cultures of *Vitis vinifera* L. cv Italia
J. Agric. Food Chemistry 59: 9094-9101, 2011.

Shahidi F.
Antioxidants in food and food antioxidants
Nahrung Vol. 44, Nr. 3: 158-163, 2000.

Schou S.A.
Über die Lichtabsorption einiger Anthocyanidine
Helvetica Chimica Acta 10 (1): 907-915, 1927.

Soleas G.J., Diamandis E.P., Karumanchiri A., Goldberg D.M.
A Multiresidue Derivatization Gas Chromatographic Assay for Fifteen Phenolic Constituents with Mass Selective Detection
Anal. Chem. 69: 4405-4409, 1997.

Stoclet J-C, Chataigneau T., Ndiaye M., Oak M-H, El Bedoui J., Chataigneau M., Schini-Kerth V.B.
Vascular Protection by dietary polyphenols
Eur. J. of Pharmacology 500: 299-313, 2004.

Sun B., Neves A.C., Fernandes T.A., Fernandes A.L., Mateus N., De Freitas V., Leandro C., Spranger M.
Evolution of Phenolic Composition of Red Wine during Vinification and Storage and Its Contribution to Wine Sensory Properties and Antioxidant Activity
J. Agric. Food Chemistry 59: 6550-6557, 2011.

Suriano S., Tarricone L., Savino M., Rossi M.R.
Caratterizzazione fenolica di Uve di Aglianico e Uva di Troia coltivate nel nord barese
L'enologo, December 2005.

Tan Y., Lim L.H.K.
***trans*-Resveratrol, an extract of red wine, inhibits human eosinophil activation and degranulation**
British Journal of Pharmacology 155: 995-1004, 2008.

Tarricone L., Suriano S.
Performance viticole ed enologiche di cloni e biotipi di Nero di Troia coltivati nell'area DOC Castel del Monte (Puglia, Italia Meridionale)

Thimothe J., Bonsi I.A., Padilla-Zakour O.I., Koo H.
Chemical characterization of red wine grape (*Vitis vinifera* and *Vitis* interspecific hybrids) and pomace phenolic extracts and their biological activity against *Streptococcus mutans*
J. Agric. Food Chemistry 55: 10200-10207, 2007.

Valls J., Millán S., Martí M.P., Borràs E, Arola L.
Advanced separation methos of food anthocyanins, isoflavones and flavanol
J., of Chromatography A 1216: 7143-7172, 2009.

Veprik A., Khanin M., Hermoni K.L., Danilenko M., Levy J., Sharoni Y.
Polyphenols, isothiocyanates and carotenoid derivatives enhance estrogenic activity in bone cells but inhibit it in breast cancer cells
Am. J. Physiol. Endocrinol. Metab. IN PRESS August 30, 2011.

Vigna G.B., Costantini F., Aldini G., Carini M., Catapano A., Schena F., Tangerini A., Zanca R., Bombardelli E., Morazzoni P., Mezzetti A., Fellin R., Maffei Facino R.
Effect of a Standardized Grape Seed Extract on Low-Density Lipoprotein Susceptibility to Oxidation in Heavy Smokers
Metabolism, Vol. 52, Nr 10: 1250-1257, 2003.

Wang H., Liu L., Guo Y-X, Dong Y-S, Zhang D-J, Xiu Z-L
Biotransformation of piceid in Polygonum cuspidatum to resveratrol by Aspergillus oryzae
Appl. Microbiol. Biotechnol. 75: 763-768, 2007.

Waterhouse A.L.
Wine Phenolics
Ann. N.Y. Acad. Sci. 957: 21-36, 2002.

www.indena.it/pdf/leucoselect.pdf

Xia E-Q, Deng G-F, Guo Y-J, Li H-B
Biological Activities of Polyphenols from Grapes
Int. J. of Molecular Sciences 11: 622-646, 2010.

Xie De-Yu, Dixon R.A.
Proanthocyanidin biosynthesis – still more questions than answers?
Phytochemistry 66: 2127-2144, 2005.

Ying Yang and Mingjien Chien
Characterization of Grape Procyanidins Using High-Performance Liquid Chromatography/Mass Spectrometry and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
J. Agric. Food Chemistry 48: 3990-3996, 2000.

Yong Ju Z., Howard L.R.
Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red Grape Skin
J. Agric. Food Chemistry 51: 5207-5213, 2003.

Zhao L., Wu J., Yang J., Wei J., Gao W., Guo C.
Dietary quercetin supplementation increases serum antioxidant capacity and alters hepatic gene expression profile in rats
Experimental Biology and Medicine 236: 701-706, 2011.