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Bioenergy and industrial applications of grape pomace from “Vinho Verde”

Bioenergia e aplicações industriais do bagaço de uva do "Vinho Verde"

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"The secret of change is to focus all of your energy not on fighting the old, but on building the new"

Socrates

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Abstract

Portugal is the fifth largest wine producer in the EU-28 and eleventh in the world, with a wine production estimated at 5,886 khL in 2014. In this year, the Portuguese Minho region had a production over 530 khl of "Vinho Verde" wine. Grape pomace (GP) is an agro-industrial residue from this process, consists of stalks, skins and seeds, and accounts for about 20-25% weight of the grape crushed for the wine production. In Minho region almost $18x10^3$ tons of GP are generated, which are mainly employed for animal feed, fertilizer or depositing in the open fields and can cause environmental problems. The uncontrolled dumping of these by-products causes phytotoxicity phenomena and contributes to the green house gas emissions. The objective of this study was to evaluate the potential application of grape pomace, from Vinhão and Loureiro grape varieties, as bioenergy or as raw material in other industrial processes.

Analyses were made in one white and one red GP variety, named Loureiro and Vinhão, respectively, from Adega Cooperativa de Ponte da Barca. The chemical properties studied were moisture, ash, Kjeldahl N, fat, carbohydrates, fiber, minerals, total phenolic content (TPC), antioxidant activity, and identification/quantification of nine polyphenols applying standard methods of analysis. The high heating value (HHV) was determined by the method from Parr instruments. The moisture in GP varies from 42.2% to 72.8%. Results in dry matter show that the contents are 3.3% to 7.9% in ash, 1.0% to 2.1% in Kjeldahl N, 2.0% to 16.4% in fat, 15.4% to 29.2% in carbohydrates, 11.9% to 31.0% in fiber, 23.9 to 120 mg GAE/g for TPC and 12.2 to 83 mg AAE/g for antioxidant activity. Comparing the two varieties of GP for the polyphenols, Vinhão has a higher content than Loureiro in eight identified polyphenols, except for resveratrol, which is higher in the Loureiro stalks (0.244 mg/g DM) and is close to the highest value in literature. The HHV of GP before and after extracting the bioactive products is between 17-21 MJ/Kg, which compares to the best hardwood biomass.

Considering the results obtained, GP is a very valuable raw material with potential to be used in industrial processes in a profitable way. The process should start with the bioactive extractions, followed by the production of grape seed oil and grape flour for human nutrition and cosmetology. Finally, the remaining stalks could be used as fertilizer, animal feed or bioenergy, producing pellets.

Resumo

Portugal é o quinto maior produtor de vinho da UE-28 e o décimo primeiro no mundo, com uma produção estimada de 5,886 khL em 2014. Neste ano, na região Portuguesa do Minho a produção de "Vinho Verde" foi de 530 khL. O bagaço de uva (GP) é um resíduo agro-industrial composto de engaço, películas e grainhas, com peso de 20-25% do total das uvas utilizadas para a produção do vinho. No Minho são geradas por ano quase $18x10^3$ ton de GP, resíduo que é utilizado para a alimentação animal, fertilizante ou depositado em campo aberto para biodegradação, causando problemas ambientais. A deposição não controlada do bagaço provoca fenómenos de fitotoxicidade e contribui para as emissões de GEE. O objetivo deste estudo foi avaliar o potencial de aplicação do bagaço de uva como bioenergia e matéria prima na indústria transformadora para obter produtos com valor acrescentado.

Este estudo foi realizado no GP das variedades Loureiro e Vinhão, provenientes da Adega Cooperativa de Ponte da Barca. A composição química foi determinada através da análise do conteúdo de humidade, cinza, N-Kjeldhal, gordura, hidratos de carbono (HC), fibra, minerais, conteúdo fenólico total (TPC), atividade antioxidante, e identificação/quantificação de nove polifenóis, aplicando métodos analíticos de referência. O poder calorífico (HHV) foi determinado pelo método da Parr Instruments. A humidade no bagaço de uva varia de 42.2% a 72.8%. Na matéria seca foram obtidos os seguintes resultados, 3.3% a 7.9% em cinza, de 1.0% a 2.1% em N-Kjeldahl, de 2.0% a 16.4% em gordura, de 15.4% a 29.2% em HC, de 11.9% a 31.0% em fibra, 23.9 a 120 mg GAE/g em TPC e 12.2 a 83 mg AAE/g em DPPH. Comparando as duas variedades de GP, o Vinhão apresenta um teor mais elevado que o Loureiro em oito polifenóis, sendo exceção o resveratrol com 0.244 mg/g MS no Loureiro, valor próximo do mais elevado encontrado na literatura. O HHV do GP antes e após extração dos bioativos varia entre 17 e 21 MJ/kg, valor comparável com o mais elevado de biomassa florestal.

Tendo em conta os resultados obtidos, conclui-se que o GP é um sub-produto muito valioso, podendo ser obtidos outros produtos num processo muito rentável. Na transformação do GP, primeiro devem ser isolados os bioactivos, posteriormente óleo de semente de uva e farinha sem glúten de uva para a nutrição humana e cosmetologia. Finalmente, o engaço pode ser usados como fertilizante, alimento para animais ou bioenergia (pelletes).

Σύνοψη

Η Πορτογαλία είναι η πέμπτη μεγαλύτερη χώρα παραγωγής κρασιού στην ΕΕ-28 και ενδέκατη στον κόσμο, με παραγωγή που εκτιμάται σε 5,886 khL το 2014. Την ίδια χρονιά στο νομό Minho η παραγωγή κρασιού «Vinho Verde» ήταν πάνω από 530 khL. Τα στέμφυλα, είναι αγροβιομηχανικά υπολείμματα που αποτελούνται από μίσχους, φλοιούς και σπόρους και αντιπροσωπεύουν περίπου το 20-25% του βάρους του σταφυλιού έπειτα από τη συμπίεση του κατά την οινοποίηση. Στον νομό Minho παράγονται ετησίως σχεδόν $18x10^3$ τόνοι στέμφυλων, τα οποία χρησιμοποιούνται κυρίως για ζωοτροφές, λιπάσματα ή εναποτίθενται σε ανοιχτούς χώρους για βιοαποικοδόμηση, η οποία μπορεί να προκαλέσει σημαντικά περιβαλλοντικά προβλήματα. Η ανεξέλεγκτη απόρριψη αυτών των υποπροϊόντων προκαλεί φαινόμενα φυτοτοξικότητας και συμβάλλει στις εκπομπές αερίων του θερμοκηπίου. Ο στόχος της παρούσας μελέτης ήταν να αξιολογήσει τις δυνητικές εφαρμογές των στέμφυλων, σε βιοενέργεια και ως πρώτη ύλη για βιομηχανικές διεργασίες.

Τα στέμφυλα που χρησιμοποιήθηκαν για τις αναλύσεις ήταν μια λευκή και μια ερυθρή ποικιλία ονόματι Loureiro και Vinhão, αντίστοιχα, που προήρθαν από τον οινοποιητικό συνεταιρισμό της Ponte da Barca. Ο χημικός χαρακτηρισμός έγινε πραγματοποιώντας αναλύσεις για υγρασία, τέφρα, Kjeldahl άζωτο, λίπος, υδατάνθρακες, ιχνοστοιχεία, ολικό φαινολικό περιεχόμενο (TPC), αντιοξειδωτική δράση, καθώς και ταυτοποίηση και ποσοτικοποίηση ορισμένων πολυφαινολών, εφαρμόζοντας πρότυπες μεθόδους ανάλυσης. Η υψηλή θερμογόνο δύναμη (HHV) προσδιορίστηκε με την μέθοδο που συνιστάτε από την εταιρία Parr. Η υγρασία των στέμφυλων κυμάνθηκε από 42.2% έως 72.8%. Τα αποτελέσματα επί ξηρού έδειξαν ότι το περιεχόμενο σε τέφρα κυμαίνεται μεταξύ 3.3% έως 7.9%, σε άζωτο 1.0% έως 2.1%, σε λίπος 2.0% έως 16.4%, σε υδατάνθρακες 15.4% έως 29.2%, σε φυτικές ίνες 11.9% σε 31.0%, σε ολικό φαινολικό περιεχόμενο (TPC) 23.9 έως 120 mg GAE / g και σε αντιοξειδωτική δράση 12.2 έως 83 mg AAE/ g. Συγκρίνοντας τις δύο ποικιλίες των στέμφυλων για το περιεχόμενο τους σε πολυφαινόλες, συμπεραίνεται ότι η ποικιλία Vinhão είχε υψηλότερη περιεκτικότητα στις οκτώ από τις εννέα ταυτοποιημένες πολυφαινόλες σε σχέση με την ποικιλία Loureiro. Η ρεσβερατρόλη, παρουσίασε υψηλότερη τιμή στους μίσχους της Loureiro, 0.244 mg / g επί ξηρού, που συνάδει με τις υψηλότερες τιμές στην βιβλιογραφία. Η υψηλή θερμιδογόνο δύναμη των στέμφυλων πριν και μετά την εκχύλιση των βιοενεργών προϊόντων (πολυφαινόλες) κυμάνθηκε μεταξύ 17 και 21 MJ/Kg, τιμές οι οποίες συγκρίνονται με βιομάζα προερχόμενη από το καλύτερο σκληρό ξύλο.

Λαμβάνοντας υπόψη τα αποτελέσματα, τα στέμφυλα είναι μια πολύτιμη πρώτη ύλη με δυναμική εφαρμογής σε διάφορες βιομηχανικές διεργασίες. Για να είναι επικερδής οι διεργασίες θα πρέπει να γίνει πρώτα η απομόνωση των βιοενεργών προϊόντων (για φαρμακευτικές, κ.α. χρήσεις) και εν συνεχεία η χρησιμοποίηση των υπολειμμάτων για την ανθρώπινη διατροφή και την κοσμετολογία (λάδι από σπόρους σταφυλιών και αλεύρι από σταφύλια). Τέλος, τα υπολείμματα των μίσχων θα μπορούσαν να χρησιμοποιηθούν ως λιπάσματα, ζωοτροφές ή πέλλετ.

Index

List of Tables:

List of Figures:

List of abbreviation

Abbeviation	**Full form**
(-)E	(-) Epicatechin
(+) C	(+) Catechin
AAE	Ascorbic acid equivalent
AAS	Atomic absorption spectophotometer
ACPB	Adega de cooperativa Ponte da Barca
BHA	Butylated-hydroxianisole
BHT	Butylated- hydroxytoluene
BLE	Branco loureiro engaço
BLG	Branco loureiro grainha
BLM	Branco loureiro mixture
BLP	Branco loureiro película
CA	Caffeic acid
CH	Carbohydrates
CHD	Coronary heart disease
COD	Chemical oxygen demand
DCP	Declaração de Colheita e Produção
DL	Detection limit
DM	Dry matter
DNS	3,5- Dinitrosalicylic acid
DPPH	2,2 Diphenyl-1-picrlhydrazyl
EBLE	Extracted branco loureiro engaço
EBLM	Extracted branco loureiro mixture
ETVE	Extracted tinto vinhão engaço
ETVM	Extracted tinto vinhão mixture
FA	Ferrulic acid
FC	Folin- ciocalteu
GA	Gallic acid

GAE	Gallic acid equivalent
GP	Grape pomace
HPLC	High pressure liquid chromatography
HHV	High heating value
ID	identification
LDL	Low-density lipoproteins
ME	Metabolised energy
MUFA	Monounsaturated fatty acid
p-CA	p-Coumaric acid
PEG	Polyethylene glycol
PUFA	Polyunsaturated fatty acid
Q	Quercetin
QL	Quantification limit
RGP	Red grape pomace
RP-HPLC	Reverse phase high liquid chromatography
RT	Retention time
SA	Syringic acid
SD	Standard deviation
TPC	Total phenolic content
t-R	trans-Resveratrol
TVE	Tinto vinhão engaço
TVG	Tinto vinhão grainha
TVM	Tinto vinhão mixture
TVP	Tinto vinhão película
WGP	White grape pomace

1 Introduction

The world global population growth causes competition for natural resources and increased pressure on agricultural production for food, energy, and various high value raw materials. Competition for natural resources will continue to increase globally with projected world populations set to reach 9.6 billion by 2050 (Figure 1.1). The increased needs will lead to limited and deficient land in mineral nutrients, shortage in fuels, and so forth. To sustain the human population growth it will requires an increased food production and biomass energy products within the bounds of these limited resources while reducing associated environmental impacts. Also, the future end of petroleum, will lead to lack of raw materials for industry. A broader use of renewable resources and the substitution of fossil based materials and products by renewable raw materials, like agriculture by-products will be an important and necessary step. This solution could reduce the problem, since the renewable material is a source to feed other industry raw material needs. The European Commission in its Lead Market Initiative (EC, 2007) identified bio-based products, made from renewable raw materials such as plants and trees, as one of the promising future markets. This is a closed cycle of transformation of matter. Nothing is lost, nothing is created, everything is transformed, as it was said by Lavoisier.

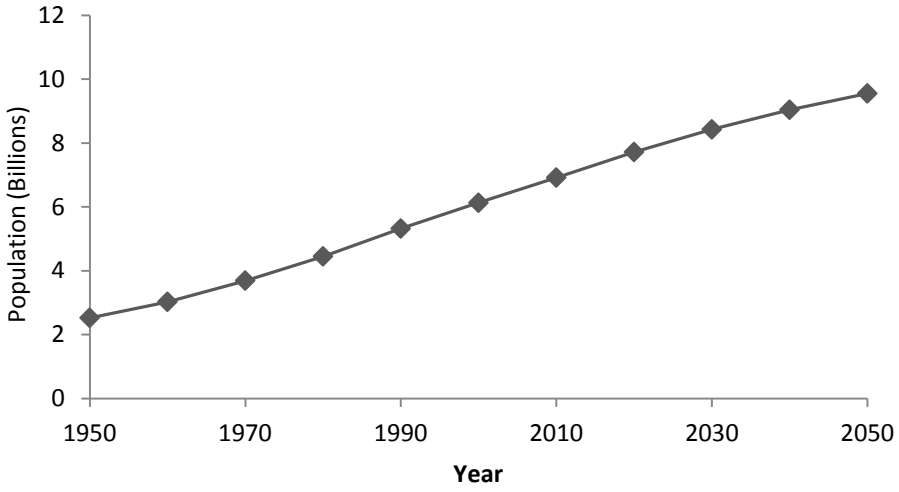

Figure 1.1 World population growth 1950-2050

Source: (ESA, 2015)

1.1 Why this project

Grape is the world's largest fruit crop with an annual production of more than 67 million tons. Grapes and products obtained from them, such as wine, grape juice, jams, and raisins, have then an obvious economic importance. Eighty percent of the worldwide grape production is used in winemaking (Fontana Ariel R., 2013).

The wine industry produces millions of tons of by-products that are named grape pomace, GP, which mainly consists of stalks, skins, and seeds and represents a waste management issue both ecologically and economically. Economically because the most wineries are small enterprises, scattered in the territory, which do not have the tools and the ability to properly manage and dispose them. So, from the ecological point of view, a common practice for GP is to lay down in the fields, in order to be composted by aerobic decomposition, because of the lack of a proper management. During this process (biodegradation), GP remains exposed and is a source of contamination, mainly because of the volume and the many natural products that they contain. The organic load has significant antimicrobial and phytotoxic activity, which significantly limits the action of microorganisms involved in the process of biodegradation. As a result, the process of biodegradation is slowed down. Thus, the uncontrolled dumping of GP causes phytotoxicity phenomena including plant growth interference, contamination of the aquifer, degradation of the quality of drinking water, kills sensitive marine organisms etc (Haroutounian, 2007-2008). Another ecological problem is the high levels of phenolic compounds that inhibit seed germination. Regarding GP use in livestock feed, some animals show intolerance to certain components, such as condensed tannins and polymeric polyphenols (lignin), which negatively affect digestibility because they inhibit cellulolytic and proteolytic enzymes as well as the growth of rumen bacteria (Fontana Ariel R., 2013).

Actually, the main industrial recovery of GP is for tartaric acid extraction or ethanol production, and the final solid residue is sometimes cast off as fertilizer. Also, GP has been utilized in animal feeding. Additionally, the high content of dietary fiber, especially glycans and pectins, emphasizes the possible nutritive value of GP with a wide range of applications as food ingredients (Fontana Ariel R., 2013). With the increase of

consumers' awareness for the use of additives in foods and the attention that functional foods have acquired in recent years, there is a need for the identification of alternative natural and safer sources of food additives, namely antioxidants, colorants and preserving additives, as well as natural functional foods. Adding to this, modern industries are focused on diminishing the environmental impact of industrial byproducts. Therefore, most attention has been paid to the recovery of bioactive phenolic compounds from grape byproducts from the winemaking industry.

The production of wine in Portugal is known since 2000 B.C and from last century it has been developed as an industrial activity with the construction of modern units (IVV, 2015). Because these units produce GP as by-products in large quantities, this project appeared in this context to study the possibilities for recovering grape pomace.

1.2 Historical and cultural context of wine production in Portugal

The vineyard and wine are a cultural and economic asset for Portuguese, as for most Mediterranean civilizations, one of the fundamental features of cultural identity of the people and the nation as well, which is worth preserving and is value to pass on to future generations. In the mid-sixteenth century, Lisbon was the largest center of consumption and distribution of the wine empire. In fact, the wines travelled by ships and caravels to Brazil and Asia with the discoveries and the expansion of Portuguese maritime in the centuries XV and XVI. After that long trips the wines characteristics changed, improving them substantially. In centuries XVII and XVIII, the intensification of international trade and the establishment of important trade agreements combined with the prestige of Portuguese wines, created a huge increase in consumption and exportation of wine and the development of several wine regions. Nevertheless, it was in 1907/1908 that began the process of official regulation for various designations of origin. It was defined not only as "fortified wines of private regional type" recognized by tradition with the usual names of "Porto", "Madeira", "Carcavelos" and "Moscatel de Setúbal" but as the so-called "regional type of pasture wines", identified as "Colares", "Bucelas", "Dão", "Bairrada", "Borba", "Torres", "Cartaxo", "Alcobaça", "Douro" (virgens), "Minho" (Vinhos

Verdes), "Amarante", "Basto", "Monção" and "Fuzeta". Today, some of these names are recognized worldwide while others are sub-regions or are already extinct (IVV, 2015).

The strengthening of a quality policy for Portuguese wines allowed the recognition and protection of 29 designations of origin and 11 of geographical indications to be incorporated in a European Community register, following the harmonization of those designations: wines with a protected designation of origin and wines with a geographical indication are protected (EC:187, 2009).

In general, there are many people who consider the diversity as the main feature of the Portuguese wine industry, and this diversity is mainly due to three factors: climate, soil and wine varieties. On the other hand, the great technological advances that have been experiencing, lead to good balance between the "culture of the vine" and the "art of making wine," of ancient tradition, which has allowed the production of so many different good quality wines. (IVV, 2015)

1.3 "Vinho Verde"

This study was developed with samples from Adega Cooperativa de Ponte da Barca in Minho region, where mostly of the wine produced are with the appellation of origin "Vinho Verde".

1.3.1 Region

The demarcated region of Vinho Verde is located in the northwest of Portugal, Figure 1.2, in an area traditionally known as Entre-Douro-e-Minho. Its limits are the Minho River in the North (border with Galicia, Spain), the mountainous areas in the East and South, forming the natural border between the Atlantic entre-Douro-e-Minho and the Atlantic Ocean as its western limit.

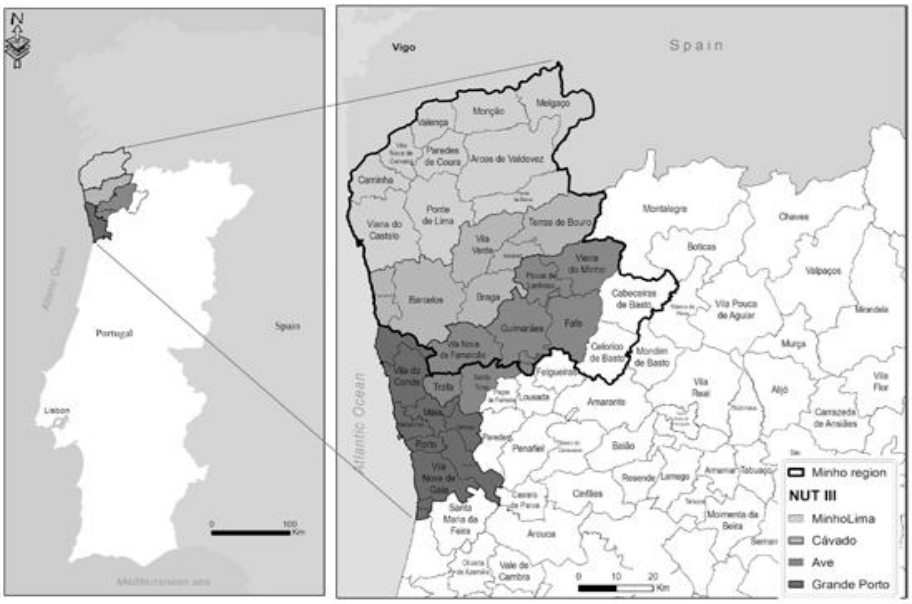

Figure 1.2 Map representing the demarcated region of Vinho Verde

Source: (IGP, 2010)

The vineyards of Vinho Verde in Minho region, distinguished by their great vegetative expansion in severe conditions, occupy an area of almost 27,432 thousand hectares (2014), corresponding to 12.5 % of the national viticulture area that is 218,677 hectares (IVV, 2015). Orografically the region presents itself as "a wide amphitheater rising gradually from the maritime border to the inland " (Amorim Girão), exposed to the Atlantic Ocean influence, being this even more reinforced by the main river valleys orientation, that facilitates the maritime winds' penetration. (CVRVV, 2015)

1.3.2 Climate

The region's climate is greatly influenced by the orographic characteristics and the already mentioned fluvial network. The most significant feature is the annually rain levels - an average of 1,500 mm - and its irregular distribution along the year, concentrated in winter and spring. On the other hand, the air temperature along the year develops similarly with the precipitation. The higher temperatures occur at the same time as the lowest precipitation - hot and dry end of spring and summer - and the lower temperatures with the highest precipitation - cold and rainy winters. Concerning the annual average temperature, the average of high temperatures and the average of low temperatures are not too high, reflecting a mild climate. (CVRVV, 2015)

1.3.3 Geology and soils

Regarding the terrain, the region has a rather irregular topography, characterized by a compact valley's system combined with the fluvial network, developing from the seaside to the inland. Most of the region lays on a granitic structure, with the exception of two narrow strips that cross it from NW-SE, one of silurian with carboniferous and gravestone structures and another one of archaic schist. The soil's main origin is the granite disaggregation. Generally, it is characterized by its low depth, predominantly sandy to franco-sandy (superficial) textures, a natural high acidity and poor in phosphorus. The fertility levels are naturally low, as one may easily conclude from the characteristics already mentioned. However and due to the nature of the agrarian systems used in the region since the old times, the soils gained a considerable fertility that allowed supporting one of the highest population densities in the country. The secret for this fertility may be attributed to the two main types of human intervention in the natural conditions: the control of the terrain, through the construction of terraces and the intensive and persistent penetration of organic substances. (CVRVV, 2015)

1.4 Portugal's place in world wine production

European Union (EU-28) is the world's leader in wine production, with almost half of the global vine-growing area and approximately 60 percent of wine production by volume. Data presented in Table 1.1 and Table 1.2 shows that France, Italy and Spain are the largest EU wine producing countries, representing 80 percent of total output, followed by Germany, Portugal, Romania, Greece, and Austria. Wine is an important sector also in Hungary, Bulgaria, Croatia, and Slovenia.

Table 1.1 World wine production (khL) excluding juice and fruit [(a)]

	2010	2011	2012	2013	2014	Variation	Variation	Rank
France	44,381	50,757	41,548	42,004	46,151	4,147	10%	1
Italy	48,525	42,772	45,616	52,429	44,424	-8,005	-15%	2
Spain	35,353	33,397	31,123	45,650	37,000	-8,650	-19%	3
United States [(b)]	20,890	19,140	21,740	23,500	22,500	-1,000	-4%	4
Argentina	16,250	15,473	11,780	14,984	15,200	216	1%	5
Australia	11,420	11,180	12,260	12,310	12,560	250	2%	6
China [(c)]	13,000	13,200	13,810	11,780	11,780	0	0%	7
South Africa	9,327	9,725	10,568	10,980	11,420	440	4%	8
Chile	8,844	10,464	12,554	12,846	10,029	-2,817	-22%	9
Germany	6,906	9,132	9,012	8,409	9,725	1,316	16%	10
Portugal	**7,148**	**5,622**	**6,327**	**6,238**	**5,886**	**-352**	**-6%**	**11**
Roumania	3,287	4,058	3,311	5,113	4,093	-1,020	-20%	12
New Zeeland	1,900	2,350	1,940	2,480	3,200	720	29%	13
Greece	2,950	2,750	3,115	3,343	2,900	-443	-13%	14
Brazil	2,459	3,460	2,967	2,710	2,810	100	4%	15
Hungary	1,762	2,750	1,776	2,666	2,734	68	3%	16
Austria	1,737	2,814	2,125	2,392	2,250	-142	-6%	17
Bulgaria	1,224	1,237	1,442	1,755	1,229	-526	-30%	18
Switzerland	1,030	1,120	1,000	840	900	60	7%	19
Croatia	1,433	1,409	1,293	1,249	874	-375	-30%	20
OIV World Total [(d)]	264,372	267,243	256,222	287,600	270,864	-16,736	-6%	

a) Countries for which information has been provided with a wine production of more than 1 khL
b) OIV estimate (USDA basis)
c) Report for the year 2013, 2014 figures not yet available
d) Range used for 2014 world production: 266,200 hL to 275,500 hL

Source: (OIV, 2015)

Table 1.2 Wine production [(a)] trend in the EU-28 (khL)

	2011/12	2012/13	2013/14	Rank
Italy	43,072	40,057	44,900	1
Spain	33,397	31,123	44,600	2
France	50,890	40,609	44,100	3
Germany	9,258	9,000	8,500	4
Portugal	**5,609**	**6,140**	**6,740**	**5**
Romania	4,700	4,100	5,400	6
Greece	2,750	3,150	3,700	7
Hungary	2,822	2,243	2,450	8
Austria	2,814	2,155	2,252	9
Other EU-28 countries	3,214	2,558	4,911	10
EU-28	158,527	141,135	167,553	11

a) Volume of product removed from fermenters after the first natural fermentation of the must of fresh grapes (juices and other musts were excluded)

Source: (GAIN-FAS, 2014)

Portugal is the fifth largest wine producer in the EU-28 (Table 1.2) and eleventh in the world (Table 1.1), with a production estimated at 5,886 khL in 2014, six percent lower

than the previous campaign, 6,238 khL, because of adverse weather conditions during flowering, especially in the regions of Minho (-14 percent) and Douro (-8 percent).

1.4.1 Vinho Verde production

The wine sector of "Vinho Verde" in Portugal has a total production of 530 khL, (Table1.3 and Figure 1.3), of wine in 2014/1015 that means 9 % of the total wine production in Portugal, since the annual wine production of Portugal in 2014/2015 was 5,886 khL of wine (Table 1.1). This production in the different sub-region (Figure 1.4) is divided in 23 % red and 77 % white Vinho Verde wine (Figure 1.5).

Table1.3 Campaigns of "Vinho Verde" wine (in L) (CVRVV, 2015)

Campaign	White	Red	Total
2003/2004	54,115,085	25,927,194	**80,042,279**
2004/2005	61,684,640	29,303,980	**90,988,620**
2005/2006	58,653,274	27,813,744	**86,467,018**
2006/2007	53,631,404	30,199,897	**83,831,301**
2007/2008	45,409,386	18,261,915	**63,671,301**
2008/2009	45,996,516	19,248,647	**65,245,163**
2009/2010	49,446,415	20,442,821	**69,889,236**
2010/2011	49,739,442	22,499,815	**72,239,257**
2011/2012	45,509,748	17,555,542	**63,065,290**
2012/2013	37,515,555	15,180,753	**52,696,308**
2013/2014	44,216,105	17,120,168	**61,336,273**
2014/2015	40,901,550	12,358,814	**53,260,364**

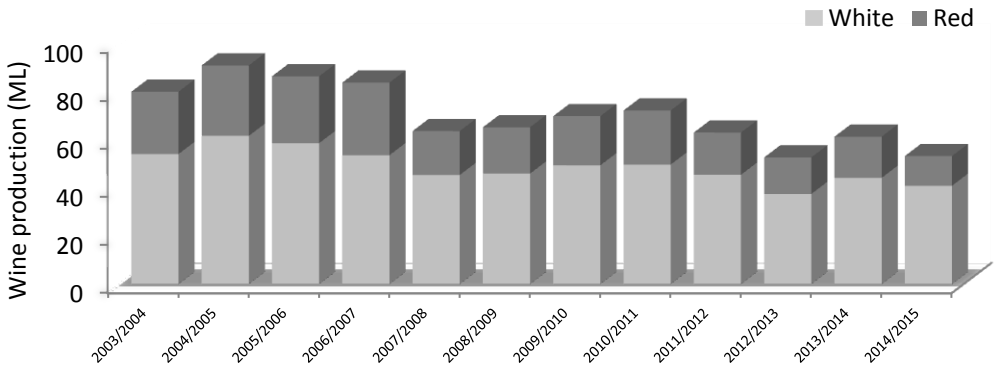

Figure 1.3 "Vinho Verde" wine production campaigns from 2003/2004 to 2014/2015 (ML)

Source: (CVRVV, 2015)

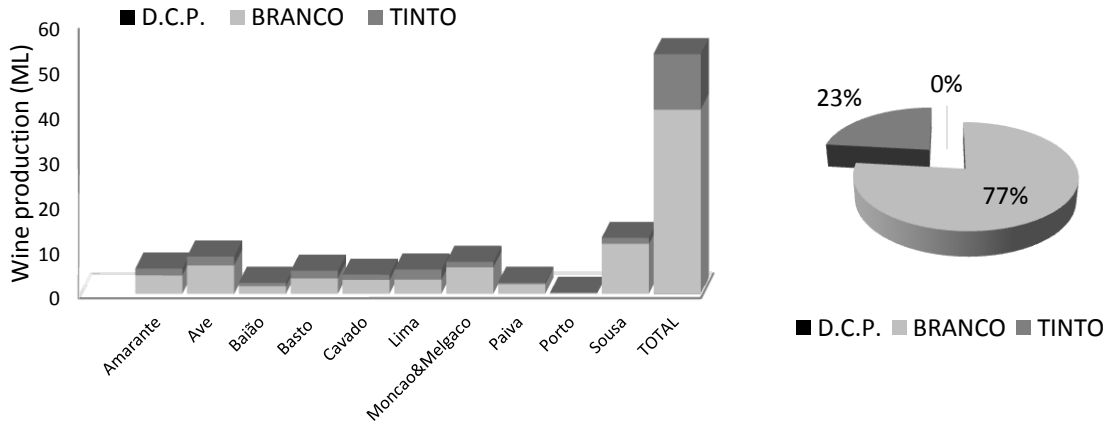

Figure 1.4 "Vinho Verde" wine production per sub-region in 2014/2015

Figure 1.5 Percentage of red and white Vinho Verde wine in 2014/2015

1.5 Winemaking process and the grape pomace (GP) production

The transformation from grapes to wine can be compared to a material flow. If we consider that the inputs of this flow are, grapes, water, oenological products, cleaning and disinfection products, we can predict that in addition to the wine it will be formed a water effluent, and a large number of solid by-products that should be well managed.

Wine production is a process that involves many steps where the quantity and quality of produced GP and other by-products is diverse. The winemaking process varies from each producer and this is a factor which contributes to the specificity of each wine. Although, there are some basic steps that are followed by all the wineries and are described below. The vinification process is different for red and white wines and in Figure 1.6 is represented the two processes followed in Adega Cooperativa de Ponte da Barca.

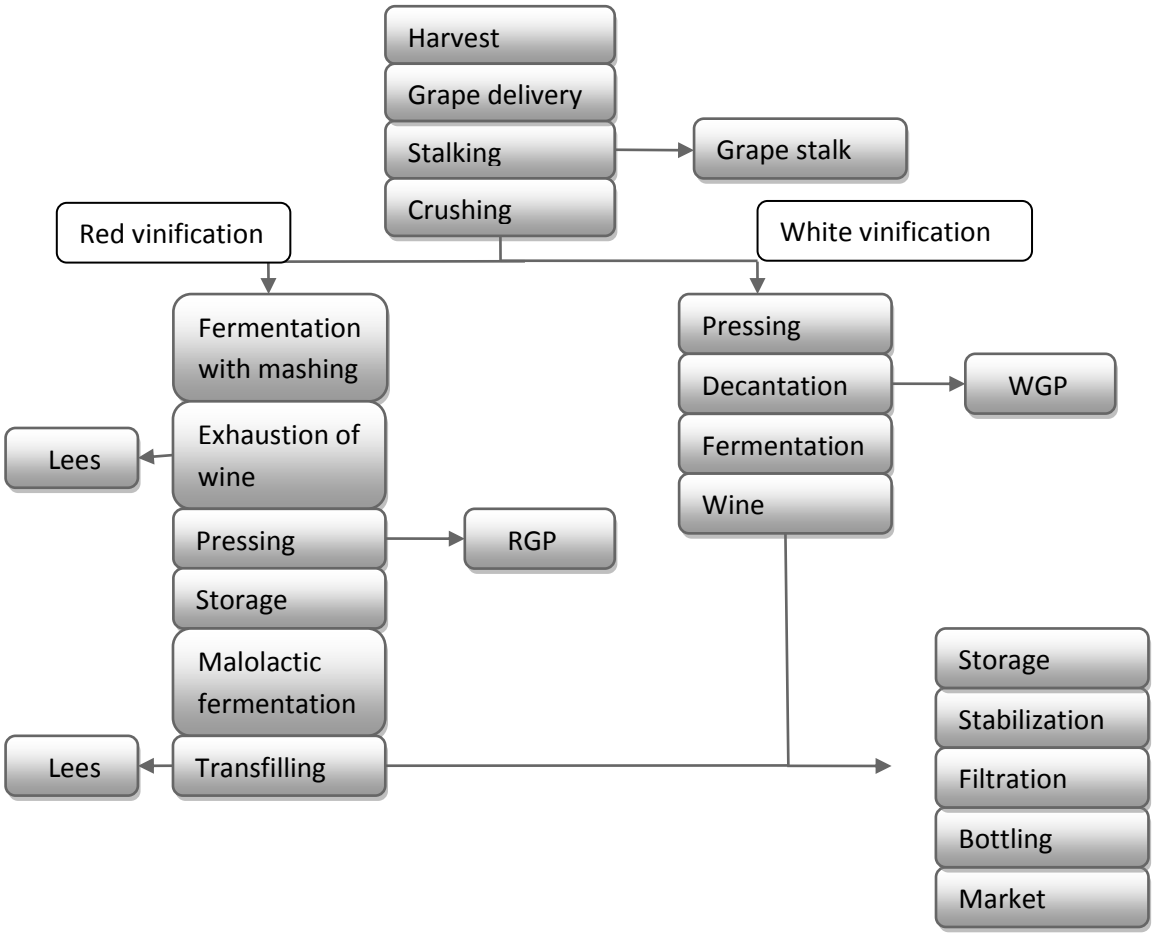

Figure 1.6 Vinification process and GP production in Adega Cooperativa de Ponte da Barca

Reception of grapes

The delivery of grapes occurs during the period of harvest, between September and October (Figure 1.7). There are several methods for transferring the grapes to the winery and is always important to consider some precautions because the grapes are partially crushed and with the high temperature, microbial contamination can occur. Therefore it is important to ensure the arrival of the grapes to the winery as soon as possible keeping the temperature during the transportation below 25^{o}C. (Vieira, 2009)

Figure 1.7 Reception of grapes in ACPB in 2014

Stalking and crushing

The stalking is the separation of the stalks (woody part) from the rest of the bunch, and it takes place before crushing (Figure 1.8). Frequently these steps are performed in equipment combining the two operations. The stalking is a recommended process because it can influence the quality of wine. The crushing of grapes merely breaks open the skin allowing the "free run" of the juice to pour forth, Figure 1.8. (Vieira, 2009)

Figure 1.8 Crushing and stalking of grapes in ACPB in 2014

Pressing and decantation

Decantation only occurs for white wine before the fermentation. In the pressing, it is added a certain amount of sulfur disinfectants to the must. The application of increasing concentrations of sulfur dioxide leads to death firstly the bacteria, then the apiculate yeast (*Kloeckera apiculata*) and finally of elliptic yeast (*Saccharomyres ellipsoideus*) that are most resistant. Slowing down the start of fermentation, sulfur dioxide promotes more or less the formation of a rapid deposit of suspended solids in the must. The process of sulfur dioxide slows the oxidation of wine, paralyzing the tirocinase and lactase enzymes, which are present in most grapes. It is important to avoid pre fermentative changes that will damage the quality of the wine. Sulfur dioxide also turns the mixture acid and attacks the plant cells promoting the dissolution of the organic acids present. On the other hand, it prevents the development of bacteria that are capable of attacking the acids, mainly the malic acid, so contributes to maintain the must acidic (Figure 1.9). After this step, the GP from white varieties is produced (Vieira, 2009).

Figure 1.9 Pressing of white grapes and addition of sulfur dioxide in ACPB in 2014

Fermentation

Once the must is in a fermentation vessel, yeast that is naturally present on the skins of the grapes, or in the environment, will sooner or later start the alcoholic fermentation, in which sugars present in the must are converted into alcohol with carbon dioxide and heat as by-products, as presented in the following reaction:

$$C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2.$$

Many winemakers, however, prefer to control the fermentation process more closely by adding specially selected yeasts in order to accelerate the fermentation process. After the must is placed in the fermentation vessel, a separation of solid and liquid phases occurs. Skins float to the surface, forming a cap (Figure 1.10). In order to encourage efficient extraction of colour and flavor components it is important to maximize contact between the cap of skins and the liquid phase. This can be achieved by pumping over, which means pumping liquid from the bottom of the tank and spraying it over the floating cap, normally this would be done several times per day during fermentation. It can also be achieved by punching down the cap, either manually or using an automated mechanical system, namely submerging the cap. The cap is kept beneath the surface of the liquid phase by a physical restraint. The above techniques can all be supplemented by a drain and return operation, in which the liquid phase is drained off the skins into another vessel and then pumped back over the skins. (Vieira, 2009)

Figure 1.10 Fermentation tank with solid parts and liquid from red grapes on ACPB in 2014

Exhaustion or first transfilling of wine

This step only occurs in red wines. The transfilling of the fermented must from the fermentation tanks to the settlement tank aims to separate the clear wine from the deposits that are formed on the bottom of the barrels or tanks. The deposit is not

instantaneous, since it depends on the diameter, the weight of the particles, the nature of the wine and the container. Approximately 60-70% of the available juice within the grape berry, the free-run juice, can be released by the crushing process and does not require the use of the press. The remaining 30-40% that comes from pressing (Figure 1.11) can have higher pH values, lower titratable acidity, potentially higher volatile acidity and higher phenolics than the free-run juice depending on the amount of pressure and tearing of the skins and will produce more astringent, bitter wine. In white wine production, pressing usually takes place immediately after crushing and before primary fermentation (Figure 1.6). In red wine production, the pressing usually takes place after or near the end of fermentation. The time of contact between the skin and the juice from the grapes enables the lacking out of color, tannins and other phenolic compounds from skin. (Vieira, 2009)

Figure 1.11 Pressing the red grape pomace in ACPB in 2014

Malolactic fermentation

This step normally occurs only in red wines, in which malic acid, naturally present in grape juice, is converted into lactic acid under the influence of bacteria. It is an important quality factor because it originates the reduction of acidity, the greater the reduction is, the more rich are the grapes in malic acid. (Vieira, 2009)

Second and third transfilling, stabilization and clarification (filtration)

The second transfilling is performed after malolactic fermentation in the early winter, after the first cold causes the precipitation of potassium bitartrate. The third transfilling is made immediately after the treatment for stabilization which serves to correct the pH and adjust the sulfur dioxide (SO_2) concentration.

Clarification stage mostly occurs in white wines, because red wines are not always clarified, by which insoluble matter suspended in the wine is removed before bottling. This matter may include dead yeast cells (lees), bacteria, tartrates, proteins, pectins, various tannins and other phenolic compounds, as well as pieces of grape skin, pulp, stems and gums. Clarification can be done by fining or by filtration. In fining it is added gelatin, bentonite, or other substance, which coagulates and settles the dragging impurities. In filtration, the wine passes through a filter where the impurities are retained. (Vieira, 2009)

Bottling

This process consists of depositing a precise amount of wine in bottles that are properly labeled and closed with stoppers usually cork. (Vieira, 2009)

Grape pomace production steps

In Figure 1.6 are outlined the steps in vinification process that are responsible for producing solid waste, including the grape pomace. The main solid waste is GP, which includes grape stalk, grape skin and seed and wine lees (biosolids). Grape stalks, skins and seeds are left after the stalking, pressing, and fermentation stages of wine production, depending of the type of wine (white or red).

1.6 Grape pomace applications

Grape fruit contains various nutrients, such as carbohydrates, edible fibers, vitamins, minerals, and phytochemicals. Nowadays, there is a growing interest in the exploitation of the by-products generated by the wine industry (Table 1.4). Grape pomace represents a rich source of various high-value products such as ethanol, tartrates and malates, citric acid, grape seed oil, grape seed flour and dietary fiber. Also, it can be

potentially used like a fertilizer. Moreover, grape pomace could be an alternative source to obtain natural antioxidants, which are considered completely safe in comparison with synthetic antioxidants, (Arvanitoyannis Ioannis S., 2006), as well as other natural food additives, like colorants and pullulan.

Table 1.4 Treatment and applications of grape pomace

Pomace sub products	Treatment	Physicochemical characteristics	Utilization
Grape waste	Composting of grape waste and hen droppings	Organic matter content	Fertilizer for corn seed
Grape seed and skin extracts	Fractionation of grape seed and skin extracts from grape waste	Phenol content	Dietary supplements for disease prevention
Grape waste	Gasification of waste products from grape	Concentrations of unused residues	Gas production for heating purpose
Pressed grape skin	Composting of solid waste and wastewater	Organic matter content	Fertilizer
Wine pomace and grape seeds	Lyophilisation and extraction of flavanols	Flavanol content	Dietary supplements, production of phytochemical
Grape marc, stalks and dregs	Lyophilisation and extraction of polyphenols	Polyphenolic content	Source of flavanols
Grape skins, seeds and stems	Acidolysis of a polymeric proanthocyanidic fraction of grape pomace in the presence of cysteamine	Flavanol content	Source of flavanols
Grape seed extract (GSE)	Pre-and post-mortem use of grape seed in feeding experiment	Phenol content	Feedstuff for dark poultry meat
Grape skin pulp	Fermentation by *Aureobasidium pullulan*	Ethanol precipitate	Pullulan production
Grape seeds	Solid-state cultivation by *Pleurotus* sp.	Lignocellulosic content	Laccase production
Grape pomace	Solid-state cultivation by Pleurotus sp.	Pruning content, high phenolic components and total sugars	Feedstuff for animals

Source: (Arvanitoyannis Ioannis S., 2006)

1.6.1 Grape seed oil and grape-seed flour

- Characteristics

The oil content of grape seeds was reported in the range of 11.6–19.6% depending on the variety and maturity of grapes (Ahmedna, 2013). The fatty acid composition of grape seed oil is also variety and maturity dependent. Major fatty acids of grape seed oil are linoleic acid 66.8–73.6%, oleic acid 17.8–26.5%, palmitic acid 6.4–7.9% and stearic

acid 3.6–5.3% (Beveridge Th, 2005). Comparing the fatty acid composition of grape seed oil with the olive oil is observed that the unsaturated fat in olive oil is primarily from monounsaturated fatty acids (MUFA), while in grape seed oil is mostly from polyunsaturated fatty acids (PUFA) (Table1.5). It was found that polyunsaturated fatty acid of oils from Cabernet Sauvignon and Royal Rouge pomace ranged from 60.9 to 64.4% (Chun Yi, 2009). The total unsaturated fatty acid accounts for more than 86% of the grape seed oil. (Baydar Gokturk Nilgün, 2001). In olive oil the content of unsaturated fatty acids is almost in the same range (Table1.6).

Table 1.5 Fatty acid composition of grape seed oil and olive oil (% w/w)

Fatty acids	Olive oil [(a)]	Grape seed oil [(b)]
myristic acid C14:0	0	0 - 0.1
palmitic acid C16:0	11.5	6.6 - 11.6
palmitoleic acid C16:1	1.5	0.1 - 0.2
stearic acid C18:0	2.5	3.5 - 5.4
oleic acid C18:1	75.5	14.0 - 20.9
linoleic acid C18:2	7.5	61.3 - 74.6
linolenic acid C18:3	1.0	0.3 - 1.8
arachidic acid C20:0	0.5	0.1 - 1.7

Source: (a) (Belitz H.-D., Olive oil, 1986)

(b) (Crews Colin, 2006)

Most oils high in PUFA, like grape seed oil, are naturally abundant in phytosterols, whereas MUFA-rich oils such as olive oil, generally possess lower plant sterol concentrations (Howell Tanya J., 1998). Indeed, the content of olive oil in phytosterol is 221 mg/100g oil (Abidi, 2001) while in grape seed oil range from 258-1,125 mg/100g oil with an average value of 571mg/100g oil (Crews Colin, 2006). Phytosterols are well known to contribute to antiarteriosclerotic activity. Among them, in grape seed oil, β-sitosterol is the most abundant (61.5–69.8%) followed by stigmasterol (11.9–16.0%), campesterol (9.3–10.8%), and sitostanol (3.5–4.0%) (Ahmedna, 2013). The concentration of each sterol changes with the variety and the maturity of grapes (Beveridge Th, 2005). Research about human metabolism has demonstrated that cholesterol absorption efficiency varies inversely with the injected phytosterols (Fernandez Maria Luz, 2005).

Plants are known to have high levels of antioxidant constituents and in particular grape seed oil has been studied mainly in its polyphenol composition. The antioxidant and fatty acid compositions of grape seed oil and thus its nutritional and cosmetic properties may be significantly affected by the grape variety, growing conditions, oil extraction methods and degree of refining (Ahmedna, 2013). In addition to phenolic antioxidants, grape seed oil also contains non-phenolic antioxidants such as tocopherols, β-carotene, both vitamins E and A are potent antioxidants and are critical to human health. The content of tocopherols in grape seed oil ranges from 265 to 454 mg/kg depending on the extraction method, on grape variety, on growing location and on growing conditions (Baydar Gokturk Nilgün, 2001). For this reason grape seed oil is a preferred cosmetic ingredient for damaged and stressed skin tissues. The regenerative and restructuring qualities of grape seed oil are most likely due to its high antioxidant and sterol contents that may convert into an attractive product for direct food consumption and cosmetic and pharmaceutical applications.

In the last few years grape flour produced from the pomace, seeds and skin, was a by-product generated during winemaking. This product has gained more and more attention from nutritionists. In addition to antioxidant content, grape flour is gluten-free and comes as a fine powder that can be added to baked goods like breads, cereal bars, scones, crackers etc, to add flavor colour and extra nutrition properties. It has a slightly astringent taste and so chefs recommend the addition to up no more than 3-5% of the total flour in the recipes. Pomace flour also is a good source of magnesium, calcium, iron, healthy fats, protein and fiber (NYR, 2015).

- Process

Grape seed oil is obtained from the seeds left after pressing of the juice from grapes for wine making. Many researchers investigated the possibility of using supercritical carbon dioxide (CO_2) extraction method to produce high-quality grape seed oil (Beveridge Th, 2005). However, owing to the high cost of supercritical fluid extraction, commercial grape seed oil is mainly produced by traditional oil extraction methods such as mechanical pressing, solvent extraction and cold-pressing, which is a method of oil extraction that involves low heat and no chemical treatment.

The production of grape seed oil by mechanical pressing can be relatively easy by grinding, flaking, or rolling and then subject the mass to a mechanical press to liberate the oil as it occurs with the olive oil.

A solvent extraction method uses solvent, like hexane to remove the edible oil from the grape seed. The grape seeds undergo a process called laminating, which crushes the seeds in a roller and cuts them into pieces so that the surface area is increased. The crushed seeds are injected with steam and then advance through an extruder. The laminated grape seeds are then immersed in a bath of cascading hexane, which washes out the grape seed oil. The new combination of oil and hexane is then purified in a distillation still. The hexane boils off and is recaptured, leaving crude grape seed oil behind. At this point, the crude oil will be refined. First, the oil is neutralized with caustic soda and phosphoric acid. Second, the caustic and phosphoric process causes the waxes to set up. This allows the waxy soap stock and oil to be pulled apart through a process called separation. Next, the separated oil is washed and dried to evaporate any excess water. Then, the oil is bleached with bleaching earth and activated carbon to remove any residual green color. The oil is then filtered and sent to a deodorizer to remove any smell. Finally, this method gives clear oil that looks like any robust refined olive oil (Kashrus Kurrents, 2015).

Another method to extract grape seed oil from ground seeds is the "cold pressing". Processing of seed is made under lower temperature (as 80^{o}C) in mechanical presses which extracts oil by exerting pressure on the seeds. This method is preferable in small production scales (less than 200 tons of seeds daily) (Al Kurki, 2008).

In recent years it was studied the use of supercritical carbon dioxide as an extraction which is achieved under high pressure and low temperature. As an extractant this solvent is nontoxic, nonflammable, noncorrosive, and available in large quantities at high purity in contrast to other extractants such as hexane, which is commonly used in conventional oil processing and which provides only some of these benefits. This recent method, on a laboratory scale has similar performance to conventional solvent extraction, but the quality of the oil is better. Since the oil produced by this method does not contain solvent, the step of distillation for removing the solvent is not needed. The quality of oil

produced when using liquid carbon dioxide (CO_2) is similar to the oil obtained by organic solvent extraction after refining. Despite the high operating cost of the process, the extraction of seed oil by liquid carbon dioxide (CO_2) could be less expensive than the conventional extraction with solvents because the last two stages of refining and removal of the solvent, which consume the most energy, can be omitted (Beveridge Th, 2005).

The main countries that produce grape seed oil are France, Spain, Italy, Chile, USA and Australia. After oil processing any remaining solids may be used as seed flour, feedstock, or fertilizer.

1.6.2 Grape pomace as fertilizer, feedstock and bio fuel

- Characteristics

The use of GP as fertilizer in the vineyards is of growing interest because it is an organic fertilizer. However the direct incorporation of grape pomace into agricultural land has caused serious problems since degradation products can inhibit root growth, due to the long time of decomposition. Simultaneously the exclusive addition of chemical fertilizers is no longer considered the best method to feed plants and keep the plant pathogens under control. So in order to use GP as organic fertilizer it needs a pretreatment like composting processes. Extensive research has demonstrated that many biodegradable organic by-products can be composted in a convenient and economical way. Due to its high concentration of macro and micro-nutrients, nitrogen, potassium and phosphorus, grape pomace can be used as a crop fertilizer. The value of grape pomace as fertilizer depends upon the presence of the proportion of organic matter which can be converted into humus with favorable effect to the soil. The high C:N provides nutrients for the microbes to survive and continue degradation (Dwyer Kyle, 2014). The application of compost from GP increases the percentages of organic matter, nutrient levels, providing a slow fertilization action over a long-period time, microbial biomass and improves the soils physical properties, aeration, water-holding capacity, etc. In addition, it has been reported that the compost, derived from pressed grape skin, produces one of the best qualities composts both in terms of its physicochemical characteristics and agronomic value (Ahmedna, 2013). M.J Diaz, reported that the grape pomace, could be

recycled as a soil conditioner in view of its organic and nutrient contents (Diaz M.J, 2002). Moreover, a comparison of the best compost obtained from winery by-products with those from other organic wastes showed that its chemical values fell within the same range in most cases, with the exception of a high-calcium value owing to the nature of wine-making process (Ahmedna, 2013). Because fresh grape pomace is acidic, it is necessary to mix it with an amendment such as slaked lime. Pomace, like other composted materials, helps filter environmental pollutants and also helps plants to improve their drought tolerance (Nistor Eleonora, 2014).

Grape pomace can also be available as feedstock. A major limitation of using this by-product as a ruminant feed is the presence of a high level of lignified grape cell wall fraction. Another barrier in the utilization of this by-product is its high tannin content which has adverse effects on nutrient utilization by animals, and are toxic at high intake levels due to their ability to bind proteins, minerals and carbohydrates (Alipour D., 2007). Although, dried or ensiled GP is low in metabolizable energy (ME) and it has been used in diets of ruminants fed close to maintenance levels, especially sheep. Several authors have noticed that tannins at low concentrations altered rumen fermentation and microbial protein synthesis to the benefit of ruminants. In contrast, similar levels of these phenolics had a negative effect on rumen fermentation. Data reported by these authors suggested that the effect of tannins on ruminal parameters depended on their level, type (condensed or hydrolysable tannins) and nature of plant. Polyethylene glycol (PEG, molecular weight 6000) which possesses a very high affinity for tannins has been used to deactivate them (Abarghuei M.J., 2010).

The solid by-products from the vinification process can alternatively be dried and burned in special burners in order to recover the energy. The increase of fuel price has shown that dried products, skin, seeds and stalks could compete favorably with traditional liquid fuels. The energy content of the dried grape pomace amounts to 18 to 22.7 MJ /kg depending on the part of grape pomace (Toscano G., 2013).

- Process

A decomposition process should be applied to prepare the GP as a material suitable for soil application. Two ways of decomposition are known: composting and

vermicomposting. Composting of organic matter is a simple and efficient method to transform agro-industrial by-products into products suitable soil amendment (Ferrer J., 2001). For optimal composting, the material being composted must have a high moisture content and contain a sufficient carbon-to-nitrogen (C:N) ratio. Vermincompost is an aerobic composting process that leads to a nitrogen mineralization increased humic materials, hormones and pH. (Nerantzis Panagiotis, 2006).

To prepare the GP as a material suitable for animal feed, deactivation of tannins and reduction of lignin are necessary. Anaerobic storage, ensilage, can be an inexpensive and simple way to inactivate tannins and decreased the concentration of phenolic compounds. Another method is addition of polyethylene glycol (PEG), which has a high affinity to tannins and makes them inert by forming tannin–PEG complex, thereby preventing tannins from binding with proteins. Several authors have noted an improvement in the nutritive value of high tannin feeds due to use of PEG, which is not expensive. (Abarghuei M.J., 2010).

Grape pomace by-products can be pelletized for bioenergy production running through a process of drying and storage firstly the raw material. Followed by dryness of the cleaned grape pomace by using mechanical drainage and thermal drying and crushing with mechanical release of the grape seed oil contained in the grape seeds. Finally the crushed grape pomace is pelletized by using a ring die press.

Grape pomace pellets can fulfill the requirements of the draft of the European standard for solid biofuels (EN 14961-6) (Volker Lenz, 2012).

1.6.3 Alternative applications for bioactive compounds

When the grape pomace is used directly as animal feed, fertilizer or fuel as described above, the valuable bioactive components contained in it, are lost. As alternative, the grape by-products can be available either as animal feed, fertilizer or fuel but after the recovery process of tartate, alcohol, phenolic compounds, colorants, seed-oil or pomace flour.

- Characteristics

Polyphenols are the most important phytochemicals in grapes because they possess many biological activities and health-promoting benefits to the plants. It is known that phenolics are the most important compounds affecting flavor and color among white, pink and red wines; they react with oxygen and are critical to the preservation, maturation and aging of the wine. Grape pomace is characterized by high-phenolic contents because of poor extraction during winemaking. The bioactive substances are used worthwhile and thus support sustainable agricultural production (Kammerer Dietmar, 2004). Although, the absolute amount as well as the relative proportion of these beneficial compounds in GP is conditioned by a myriad of factors including genetic load of the separate grape varieties, agro-climatic conditions, fertilization procedures, and soil properties, among others. On the other hand, the specific wine-making processes as well and the time between the generation of by-products and valorization activities, as well as the characteristics of the recycling and recover procedures have a direct impact on the final concentration of phenolic compounds in the GP and, therefore, on the potential use as a source of bioactive phytochemicals.

In food science research, natural phenolics are generally classified into classes and sub-classes based on the similarity of their chemical structures, that is, the types of building blocks that appear as repeated units. Four major classes of polyphenols are found in foods, flavonoids, phenolic acids, stilbenes and lignans (Figure 1.12). In Figure 1.13 is presented the tannins classifications (Ahmedna, 2013).

The by-products obtained after winery exploitation, constitute a very cheap source for the extraction of antioxidant flavanols, which can be used as dietary supplements, or in the production of phytochemicals, thus providing an important economic advantage (Ahmedna, 2013). In grapes, the phenolic compounds reside mainly in the skins, seeds and less on the stalks. GP is rich in extractable phenolic antioxidants (10–11% of dry weight) (Makris Dimitris P., 2007). Flavonol anthocyanins, flavan-3-ols, phenolic acids and stilbenes are the principal phenolic constituents found in GP (Ahmedna, 2013).

Anthocyanins are the highly soluble flavonoids directly responsible for color in red grapes and wines, existing mainly in the stable red colored form and mainly exist in red grape skins. Flavonoids are widely distributed in grapes and the principal compounds found are (+)-catechins, (−)-epicatechin and procyanidin polymers. The most common flavonols are quercetin and kaempferol. The distribution of flavonols in the different parts of vinification by-products, according to the bibliography, shows differences concerning the individual flavonols and, in general, the parts from red varieties present higher amounts of these compounds than white byproducts (Teixeira Ana, 2014). Phenolic acids include benzoic and cinnamic acid derivatives, with hydroxycinnamic acids being the most abundant class in wine industry by-products. To date, the relative proportion of the phenolic acids in winery by-products compounds have been calculated by HPLC analyses (Teixeira Ana, 2014). Stilbenes are phenolic compounds which can be found in grape skin, but also in stalks and in seeds. Resveratrol is a phytoalexin produced in the plant in response to pathogen attack. It has a low toxicity in humans and is a naturally occurring fungicide. Grapes produce stilbenes (resveratrol) in response to a number of physiological stressing factors, including ozone and UV-C radiation. Tannins are complex phenolic compounds of high molecular weight that can be divided in two distinct groups: condensed tannins and hydrolysable tannins. Condensed tannins, also known as proanthocyanidins, are constituted by subunits of flavan-3-ols monomers and their structures vary depending on their constitutive subunits, the degree of polymerization, and the linkage position, whereas hydrolyzable tannins are complex (poly)phenolics that can be degraded into smaller units, mainly sugars and phenolic acids (Dai Jin, 2010).

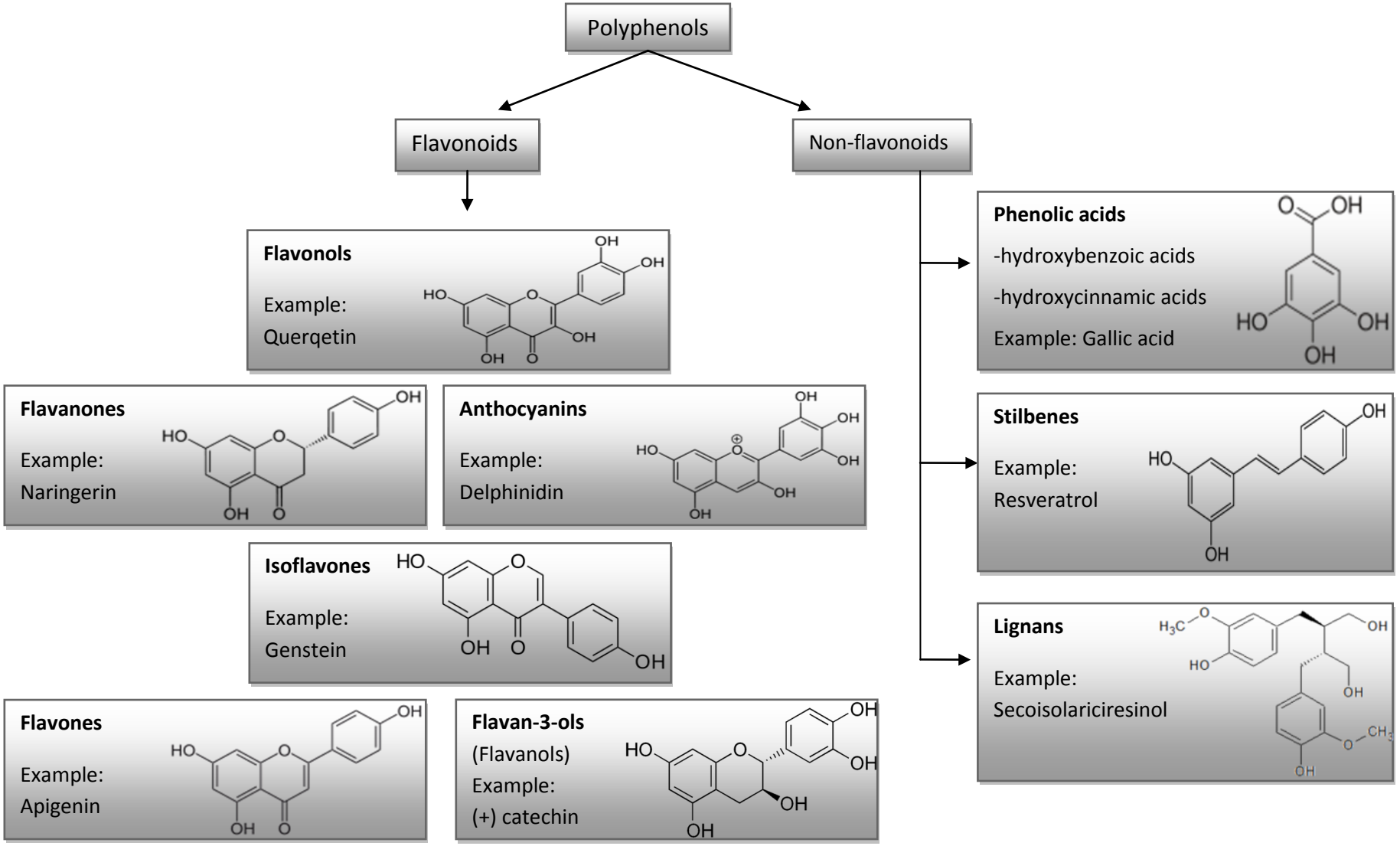

Figure 1.12 Classification of polyphenols

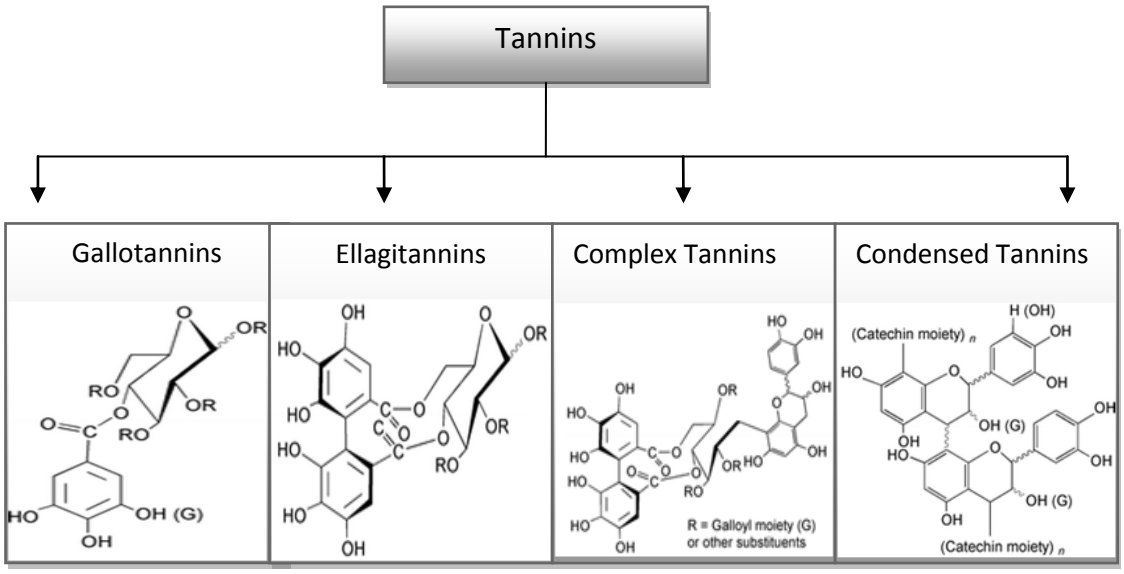

Figure 1.13 Classification of tannins

The benefits from the properties of the bioactive compounds found in grape pomace are noticeable. It was believed that the high consumption of red wine in France reduced the prevalence of coronary heart disease (CHD) even though with diets containing large amounts of saturated fats. Later studies believed that the "French paradox" was due to the phenolic compound, resveratrol found in red wine. Phenolic compounds have been proven to inhibit LDL (low-density lipoproteins) oxidation and reduce the risk of CHD (Dwyer Kyle, 2014). The reported evidences of beneficial health effects of phenolic compounds include inhibition of some degenerative diseases, such as cardiovascular diseases, and certain types of cancers, reducing plasma oxidation stress and slowing aging, having also anti-ageing, antioxidants, antimicrobial and anti-inflammatory activities. (Xia En-Qin, 2010).

- Process

Extraction techniques have been widely investigated to obtain high recoveries of valuable natural compounds for commercialization, because of sample complexity, which includes high quantity of target analytes with different chemical nature. So, highly selective and sensitive analytical methods are necessary for the characterization of GP extracts. Traditional extraction techniques have been gradually switched to novel extraction methods with reduced extraction times and low consumption of organic solvents, which

increase the sustainability of the process. After extraction, chromatographic techniques, especially high-performance liquid chromatography (HPLC), have been the choice for the analysis of phenolics in GP extracts. The method for isolation and identification of polyphenols extracts should be developed for each plant variety and depending on the target analytes.

The food industry has proposed many theoretical applications for the use of grape pomace bioactives. An application is the production of pullulan. Pullulan is a non-ionic exo-polysaccharide produced by the fungus *Aureobasidium* pullulans. Pullulan has high film-forming capabilities. Pullulan can be added to food to increase texture and provide low-calorie bulk. Also, it is applicable to health care like lotions and shampoos and pharmaceutical products like denture adhesives and capsules for supplements. A future application currently being developed is a pullulan-based antimicrobial active packaging system (Dwyer Kyle, 2014). Another area of grape pomace bioactive applications is food preservation. The bio-film previously discussed was externally added to foods, but other bioactives such as antioxidants can be added internally to preserve food. With respect to meat and meat products, the use of natural antioxidants such as the phenolics contained in grape pomace, are greatly preferred and are considered completely safe in comparison with synthetic antioxidants such as butylated-hydroxyanisole (BHA) and butylatedhydroxytoluene (BHT), compounds now largely used in the food industry with undesirable effects on the enzymes of human organism (Arvanitoyannis Ioannis S., 2006). The antioxidants and oils extracted from grape pomace can also be used in the cosmetic industry (Dwyer Kyle, 2014). Vitamin E has also been found to be effective as a dermatological treatment. This is significant due to the amount of vitamin E that can be extracted from grape seeds. It has also been determined that grape seed oil rich in fatty acids are beneficial to the skin (Dwyer Kyle, 2014).

1.7 Legislation

1.7.1 European regulations

Wine is one of the agricultural products covered by the Common Agricultural Policy (CAP, 2012) and therefore has been established Community Legislation that covers

all the activities in the production and distribution of wine. It is also known that the European Union is in surplus at the wine production, thus established intervention measures to support the product and improve its quality. Such measures include the storage, distillation and application of wine deliveries. According to the latter, each producer is obliged to deliver to distilleries the by-products produced, for distillation of ethyl alcohol. The amount of the by-products cannot be less than 10% of the alcohol contained in the overall production of wine products from the winemaker. Other measures taken by the European Union aimed to control the production and eliminate the surpluses, such as banning plantations and subsidizing grubbing vineyards, especially in areas where the quality of the wine is not good, nor traditional.

According to EU regulations 1493/99 and 1623/2000, it was set a minimum purchase price for the distillers of wine by-products. Today, the lower price is set at 0,995 € per alcoholic degree and hectoliter of pomace or lees. To keep distillation costs within acceptable limits, Article 46 of Regulation 1623/2000 of the EU defined the minimum contents of ethyl alcohol to be 2 – 2.8 L per 100 kg of pomace and 3 - 4 L ethyl alcohol for every 100 kg of lees, depending on the wine-growing zone. If there are no facilities in the winery area for the reception and processing the by-products, and also the transportation costs are excessive, the winemaker has the right to withdraw the sub-products from the market, under the supervision and control of the local Agriculture Department. By withdrawing, the by-products can be disposed as animal feed or for the production of compost soil conditioner. The by-products withdrawn can have minimum contents of ethyl alcohol about 3 L alcohols per 100 kg of pomace and 5 L of alcohol per 100 kg of lees. For the purposes of Article 27 of Regulation (EC) No 1493/1999, the by-products shall be withdrawn without delay and no later than the end of the wine year in which they were obtained. Withdrawal, together with an indication of the estimated quantities, shall be either entered in the registers kept in accordance with Article 70 of Regulation (EC) No 1493/1999 or certified by the competent authority. The winemaker fulfills the obligation to deliver the by-products for distillation, or part of it, by making the alternative solution of withdrawing.

Distillers that receive and process winemaking by-products are financially supported by the Article 48 of the EU 1623/2000. The payment of financial support is determined according to the classification of the product produced, from 0.6279 € per alcoholic degree and hectoliter of neutral alcohol (pomace, lees or wine), to 0.3985 € per alcoholic degree and hectoliter of pomace spirit or raw alcohol pomace, and finally to 0.277 € per alcoholic degree and hectoliter wine distillate or raw alcohol from wine and lees. As a result of this situation, it remains virtually unexploited the main volume of grape pomace that is produced in the large wineries. It is not unusual a large part of the grape pomace to remain forever at open fields and not utilized nor for fertilizer, neither for animal feed, thus further environmental problems are created. This particularly happens when the winemakers ask the livestock farmers or farmers some compensation to collect the grape pomace. In practice this leads many winemakers to allocate it by free just to get rid of them from their establishments. In any case, price for these uses are 0 – 0.15 € / kg incomparably lower than those of distillers.

The present practices for GP deposition result in the destruction of many components with high commercial value. As was discussed in section 1.6, the recovery components from grape pomace (beyond alcohol and potassium tartrate) are valuable, and the demand for these products in the future is expected to increase significantly. The grape pomace that comes out from the winemaking process has a significant residual value which currently remains unexploited. Indeed, there is a growth in the area of utilization the grape pomace, which may benefit a range of agricultural industries (wineries, distilleries, oil factories), pharmaceutical companies, food and dietary supplements industries, the growers themselves because it will upgrade their product . Finally, it will benefit the national economy by increasing the added value products and the replacement of imported goods with domestic production.

1.7.2 EMAS-European Organization (EMAS, 2015)

In order to approach the management of environmental problems by the vine-growing and wine-producing sector there is a European Organization called EMAS. *EMAS* is an environmental management scheme that aims to improve the environmental

performance of organizations by committing them to evaluating and reducing their negative environmental impacts. It is a voluntary framework that provides the basis for a published environmental report and it aims to recognize and reward those organizations that go beyond minimum legal compliance and continuously improve their environmental performance. *EMAS* incorporates *ISO14001* and is externally evaluated. Once accredited, participants can publicize their participation in the scheme through use of the *EMAS* logo. The core of *EMAS* is a 'continuous improvement cycle' or the plan-do-check-act process.

The environmental review is an initial comprehensive analysis of the environmental problems caused by an organization's activities. The outcome is a report that includes hard data about consumption of raw materials and energy and the production of wastes and emissions; information on the indirect environmental impacts of the organization's activities and an outline of the management structures in place to deal with these impacts. The purpose of the initial review is to identify the most significant environmental impacts – and thereby identify possible priorities to be set in the environmental program – and to lay down a benchmark to measure future success in reducing these impacts.

Objectives

The objective of this study was to evaluate the potential application of grape pomace, from Vinhão and Loureiro grape varieties, as bioenergy or as raw material in other industrial processes. To achieve this, four specific goals were established:

- Study the chemical characteristics of the two grape pomace varieties obtained from Portuguese grapes, Loureiro and Vinhão;
- Determine the GP bioactive content, through the assays of total phenolic content (TPC), antioxidant activity (DPPH) and also the identification and quantification of nine selected phenols;
- Evaluate the high heating value (HHV) in GP and extracted GP as a form of bioenergy;
- Develop a financial analysis for evaluating some industrial processes with GP as raw material.

2 Experimental development

The study for the by-products recovery after the winemaking process with Loureiro and Vinhão varieties was an innovative theme, developed for the first time in ESTG-IPVC. Therefore, it was necessary to implement the process methodology and explore appropriate experimental conditions. It began with a general characterization of the grape pomace, including the extraction and identification/quantification of the bioactive compounds and finally the determination of the calorific value of grape pomace and extracted samples. In the present chapter, reagents, standards and solutions used throughout all the experiments are described. Furthermore, aspects regarding the sample preparation and latter analysis are also explained.

2.1 General experimental conditions

Analytical grade reagents were purchased from different suppliers and used for all experiments. They were stored according to the supplier specifications. For the preparation of all solutions, water from Barnstead™ E-Pure™ Ultrapure Water Purification Systems was used throughout the work. All the standards and the samples were weighted in a Mettler Toledo analytical balance (model AE 200) with the accuracy of decimal of miligram. Standard stock solutions were prepared by rigorous weighing the respective reagent, followed by dissolution in the appropriate solvent (water, buffer solution, methanol, or mixture of the previous solvents). All reagents that appear in the experimental procedure, without the reference grade, were pa grade. All working solutions were freshly obtained through rigorous dilution of standard stock solutions with pipettes, using volumetric flasks of different volumes.

2.2 Sampling and transportation

Samples of grape pomace resulting from winemaking with the species *Vitis Vinifera L.* of the varieties Loureiro and Vinhão cultivated and harvested in the region of Lima, Alto Minho, Portugal were provided by Adega Cooperativa de Ponte da Barca, in September 2014. At the Adega, the GP from the white Loureiro was collected immediately after separation from the must in the step of decantation, before

fermentation as a mixture (skin and seed) from big opened air tanks shown in Figure 2.1. The GP from the red grapes Vinhão was collected after the fermentation of the must, in two types of samples; one was collected in the form of separated seed and skin. However this is obtained in a limited amount. The other sample was collected as a mixture of skin and seed. The stalks from both varieties were collected in the back of the factory, Figure 2.2, after the stalking step. Many authors consider GP consisting of a mixture of all the fractions, stalk, skin and seed, while others consider only the skin and the seed. In this work, GP means the stalks, skin and seed and GP mixture refers to the skin and seed.

The sampling for both varieties was done during three different periods of time in September, collecting about 3 kg of GP in each sampling visit, making a total of 9 kg of each variety. All the samples were transported in fully and closed vessels at room temperature for about one hour (duration of trip from Adega Cooperativa de Ponte da Barca to the laboratory of the school) and then followed the pretreatment.

Figure 2.1 Storage of grape pomace in the factory

Figure 2.2 Storage of stalks in the factory

2.3 Pretreatment and codification of samples

In the lab, fresh grape pomace was divided as presented in Figure 2.3. Half amount was dried at 30 °C in a drying oven, Heraeus Model UT 6060 for four days. After, it was vacuum packed and stored until analysis. Before analyses, the seeds and skins from each sampling visit were manually separated and the same fraction from the three visits was mixed together and dried at 103°C. The other half amount of each variety was vacuum packed and it was stored at -20°C, after manual separation of the skin and the seed of white Loureiro. In Vinhão variety, it was obtained seed separated from skin in the Adega. The analyses of the major compounds, except moisture, and the analyses of the heating value were determined on samples dried at 103 °C. The samples stored at -20 °C were lyophilized in a Christ Alpha 1-2 LD Freeze Dryer with a Rotary vane pump RZ 2.5, the samplings visits were mixed and were used for the studies on polyphenols extractions. For all the analyses the samples were ground in a Taurus blender (Aromatic Model, Type: SP- 7407) until homogenous flour to approximately 8-meshes (Figure 2.4).

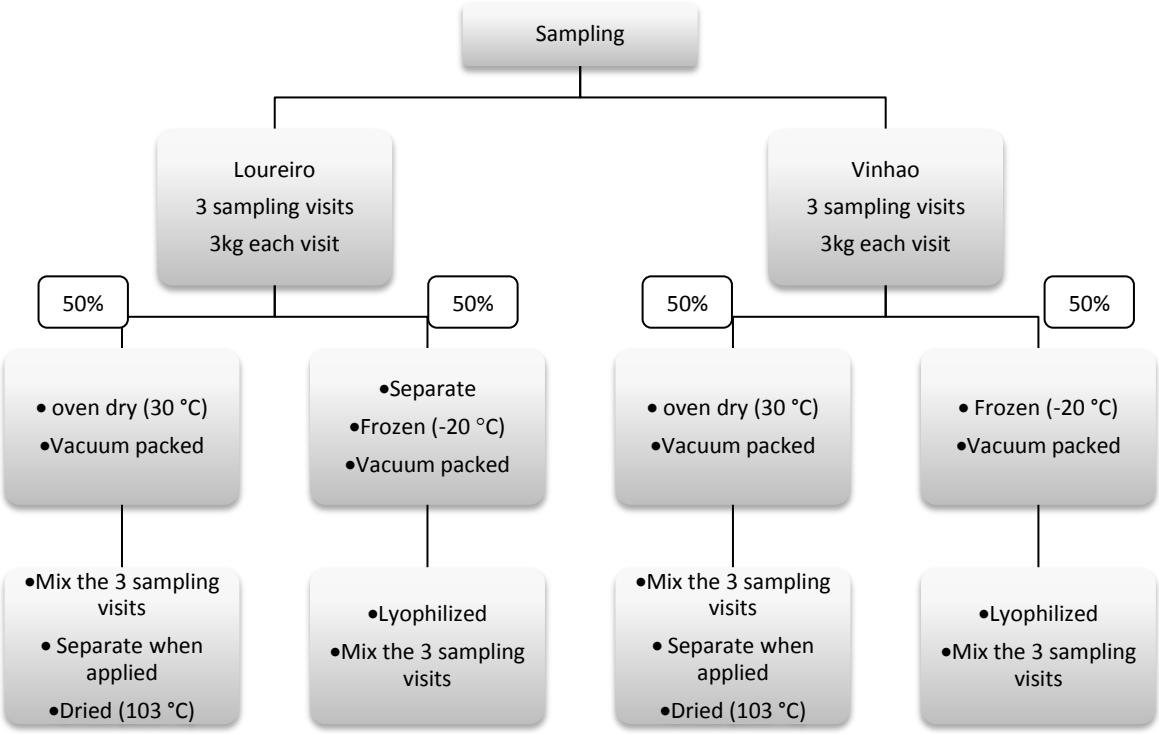

Figure 2.3 Sampling and pretreatment flow

Samples were codified by B and T standing for white ("Branco") and red ("Tinto") GP, respectively, followed by L for Loureiro and V for Vinhão varieties. Finally the letters E, P and G stands for stalks ("Engaço"), skin ("Película") and seed ("Grainha") respectively. Samples of GP with a fourth letter, E, in the beginning stands for extracted samples (Table 2.1).

Table 2.1 Codification of samples from Adega Cooperativa de Ponte da Barca

CODE	PART OF GRAPE	COLOUR	VARIETY
BLE	Stalk	White	Loureiro
BLM	Mixture*		
BLP	Skin		
BLG	Seed		
TVE	Stalk	Red	Vinhão
TVM	Mixture		
TVP	Skin		
TVG	Seed		
	Samples after extraction		
EBLE	Extracted Stalk	White	Loureiro
EBLM	Extracted Mixture		
ETVE	Extracted Stalk	Red	Vinhão
ETVM	Extracted Mixture		

*The mixture contains the seed and the skin of the grapes

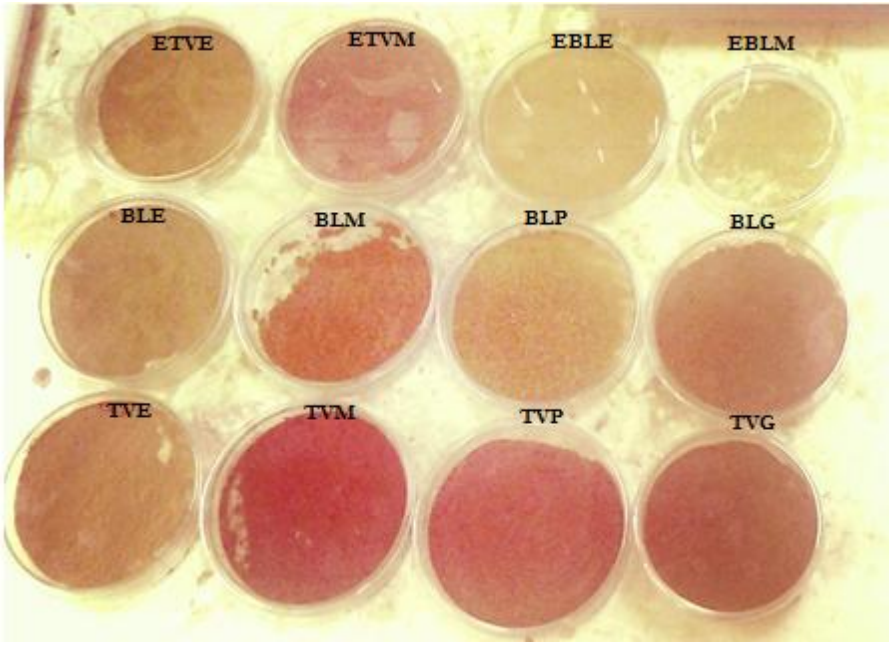

Figure 2.4 Samples after the pre-treatment, powered to 8-mesh

2.4 Major chemical characterization

2.4.1 Moisture content

Moisture content of GP samples was determined according to the method AOAC 934.01- 4.1.02 (AOAC, 1995)

- Principle

A well homogenize sample was dried in a porcelain crucible to constant weight in an oven at 103 - 105°C. The method is based in a gravimetric method.

- Equipment and material

Drying oven line function Heraeus, Model UT 6060

Porcelain crucible

- Procedure

Sample, about 5 g, was placed in a previously dried porcelain crucible and dried in a drying oven to a constant weight.

- Calculation:

The moisture content was determined by measuring the mass of sample before and after the removal of water by evaporation:

$$\%\, moisture = \frac{\mathrm{m_{initial}} - \mathrm{m_{dried}}}{\mathrm{m_{initial}}} \times 100$$

where:

$\mathrm{m_{initial}}$ is the mass of fresh sample as it was collected from Adega

$\mathrm{m_{dried}}$ is the mass of dried sample

2.4.2 Ash content

Ash content in dry GP sample was determined according to method 942.05- 4.1.10 (AOAC, 1995)

- Principle

The dry residues resulting from the moisture determination were ignited to constant weight at 550 °C. The remaining residues represent the inorganic matter, while the weight lost on ignition is the volatile solids. The method is based in a gravimetric method.

- Equipment and material

Muffle furnace line function Heraeus, Model M110

Porcelain crucible

- Procedure

Grape pomace dry residues were heated in a muffle furnace at 550°C till a constant weight.

- Calculation:

The ash content was determined by measuring the mass of sample before and after the ignition:

$$\%\ ash\ (DM) = \frac{\mathrm{m_{ash}}}{\mathrm{m_{dried}}} \times 100$$

Where:

$\mathrm{m_{ash}}$ is the mass after ignition at 550 °C

$\mathrm{m_{dried}}$ is the mass of GP dry residue

2.4.3 Minerals

All minerals, including phosphorus, were determined from the ash. The sample preparation described in this section for the dissolution of the ash in a 50 mL concentrated sample solutions, is the same for the phosphorus determination.

2.4.3.1 Minerals by atomic absorption spectrometry

Mineral content in GP dry matter were determined according to SMEWW, method 3111 B (SMEWW, 1998)

- Principle

In atomic absorption spectrometry (AAS), a sample is aspirated into a flame and atomized. A light beam is directed through the flame, into a monochromator, and onto a detector that measures the amount of light absorbed by the atomized element in the flame. Each metal has its own characteristic absorption wavelength, so a source lamp composed of that element is used.

- Equipment and material

Varian SpectrAA-300 Atomic Absorption Spectrometer, Model 1111807

Hollow Cathode Lamps (Photron PTY.LTD Australia and S&J JUNIDER and Co)

Hot plate

Glass filter (FisherBrand QL100, 185mm)

Glass material: volumetric flasks, beakers 100mL, buchner funnel, filtering flasks and pipettes

- Reagents

Deionized water

Calcium carbonate solution ($CaCO_3$): 630mg of calcium carbonate was dissolved in 50 mL of 1 + 5 HCl and then diluted to 1000 mL with water

Lithium carbonate solution ($LiCO_3$): 0.5332g of lithium carbonate was dissolved in 100 mL of water

Oxidant lanthanium solution (La_2O_3): 58.65 g of lanthanum oxide was dissolved in 250mL concentrated HCl and then diluted to 1000 mL with water

Nitric acid solution (HNO_3) 65% w/v (Panreac, PA-ISO)

The different standard metal solutions were prepared in the optimum concentration range for calibration curves by diluting stock solutions (1,000 mg/L) in deionised water. The concentration of the standard solutions for the calibration curves of each element are listed in Table 2.2:

Table 2.2 Concentration of the standard solutions for the calibration curves of different elements

Element	Concentration mg/L					
Potassium	0.000	0.200	0.400	0.600	1.000	1.400
Calcium	0.000	1.000	2.000	3.000	4.000	5.000
Phosphorus	0.000	0.100	0.200	0.300	0.400	0.500
Magnesium	0.000	0.060	0.160	0.240	0.320	0.400
Sodium	0.000	0.600	1.000	1.200	2.000	2.400
Iron	0.000	0.400	1.000	1.600	2.000	3.000
Copper	0.000	0.200	0.400	1.000	1.600	2.000
Zinc	0.000	0.100	0.200	0.300	0.400	0.500
Manganese	0.000	0.400	1.000	1.600	2.000	3.000
Chromium	0.000	0.200	0.400	1.000	1.600	2.000
Nickel	0.000	0.200	0.400	0.600	0.800	1.000
Lead	0.000	0.500	1.000	2.000	3.000	4.000
Cadmium	0.000	0.100	0.200	0.500	0.800	1.000

- Procedure

Sample Preparation: The ash (from section 2.5.2) was transferred to the beaker. The crucible was cleaned one time with deionised water, a second time with a portion of water and 2 mL of nitric acid and a third time with a portion of water and 1 mL of nitric acid. Deionised water was added to the beaker until 50 mL. The beaker was heated on the hot place. It boiled slowly until evaporation to the lowest volume possible, around 10 mL, before precipitation occurred. After the beaker had cooled, the solution was filtrated and transferred to a 50 mL volumetric flask and adjusted to volume with deionised water. The concentrated sample solution was then diluted appropriately (Table 2.3), to different volumes. For eliminated interferences in the determination of calcium (Ca) and magnesium (Mg), 10 mL of the diluted samples were mixed with 1 mL of lanthanium solution. For determination of iron (Fe) and manganese (Mn), 10 mL of the diluted samples mixed with 2.5 mL of carbonate solution. Finally for determination of sodium (Na) and potassium (K), 10mL of the diluted samples were mixed with 10 µL of lithium carbonate solution. The same conditions were applied to the standard solutions of calibration curves.

Table 2.3 Dilution factor for each element in the different parts of GP

	BLE	BLM	BLP	BLG	TVE	TVM	TVP	TVG
Potassium	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Calcium	100	50	100	100	100	50	50	100
Phosphorus	250	500	250	500	250	500	250	500
Magnesium	250	250	100	250	250	200	100	250
Sodium	50	50	50	50	50	50	50	50
Iron	5	5	2	5	5	5	2	2
Copper	-	-	-	-	-	5	5	-
Zinc	5	2	2	2	5	5	5	2
Manganese	2	-	-	-	-	-		

Analysis: The first solution to be aspirated was the blank solution to make the zero determination value of the equipment. The calibration curve using the solutions prepared as in table 2.2 was obtained, from the absorbance recorded in the AAS. Then the aspiration of the diluted solutions was done to determine the corresponding absorbances. The same sequence followed for all the metals.

• Calculation

From each calibration curve it was obtained an equation of first degree:

$$Abs = m \times C + b$$

From the equation above, it was calculated the concentration (mg/L) using the formula:

$$C\ (mg/L) = \left[\frac{Abs_{sample} - b}{m}\right] \times d.f$$

Then, to present the results in dry matter (mg/kg), it was applied the following formula:

$$mg/kg = \frac{C_{mg/L} \times 0{,}05\ L}{m_{sample}(g)} \times 1000$$

where:

Abs is the absorbance obtained from the AAS,

m is the slope of the line

C is the concentration in mg/L,

b is the y-intercept of the line,

d.f is the dilution factor,

$m_{sample}$is the mass of dried sample of GP

2.4.3.2 Phosphorus by UV-Vis spectrometry

Phosphorus content in GP was determined according to SMEWW, method 4500-P E (SMEWW, 1998)

• Principle

Ammonium molybdate and potassium antimonyl tartate reacts in acid medium with orthophosphate to form a heteropoly acid, phosphomolybdic acid, that is reduced to intensely colored molybdenum blue by ascorcic acid.

• Equipment and material

Double-beam ultraviolet visible spectrophotometer, Hitachi U-3210

Glass material: volumetric flasks 50 mL and pipettes

- Reagents

Sulfuric acid 5N: 70 mL of concentrated H_2SO_4 dilluted to 500 mL with distilled water

Pottasium antimonyl tartate solution: 1.3715 g $K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$ dissolved in 400 mL deionised water in a 500 mL volumetric flask and diluted to volume

Ammonium molybdate solution: 20 g of $(NH_4)_6Mo_7O_{24}$ was dissolved in 500 mL deionised water

Ascorbic acid 0.1 M: 1.76 g ascorbic acid was dissolved in 100 mL water

Mixed solution: a solution from sulfuric acid 5N, pottasium antimonyl tartate solution, ammonium molybdate solution, ascorbic acid 0.1 M was prepared in the proportion of 50:5:15:30 respectively in a 100 mL volumetric flask.

NaOH 1.0 M

Phenolphthalein 2%

- Procedure

The concentrated sample solutions prepared in section 2.4.3.1 (AAS) were diluted appropriately (Table 2.3) and 25 mL of the diluted solution was transferred to a volumetric flask of 50 mL. One drop of phenolphthalein and some drops of NaOH were added until the solution became pink and it was adjusted to volume with water. Over this volume 2 mL of the Mixed solution were added. After 17 min the absorbance was read in a spectrophotometer at 880 nm.

- Calculation

The calculations were made as in the other elements, except of the following formula:

$$mg/kg = \frac{C_{mg/L} \times 0{,}025\ L}{m_{sample}(g)} \times 1000$$

where:

C is the concentration in mg/L,

m_{sample} is the mass of dried sample of GP

2.4.4 Kjeldahl nitrogen determination

The Kjeldahl nitrogen was determined according to SMEWW, method 4500-N_{org} C (SMEWW, 1998)

• Principle

In the presence of concentrated sulfuric acid (H_2SO_4), potassium sulfate (K_2SO_4) and selenium catalyst, amino nitrogen of organic material is converted to ammonium. Free ammonia is also converted to ammonium. After addition of base, the ammonia is distilled from an alkaline medium and absorbed in boric acid. The ammonia borate complex is determined by titration with standardized hydrochloric acid solution (HCl).

$$NH_3 + H_3BO_3 \rightarrow NH_4^+ : H_2BO_3^- + H_3BO_3$$

• Equipment and material

Digestion unit, Tecator 2006

Distilling unit, Kjeltec ™ 1002 System

Glass material: kjeldahl tubes, borosilicate glass flask, burette

• Reagents

Kjeltabs S/3.5 with a composition of 3.5 mg Selenium (Se) as a catalyst and 3.5 mg K_2SO_4 to prevent sample bumping (173348.1213, Panreac)

Concentrated H_2SO_4 36 N, 18M

Hydrogen peroxide, 30% w/v (VWR chemicals)

Deionised water

NaOH 40% w/v: 1.0 kg of NaOH dissolved in 2.5 L water

Boric acid solution 4% w/v with indicators: 40 mg H_3BO_3 dissolved in water, added 10 mL of the mixed indicator solution and diluted to 1L

Mixed indicator solution: 200 mg of methyl red dissolved in 100 mL ethanol and 100 mg of methylene blue dissolved in 50 mL of ethanol. Then the two solutions were mixed.

HCl 0.1M (0.0970 M from the standardization) (HCl 37% w/v Fischer Scientific)

Na_2CO_3 0.01 M: 0.2503 g of previously dried Sodium carbonate was dissolved in 200 mL deionised water

Methyl orange 0.5g/L

• Procedure

Dried grape pomace sample, about 1 g, was placed in a Kjeldahl tube with the addition of 10 mL of deionized water. Two Kjeltabs, 12.5 mL concentrated H_2SO_4 and 5 mL of hydrogen peroxide was added to each tube. Sample digestion was done at 420°C, until a clear solution was obtained which ensured complete oxidation of all organic matter. The digest was diluted with 75 mL of deionised water and the Kjeldahl tubes were attached to the unit for distillation. After addition of 50 mL NaOH 40%, sample distillation started until collecting the released ammonia (300 mL) into boric acid solution (25 mL) with indicators. Borate anion ($H_2BO_3^-$:NH_4^+) (proportional to the amount of nitrogen) was titrated with HCl standardized solution. A reagent blank was run simultaneously.

• Calculation

Kjeldahl nitrogen content was calculated using the following formula:

$$\% \, \mathrm{N} = \frac{\mathrm{V} \times \mathrm{C} \times 10^{-3}}{\mathrm{m_{sample}}} \times 14.007 \times 100$$

The value obtained for nitrogen content was converted into protein with the factor of 6.25 according to (González-Centeno M.R., 2010):

$$\% \, \mathrm{protein} = \%\mathrm{N} \times 6.25$$

where:

V is the mL of HCl used in sample titration - mL of HCl used for blank titration

C is the normality of HCl

m_{sample} is the weight of dried sample

2.4.5 Determination of total fat

Total fat was determined according to method 948.22- 40.1.05 (AOAC, 1995)

• Principle

The method is based in a gravimetric quantification. The sample was firstly hydrolyzed with HCl followed by solid-liquid extraction in a Soxhlet apparatus. A constant flow of organic solvent over the solid was used. The boiling solvent condenses and passes the tissues several times thereby extracting the lipids. After a suitable time the process is stopped, the solvent evaporates and the fat is weighted

- Equipment and material

Soxhlet extractor, JP SELECTA s.a

Rotary evaporator Büchi, Flawil

Hot plate

Buchner funnel

Filter paper (Fisher Brand QL100, 90 mm and 185mm)

Glass material: planned bottom flasks (250 mL), erlenmeyer flask boro 3.3, 250 mL, filtering flasks of 500 mL, watch glasses

- Reagents

HCl solution 4N

HCl 37% w/v (Fischer Scientific),

Deionized water

Petroleum ether (Fischer Scientific, boiling point range 39.0-53.8^{o}C)

- Procedure

Dried grape pomace sample, about 10 g, was placed in a flask with 50 mL of HCl 4.0 N and it was covered with the watch glass and it was heated for one hour. After hydrolysis, the samples were filtrated and the filters were washed with boiled deionized water. The filters with the samples were dried at 103°C for 2 hours and added to the Soxhlet apparatus. The lipid extraction was completed using petroleum ether (sample to solvent ratio of 1:20 w/v) for six hours. The solvent was evaporated in a rotary evaporator and the flasks with residue were then dried (two to four hours) in the oven (103°C) and weighed to calculate the lipid content.

$$\%\ Lipid\ content = \frac{m2_{flask\ (g)} - m1_{flask\ (g)}}{m_{sample\ (g)}} \times 100$$

where:

$m1_{flask}$ is the initial weight of empty flask in g

$m2_{flask}$ is the weight of the flask with dry residue after the lipid extraction in g

m_{sample} is the weight of dry sample

2.4.6 Determination of total carbohydrates

The carbohydrates content were determined by the method developed by the author James (JAMES, 1995)

• Principle

The available carbohydrates content was firstly hydrolyzed to reducing sugars. Added, alkaline 3,5-dinitrosalicylic acid (DNS) forms a red-brown reduction product, 3-amino-5-nitrosalicylic acid, when heated in the presence of a reducing sugar. The intensity of the colour developed at 540 nm may be used to determine the available carbohydrates content.

• Equipment and material

Double-beam ultraviolet visible spectrophotometer, Hitachi U-3210

Water bath

Glass material: volumetric flasks of 100 mL, test tubes, pipettes

• Reagents:

H_2SO_4 1.5 M (concentrated H_2SO_4 Fisher Scientific S/9240/PB17)

NaOH 10% w/v

NaOH 2 M

Deionized water

Dinitrosalicylic reagent (3,5 dinitrosalicylic acid 98%, Acros organics):

Solution 1: 5 g DNS were dissolved in hot water and 100mL NaOH 2M were added.

Solution 2: 150 g of sodium potassium tartarate were dissolved in 250 mL of water and heated. Then solution 1 and 2 were mixed in a 500 mL volumetric flask and the volume adjusted with water.

Stock glucose solution 5 g/L (D(+)-glucose anhydrous, extra pure, Riedel-de Haën®)

• Procedure

Dried grape pomace sample, about 0.5 g, was placed in test tubes in the presence of 10 mL H_2SO_4 1.5 M and heated in a water bath for 40 min at 100 °C stirring occasionally, to hydrolyze polysaccharides and other non-reducing sugars. After samples have cooled to room temperature, 12 mL of NaOH 10 % was added carefully. The samples were stirred and filtered into 100 mL volumetric flask, by washing the tubes into the flask with

deionized water. The volume was made up by deionized water and mixed well by inversion. A volume of 1.0 mL from the hydrolyzed samples was transferred by pipette into a test tube and it was added 2.0 mL of water and 1.0 mL of DNS reagent and heated in a boiling water bath for 20 min to allow the reaction between the reducing sugars and DNS to occur. After cooling, each volume was adjusted to 20 mL accurately with deionized water and stirred well. The absorbance of each solution was read at 540 nm.

The construction of the standard curve was made with the following concentrations 0.25; 0.5; 1.0; 1.25 and 1.5 g glucose per L. The standard glucose solutions and the blank were running simultaneously with the samples after the hydrolysis step.

•Calculation:

Referring to the calibration curve of glucose, it was obtained an equation of first degree:

$$Abs = m \times C + b$$

From the equation above, was calculated the concentration (g/L) using the formula:

$$C\ (g/L) = \left[\frac{Abs_{sample} - b}{m}\right]$$

Then, for presenting the results in % dry matter, it was used the following formula:

$$\% = \frac{C_{g/L} * V\ (L)}{m_{sample}} \times 100$$

Where:

Abs is the absorbance obtained from the spectrophotometer,

m is the slope of the line

C is the concentration in g/L,

b is the y-intercept of the line,

V is the volume for dissolving the hydrolyzed samples in L (0.1 L)

$m_{sample}$is the mass of dry sample

2.4.7 Determination of crude fiber

Crude fiber was determined according to Commission Regulation (EC) No. 152/2009 laying down the methods of sampling and analysis for the official control of feed. (EC152/2009, 2009)

- Principle

The sample is treated successively with boiling solutions of sulphuric acid and potassium hydroxide of specified concentrations. The residue is separated by filtration on glass filters, washed, dried, weighed and ashed within a range of 475 to 500 ^{o} C. The loss of weight resulting from ashing corresponds to the crude fiber present in the test sample.

- Equipment and material

Drying oven line function Heraeus Model UT 6060

Muffle furnace line function Heraeus, Tmax = 1100 °C, Model M110

Heating mantle

Buchner funnels

Crucibles

Glass filter (Whattman, GF/C, 47mm), heated at 500 ^{o}C for four hours

Paper filters (Fisher Brand QL100, 185mm)

Glass material: round bottom flasks of 250 mL, condensers, filtering flasks

- Reagents

Sulphuric acid, 0.13 mol/L: 22 mL of concentrated H_2SO_4 dissolved in 3 L of water

Potassium hydroxide solution, 0.23 mol/L: 38,72g of KOH dissolved in 3 L of water

Acetone

Petroleum ether (Fischer Scientific, boiling point range 39.0-53.8^{o}C)

- Procedure

Dry grape pomace sample, about 1 g, was placed in a round bottom flask. It was added 150 mL of diluted sulfuric acid in the flask. The heating unit was assembled and the condenser was attached to the flask. The liquid was brought to boil within 5 ± 2 minutes and boiled vigorously for exactly 30 minutes. After, the sulphuric acid solution was filtered through vacuum with a Buchner using the conditioned filters. The residue was washed with three consecutive 30 mL portions of boiling water, ensuring that the residue is filtered dry after each washing. The filters with the samples were transferred again to the flask and 150 mL of potassium hydroxide solution was added. The flask was assembled again and boiled vigorously for exactly 30 minutes. The filtration step was repeated by using a second filter and the washing procedure was done with three consecutive 30 mL portions of boiling water and 25 mL portions of acetone ensuring that

the residue filtered dried after each washing. The two filters with the sample were transferred to conditioned crucibles (previously heated at 500 ^{o}C and weighted), dried in the oven at 130 ^{o}C and weighted to constant weight. The crucibles were placed in a muffle furnace to ash the content to constant weight (loss in weight between two successive weightings must be less than or equal to 2 mg) at 500^{o}C for at least 1h.

- Calculation

The crude fibre content is given by the expression as a percentage of dry sample:

$$\%\ crude\ fiber = \frac{m1_{crucible\ (g)} - m2_{crucible\ (g)}}{m_{sample\ (g)}} \times 100$$

where: $m1_{crucible}$ is the weight of the crucible after drying at 130 ^{o}C in g

$m2_{crucible}$ is the weight of the crucible after drying in 500 ^{o}C in g

m_{sample} is the weight of dry sample

2.4.8 Determination of Chemical Oxygen Demand (COD)

The chemical oxygen demand was determined by the method 5220 D from the standard methods for the examination of water and wastewater (SMEWW, 1998).

- Principle

The COD analysis indicates the equivalent amount of oxygen to be consumed in the oxidation under certain conditions of oxidable organic substances. In this method a strong oxidant is used, potassium dichromate ($K_2Cr_2O_7$) and the oxidation is carried out under strongly acid medium (H_2SO_4), in the presence of silver sulfate (Hg_2SO_4) as a catalyst, and high temperature (148^{o}C). During the oxidation the organic matter is transformed into water and carbon dioxide (CO_2) and the chromium hexavalent is reduced to trivalent as the reaction below:

$$C_aH_bO_c + dCr_2O_7^{2-} + 8H_{(aq)}^{+} \rightarrow 2dCr_{(aq)}^{3+} + aCO_2 + (d + \frac{8d}{2})H_2O$$

Where $d = \frac{2}{3}a + \frac{1}{6}b - \frac{1}{3}c$

In this analysis, the amount of chromium necessary in the oxidation of organic matter is measured spectrophotometrically. In the high range, 50-800 mg COD/L, measures the

formation of trivalent chromium and in the low range 5-50 mg COD/L, measures the loss of hexavalent chromium.

- Interferences and limitations

Compounds like pyridine and saturated hydrocarbons are resistant to oxidation. Volatile organic compounds are not oxidized to any great extent, since they are present in the vapor spaces and are not being in contact with the oxidizing liquid. Volatile compounds can be lost during the mixing of reagents and sample, before closing the tube. This can be avoided by careful addition of the sample in order to form two phases, avoiding the continuous heating of the solution until closing the tube. Linear chain aliphatic compounds like fatty acids and low molecular weight alcohols are not oxidizable in great extent. For this oxidation silver sulfate is used, as an oxidation catalyst, chlorides, bromides and iodides may precipitate with silver ion taking of the catalytic properties as well. For instance the chlorides are eliminated by the addition of silver sulfate in excess, this addition results in a soluble complex of mercury chloride. The presence of chlorides is harmful, since it can reduce the dichromate to chromium (III). The oxidation of inorganic ions such as nitrites, sulfites and iron (II) sulfide which reduce chromium (VI) to chromium (III), is eliminated by adding sulfamic acid. Photometric interference due to turbidity with precipitate formation can be removed by centrifugation or titrating with ferric ammonium sulphate. The ammonia only can be oxidized in the presence of significant concentration of chloride ions

- Equipment and material

Double-beam ultraviolet-visible spectrophotometer, Hitachi U-3210

Digestor of COD, C9800 reactor Hanna instruments

Glass material: pipettes and tubes 16 x 100 mm

- Reagents

Digestion solution for high range determination: potassium dichromate ($K_2Cr_2O_7$), 10.216 g, primary standard grade, previously dried at 103^{o}C for two hours, concentrated sulfuric acid (H_2SO_4) 167 mL and mercuric sulfate (Hg_2SO4) about 33.3 g, were added in approximately 750 mL of water. The mixture was stirred, until dissolved, cooled to room temperature and diluted to 1000 mL.

Potassium hydrogen phthalate standard: potassium hydrogen phthalate 0.851 g, previously dried at 120°C, dissolved in 1,000 mL of water 1.00 mL = 1.0 mg O_2 (COD)

Catalyst solution: silver sulfate (Ag_2SO_4) about 10 g dissolved in 1L of concentrated sulfuric acid

- Procedure

Reaction mixture preparation: in digestion tube, 1.5 mL of digestion solution and 3.5 mL of catalyst solution were added and mixed. Dried sample, about 0.0015 g, was placed in the tube and then it was added 2.5 mL of water and mixed. The tubes were placed in the digester for two hours at 148°C. After, they were cooled for ten minutes until room temperatures and mixed. Each sample and standard was measured in a spectrophotometer at 600 nm. A standard curve with the following concentrations was prepared: 50; 100; 200; 400; 600; 800 and 1000 mg 0_2/L. Each standard was added in the tube with the same procedure as for the sample, adding 2.5 mL of each standard solution instead of water.

- Calculation

Referring to the calibration curve it was obtained an equation of first degree:

$$Abs = m \times C + b$$

From the equation above, it was calculated the concentration (mg/L) using the formula:

$$C\ (mgO_2/L) = \left[\frac{Abs_{sample} - b}{m}\right]$$

The concentration was then introduced in the following formula to obtain the results in mg O_2/g DM sample:

$$\text{mg } O_2/\text{g DM} = \frac{C\ (mgO_2/L) \times V(L)}{\text{m}_{\text{sample}}(\text{g})}$$

Where:

Abs is the absorbance obtained from the spectrophotometer,

m is the slope of the line

C is the concentration in mg/L,

b is the y-intercept of the line,

V is the volume in L for the sample dissolution ($2.5x10^{-3}$ L)

m_{sample} , mass of dry sample in g

2.5 Polyphenols characterization

Fresh grape pomace samples, stored at -20°C, were taken directly from the freezer to the lyophilizer without defrosting and after lyophilized they were milled. In Figure 2.5 is presented the procedure that was followed for all studies of identification and quantification of polyphenols in the GP samples.

After extraction it was obtained the dry residue (m1), which after weighting was immediately re-dissolved to protect from degradation and stored at -20°C. The frozen sample was brought to RT and 2.0 mL of solution were vacuum dried to give m2. This mass was dissolved exactly to 50 mL in methanol and these concentrated sample solutions were used to prepare diluted solutions for the determinations of TPC and antioxidant activity as described in procedures. For HPLC identification and quantification of polyphenols, the total mass of m1, subtracted the m2 (taken for TPC and DPPH), was diluted to 25 mL, except for TVP and TVG which were diluted to 50 mL. These concentrated sample solutions were used to prepare HPLC injection solutions.

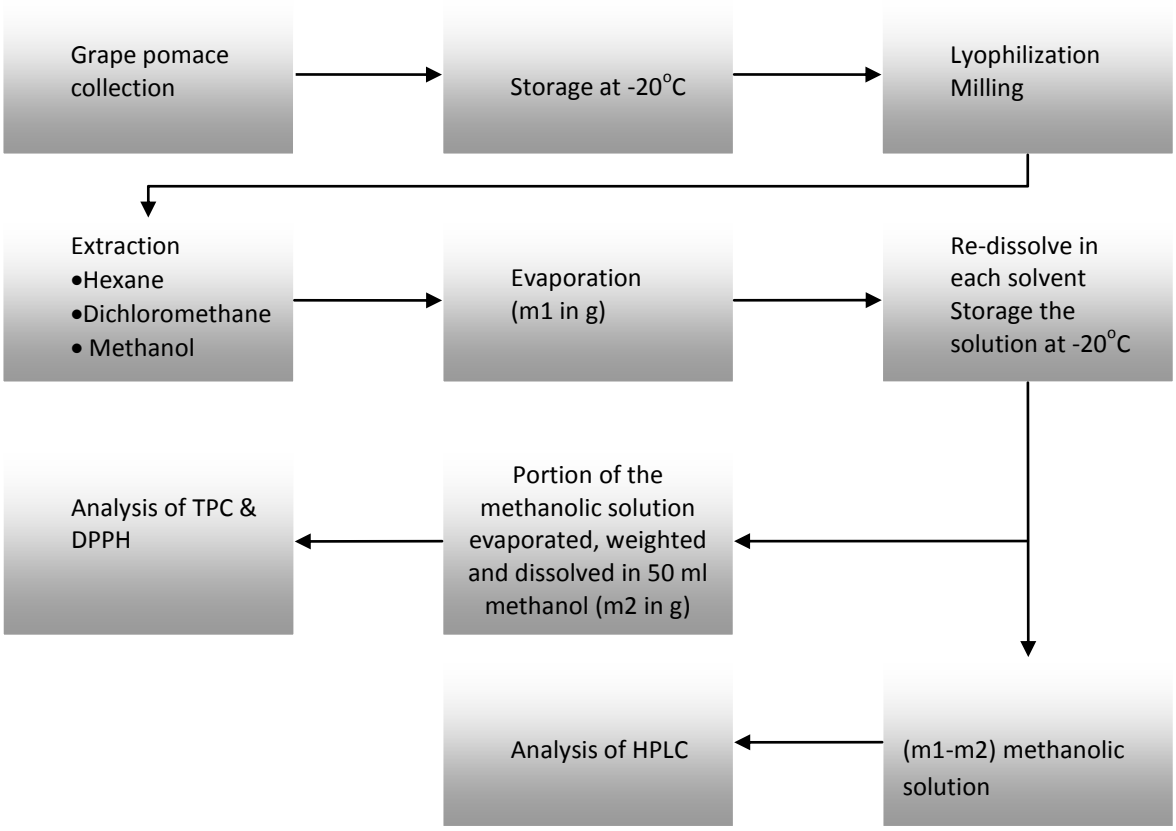

Figure 2.5 Flow diagram of GP processing, for the entire characterization of polyphenols

2.5.1 Polyphenols extraction

The extractions were made according to author Shoeb (Shoeb Mohammad, 2005).

• Principle

A Soxhlet extractor is a laboratory apparatus invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. However, a Soxhlet extractor is not limited to the extraction of lipids. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent.

• Equipment and material

System of soxhlet extractor, JP SELECTA s.a

Rotary evaporator Büchi, Flawil

Drying oven line function Heraeus Model UT 6060

Thimble for Soxhlet extractor, filter paper (QL 100, 185mm Fisher Scientific)

Glass material: planned bottom flasks of 250 mL

• Reagents

n-hexane 95% (J.T.Baker, analytical reagent standards)

Dichloromethane (J.T.Baker analytical reagent standards)

Methanol (Riedel-deHaën, Analytical Reagent and J.T Baker analytical reagent standards)

• Procedure:

Lyophilized grape pomace sample, about 20 g, was placed inside a thimble made from thick filter paper, which was loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor was placed onto a 250 mL glass flask with hexane as the first extraction solvent (220 mL). Dichloromethane and methanol also 220 mL each were used as the second and third extraction solvents by this order. The Soxhlet was then equipped with a condenser. Each solvent was heated to reflux for 6h at the temperature of 60, 40 and 60^{o}C, respectively. After extraction, the solvent was removed, in a rotary evaporator, yielding the extracted compounds. After weighting (m1) the solid residue was re-dissolved immediately in methanol and stored at -20^{o}C until analyses. After the three solvent extractions the non-soluble portion of the extracted solid remaining in the thimble, was collected for further analyses (phosphorus, proteins and heating value).

• Calculation:

$$\%\ yield = \frac{m2_{flask\ (g)} - m1_{flask\ (g)}}{m_{sample\ (g)}} \times 100$$

where:

$m1_{flask}$ is the initial weight of empty flask in g

$m2_{flask}$ is the weight of the flask after each solvent extraction (hexane, dichloromethane, methanol) in g

m_{sample} is the weight of dry sample

2.5.2 Total phenolic content- Folin & Ciocalteu

The total phenolic content in GP was determined by the Folin-Ciocalteu colorimetric method. (Lafka Theodora-Ioanna, 2007)

• Principle

The colorimetric total phenolic assay with Folin & Ciocalteu reagent relies on the transfer of electrons, in alkaline medium, from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes, which act as oxidant. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The exact chemical composition of the reagent is not known, but the reaction that occurs is the reduction from Mo (VI) to Mo (V) by transfer of one electron.

• Equipment and material

Double-beam ultraviolet– visible spectrophotometer, Hitachi U-3210

Glass material: volumetric flasks of 25 mL

• Reagents

Folin & Ciocalteu reagent, 2M (Sigma Aldrich)

Saturated solution of Na_2CO_3 (anhydrous, J.T.BAKER),

Deionized water,

Gallic acid monohydrate (Sigma Aldrich),

Methanol (Riedel-deHaën, analytical reagent)

• Procedure:

Methanolic GP extract (m2 in 50 mL) was appropriately diluted (Table2.4) and 0.5 mL of this solution was transferred to a volumetric flask of 25 mL and it was added 20 mL deionized water and 0.625 mL of Folin–Ciocalteu reagent. After mixing, it was left standing for 3 min and then 2.5 mL of saturated solution of Na_2CO_3 was added. The content was mixed again and diluted to volume with deionized water. After two hours, the absorbance of the sample was measured against a blank by using a spectrophotometer. Gallic acid served as the standard for preparing the calibration curve with the concentrations of 10; 25; 50; 75; 100; 125; 150; 175; 200; 250 and 300 mg/L, following the same procedure as the samples(0.5 mL of standard solutions and blank in a volumetric flask of 25 mL).

Table 2.4 Dilution factor for the TPC determination

Sample	BLE	BLM	BLP	BLG	TVE	TVM	TVP	TVG
d.f	2	20	10	20	4	20	2	2

• Calculation

The calibration curve of gallic acid was obtained as an equation of first degree:

$$Abs = m \times C + b$$

From the equation above, was calculated the concentration (mg/L) using the formula:

$$C\ (mg/L) = \left[\frac{Abs_{sample} - b}{m}\right] \times d.f$$

The following formula was used to obtain the results in mg gallic acid equivalent/g DM:

$$\text{mg GAE/gDM} = \frac{C\ (mg/L) \times V(L) \times (\frac{m_1(g)}{m_2(g)})}{\text{m}_{\text{sample}}(\text{g})}$$

where:

Abs is the absorbance obtained from the spectrophotometer,

m is the slope of the line

C is the concentration in mg/L,

b is the y-intercept of the line,

d.f is the dilution factor

V is the volume in L, for the m_2 dissolution (0.05 L)

m_1 is the mass of the, in g

m_2 is the mass of the, in g

m_{sample} is the mass of the GP lyophilized sample in g, used in the extraction

2.5.3 Antioxidant Activity- DPPH assay

The free radical scavenging activity of the GP fractions was measured in vitro by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described below. (Qian Deng, 2011)

• Principle

The method is based on the molecular absorption of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The solution of this radical, which has a blue color, is measured spectrophotometrically at 517nm. When this solution is added to a substance with antioxidant activity (A-H), then the DPPH radical is reduced with the recruitment of a hydrogen atom or an electron and is converted to 1,1-diphenyl-2-picrylhydrazyl which is colorless (Figure 2.6). This reaction takes place in the dark during 30 min and gives a yellow color, depending on the extension of the reaction.

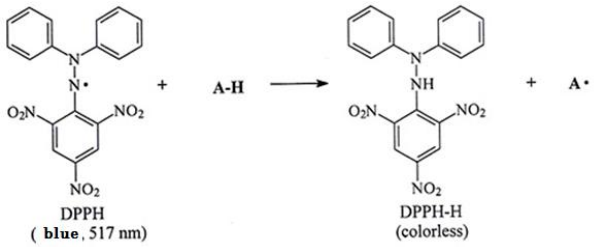

Figure 2.6 Reaction of DPPH reduction in the presence of an antioxidant (AH)
Source: (Medeiros, 2009)

• Equipment and material

Double-beam ultraviolet– visible spectrophotometer Hitachi U-3210

Glass material: volumetric flask

• Reagents:

DPPH solution: 9 mg of DPPH dissolved in 100 mL methanol and then diluted 30/50 (DPPH, Sigma-Aldrich)

(+) L -Ascorbic acid (Panreac)

Methanol (Riedel-deHaën, analytical reagent)

• Procedure:

Methanolic GP extract (m2 in 50 mL) was appropriately diluted (Table 2.5) and 0.5 mL of this solution was transferred in a cuvvette and mixed thoroughly with 1.5 mL of DPPH–methanol reagent and allowed to stand at room temperature for 30 min prior to measure the solution absorbance at 517 nm. Ascorbic acid was used as standard for preparing the calibration curve with concentrations 1.00; 2.50; 5.00; 7.50; 10.00; 20.00; 30.00; 35.00 and 40.00 mg/L followed the same procedure as the samples.

Table 2.5 Dilution factor for the DPPH determination

Sample	BLE	BLM	BLP	BLG	TVE	TVM	TVP	TVG
d.f	5	100	10	100	20	20	2	10

• Calculation

The calibration curve of ascorbic acid was obtained as an equation of first degree:

$$Abs = m \times C + b$$

From the equation above, it was calculated the concentration (mg/L) using the formula:

$$C\ (mg/L) = \left[\frac{Abs_{sample} - b}{m}\right] \times d.f$$

The concentration then introduced in the following formula to obtain the results in mg Ascorbic Acid Equivalent/gDM:

$$\text{mg AAE/g DM} = \frac{C\ (mg/L) \times V(L) \times (\frac{m_1(g)}{m_2(g)})}{\text{m}_{\text{sample}}(\text{g})}$$

where:

Abs is the absorbance obtained from the spectrophotometer,

m is the slope of the line

C is the concentration in mg/L,

b is the y-intercept of the line,

d.f is the dilution factor

V is the volume in L, for the m2 dissolution (0.05 L)

m_1 is the mass of the, in g

m_2 is the mass of the, in g

m_{sample} is the mass of the GP lyophilized sample in g, used in the extraction

2.5.4 Development of HPLC-UV method for qualitative and quantitative polyphenols determination

The identification and quantification of polyphenols was made according to the method described by Iora. (Iora Sandra R. F., 2015)

- Principle

High-performance liquid chromatography (HPLC) is a separation technique that can be used with mixtures of organic molecules and ions using various detectors. HPLC is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. It involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases.

- Equipment and material

The system used to perform the analyses of polyphenols in this work was HPLC equipment from Hewllett Packard Series 1100 with a high pressure pump, chamber mixing with four channels (P4000), a degasser, a UV-Vis detector and an injector. The software package used for processing the data was Chemstation version Hewlett Packard 1998. The column used was from Agilent company, type Hypersil ODS – C18 with a 250 mm x 4,6 mm internal diameter and particle diameter 5µm.

Ultrasonic bath, SONICA S3- SCANSCI

Micro glass fiber paper (Munktell Filter AB, 0.47µm)

Disposable hydrophilic syringe filter unit (Advantec, 0.20 µm)

Syringe 5 mL

Glass materials: buchner funnels, volumetric flasks of 10 mL, pipettes, filtering flasks

- Reagents

Phosphoric acid (Merck, 85% w/v)

Methanol (Fisher Scientific, HPLC grade)

The following standard compounds were used for assays and were purchased from Sigma-Aldrich: 1) gallic acid, 2) epicatechin, 3) (+) – catechin, 4) syringic acid, 5) caffeic acid, 6) p-coumaric acid, 7) *trans*-resveratrol, 8) ferrulic acid and 9) quercetin.

Mobile phase:

The preparation of solvents for the mobile phase followed the rules by vacuum filtration on a Buchner funnel with micro glass fiber paper, 0.47µm, and an ultrasonic bath was applied to degas.

Solvent A: aqueous buffer phosphoric acid, 1.0% v/v. Dissolved 11.8 mL H_3PO_4 in 1L deionized water nanopure purity.

Solvent B: methanol

Solvent C: deionized water nanopure purity

The solutions of standards were prepared in the optimum concentration range for calibration curves by diluting stock solutions (250 mg/L) in methanol. The standard solution concentrations of each compound are listed in Table 2.6:

Table 2.6 Solution concentrations for calibration curves of each phenol

Phenolic compounds	**Concentration mg/L**									
Gallic acid	0.514	0.771	1.29	2.57	5.14	10.3	20.6	51.4	103	257
(+)Catechin	0.600	0.900	1.50	3.00	6.00	12.0	24.0	60.0	120	300
(-)Epicatechin	0.824	1.24	2.06	4.12	8.24	16.5	33.0	82.4	165	412
Syringic acid	0.406	0.609	1.02	2.03	4.06	8.12	16.2	40.6	81.2	203
Caffeic acid	0.406E-01	0.812E-01	0.203	0.406	0.609	1.02	2.03	4.06		
p-Coumaric acid	0.296E-01	0.592E-01	0.148	0.296	0.444	0.740	1.48	2.96		
Ferrulic acid	0.316E-01	0.632E-01	0.158	0.316	0.474	0.790	1.58	3.16		
trans-Resveratrol	0.408E-01	0.816E-01	0.204	0.408	0.612	1.02	2.04	4.08		
Quercetin	0.398E-01	0.798E-01	0.199	0.398	0.597	0.995	1.99			

- Procedure

Methanolic GP extract (m1-m2 in 25 mL, except of TVP and TVG, section 2.5) was transferred to a 10 mL volumetric flask and diluted to volume with methanol (dilution factor of 100). The solution was stirred, filtered with disposable syringe filters and kept in the dark.

Each separation of phenolic compounds from GP extracts was made with a gradient elution during 70 min. The gradient elution applied is presented in Table 2.7. The volume of sample introduced in the injector was 50 µL with a loop of 20 µL. The flow of solvent remained constant at 1.0 mL/min during the analysis. The absorbance of the components was monitored at three different wavelengths, 280 nm, 320 nm and 370 nm in three

independent runs. The net time of the analysis was 65 min but the total time of the running was approximately 70 min, because after the separation it restores the original solvent ratio (80% A and 20% C) for 5 min in order to equilibrate the column prior to the next running.

Table 2.7 Mobile phase gradient of the HPLC method

Time (min)	Flow mL/min	Solvent A%	Solvent B %	Solvent C%
0	1	80	0	20
2	1	65	15	20
5	1	55	25	20
10	1	50	30	20
15	1	45	35	20
25	1	30	50	20
30	1	20	60	20
35	1	0	80	20
45	1	0	90	10
65	1	0	100	0
70	1	80	0	20

- Calculation:

Referring to the calibration curve of each phenolic standard it was obtained an equation of first degree:

$$Abs = m \times C + b$$

From the equation above, it was calculated the concentration (mg/L) using the formula:

$$C\ (mg/L) = \left[\frac{Abs_{sample} - b}{m}\right] \times d.f$$

This concentration was then introduced in the following formula to obtain the results in mg phenolic compound/g DM:

$$\mathrm{mg_{phenolic\ compound}/g\ DM} = \frac{C\ (mg/L) \times V(L)}{(\frac{\mathrm{m_1(g)} - \mathrm{m_2(g)}}{\mathrm{m_1(g)}}) \times \mathrm{m_{sample}(g)}}$$

where:

Abs is the absorbance obtained from the spectrophotometer,

m is the slope of the line

C is the concentration in mg/L,

b is the y-intercept of the line,

d.f is the dilution factor

V is the volume in L, (0.025 L, except of TVP and TVG, where is 0.05 L)

m_{sample} is the mass of the lyophilised sample in g

2.6 High heating value

- Principle

Heat released in a chemical reaction can be determined experimentally by using an adiabatic bomb calorimeter, as the case of heat of combustion. The reaction must proceed without any side reactions and sufficiently fast, so the heat exchange with the surroundings is negligible. In such calorimeter, the combustion reaction occurs in a closed container under constant volume ("bomb"). The bomb is immersed in a weighted quantity of water and surrounded by an adiabatic shield that serves as a heat insulator. Continuous bath stirring ensures that heat is distributed evenly in the calorimeter. The bomb and the water bath, which are in direct thermal contact, constitute an adiabatic bomb calorimeter and the increase in the water temperature is used to calculate the heat of the reaction.

- Equipment and material

Parr Calorimeter 6772 (Figure 2.7 A)

Press pellet, Parr (Figure 2.7 B)

Muffle furnace line function Heraeus, Tmax = 1100 °C

Wire (Parr)

- Reagents:

Deionized water

Benzoic acid (one gram pellets standardized for bomb calorimeter, Parr)

Benzoic acid (Panreac)

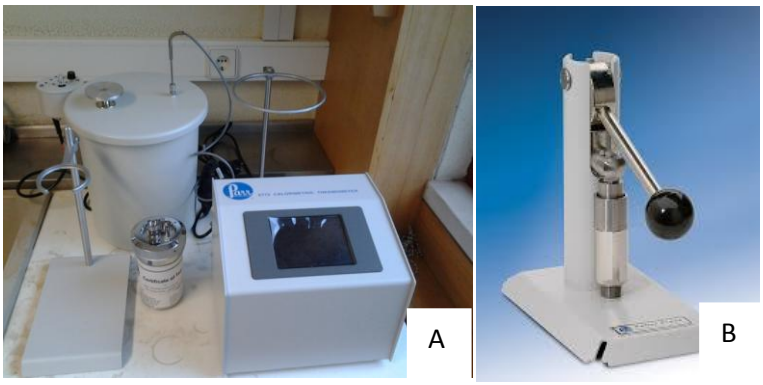

Figure 2.7 (A) Parr Calorimeter 6772 (B) Press pellet

• Procedure

Grape pomace sample, about 0.5 to 1.0 g, was placed in the pellet press (Figure 2.7 A) to preapre the pellet. The final pellet was weighted accurately. Carefully, it was placed in the sample cup with tweezers (Figure 2.8 B). It was measured approximately 10 cm of ignition wire and attached to the electrodes (Figure 2.8 A) and each cap was slide downward to complete the connection. The sample cup (with the sample sitting in the center) was placed in the cup holder and the wire bended in a V-shape so that it almost touches the surface of the pellet (about 1 mm separation) and do not touch the cup surface. Figure 2.8 B illustrates the proper installation and sample placement. Deionized water (1.0 mL) was pipetted into the bomb to absorb the oxides of nitrogen and sulfur formed from nitrogen and sulfur present in air and sample.

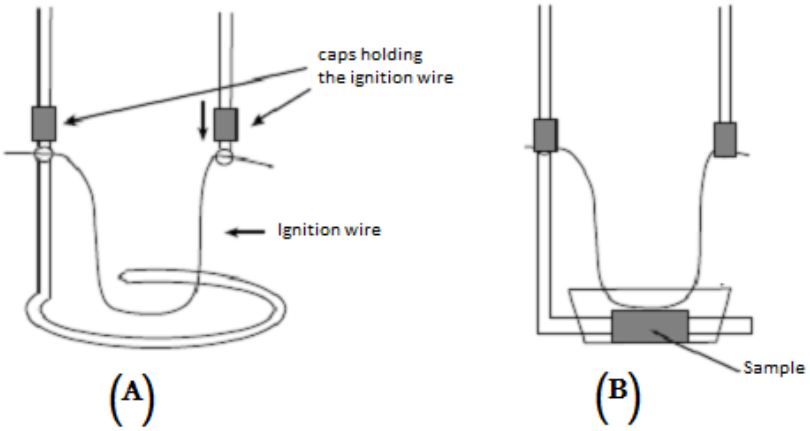

Figure 2.8 (A) Attachment of the ignition wire.(B) Schematic of the sample support stand.

(Jussi, 2010)

Care must be taken not to disturb the sample when sealing and charging the bomb. The head assembly slides into the bomb cylinder, followed by screw opening the vent cap on the head assembly to allow air to be expelled, and the head pushed down and after the vent cap was tightly closed. Later on, the oxygen connection was installed by secure the bomb in the bench clamp and slipped on the oxygen tank connection hose to the pin on the head assembly. The oxygen tank valve was opened to fill the bomb slowly by watching the gauge in the oxygen bottle as the bomb pressure was rising to the desired filling pressure (30 atm). Once this pressure was reached, the control valve and

then the tank valve were closed. The filling of the bomb should not be very fast because it can blow the sample out of the sample cup.

After the bucket was filled with 2,000 ± 0.5 g of water it was checked to see that it is resting properly in the jacket, confirming the four pegs on the bottom of the jacket, which hold the bucket in place. Carefully the charged bomb was placed in the bucket, noting that it rests on the raised circular area on the bottom of the bucket and making sure that there are no oxygen leaks, by watching if there are bubbles through the warer. The ignition wires were connected to the terminal socket on the bomb head, avoiding touching with the fingers the water. The cover on the jacket was placed and the screw attached to the lid fitted into the screw hole in the ledge of the jacket. Finally the stirrer was turned on by hand to be sure that it runs freely, and then the drive belt slipped onto the pulley. In the operating mode of the equipment it should be selected start, to enter the sample ID, the Bomb ID and the sample weight. In some samples it is also needed the spike weight. If a sample does not ignite easily or press well, then the spiking method of ignition can be used to promote complete combustion of the sample. The heat produced by the combustion aid must be subtracted from the total energy release.

In this work a known amount of benzoic acid was added with the sample as a medium to compress easily the sample and make pellet. The benzoic acid is removed from the calculation of the heating value. When the calorimeter was finished any unburned fuse wire length still attached to the electrodes was measured and subtracted from 10 cm, in the calculation for the heating value. Also after the end of each analysis the cup was heated in a muffle furnace, maintained at 550 ^{o}C till a constant weight to weight the unburned sample. Ten independent measurements of the benzoic acid standard were carried out and six independent measurements of each GP samples.

• Calculation:

The heat produced by the reaction $q_{rxn} = -(q_{water} + q_{bomb})$ can be accurately determined by measuring the temperature increase in the known amount of surrounding water. This result obtained automatic from the equipment.

2.7 Statistical analyses

In this study three types of statistical analysis were carried out. For the comparison between the same fraction from the two varieties (e.g. BLE versus TVE) a t-test was used after the evaluation of homogeneity variances of the two samples. A paired t-test was used for the comparison for a lot of pairs, between the same fraction from the two varieties when the results were very different between one pair to another. Finally, the comparison of averages of three or more samples (e.g. BLE versus BLG versus BLP) in the same variety, was made using ANOVA followed by the Tukey's post hoc test. Significant differences were defined for a $p < 0.05$ and very significant differences were defined for a $p < 0.01$. The calculations were made with XLSTAT 2014.4.10 add-in (Addinsoft, Paris, France) for Microsoft Excel 2010 (Microsoft, Redmond, United States of America).

3 Results and Discussion

Considering the different studies on grape pomace in the literature, this study is innovative about these varieties, Loureiro and Vinhão. Two varieties of GP, composed of stalks, skins and seeds collected from Adega as fresh samples were studied, however the mixture (seed and skin) was also studied because the main results presented in the literature are about the mixture. The analyses of fiber, TPC and DPPH were done in duplicate, while the analysis of HHV was done in six replicates. For the identification and quantification of polyphenols by HPLV-UV, one injection was done for the wavelength of 320nm and 370nm. The analyses in the wavelength of 280 nm were made with two different sample concentrations and a third injection was applied with a spike addition. Because only this last situation, analysis at 280nm, was made with replicates, all the results were expressed without standard deviation. All the other analyses were made in triplicate. Results were expressed as mean ± standard deviation ($\bar{x} \pm sd$) .

The results for the physicochemical characteristics of GP were compared by the statistical methodology described in section 2.7. For moisture and major components, it will be presented the statistical comparison between the three fractions in each variety, and also the comparison between the same fractions from both varieties. For the minerals, total phenolic content, antioxidant activity and HHV, the statistical comparison was applied between the same fractions from both varieties.

3.1 Physical characteristics of Loureiro and Vinhão GP

Loureiro is a very vigorous, high-yielding variety of grape that has only recently been recognized as "noble". The bunches are elongated and relatively compact, bearing medium-sized, yellowish-greenish grapes. Vinhão is famous for its biting acidity and dark red grapes, opaque color; it is the most-planted grape of the denomination "Vinho Verde" in Minho region (wikipedia, 2015). In Figure 3.1 are presented some pictures of the three fractions, stalk, skin and seed, of each variety, which characteristics are in accordance with the description from the literature. However the determination of the yield of seed and skin on each variety was not made in this study, but some information from the literature, about other varieties. will be presented in section 4.1.

Figure 3.1 Different parts of white and red GP from Adega Cooperativa de Ponta da Barca

3.2 Moisture and major nutrients content in two varieties of grape pomace, Loureiro and Vinhão

The determination of moisture content in GP is very important for the evaluation of safe storage as raw material for the industrial processes, to prevent undesired changes and growth of fungi and other microorganisms. In industrial applications, moisture content has economic impact from the costs of transportation and the yield of the processes. The results for moisture content and statistical comparison in the fresh GP are presented in Table 3.1 and 3.2. These results refer to the samples collected immediately after the winemaking as it is described in section 2.3.

Table 3.1 Moisture content of fresh grape pomace samples (% w/w)

Sample	Moisture
BLE	72.8 ± 0.20
BLM	55.8 ± 0.55
BLP	68.8 ± 0.66
BLG	42.2 ± 0.09
TVE	66.8 ± 0.80
TVM	57 ± 1.4
TVP	62.0 ± 0.87
TVG	44.5 ± 0.88

Table 3.2 Statistical analyses fror the results of moisture and major components

Analyses	Fractions	Moisture	Ash	Protein	Fat	CH	Fiber	
ANOVA, *p*		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.087	0.0003	Loureiro
Tukey analysis								
	E vs P	0.001	0.001	< 0.0001	< 0.0001	0.78	0.019	
	E vs G	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.085	0.0007	
	P vs G	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.20	0.0003	
ANOVA, *p*		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0008	0.0002	Vinhão
Tukey analysis								
	E vs P	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.030	0.0064	
	E vs G	< 0.0001	0.0001	< 0.0001	< 0.0001	0.0006	0.0002	
	P vs G	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.014	0.0006	
t-test								
	BLE vs TVE	0.00050	0.44	0.0003	0.040	0.45		
	BLP vs TVP	0.0009	0.005	0.0006	< 0,0001	0.94		
	BLG vs TVG	0.17	0.071	0.0057	0.025	0.17		

The fractions of Loureiro GP have very significant differences (p<0.01) with more moisture in the stalks than the skin and finally the seed (72.8%, 68.8% and 42.2%, respectively) and the same was observed on the fractions of Vinhão GP with very significantly differences (p<0.01) with more moisture in the stalks than the skin and finally the seed (66.8%, 62.0% and 44.5%, respectively). When comparing the same fraction between the two varieties of GP, percentages of moisture had very significantly differences (p< 0.01), higher in stalks and skin from Vinhão GP than stalks and skin from Loureiro GP (72.8%, 68.8% and 66.8%, 62.0%, respectively) while moisture content in seed was significantly the same in Loureiro and Vinhão (42.2% and 44.5%, respectively; p= 0.17). The moisture content found in these varieties is in agreement with those found in some other articles (Table 3.4 and 3.5). Specifically, the GP mixture has a value of about 56-57% in which is between the ranges 50-74% found in other studies. These differences could be explained because of the type of grapes, the way the sample is stored in the factory before it is collected as it mentioned in section 2.2, the time of storage etc.

The ash content of a sample represents the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of inorganic matter in a food. Ash

in the two varieties of grape pomace was determined after determination of moisture, and the results are presented in Table 3.3 and the statistical comparison in Table 3.2.

Table 3.3 Major nutrients of grape pomace samples (% w/w DM)

Sample	Ash	Proteins	CH	Fiber	Fat
BLE	5.3 ± 0.13	8.62 ± 0.03	29.2 ± 0.38	11.9 ± 0.28	2.0 ± 0.15
BLM	4.8 ± 0.77	10.75 ± 0.01	22.5 ± 0.75	20 ± 1.4	11.3 ± 0.15
BLP	7.9 ± 0.22	11.95 ± 0.02	23.4 ± 0.63	17.2 ± 0.40	7.2 ± 0.12
BLG	3.8 ± 0.20	9.89 ± 0.02	15.4 ± 0.11	29.0 ± 0.94	15.7 ± 0.12
TVE	5.5 ± 0.26	6.38 ± 0.04	21 ± 1.5	19.5 ± 0.55	1.65 ± 0.04
TVM	5.6 ± 0.28	11.92 ± 0.01	19.8 ± 0.66	19 ± 1.2	8.1 ± 0.24
TVP	6.8 ± 0.19	13.18 ± 0.02	22.0 ± 0.20	15.8 ± 0.38	4.93 ± 0.07
TVG	3.3 ± 0.22	10.71 ± 0.03	17.3 ± 0.38	31.0 ± 0.85	16.4 ± 0.24
EBLE		7.69 ± 0.01			
EBLM		13.5 ± 4.7E-03			
ETVE		5.60± 3.3E-03			
ETVM		14.92 ± 0,04			

In Loureiro GP, it was observed that the fractions have very significant differences (p<0.01) with more ash content in the skin than the stalk and finally the seed (7.9%, 5.3% and 3.8%, respectively) and the same was observed on the fractions of Vinhão GP which had very significantly differences (p<0.01) with more ash content in the skin than the stalk and finally the seed (6.8%, 5.5% and 3.3%, respectively). Between the two GP varieties, the same fraction showed no significant differences for the stalk and the seed of Loureiro and Vinhão GP (5.3%, 3.8% and 5.5%, 3.3%; p=0.44 and p=0.071, respectively) while ash content in skins showed very significantly differences (p<0.01), higher in Loureiro than Vinhão (7.9% and 6.8%, respectively). The ash values in the mixture of white and red GP are similar to values reported by (Tseng Angela, 2013) and (Llobera A., 2007) of 5.07 % and 5.50 %, but lower than values of Pinot noir 8.8 % and Pinot blanc 12.1 % from (Winkler Anne, 2015). These differences may be due to harvest methods and grape varieties.

In Table 3.3 are presented the values of protein in the GP samples and the extracted GP as described in section 2.5.1. The extracted samples were analyzed for the Kjeldahl nitrogen because it is an important parameter to evaluate the application of GP as food, feedstock and fertilizer after extracting the polyphenols. The fractions of Loureiro GP have very significant differences (p< 0.01) with more protein in the skin than the seed and finally the stalk (11.95%, 9.89% and 8.62%, respectively) and the same was observed

on the fractions of Vinhão GP which have very significant differences (p< 0.01) with more protein in the skin than the seed and finally the stalk (13.18%, 10.71% and 6.38%, respectively). When comparing the same fraction in both GP varieties, percentages of protein have very significant differences (p< 0.01), higher in skin and seed from Vinhão than skin and seed of Loureiro (13.18%, 10,71% and 11.95%, 9,89%, respectively) while in stalk was higher in Loureiro than Vinhão (8.62% and 6.38%, respectively). The protein content in GP mixture presents a value between 11-12% which is in the range of 6.11-17.27 % from other studies (Figure 3.3).

The results of carbohydrates are presented in Table 3.3 (calculation data in Table A in Annex 1). The present study revealed that the fractions of Loureiro GP variety have significant differences (p< 0.05) with more carbohydrate content in the stalks than the skin and finally the seed (29.2%, 23.4% and 15.4%, respectively), while the fraction of Vinhão GP variety have no differences between the skin, stalk and seed (22.0%, 21% and 17.3% respectively; p=0.74). Between the two GP varieties, the same fraction showed no significant differences for the stalk, the skin and the seed of Loureiro and Vinhão (29.2%, 23.4%, 15.4% and 21%, 22.0, 17.3%, respectively; p=0.45, p= 0.94 and p=0.17, respectively). The carbohydrates content of these varieties present a value of about 20-23% in the mixture which is in the range 7-32% published in other studies.

In Table 3.3 are presented the results of crude fiber in the grape pomace samples. The fractions of Loureiro GP have very significant differences (p<0.01) with more crude fiber in the seed than the skin and finally the stalk (29.0%, 17.2% and 11.9%, respectively) while the fractions of Vinhão GP have very significant differences (p<0.01) with more fiber content in the seed than the stalk and finally the skin (31.0%, 19.5% and 15.8%, respectively). The range for the crude fiber in the bibliography is between 15.8- 25.5% in the mixture and in this study the two varieties present an interval of about 19-20% in the mixture, but in the RGP mixture, the fiber content was lower than the minimum value from other authors (Figure 3.3).

Analyzing the fat content of grape pomace presented in Table 3.3, the fractions in Loureiro GP have very significant differences (p<0.01) with more fat in seed than the skin and finally the stalk (15.7%, 7.2% and 2.0%) and the same was observed in Vinhão GP

variety where very significantly differences were observed (p<0.01) with more fat content observed in seed than skin and finally the stalk (16.4%, 4.94% and 1.65%). When comparing the same fraction in both GP varieties, percentages of fat were showed to have significant differences (p< 0.05) with higher content in seed from Vinhão than seed of Loureiro (16.4% and 15.7%, respectively) while fat content in skin and stalk were higher in Loureiro than Vinhão (7.2%, 2.0% and 4.93%, 1.65%, respectively) with significant differences (p< 0.05). Comparing with the oil content in GP reported by other authors, the percentages in grape seeds are in the range of 11.6–19.6% depending on the variety and maturity of grapes (Ahmedna, 2013). Loureiro and Vinhão varieties present values close to the maximum (16%) and a value between 8-11% for the mixture which is between the ranges 0.68-13.53 % from other studies. In white GP the fat content appeared to be more than the maximum content found by other authors. In this work, it was also performed a Soxhlet extraction with hexane (section 2.5.1) for the polyphenols quantification. Because this also extracts the fat, the results were compared. No differences (p= 0.75) and a good correlation with r= 0.9433 was observed between these two fat extraction methods (Figure 3.2).

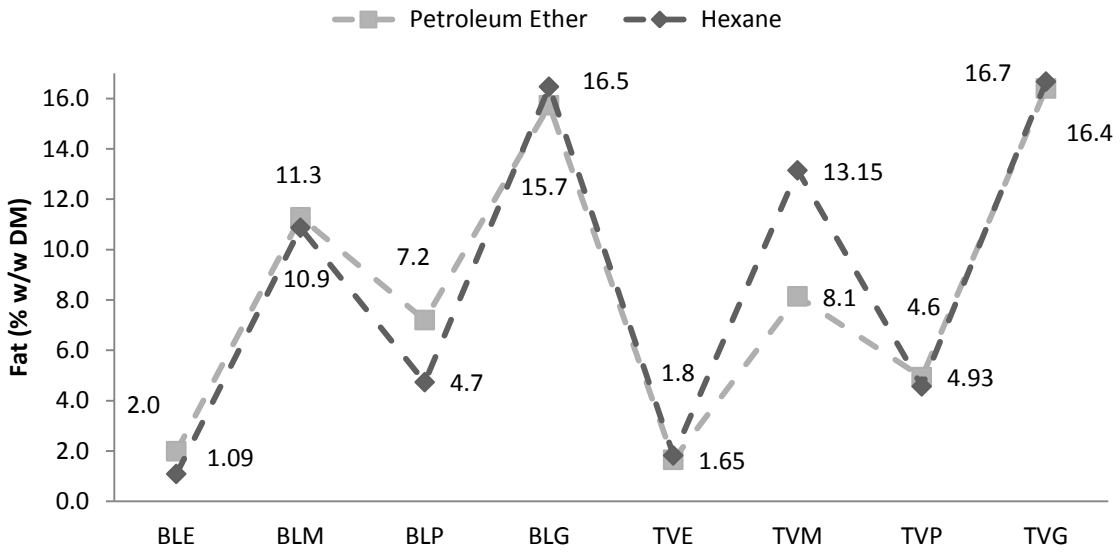

Figure 3.2 Comparison of fat percentages with two different methods

The results of the major nutrients found in this work for the studied varieties were compared with different grape pomace varieties from other authors Table 3.4 and Table 3.5 and showed that the values are between the ranges. In Figure 3.3 is presented the comparison between the results found in this study with the minimum and maximum values found from other authors in white and red GP. As it is presented in Figure 3.3, the fat content in Loureiro variety is higher than the maximum values from the literature, while the ash content in both varieties are close to minimum values found from the other authors.

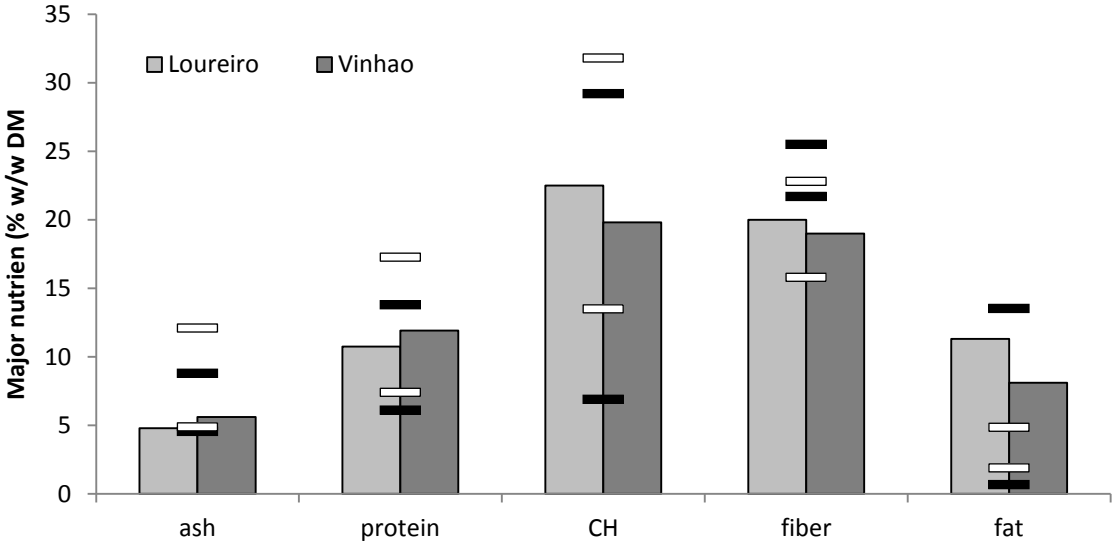

Figure 3.3 Comparison of major nutrient of WGP and RGP with min and max values from other studies

Table 3.4 Results for moisture content and major nutrients in red GP varieties from other studies (% w/w DM)

Red varieties	Moisture				Ash				Protein				CH	Fiber	Fat				
	stalk	mix	skin	seed	stalk	mix	skin	seed	stalk	mix	skin	seed	mix	mix	stalk	mix	skin	seed	
Turkey varieties	68.6	65.16	81,2*	51.2	7.64	5.3	11,72*	2.76	6.92	10.84	11,97*	9.33	-	-	1.22	4.62	4,68*	6.26	(Basalana M., 2011)
Pinot noir	-	-	-	-	-	5.07	-	-	-	10.32	-	-	-	-		11.09	-	-	(Tseng Angela, 2013)
Cabernet S.	65.80	61.50	-	-	10.75	5.45	-	-	5.81	8.05	-	-	-	-	2.62	1.56	-	-	(González-Centeno M.R., 2010) **
Callet	66.3	55.6	-	-	7.18	4.98	-	-	8.38	6.11	-	-	-	-	2.09	0.68	-	-	(González-Centeno M.R., 2010) **
Manto Negro	59.7	63.6	-	-	6.84	5.79	-	-	6.60	8.82	-	-	-	-	0.98	1.93	-	-	(González-Centeno M.R., 2010) **
Merlot	61.5	53.9	-	-	11.24	4.56	-	-	5.75	8.25	-	-	-	-	2.35	1.09	-	-	(González-Centeno M.R., 2010)
Tempranillo	56.8	55.7	-	-	10.01	5.22	-	-	4.89	7.04	-	-	-	-	0.93	1.36	-	-	(González-Centeno M.R., 2010) **
Syrah	64.9	50.2	-	-	4.80	6.03	-	-	6.78	6.63	-	-	-	-	2.54	1.41	-	-	(González-Centeno M.R., 2010) **
Manto negro	-	-	-	-	5.48	5.50	-	-	7.29	12.20	-	-	-	-	1.65	13.53	-	-	(Llobera A., 2007)
Friuli Venezia Giulia	-	-	-	-	-	-	-	3.9	-	-	-	12.5	-	-	-	-	-	11.7	(Spanghero M., 2009)
California	-	-	-	-	-	-	-	5.8	-	-	-	11.9	-	-	-	-	-	9.5	(Spanghero M., 2009)
Benitaka	-	-	-	-	-	4.65	-	-	-	8.49	-	-	29.2	-	-	8.16	-	-	(Sousa Eldina Castro, 2014)
Cabernet S.	-	-	-	-	-	-	7.59	-	-	-	12.34	-	-	-	-	-	6.33	-	(Deng Qian, 2011)
Merlot	-	-	-	-	-	-	7.19	-	-	-	11.26	-	-	-	-	-	3.35	-	(Deng Qian, 2011)
Pinot Noir	-	-	-	-	-	-	6.17	-	-	-	12.13	-	-	-	-	-	4.74	-	(Deng Qian, 2011)
Agiorgitiko	-	73.6	-	-	-	4.6	-	-	-	-	-	-	-	-	-	6.3	-	-	(Lafka Theodora-Ioanna, 2007)
Dornfelder	-	62.6	-	-	-	5.7	-	-	-	13.8	-	-	6.9	25.5	-	3.6	-	-	(Winkler Anne, 2015)
Pinot noir	-	57.9	-	-	-	8.8	-	-	-	10.2	-	-	9.4	21.7	-	3.8	-	-	(Winkler Anne, 2015)
Portugais bleu	-	60.5	-	-	-	4.7	-	-	-	9.9	-	-	21.3	24.4	-	3.2	-	-	(Winkler Anne, 2015)

Table 3.5 Results for moisture content and major nutrients in white GP varieties from other studies (% w/w DM)

White varieties	Moisture		Ash				Protein				CH	Fiber		Fat				Reference
	stalk	mix	stalk	mix	skin	seed	stalk	mix	skin	seed	mix	mix	skin	stalk	mix	skin	seed	
Turkey varieties		70.1	-	6.3	-	-	-	8.31	-	-	-	-	-	-	4.86	-	-	(Basalana M., 2011)
Iran varieties			-	5.7	-	-	-	17.27	-	-	13.5	22.8	-		3.7	-	-	(Afshar Mirzaei-Aghsaghali, 2011)
Chardonnay	67.5	63.9	8.73	4.94	-	-	7.79	7.41	-	-	-	-	-	2.18	2.75	-	-	(González-Centeno M.R., 2010) **
Macabeu	72.7	72.2	5.45	6.85	-	-	6.54	11.53	-	-	-	-	-	1.82	3.24	-	-	(González-Centeno M.R., 2010) **
Parellada	76.7	62.8	6.50	4.89	-	-	11.27	9.24	-	-	-	-	-	3.47	1.90	-	-	(González-Centeno M.R., 2010) **
Premsal B.	69.7	69.3	5.93	6.46	-	-	9.23	10.01	-	-	-	-	-	2.31	2.26	-	-	(González-Centeno M.R., 2010) **
Venezia Giulia	-	-	-		-	3.8	-	-	-	12.6	-	-	-	-	-	-	14.5	(Spanghero M., 2009)
Riesling	-	63.7	-	14	-	-	-	9.30	-	-	28.3	17.5	-	-	2.70	-	-	(Winkler Anne, 2015)
Pinot blanc	-	63.5	-	12.10	-	-	-	8.90	-	-	31.8	15.8	-	-	3.10	-	-	(Winkler Anne, 2015)
Muller Thurgau	-	-	-	-	2.53	-	-	-	6.54	-	-	-	-	-	-	2.64	-	(Deng Qian, 2011)
Morio Muscat	-	-	-	-	3.31	-	-	-	5.38	-	-	-	-	-	-	1.14	-	(Deng Qian, 2011)
Red+White	-	-	-	9	7.2	-	-	14	16.4	-	-	24.4	27.2	-	6.4	7.4	-	(Feedipedia, 2015)

* skin plus pulp

** calculated from the fresh sample

3.3 Minerals content in two varieties of GP, Loureiro and Vinhão

Minerals means the elements found in the form of inorganic salts or elements obtained in this form from incineration of the samples. There are 20 minerals classified into two groups, major minerals, sodium, potassium, calcium, magnesium, phosphorus and chloride and trace elements, iron, copper, zinc, manganese, selenium, iodine, chromium, cobalt, molybdenum, fluorine, vanadium, nickel, silicon and tin (Belitz H.-D., Minerals, 1986). The equations obtained from the calibration curves were usedto calculate the mineral content and are presented in Table B in Annex 1. The results are in Table 3.6 and Table 3.7, which contains the major and trace minerals in Loureiro and Vinhão GP: potassium, calcium, phosphorus, magnesium, sodium, iron, copper, zinc, manganese, chromium, nickel, lead and cadmium. The phosphorus content refers to the original samples of GP and the extracted solid that remained in the Soxhlet thimble after the extraction process. The phosphorus was analyzed in the extracted samples because it is an important parameter to evaluate the samples as food, fertilizers and animal feedstock.

Table 3.6 Major mineral composition of grape pomace samples in g/kg DM

Sample	K	Ca	P original	P extracted	Mg	Na
BLE	25.8 ± 0.39	4.59 ± 0.04	2.17 ± 0.03	2.23 ± 0.06	1.19 ± 0.01	0.97 ± 0.07
BLM	19.9 ± 2.61	4.1 ± 0.11	2.76 ± 0.05	2.95 ± 0.12	1.06 ± 0.04	0.29 ± 0.04
BLP	36.2 ± 0.50	3.1 ± 0.33	2.37 ± 0.02	-	0.72 ± 0.02	0.47 ± 0.06
BLG	11.5 ± 0.20	5.5 ± 0.12	3.34 ± 0.09	-	1.38 ± 0.04	0.32 ± 0.01
TVE	29 ± 1.2	5.1 ± 0.17	2.59 ± 0.05	2.46 ± 0.01	1.12 ± 0.07	0.91 ± 0.01
TVM	28.1 ± 0.41	3.3 ± 0.16	2.89 ± 0.05	2.9 ± 0.26	0.69 ± 0.04	0.42 ± 0.02
TVP	37.1 ± 0.54	1.83 ± 0.08	2.63 ± 2.38E-03	-	0.39 ± 0.03	0.56 ± 0.02
TVG	9.9 ± 0.77	4.4 ± 0.13	3.29 ± 0.06	-	1.39 ± 0.03	0.36 ± 0.01

The results of the major mineral content with the statistical comparison, is presented in Table 3.8. Comparing the same fraction between the two GP varieties, the potassium content have very significant differences (p<0.01) with higher content in the stalk of Vinhão than in Loureiro (29 g/kg and 25.8 g/kg, respectively), while in the seed showed very significant differences (p<0.01) with higher content in Loureiro than Vinhão (11.5 and 9.9 g/kg, respectively). There were no significant differences in the content of potassium in the skin between the two varieties. The addition of potassium bitartrate during winemaking could influence the results (Basalana M., 2011).

Concerning the Ca and Mg content between Loureiro and Vinhão, there were no significant differences in the stalks (4.59 and 5.1 g/kg; p=0.40 and 1.19 and 1.12 g/kg; p= 0.34, respectively). Also, Mg content between Loureiro and Vinhão showed no significant differences in the seed comparing the two varieties (1.38 and 1.39g/kg; p=0.69). On the other hand, the Ca and Mg content in the skin have very significant differences (p<0.01) with higher content in Loureiro compared with Vinhão (3.1, 0.72 and 1.83, 0.39 g/kg, respectively).

The sodium content in skin have very significant differences (p<0.01) with higher content in Vinhão compared with Loureiro (0.56 and 0.47 g/kg, respectively), while in the stalks was higher in Loureiro than Vinhão (0.97 and 0.91 g/kg, respectively) with significant differences (p<0.01). No significant differences were observed in the seed between Loureiro and Vinhão (0.32 and 0.36 g/kg, respectively; p=0.65). The variability of Na content may be due to the geographic origin of the vineyard.

In phosphorus content, showed no significant differences in the skin and the seed between Loureiro and Vinhão (2.37 and 2.63 g/kg; p=0.85 and 3.34 and 3.29; p=0.50, respectively), except in the stalk where very significant differences were observed (p<0.01) with higher content in Vinhão than Loureiro (2.59 and 2.17 g/kg, respectively). The results from the initial and the extracted samples showed a good correlation with a r = 0.915 (Figure 3.4) and also they have no significant differences (lowest p=0.18) which means that the samples after the extraction have almost the same concentration. Considering the above results, it seems that these minerals remain in the GP after extraction. This could be relevant for food applications.

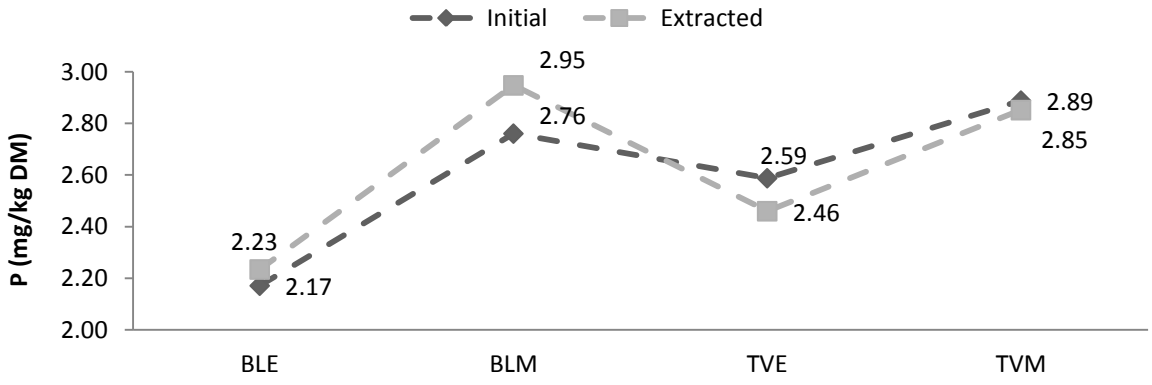

Figure 3.4 Correlation between the phosphorus content of the initial and the extracted samples

In the two grape varieties studied, the concentrations of the major minerals from high to low followed the sequence of K>Ca>P>Mg>Na, except for the skin in red Vinhão where the sequence was K>P>Ca>Na>Mg, as showed in Figure 3.5, which is in logarithmic scale. Between the two GP varieties the tendency concentrations for each mineral were similar, except for calcium. For the calcium content, in white variety the seed has the highest value with 5.5 g/kg while in red variety the highest content appeared in stalks with 5.1 g/kg. The skin had the lowest content in Ca in both GP varieties.

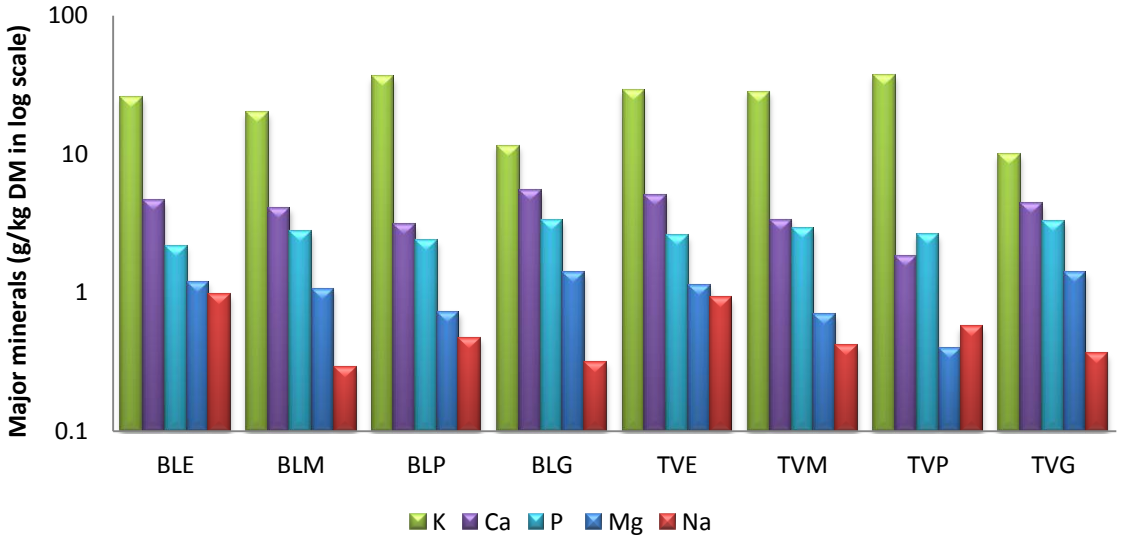

Figure 3.5 Major mineral content in GP samples (g/kg DM)

The results of trace minerals , except of Cr, Ni, Pb and Cd which were lower than the quantification limits, more specifically < 2.9 mg Cr/kg (QL), < 2.3 mg Ni/kg (QL), < 4.8 mg Pb/kg (QL) and < 1.5 mg Cd/kg (QL) respectively, are presented in Table 3.7. Comparing the content in trace minerals (Fe, Cu, Zn, Mn) and taking into consideration the statistical comparison (Table 3.8) it was observed that the mineral with the highest content in the two GP varieties was iron (Fe). Although, for the other quantified trace minerals differences among the different parts of GP were observed Figure 3.6. The copper content in the stalk and in the seed have no significant differences between Loureiro and Vinhão (46.4 and 39.2 mg/kg; p=0.18; and 17 and 18; p=0.44, respectively), while in the skin it was observed very significant differences (p<0.01) with higher content in Vinhão compared with Loureiro (115 and 41.6 mg/kg, respectively). These results may be due to different exposure to the treatments, since the copper is used in phytosanitary

treatments during grapevine growing: copper is one of the most important biopesticides, used in organic farms against grapevine insect pests and diseases.

Table 3.7 Trace mineral composition of grape pomace samples in mg/kg DM

Sample	Fe	Cu	Zn	Mn
BLE	410 ± 14	46.4 ± 0.45	30.5 ± 0.64	78 ± 1.6
BLM	61 ± 2.4	43 ± 1.1	23.5 ± 0.22	19.9 ± 0.65
BLP	78.1 ± 0.52	41.6 ± 0.29	19 ± 1.6	21.8 ± 0.65
BLG	31.9 ± 0.65	17 ± 1.6	15.0 ± 0.29	28.3 ± 0.77
TVE	79 ± 3.8	39.2 ± 0.56	22 ± 1.1	39.8 ± 0.73
TVM	101 ± 1.7	95 ± 1.8	18.0 ± 0.39	16.4 ± 0.11
TVP	126 ± 6.7	115 ± 4.2	21.5 ± 0.57	13.15± 0.08
TVG	46 ± 7.5	18 ± 1.4	16.4 ± 0.17	17.4 ± 0.35

Table 3.8 Statistical results for the mineral content in GP

Fractions	K	Ca	P	Mg	Na	Fe	Cu	Zn	Mn
t-test									
BLE vs TVE	0.0002	0.40	0.0008	0.34	0.033	< 0.0001	0.18	0.00	< 0.0001
BLP vs TVP	0.083	< 0.0001	0.85	< 0.0001	0.016	0.69	0.0053	0.84	0.0018
BLG vs TVG	0.045	0.0003	0.50	0.69	0.65	0.019	0.44	< 0.0001	< 0.0001
BLE vs EBLE	-	-	0.18	-	-	-	-	-	-
BLM vs EBLM	-	-	0.53	-	-	-	-	-	-
TVE vs ETVE	-	-	0.27	-	-	-	-	-	-
TVM vs ETVM	-	-	0.84	-	-	-	-	-	-

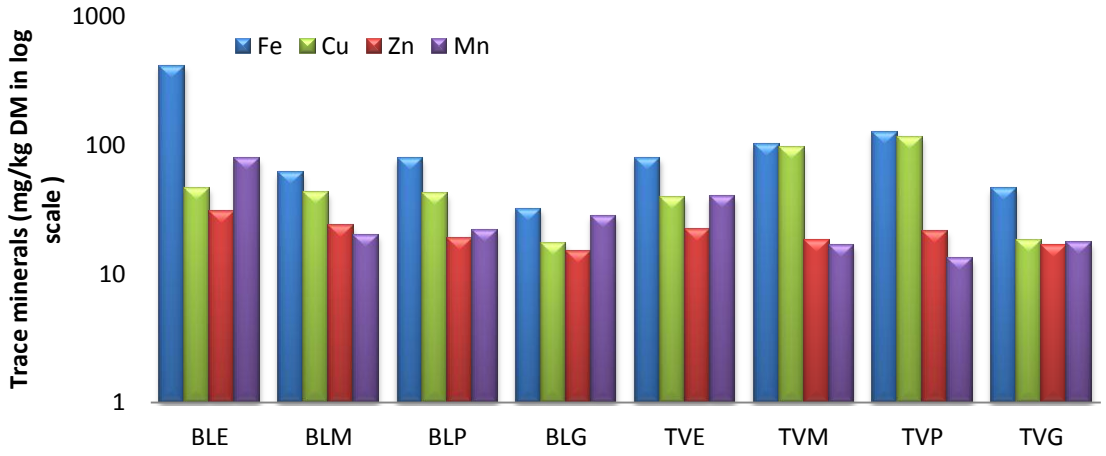

Figure 3.6 Trace content in GP samples (mg/Kg DM)

Comparing GP results of ash and total minerals quantified in this study, it can be observed a good correlation r = 0.9423 between these parameters (Figure 3.7).

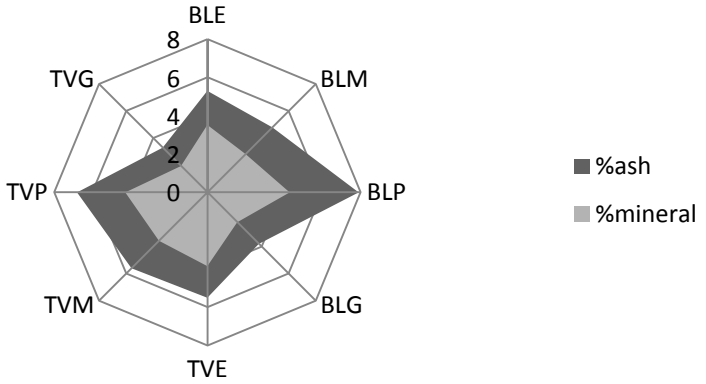

Figure 3.7 Percentage of ash and minerals in grape pomace DM

As it shows in Figure 3.8 which is in logarithmic scale, all the values from the minerals in both varieties are between those found in literature (table 3.9) except for Ca and P where the contents were lower and higher, respectively both in Loureiro and Vinhão comparing with other authors.

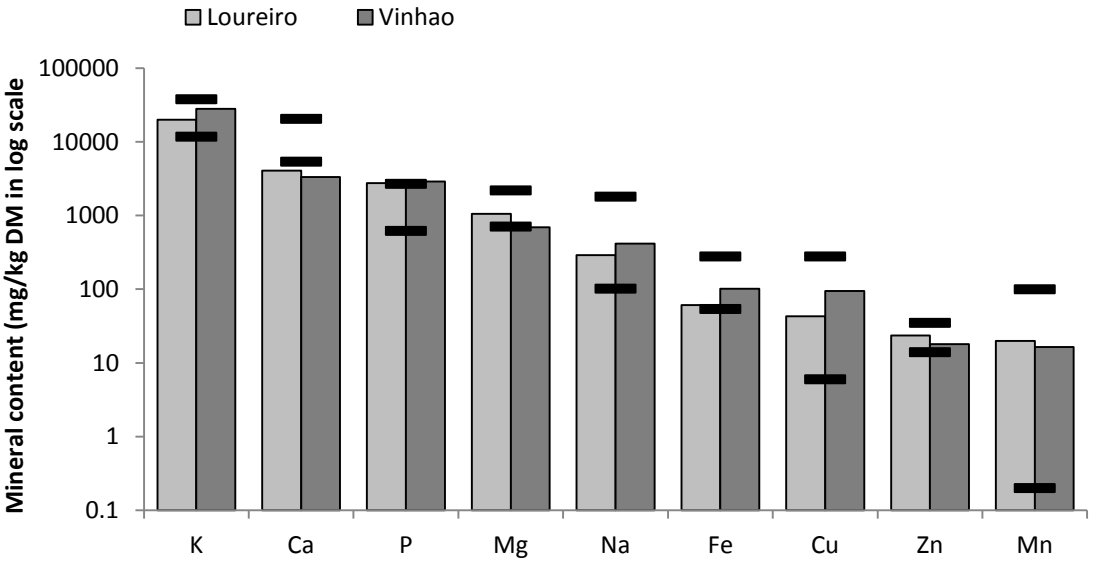

Figure 3.8 Comparison of mineral content of Loureiro and Vinhão GP with minimum and maximum values from other studies

Table 3.9 Results for minerals content in white and red GP varieties from other studies in mg/kg DM

mg/kg	Stalk		Mixture					skin	seed			
	*	*	*	*	Red	White	Range	*	Red		White	*
	Spanish 2002-2003	Italian	Spanish 2002-2003	Italian	Cabernet S.	Sauvignon Blanc		Italian	Friuli Venezia Giulia	California	Friuli Venezia Giulia	Italian
Sodium	416	86.62	357	70.62	58	61	102-1,809	56.84	-	-	-	20.17
Potassium	30000	24389	24200	19967	27333	20267	11,800-38,000	38041	7000	15400	8000	13777
Magnesium	2100	-	1200	-	987	710	710-2,200	-	1200	1200	1300	-
Calcium	9500	-	9400	-	3867	2170	5,400-20,600	-	5900	5300	5000	-
Phosphorus	940	-	1150	-	2733	2367	620-2,733	-	3200	3200	3100	-
Manganese	25	23.22	12	20.89	-	-	<0.2-100	18.03	23	17	19	22.69
Iron	128	-	136	-	85	60	54-279	-	64	174	58	-
Chromium	1.4	0.32	0.1	0.91	-	-	<0.2-5.6	0.58	-	-	-	0.78
Copper	22	65.06	28	99.84	-	-	6-279	141.92	40	16	49	72.17
Nickel	8.7	1.05	3.5	1.18	-	-	<0.2-27.6	1.77	-	-	-	1.69
Cadmium	0.8	0.37	1.1	0.19	-	-	<0.2-4.6	0.07	-	-	-	0.47
Lead	26.2	2.68	16.3	2.04	-	-	<0.4-43.9	1.07	-	-	-	3.28
Zinc	26	-	24	-	15	9	14-35	-	14	17	13	-
Reference												
	(Bustamante M.A., 2008)	(Toscano G., 2013)	(Bustamante M.A., 2008)	(Toscano G., 2013)	(Corbin Kendall R, 2015)	(Corbin Kendall R, 2015)	(Bustamante M.A., 2008)	(Toscano G., 2013)	(Spanghero M., 2009)	(Toscano G., 2013)	(Toscano G., 2013)	(Toscano G., 2013)

*White and red varieties

3.4 Polyphenols quantification and identification in GP

3.4.1 Results of the extraction process

The phenolic derivatives distributed in plant tissues are classified by their structures. Thus, the soluble polyphenols with a low molecular weight are located in the vacuoles of the cells, while the insoluble phenolic derivatives (e.g. polymeric lignins) constitute the structural components of the cell walls contributing to the stability and morphology of the plants (Santos-Buelga Celestino, 2012). The process of extraction releases phenolic derivatives from cells and tissues, leading to qualitative and quantitative determination. For samples with high water content it is recommended the refrigeration before the process of extraction, to inactivate the enzyme functions and protect the unstable polyphenols. Furthermore, the extraction of polyphenols is facilitated when preceded by refrigeration at low temperatures because the crystals of the water are formed and destroyed the cellular walls favoring the release of intracellular material. Finally, another practice followed is the lyophilization of the samples, which takes place at low temperatures and leaves intact the polyphenols, allowing the conservation of the samples for a sufficiently long time followed by the use of a homogenizer to rupture the cell tissue and reduce the size particle. Subsequently, the immersion in an appropriate solvent is carried out, to extract the phenolics derivatives by diffusion. The extraction of polyphenols from plant material can be affected by a number of factors, such as the chemical composition, the process used for the extraction, the extraction conditions, the storage time, the tissue size and the presence of undesirable substances and the sample treatment for extraction. Usually the extracts are comprised by mixtures of various polyphenols which are soluble in the solvent however they contain also molecules that are not phenols, such as fats, terpenes, and chlorophylls. To remove the latter, often it requires fractionation based on the polarity of the substances (Santos-Buelga Celestino, 2012). The solubility of the polyphenols is dependent on the polarity of solvent used, the polymerization degree and the interaction with other molecules (e.g., proteins) to form insoluble products. For these reasons there is not a single procedure to extract all the phenolic derivatives from a plant sample. The most common technique applied is the method of extraction, where the selection of the type of extraction

depends from the author, as it could be used, the solid-liquid extraction, the Soxhlet extraction, the supercritical fluid extraction etc. Also, some authors for the fractionation of the undesirable substances, use hexane to remove the fat, dichloromethane to remove the chlorophylls and other pigments and only after that, polar protic media such as hydro alcoholic solutions, to extract the polyphenols. Methanol exhibits the highest capacity to extract phenolics even though the ethanol is cheaper and safer. Also ethanol should be preferable in the case of later food applications. The published results are not conclusive about an ideal solvent, and different mixtures have been proposed (Fontana Ariel R., 2013) .

Table 3.10 Extraction yields obtained from Loureiro and Vinhão lyophilised GP samples

Type of Sample	Hexane (%)	Dichloromethane (%)	Methanol (%)
BLE	1.09 ± 0.08	0.40 ± 0.01	34 ± 3.1
BLM	10.9 ± 0.14	1.49 ± 0.14	18.1 ± 0.62
BLP	4.7 ± 0.34	3.36 ± 0.11	40 ± 4.1
BLG	16.5 ± 0.12	0.43 ± 0.01	18.6 ± 0.20
TVE	1.8 ± 0.22	0.44 ± 0.03	16.5 ± 0.11
TVM	13.15 ± 0.03	1.09 ± 0.02	11 ± 1.1
TVP	4.6 ± 0.21	1.67 ± 0.12	28 ± 1.2
TVG	16.7 ± 0.18	0.60 ± 0.06	7.4 ± 0.39

In the present study the GPE was obtained by using firstly hexane, followed by dichloromethane and finally methanol was selected in order to obtain a high yield of polyphenols. The total extraction yields obtained from Loureiro and Vinhão GP are presented in Table 3.10. The yields from hexane were discussed in the section 3.1, showed a higher percentage in the seeds of the two varieties with 16.5% and 16.7% from Loureiro and Vinhão respectively. The dichloromethane extraction showed higher yield in the skin with a percentage of 3.36% in Loureiro and 1.67% in Vinhão. The high extraction yield with methanol, which contains the polyphenols, could be explained because of the presence of other substances, than polyphenols, which were not determined. The highest yield obtained, was from the skin (40 %) and the stalk (34 %) from white Loureiro GP. The extraction yields with methanol from another study were Chardonnay 23.3%, Macabeu 32.4%, Parellada 17.2% and Premsal Blanc 33.3% (González-Centeno M.R, 2013). Usually the authors publish the results of analyses of TPC and antioxidant activity and do not present the yield, so the comparison cannot be so extensive.

3.4.2 Results of total phenolic content (TPC) and antioxidant activity (DPPH)

The method of Folin & Ciocalteu (FC), for measuring the total phenolics is a photometric method which was originally developed in 1927 for determining proteins, taking into advantage the fact that the reagent used, reacted with the phenol ring of tyrosine, forming a colored product. Thereafter Singleton and Rossi (Singleton V. L., 1965) improved the method and used it for determination of total phenols in the wine. The process became very popular and widely used since then to determine the phenolic content of various natural products. Although the FC process is used to determine the phenolic substances, actually it is used to determine the reducing ability of the sample, since the reaction carried out in this process is a redox reaction. Therefore, the above method can be considered as a method of measuring antioxidant capacity. The mechanism of this reaction belongs to the category of transport electron, so it should not be surprising that the "polyphenol profile" determined by this method shows good linear correlation with antioxidant capacity determined by other antioxidant methods which also transfer electrons, for example like the ferric reducing ability of plasma (FRAP) or 1,1-diphenyl-2-picryl-hydrazyl (DPPH).

Table 3.11 Results of TPC and DPPH in red and white lyophilized GP sample

Type of Sample	Antioxidant activity (mg AAE/g DM)	Total phenolic content (mg GAE/g DM)
BLE	16.1 ± 0.46	26 ± 1.3
BLM	40 ± 1.9	39 ± 1.2
BLP	12.2 ± 0.48	24 ± 1.8
BLG	83 ±17	86 ± 9.5
TVE	18.0 ± 0.73	23.9 ± 0.15
TVM	28.6 ± 0.84	57 ± 1.1
TVP	76 ± 7.2	120 ± 1.4
TVG	29 ± 1.4	35.1 ± 0.97

Table 3.12 Statistical analysis for the TPC and DPPH results

Fractions	Antioxidant activity (mg AAE/g DM)	Total phenolic content (mg GAE/g DM)
BLE vs TVE	0.076	0.008
BLP vs TVP	0.051	0.0003
BLG vs TVG	0.001	0.0004

The results of TPC and DPPH obtained in this study are presented in Table 3.11 and were calculated from the equations presented in Table A in Annex 1. In Table 3.12

presented the results of the statistical analysis. Comparing the same fraction between the two GP varieties, the TPC had very significant differences (p<0.01) with higher content in the stalk and the seed of Loureiro GP than Vinhão (26, 86 and 24, 35.1 mg GAE/g, respectively), while in the skin, the content of TPC was higher in Vinhão than Loureiro (120 and 24 mg GAE/g, respectively) with very significant differences (p<0.01). Figure 3.9 and Figure 3.10 show the correlation between the results of TPC and DPPH with an r= 0.988 for red Vinhão and an r= 0.975 for the white Loureiro.

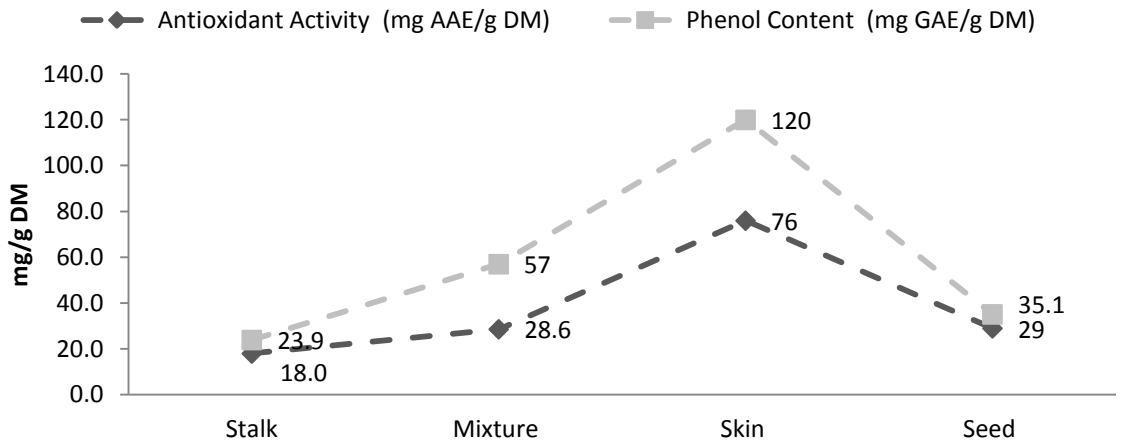

Figure 3.9 Correlation between the TPC and the antioxidant activity in red grape pomace

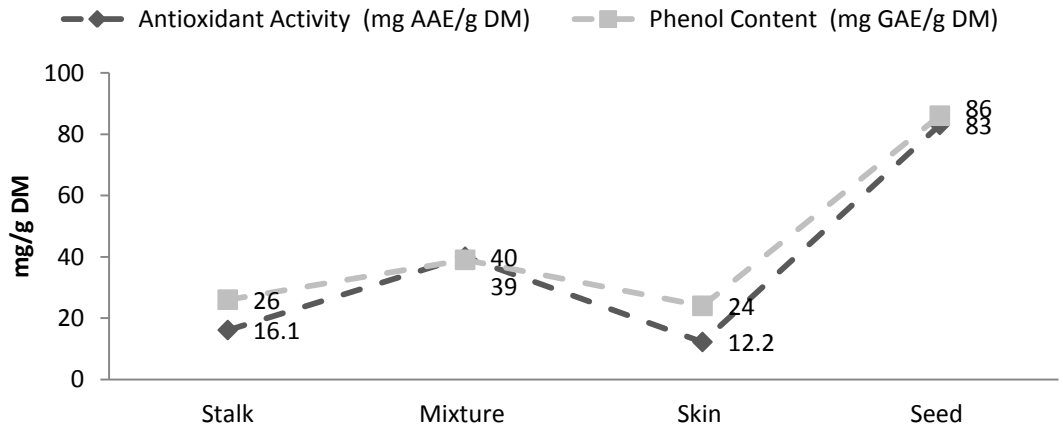

Figure 3.10 Correlation between the TPC and the antioxidant activity in white grape pomace

Comparing these results with other authors (Table 3.13), the values for the seed from Loureiro are similar to Friuli Venezia Giulia but the results of the skin from Vinhão

are higher than all the other varieties. The TPC of the mixture of these two varieties has a range of 39-57 mg GAE/g DM which is between the range of 26.3-131.7 mg GAE/g DM from the bibliography.

Table 3.13 Results for total phenolic content (TPC) in white and red GP varieties from other studies

	TPC mg/g DM				Reference
Red varieties	Stalk	Mixture	skin	seed	
Pinot noir	-	67.74	-	-	(Tseng Angela, 2013)
Manto negro	116	26.3	-	-	(Llobera A., 2007)
Friuli Venezia Giulia	-	-	-	51	(Spanghero M., 2009)
California	-	-	-	64	(Spanghero M., 2009)
Pinot Noir	-	56	-	-	(Winkler Anne, 2015)
Touriga Nacional	-	131.7	-	-	(Tournour Hernán H., 2015)
Touriga Franca	-	100.1	-	-	(Tournour Hernán H., 2015)
Tinta Roriz	-	69.3	-	-	(Tournour Hernán H., 2015)
Cabernet S.	-	-	26.7	-	(Deng Qian, 2011)
Merlot	-	-	25	-	(Deng Qian, 2011)
Pinot Noir	-	-	21.4	-	(Deng Qian, 2011)
Agiorgitiko	-	54.02	-	-	(Canellas, 2008)
Kadarka	16-19.4	-	3.2-3.8	35-43	(Llobera A., 2007)
Negro Amaro	-	41.9	33.3	85.8	(Llobera A., 2007)
Dornfelder	-	57	-	-	(Winkler Anne, 2015)
Portugais bleu	-	65	-	-	(Winkler Anne, 2015)
White varieties	Stalk	Mixture	Skin	seed	
Friuli Venezia Giulia	-	-	-	90	(Spanghero M., 2009)
Riesling	-	44	-	-	(Winkler Anne, 2015)
Pinot blac	-	54	-	-	(Winkler Anne, 2015)
Muller Thurgau	-	-	15.8	-	(Deng Qian, 2011)
Morio Muscat	-	-	11.6	-	(Deng Qian, 2011)
Chardonnay	-	38.91	-	-	(González-Centeno M.R, 2013)
Macabeu	-	30.93	-	-	(González-Centeno M.R, 2013)
Parellada	-	46.54	-	-	(González-Centeno M.R, 2013)
Premsal Blanc	-	36.39	-	-	(González-Centeno M.R, 2013)
Premsal Blanc	87.3	34.9	-	-	(Canellas, 2008)
Roditis	57.98	48.26	-	-	(Canellas, 2008)

3.4.3 Identification and quantification of polyphenols by HPLC analysis

The high performance liquid chromatography (HPLC) has proven to be particularly useful for the qualitative and quantitative determination of phenols, offering convenience

and time saving because samples are not required to undergo special treatment before analysis. For these reasons, the application of this analytical technique have been extended significantly in recent decades and has almost replaced traditional chromatographic techniques such as paper chromatography (PC) and the thin layer chromatography (TLC). Although in some cases it has been used normal phase liquid chromatography to separate phenols from fruits and vegetables, this technique is not indicated, as there is the risk of high polarity components remaining permanently bonded to the phase and alter the column characteristics and separating capacity. In contrast, the reverse phase liquid chromatography (RP-HPLC) has become the first option for separating a mixture of phenolic compounds using C8 or C18 columns, and it presents several advantages over the normal phase chromatography. The usefulness of RP-HPLC has become evident in the separation of all phenolic substances, but mainly with anthocyanins which are highly polar and their separation was not possible with normal phase chromatography (Antolovich, 1997). Sometimes before the instrumental analysis by HPLC is required a pretreatment of the extract obtained from recovery processes, for the fractionation of specific chemical groups because it includes many bioactive phytochemicals. There are different reviews reporting sample preparation strategies for cleaning up grape polyphenolics. Solid-phase extraction (SPE) has been reported from many authors, with diverse sorbent material like reverse-phase octadecylsilane (C18) or hydrophilic lipophilic-balanced (HLB) reversed-phase sorbent (Fontana Ariel R., 2013). In this study the pretreatment for the extracts was not applied. Under the RP-HPLC separation the most polar substances are eluted first. Therefore glycosides with more sugar units are eluted first, followed by the monoglycosides and finally aglycone components. Also, the order of elution of the flavonoids is flavanones, flavonols and flavones. Finally in the phenolic acids, hydroxybenzoic acids are more polar than hydroxycinnamic acids that and are eluted first (Zhang A., 2013). The detection of phenols in the HPLC is typically based on the measurement of absorption in the UV-Vis at characteristic wavelengths. For example, anthocyanins absorb at 540 nm, glycosites and flavonols absorbs at 370 nm, stilbenes and hydroxicinnamic acid at 320 nm and hydroxybenzoic acids, flavanols and procyanidins at 280 nm. The identification of these

substances is done by comparing their retention time at the characteristic absorption spectrum to that of standard compounds.

In grapes, the phenolic compounds are localized mainly in the skins, seeds and short stems. GP is rich in extractable phenolic antioxidants (10–11% of dry weight). Anthocyanins, flavanols (catechins, procyanidins), flavonol glycosides, phenolic acids and stilbenes are the principal phenolic constituents found in GP (Makris Dimitris P., 2007).

Polyphenols composition of GP is dependent on variety and is influenced by the growing location, climate, maturity and the winemaking process like the time of fermentation. Within the same variety, a different part of GP has different polyphenol composition (Ahmedna, 2013). Other studies also concluded these differences between the polyphenols composition (Xia En-Qin, 2010), (Makris Dimitris P., 2007).

The results of polyphenol content in GP obtained in this study by RP-HPLC analysis are presented in Table 3.14 as mg/g DM and were calculated from the equations presented in Table C in Annex 1.

Table 3.14 Phenolic composition (mg/g DM) of lyophilized GP samples

Phenolic compound	BLE	BLP	BLG	Total WGP	TVE	TVP	TVG	Total RGP
Gallic acid	0.087	0.073	0.112	0.272	0.097	0.111	0.148	0.356
(+)Catechin	0.176	0.059	0.971	1.206	0.177	1.068	0.629	1.874
(-)Epicatechin	0.073	0.080	0.537	0.690	0.775	0.213	0.199	1.187
Syringic acid	0.016	0.020	0.006	0.043	0.010	0.076	0.090	0.176
Caffeic acid	0.047	0.027	0.020	0.094	0.247	0.050	0.045	0.342
p-Coumaric acid	0.076	0.015	0.024	0.115	0.026	0.047	0.053	0.126
Ferrulic acid	0.014	0.010	0.005	0.029	0.010	0.083	0.044	0.138
trans-Resveratrol	0.244	0.028	0.103	0.375	0.074	0.070	0.025	0.169
Quercetin	0.064	0.055	0.018	0.137	0.042	0.114	0.055	0.211

The results showed differences in the concentration of the phenolic compounds depending on the variety but also on the different parts of the GP (Figure 3.11). The distribution of the different phenolic compounds quantified, is presented in Figure 3.13. Gallic acid that is a non-flavonoid phenolic acid, found in all samples of grape pomace was present with a highest concentration (0.148 and 0.112 mg/g DM) in the seed of Vinhão and Loureiro respectively. The phenolic content in grape seeds consists almost exclusively of flavan-3-ols such as (+)-catechin and (-)-epicatechin which are in accordance with the amounts described by other authors (Rockenbach Ismael Ivan, 2011). (+)Catechin had a

higher content in skin from Vinhão with a content of 1.068 mg/g DM, followed in seed of Loureiro and Vinhão (0.971 mg/g and 0.629 mg/g DM respectively) and (-) epicatechin was observed to have the highest content in stalks of red GP (0.775 mg/g DM) followed by the seed of Loureiro (0.537 mg/g DM). Syringic acid is a hydroxybenzoic acid which was found in highest concentrations in seed and skin of Vinhão with 0.090 and 0.076 mg/g DM respectively. Regarding the flavonols, which in this study it was quantified the quercetin, the highest concentrations was observed in the skin of the Vinhão at 0.114 mg/g DM. The data obtained for cinnamic acid derivatives namely, caffeic acid, ferrulic acid and p-coumaric acid showed differences between the parts of GP. The highest content of caffeic acid was observed in the stalks of Vinhão with 0.247 mg/g DM, while the highest content of ferrulic acid was found in the skin of Vinhão with 0.083 mg/g DM. Finally the higher p- coumaric content was detected in stalk of Loureiro with 0,076 mg/g. From the literature it is known that one of the most economic valuable phenolic compounds is trans-resveratrol (Haroutounian, 2007-2008). The grape stalks are known to contain the highest amount of trans-resveratrol comparing to skins (Figure 3.13). In a survey of trans-resveratrol contents of grape stalks from nine Vitis vinifera varieties, the levels found ranged from 0.007 to 0.48 mg/g DM (Rayne Sierra, 2008). In this study the highest percentage of resveratrol was found in the stalks of white Loureiro with 0.244 mg/g DM which is close to the maximum reported above.

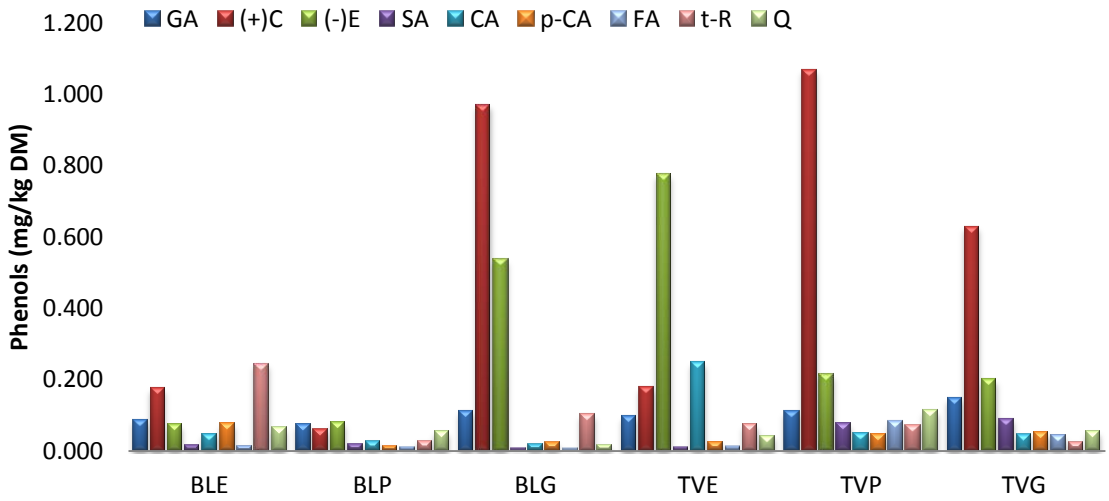

Figure 3.11 Phenolic content in mg/g DM in each part of Loureiro and Vinhão GP

Rockenbach studied the phenolic content and antioxidant activity of pomace from the vinification of grape varieties widely produced in Brazil, Cabernet Sauvignon, Merlot, Bordeaux and Isabel, and found that catechin was the most abundant non-anthocyanic compound identified in the GP (150.16 mg/100 g dry GP) for all varieties (Rockenbach Ismael Ivan, 2011). As it can be observed from Figure 3.12 in this study, the most abundant polyphenol in Loureiro and Vinhão GP is catechin, followed by the epicatechin. Also, it could be concluded that the red GP variety contains a higher content in polyphenols than the white GP, except of trans resveratrol, where the highest content was in white GP.

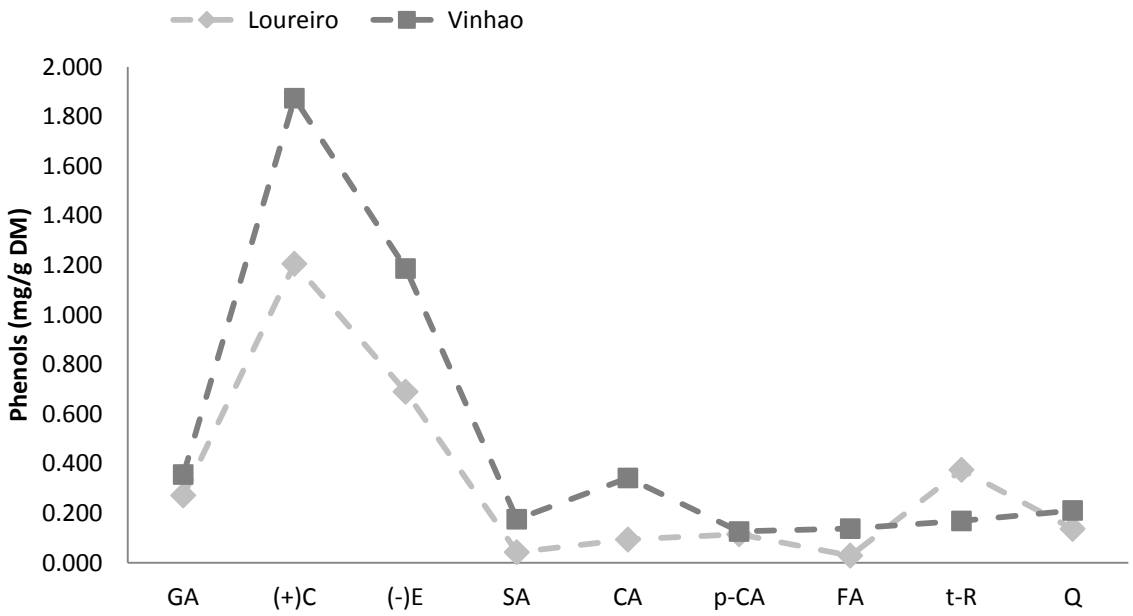

Figure 3.12 Distribution of phenolic compounds in Loureiro and Vinhão GP mixture varieties

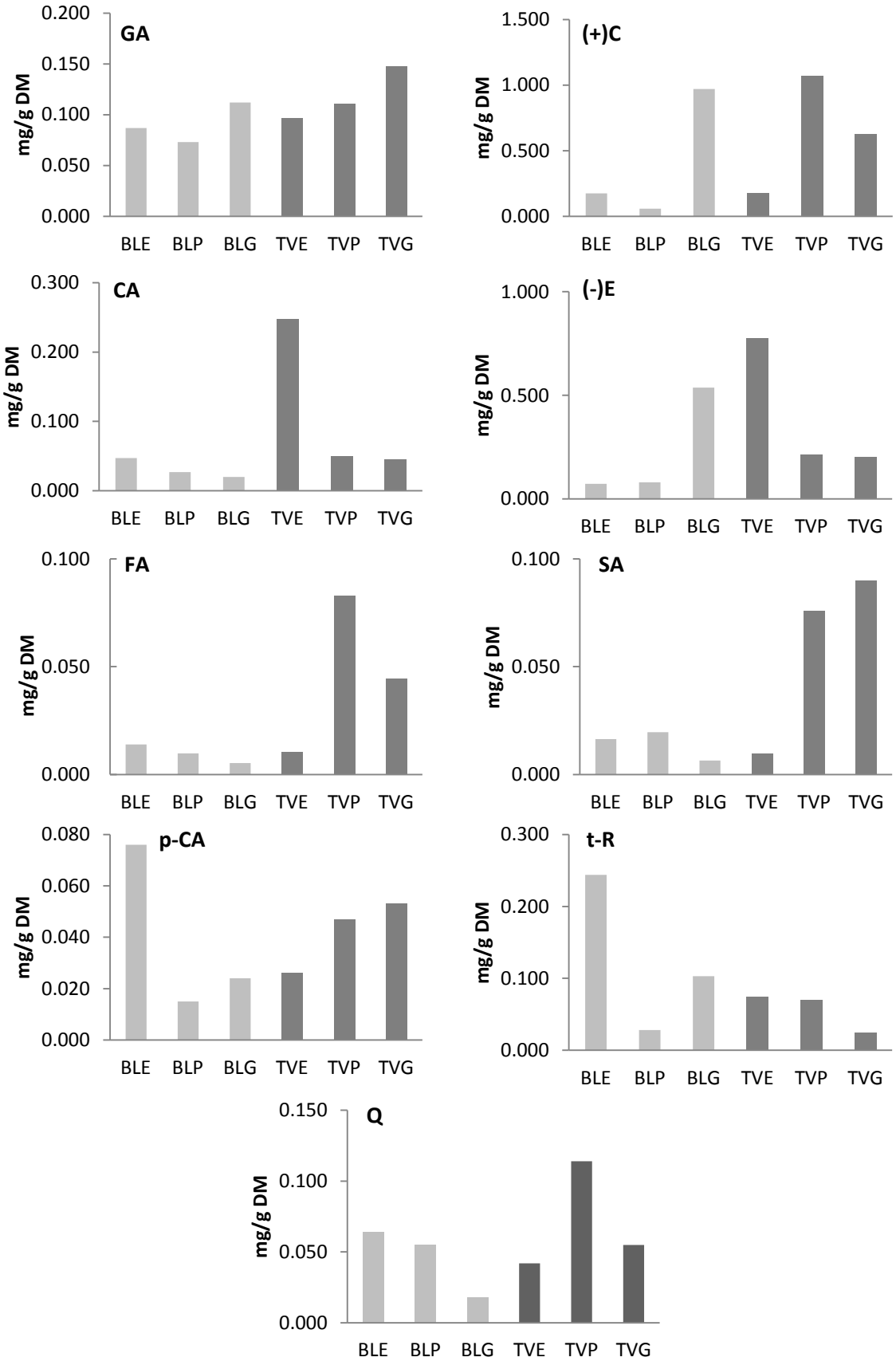

Figure 3.13 Distribution of phenolic compounds in the different parts of Loureiro and Vinhão GP

In Figures 3.14 - 3.18 are presented examples of HPLC chromatograms for the different wavelengths studied. In Figure 3.14 the chromatogram shows the analysis made at 280 nm for the identification of hydroxybenzoic acids and flavanols. In Figure 3.15 the chromatogram shows the analysis made at 320 nm for the identification of hydroxycinnamic acids and stilbenes and Figure 3.16 presents the chromatogram at 370 nm for the identification of flavonols. The chromatogram in Figure 3.17 was obtained at 280 nm with the standards for the calibration curve. The chromatogram in Figure 3.18, was obtained from an analysis made at 540 nm. From the literature the anthocyanins compounds are eluted at this wavelength. However in this work, the anthocyanins standards were not available to confirm this.

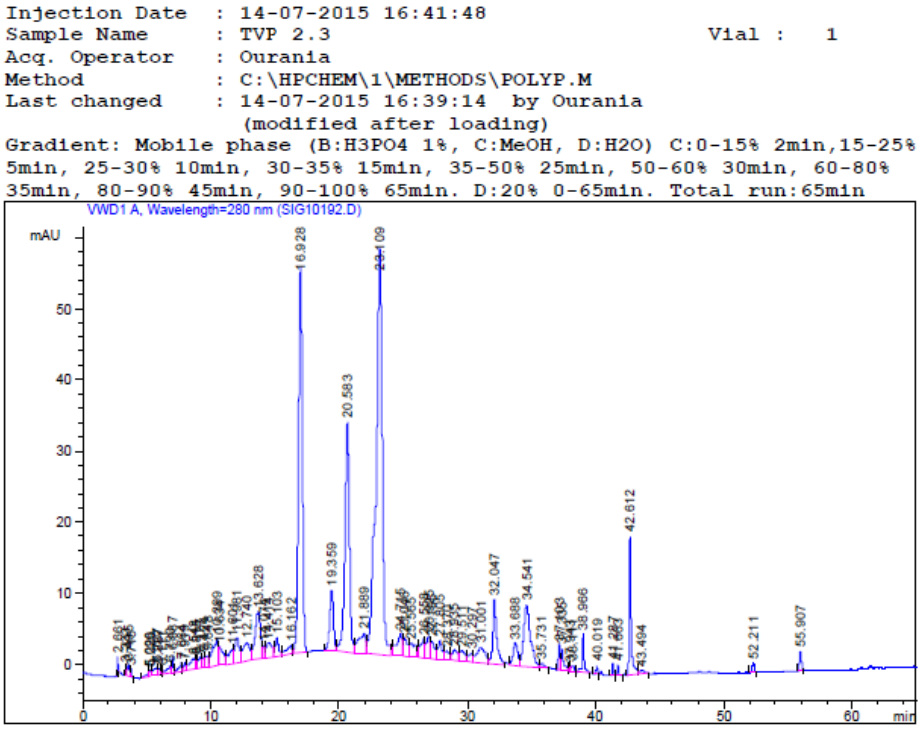

Figure 3.14 Example of an HPLC chromatogram at 280 nm

```
Injection Date   : 14-07-2015 10:54:34
Sample Name      : BLG 1.3                                  Vial :   1
Acq. Operator    : Ourania
Method           : C:\HPCHEM\1\METHODS\POLYP.M
Last changed     : 14-07-2015 11:02:54  by Ourania
                   (modified after loading)
Gradient: Mobile phase (B:H3PO4 1%, C:MeOH, D:H2O) C:0-15% 2min,15-25%
5min, 25-30% 10min, 30-35% 15min, 35-50% 25min, 50-60% 30min, 60-80%
35min, 80-90% 45min, 90-100% 65min. D:20% 0-65min. Total run:65min
```

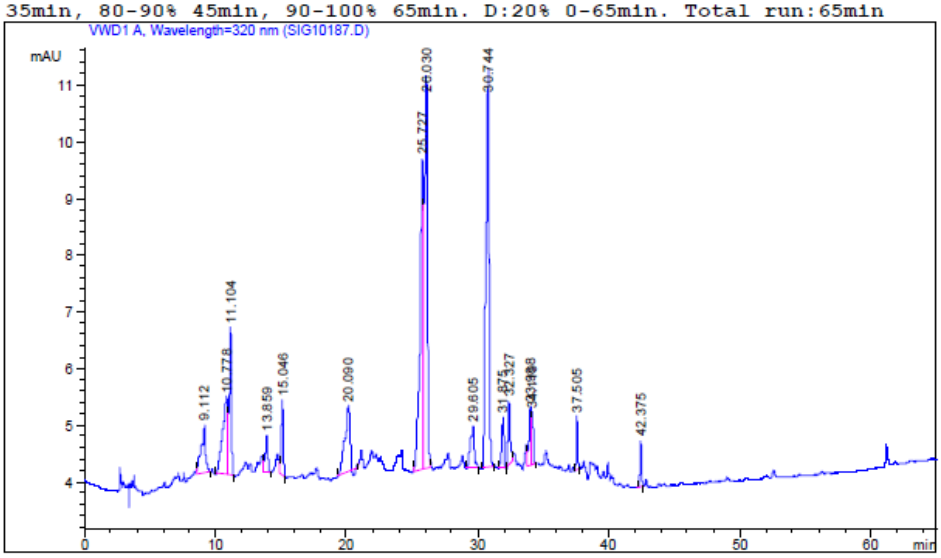

Figure 3.15 Example of an HPLC chromatogram at 320 nm

```
Injection Date   : 15-07-2015 11:11:57
Sample Name      : TVP 2.3                                  Vial :   1
Acq. Operator    : Ourania
Method           : C:\HPCHEM\1\METHODS\POLYP.M
Last changed     : 15-07-2015 11:11:56  by Ourania
                   (modified after loading)
Gradient: Mobile phase (B:H3PO4 1%, C:MeOH, D:H2O) C:0-15% 2min,15-25%
5min, 25-30% 10min, 30-35% 15min, 35-50% 25min, 50-60% 30min, 60-80%
35min, 80-90% 45min, 90-100% 65min. D:20% 0-65min. Total run:65min
```

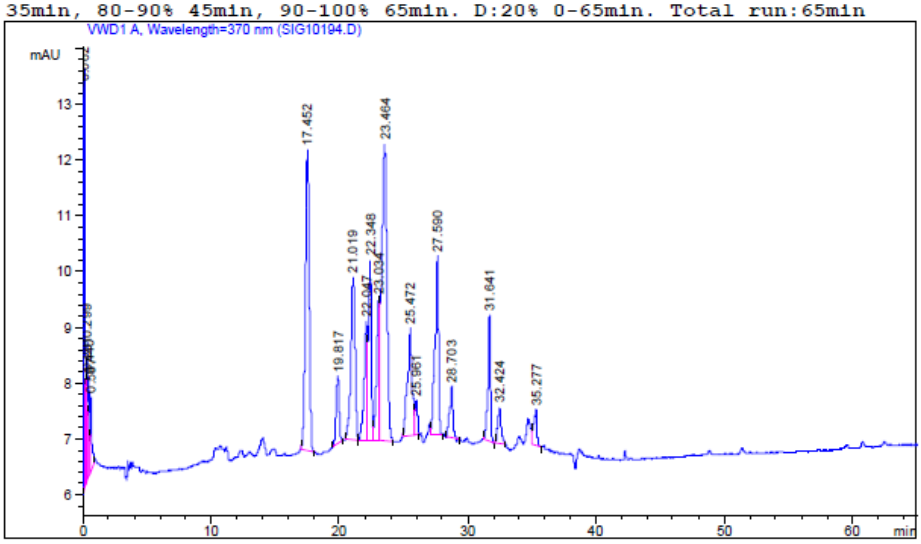

Figure 3.16 Example of an HPLC chromatogram at 370 nm

```
Injection Date   : 15-07-2015 17:50:51
Sample Name      : MixStandards(11)                    Vial :   1
Acq. Operator    : Ourania
Acq. Method      : C:\HPCHEM\1\METHODS\POLYP.M
Last changed     : 15-07-2015 17:46:01  by Ourania
                   (modified after loading)
Analysis Method  : C:\HPCHEM\1\METHODS\POLYP.M
Last changed     : 16-07-2015 11:06:31  by Ourania
                   (modified after loading)
Gradient: Mobile phase (B:H3PO4 1%, C:MeOH, D:H2O) C:0-15% 2min,15-25%
5min, 25-30% 10min, 30-35% 15min, 35-50% 25min, 50-60% 30min, 60-80%
35min, 80-90% 45min, 90-100% 65min. D:20% 0-65min. Total run:65min
```

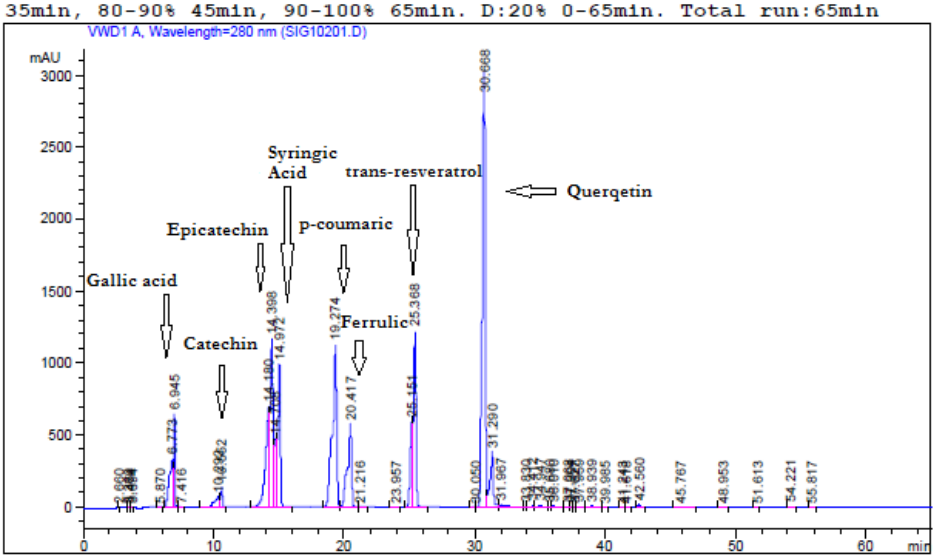

Figure 3.17 Example of an HPLC chromatogram of standards at 280 nm

```
Injection Date   : 08-05-2015 16:52:43
Sample Name      : TVP 2.3                             Vial :   1
Acq. Operator    : Ourania
Method           : C:\DEF LC.M
Last changed     : 08-05-2015 16:50:19  by Ourania
                   (modified after loading)
```

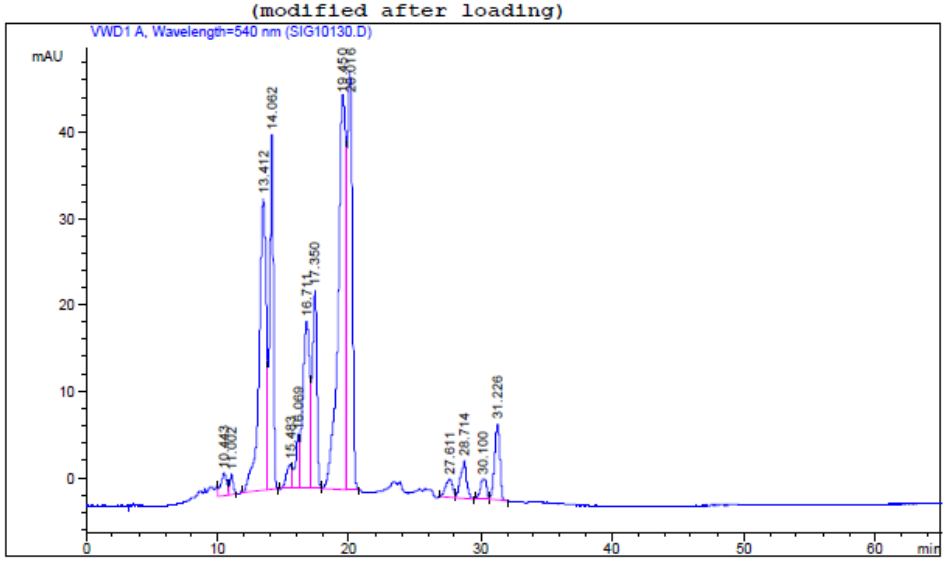

Figure 3.18 Example of an an HPLC chromatogram at 540 nm

3.5 Results of high heating value (HHV) with a constant volume bomb calorimeter

In order to investigate the possibility of energy recovery for grape pomace, it was determined the heating value of stalks and mixture from the Loureiro and Vinhão GP varieties. Additionally to the original GP samples, this property was also studied in the extracted samples, to compare the results after the extraction of the bioactive compounds. In order to investigate the amount of oxidizing matter in GP it was made the COD test to compare this wet oxidation analyses with the dry oxidation analysis by the bomb calorimeter. The results are presented in Table 3.15.

Table 3.15 COD results and high heating values for studied GP varieties in DM and other biomass values

Sample	HHV cal/g	HHV MJ/kg	COD mg O_2/g	Fuel[1]	HHV MJ/kg
BLE	4,289 ± 26	18.0 ± 0.11	884 ± 16	Corn stalks/stover	17.6 - 18.5
EBLE	4,103 ± 34	17.2 ± 0.14	-	Sugarcane bagasse	17.3 - 19.4
BLM	5,043 ± 32	21.1 ± 0.13	826 ± 22	Fruit pits	15.8 - 20.5
EBLM	4,321 ± 49	18.1 ± 0.20	-	Miscanthus	18.1 - 19.6
BLP	-	-	826 ± 11	Eucalyptus	19.0 - 19.6
BLG	-	-	1,004 ± 3	Hardwood wood	18.6 - 20.7
TVE	4,204 ± 20	17.6 ± 0.08	947 ± 67	Softwood wood	18.6 - 21.1
ETVE	4,040 ± 86	16.9 ± 0.36	-	Coal (wet basis)	23.97
TVM	4,957 ± 26	20.8 ± 0.11	731 ± 5	Petroleum coke	31.31
ETVM	2,432 ± 40	17.7 ± 0.17	-	Crude oil	45.54
TVP	-	-	1,169 ± 29	Natural gas	52.22
TVG	-	-	944 ± 19	Hydrogen	142.18

[1] From website (Boundy B., 2011)

Table 3.16 Statistical analyses for HHV and COD results

Fractions	HHV	COD		
t-test		**ANOVA, *p***	0.20	**Loureiro**
BLE vs EBLE	< 0.0001	**Tukey analysis**		
BLM vs EBLM	< 0.0001	**E vs P**	0.50	
TVE vs ETVE	0.006	**E vs G**	0.18	
TVM vs ETVM	< 0.0001	**P vs G**	0.67	
		ANOVA, *p*	0.74	**Vinhão**
		Tukey analysis		
		E vs P	0.74	
		E vs G	0.83	
		P vs G	0.98	

In Table 3.15 are presented the results for high heating values from Loureiro and Vinhão GP and from other biomass samples. The results showed that there were very significant differences between the two varieties, for all the fractions (p<0.01) (Table 3.16). Comparing high heating value for stalks and mixture the later has the highest values (21.1%) and very similar to the study of Toscano that found a heating value for grape mixture of 20.1 MJ/kg and for the stalks of 16.8 MJ/kg (Toscano G., 2013). Comparing the results for the heating value of the wood pellet from Table 3.15 it can be observed that the GP samples even after the fat and the polyphenol extraction have heating values near to the wood pellets, which indicates that this biomass has high potential for energy recovery. Both mixture and stalks from the two varieties have similar values after the extractions (Figure 3.19).

The results for COD showed no significant differences between the fractions in stalk, skin and seed from Loureiro (884, 826 and 1,004 mg O_2/g, respectively; p=0.20) and the same was observed in Vinhão (947, 1,169 and 944 mg O2/g, respectively; p=0.74). The high heating values in GP are in accordance with the high values for the chemical oxygen demand (COD) between 731-1,169 mg O2/g. This means that GP has high content in organic compounds that can be oxidized by a strong oxidizing agent (chromium dichromate).

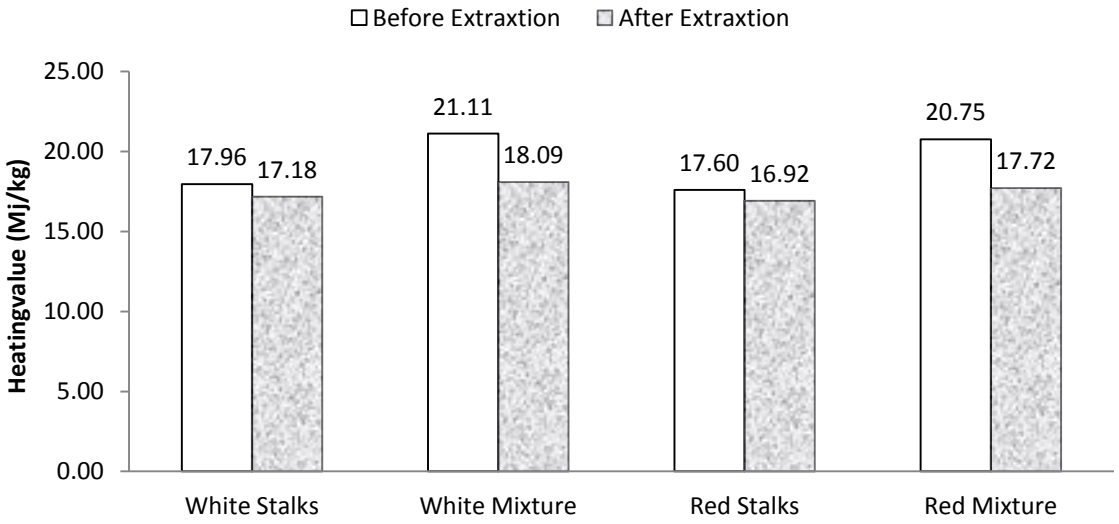

Figure 3.19 Correlation between the heating value (MJ/kg) of the original and extracted sample

3.6 Brief considerations for the grape pomace applications according to the results

The separation of GP into the different components can be useful to give a distinct value to each part according to its typical characteristics. Concerning the general composition of grape pomace from Vinho Verde, the moisture varies from 42.2% to 72.9% depending on the grape variety and the grape part considered. This high value represents a problem for the transportation and storage because this raw material is produced only once a year and it is necessary to apply some drying process before storing it. The inorganic content in a by-product with 10 % moisture ranging from 3.7% to 8.8% is an indicator of the mineral content. Comparing these values with wheat flours for example, ash values ranges from about 0.3 % for white cake flour to about 1.5% for whole wheat flour (Gisslen Wayne, 2012). This means that grape skin and grape seed could be a good source for production of flour with a high content of minerals as it was discussed in section 3.2, to be supplemented in other flour products. Another parameter that is also very important for the production of the flour is the protein content. The protein content of flours available to professional bakers may range from about 8% for cake flours to 12% to 13% (10% moisture) for bread flour (Wayne Gisslen). The protein content is between 11.9 to 13.2 % (DM) in grape seed and skin varieties, could be a valuable characteristic for flour. Also, GP flour is free of gluten as it is referred by many authors. The carbohydrates and the crude fibers showed a considerable amount for both 15.4-29.2% and 11.9-31.0 % respectively. In the white and red seed the fiber content presents very high values. Several authors have reported the important physicochemical properties of wine by-products indicating that grape pomace could be good sources of dietary fiber (Bravo, 1998). Fiber content is related not only to the quantitative but also qualitative aspect since grape pomace fibers are structurally different from those found in other cereals and other fruits since they are associated with polyphenols and antioxidant activity. With regard to the fat content, the values found between 1.65-16.4% are more relevant in seeds, known for their beneficial properties, particularly to the cardiovascular system (section 1.6.1). The fat content from the grape seeds is highly enough for play an important role in the production of oil with valuable characteristics as it was discussed in section 1.6.1 for culinary and cosmetic uses.

Grape pomace contains a noticeable amount of minerals rich in potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and iron (Fe), even after the extractions. Due to its high concentration in macro and micro-nutrients, nitrogen, potassium and phosphorus, grape pomace, like most by-products, can be used as a crop fertilizer. However, the presence of polyphenols, which are compounds related to phytotoxic and antimicrobial effects, limitates the direct application and makes conditioning treatments, such as composting, necessary before using these by-products for agricultural purposes. For optimal composting, the material must have a high moisture content and contain a sufficient carbon-to-nitrogen (C:N) ratio. The high C:N ratio provides nutrients for the microbes to survive and continue degradation (Bertran E. X. Sort, 2004). The compost obtained is recommended for application to the vineyards because: (i) the humified nature of the organic matter would facilitate its incorporation and improve the water-holding capacity of the soil, an important factor for the quality and specificity of wine production, (ii) nitrogen is released only gradually which is particularly appropriate for the vineyards that suffer from high nitrogen levels and (iii) it reports high-to-moderate values of potassium considered a quality factor in wines (Arvanitoyannis Ioannis S., 2006). Also, from the results obtained in phosphorus determination, resulted that the minerals content still remain in the samples after the extraction of the bioactive compounds. From this observation it could be concluded that the extracted GP has rich characteristics for use as an animal feed, due also to the high protein content that remains in the extractable samples.

As observed, from the HPLC study the whole fractions of grape pomace byproducts can be considered as an important source of polyphenols and, depending on the end use, the separation of skins and seeds in preliminary steps may not be always necessary. It can be concluded that the best described property of almost every group of polyphenols is their capacity to act as antioxidants. Antioxidants are used in foods to stall or hinder the oxidation of molecules. Two types of antioxidants can be used, either natural or synthetic. Examples of synthetic antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which have been used since the beginning of the 20th century. More recently, these products have been restricted because of their

carcinogenicity (Shi John, 2005). Therefore, there is a larger focus on natural antioxidants such as polyphenols that can be used as functional foods. The commercial development of natural antioxidants from plant sources for nutritional purposes is of major interest. Currently, catechin has started to be used in the market for grape seed extracts as dietary supplements. The flavonols like quercetin and flavanols like catechin and epicatechin seem to be the most powerful flavonoids for protecting the body against reactive oxygen species with various effects like antiviral, anti-inflammatory, anti-atherosclerotic, anti-ageing etc. Therefore, GP has great potential to serve as a source of functional food ingredient, to optimize the health benefits and minimize possible negative health effect by using it in the pharmaceutical sector or in the cosmetic sector.

From data obtained in heating value determinations resulted that the samples contain a high heating value that is comparable with the wood chips, even after the extraction of the bioactive compounds. Mixture from white Loureiro has lower ash content than the stalks and they also have a higher HV, due to the oil present from seeds. During combustion part of ash volatilizes leading to fly ash production. The formation of this pollutant is caused by different elements contained into grape pomace and its components, in particular high K content together with N, S and Cl. For these issues, special abatement systems are required to control emissions. Currently grape pomace for energy application is generally blended with other biomass of higher quality, like wood chips, to improve energy content and reduce ash content (MirandaT., 2012). Heavy metals concentrations in all different parts are generally lower than wood chip values; only copper is higher in all parts of GP than in wood chips (Toscano G., 2013). In particular, copper concentration is very high in mixture of red Vinhão and much lower in mixture from white Loureiro. The presence of copper can contribute in particular to the fly ash problem and the ash recycling or utilization.

4 Economic Evaluation

The "Poliempreend" is a contest of ideas and business plans that aims to evaluate and reward projects developed and presented by students of polytechnic education. The "Poliempreend" is promoted jointly by all Portuguese Polytechnic Institutes and has a regional and national component. In the final part of the development of this work it was presented an application to Poliempreend with a project named "Oil and Bioenery" which intended to develop and commercialize different products from GP.

4.1 Availability of GP from "Vinho Verde" winemaking as raw material

In this chapter it will be presented the potential market for grape pomace industrial applications, a study which was made for Poliempreend contest.

Grape pomace accounts for about 20-25% of the weight of the grape crushed for wine production (Ahmedna, 2013). One ton of grapes produce 748.5 L wine (FAO, 2009). Knowing the amount of "Vinho Verde" wine that is produced annually, it is possible to calculate the amount of grape pomace produced each year in the Minho region and also in Portugal and Europe, considering 25 % weight for GP production.

Table 4.1 Annual grape pomace production[1]

	Wine (L)	Grapes (ton)	Grape pomace(ton)
Vinho Verde	53,279,359[2]	71,181.5	17,795.4
Portugal	674,000,000	900,467.6	225,116.9
Europe	16,755,300,000	22,385,170.3	5,596,292.6

[1] Calculated in this study

[2] The value is different from the table 1.3 because it contains the amount of D.C.P wine (18,995 L)

Since the products obtained from GP recovery are new and not known for majority of population, the potential market for grape pomace products was determined using the results from this study and only a small part of the total available quantity of grape pomace from Vinho Verde winemaking, which was the 2.7 % of the 17,595.4 tons. The project proposed, included ecological processes for the recovery of grape pomace to generate final products like grape seed oil, grape pomace extract of polyphenols and gluten-free flour, which are innovative foods for human nutrition with a high economic value. Also, will be presented the potential market for the extracted grape pomace

samples, which are the samples after the removal of bioactive compounds which could be used as natural fertilizers and animal feeds.

In 2014, 53,279,359 L of "Vinho Verde" wine was produced in Minho region. The annual wet grape pomace produced from "Vinho Verde" is about 17,795.4 tons (Table 4.1). "Vinho Verde" is more known for the white wines as a result the pomace that is produced is mostly from white grapes as shown in figure 1.5. The total amount of red pomace was determined by multiplying 17,795.4 tons of total pomace by 23% (percentage of red wine produced in Minho region) giving 4,092.94 tons wet red GP. The same was done for the white grape pomace by multiplying this time with 77% that is the percentage of white wine produced in Minho region, resulting the amount of 13,704.46 tons of white wet grape pomace. An average humidity determined from this study was for the white GP 59.9 % and for the red GP is 57.5 %. According to Fiori, grape pomace composition on a dry basis is 51% skins, 47% seeds and 2% stalks (Fiori, 2010). The latter composition and moisture percentages have been used to calculate the results presented in Figure 4.1, about the availability of "Vinho Verde" grape pomace.

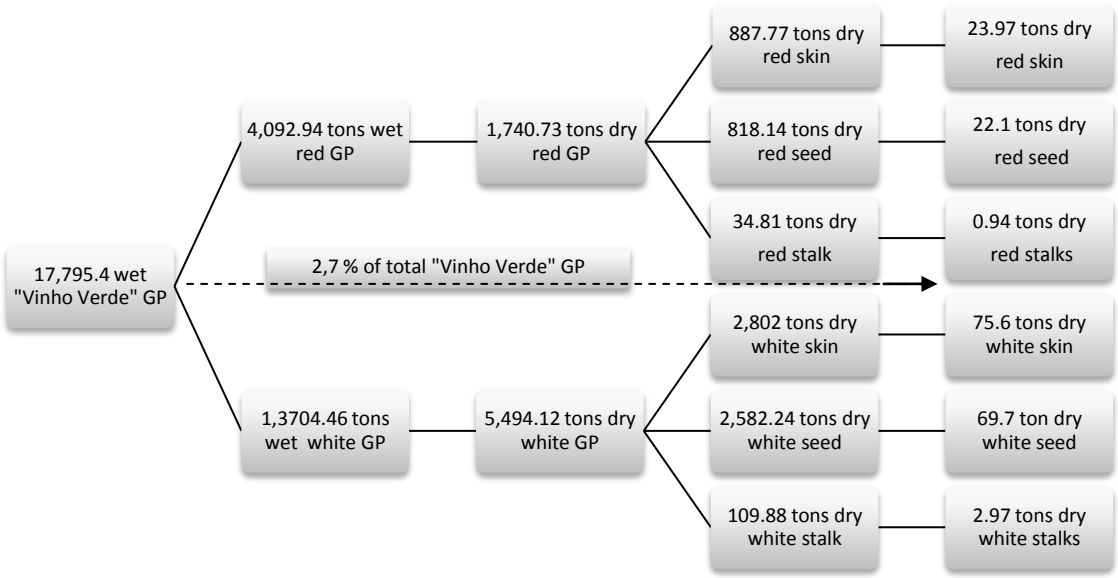

Figure 4.1 Availability of Vinho Verde grape pomace

Because of the geographic dispersion of the available raw material (section 1.3.1) presented in Figure 1.2, the initial project involves a small scale production because of the cost of the transport and likely difficulties in buying the raw materials. Because these are new products, there will be some barriers at the entrance of the products on the market.

To show the value of these GP products the scenario that was presented was pessimistic, using only the 2.7% of the available GP in Minho region that means 480 tons wet GP or 195.3 tons of dry GP that means 2 tons of wet GP to be processed per day (240 days labor in a year). Data regarding the price of the grape pomace 0.15 € per kg was collected by interviewing the production supervisor of Adega Cooperativa de Ponte da Barca, resulting a amount of 72,071 € for the purchase of the raw material.

4.2 Potential market of GP from "Vinho Verde" winemaking

In Figure 4.2 is described the operation flow after the collection of GP from the factory. The stalks, skins and seeds have to be separated by mechanical sieves, whose separation efficiency, looking at the industrial equipments available in the market is 100% (Trainor, 2006). After follows the storage which should be done with refrigerator in low temperature to protect the raw material from fungi in order to have the raw material to process during one year.

For the production of the oil the grape seed will be dried in a rotary drier where the drying temperatures are selected so as to not degrade the seed. In particular, charring or burning of the seed must be avoided, since this causes the damaging of valuable compound. Such a temperature could be approximately 80°C whereby to maintain a useful quality of grape seed. In preference, such drying is to an extent that there is 10% or less of water remaining by volume. Then, the oil will be extracted by cold press extraction that gives lower yields than the hot extraction and solvent extraction but is ecological and energy efficiency (75% yield instead 99% in solvent extraction (GreenerPro, 2009). To determine the quantity of grape-seed oil quantity that could be produced, pomace seeds from red and white (91.8 tons) were multiplied by 12.16% (75% of the 16.2% which is the average oil content of seeds, determined in the study) giving 11.16 tons of seed oil. Seed oil (11.16 tons) was then converted to grams. The density of grape-seed oil is 0.92, (Ceriani Roberta, 2008) were used to convert the grams of grape-seed oil to volume.

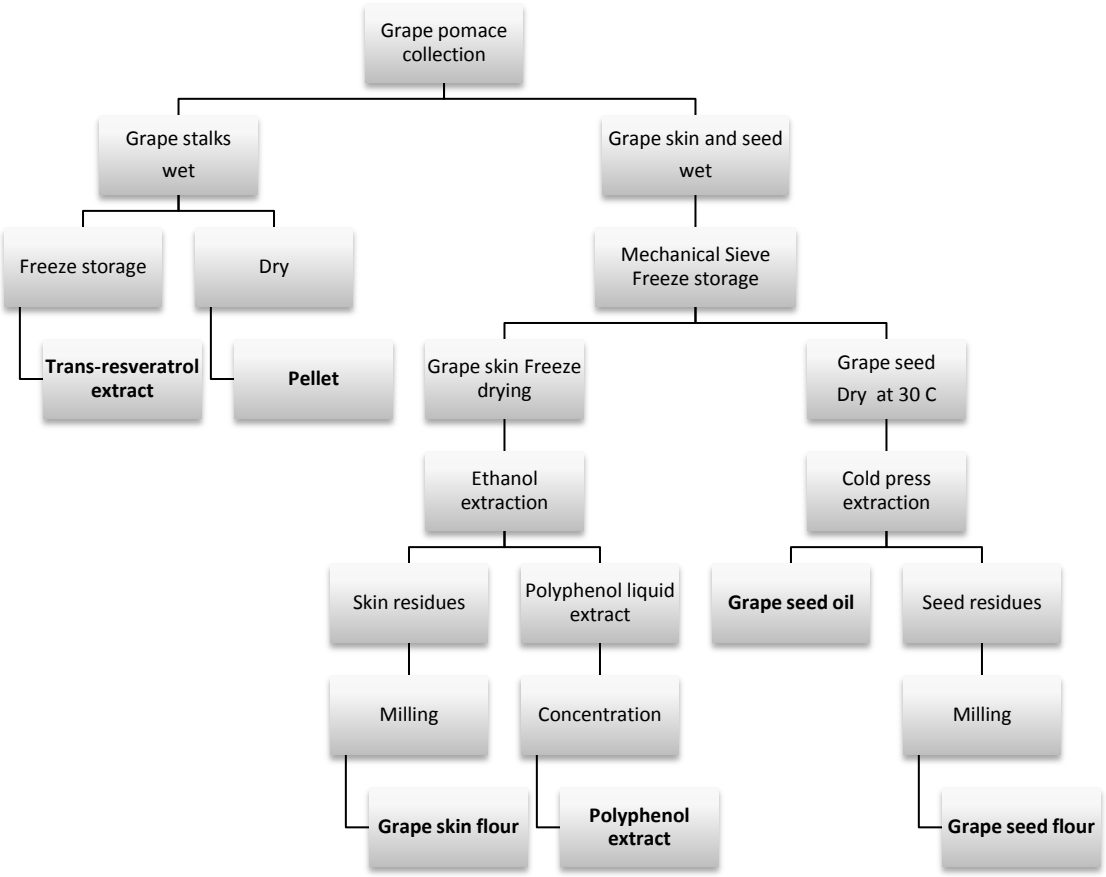

Figure 4.2 Operation flow for GP industrial processes in order to obtain the different final products

The market potential was determined based on a research about the prices of other grape-seed oils available in retail (La tourangelle, Montebaldo, Kirkland Signature, Now Foods, Pompeian 100%, Salute Sante, Napa Valley/Extra Virgin, Bel Aria, Whole Vine Products) where the prices are very diferent depending on the quality. A good quality product has a price around 10.00€ for half a liter, so for a factory price it was defined 1.5€ for half a liter. With this data, the total market value is 36,391€. After the oil extraction, the residual part of the grape seed could be milled and used for other purposes with a 10% of humidity, giving 80.7 tons of grape seed flour, after subtracting from the total amount of dry seeds, the amount of the extracted oil.

Another industry process could be applied to GP to recover the polyphenol extract. For the polyphenols extract the grape skin will be freeze dried and then extracted into horizontal rotary extractors (extraction in batches) in ethanol solution, as a more environmentally friendly solvent, recognized as safe according to the European Food Safety Authority (EFSA) and FAO/WHO Expert Committee on Food Additives (JEFCA). The

extract will be concentrated with evaporation. The concentrated extract of polyphenols, will be sold without other treatment to other industries, like pharmaceuticals, cosmetic and food. The further isolation of the bioactive compounds would produce several ingredients to be incorporated into final products. Because of the high investments needed for the equipments to isolate the different polyphenols this process will not be included in an initial start up. From this study was determined that the polyphenols content in white grape skin was about 24 g GAE/kg DM of total polyphenolics and the red grape skin 120 g GAE/kg DM, equivalent to 2.4 – 12% in white and red skin respectively. These values were used to calculate the amount of extract could be produced. From 195.3 tons of dry grapes, 51% represented skins (99.6 tons of dry grape skin), which are submitted as raw material and mixed with ethanol to obtain the polyphenolic extract. This could result a production of 4,690 kg of polyphenols extract which is obtained when added the result of multiplication of the amount of dry red grape skin (23.97 tons) with the percentage of 12 % plus the results of multiplication of dry white grape skin (75.6 tons) with the percentage of 2.4 %. After the polyphenol extraction, the residual part of the grape skin could be milled and used for other purposes, giving 94.9 tons of grape skin flour, after subtracting from the total amount of dry skins, the amount of the extracted polyphenols.

After the production of the oil and the polyphenols extract, the residual part of the skins and the seeds are 175.6 tons which could be sold as gluten free flour, but also a part of it could be sold as animal feed or fertilizer. The reference market price for GP flour is 3€ per kg, which gives an income of 526,800€. The food products (oil and flour) are distinguished from the common olive oil and the conventional cereal (wheat, barley, rye) flour because they have extra features that are beneficial to nutrition / health and can be distributed in Portugal and abroad.

From this study it was determined that the white grape stalk contain about 26 g GAE/kg DM, in total polyphenolics. From 195.3 tons of dry grape pomace, 2% represented stalks, which results 3.91 tons of dry stalks from which 2.97 tons are white stalk. Using this raw material with ethanol could give a production of 78.3 kg of polyphenols extract. The white stalk was selected for the extraction of polyphenols because from the HPLC

analysis (section 3.3.3) found that contain a noticeable amount of trans-resveratrol, 0.244g/kg DM. This compound has a high economical value and from the 2.7 % white stalks (3,011 kg) a production of 0.735 kg of trans-resveratrol, could be obtained. As an example for the importance of this compound, according to the market price 100 mg of trans-resveratrol costs 150 €, which means 1,102,500 € for 0.735 kg produced. The final amount of the polyphenol extract would be 4,691 kg which taking as reference the price 125 € per kg according to Cardona (Cardona Jorge A., 2010) gives an income of 585,786 €.

The residual part of the grape stalks could be used for the production of pellets, since the heating value of the grape stalks was high, around 17 MJ/kg as it was determined in this study. Although, the pelletization is better to combined with the production of other products, since the grape pomace pellets are more expensive than the others pellets from alternatives biomass, due to the high drying costs of the raw material. In the project of "Oil and Bioenery" did not include the economic analysis for pellet production because it is a different economic activity which needed different industrial units, although in the Table 4.2 presented profits from pellets considering the market price from wood pellets.

Table 4.2 Potential market of the products obtained from 2.7% of "Vinho Verde" GP

Products	Amount produced	Reference price	Profits
Grape-seed oil	12,130 (L)	3 € per L	36,391 €
Polyphenol extracts	4,691 (kg)	125 € per kg	585,786 €
Grape Pomace flour	175,600 (kg)	3 € per kg	526,800 €
Pellets	3,910 (kg)	3.5 € per 15 kg	912 €
Total			**1,149,889 €**

4.3 Economic Analysis

The economic analysis was conducted based on profitability, and sustainability of the final products and the analysis are presented in Annex 2. Profitability was evaluated by comparing total revenue versus total costs through a break-even analysis of prices and production volumes, assuming that prices would have an increment about 15% next year. The economic return of this business was assessed over a period of 6 years by a cash flow analysis. For the project analyses, a land with two buildings (factory and store) and the equipment needed for the industrial operations were evaluated in 1,794,000 euro. Eight

employees would be needed for this operation and it was assumed to be spent 171,251 €/year, with the workers working 8 h per day on these processes. Supplies and external services like marketing costs, electricity, cleaning etc were taken into account in this analysis with an annual cost of 128,000 €. For the cash flow calculations, 21% was used as tax rate. for electricity costs, taken into account the operating hours needed to produce the quantities describes above per day the time (extra 1 h) was added for basic maintenance and warming up of equipment. A loan was calculated based on a fixed interest rate (6%) and to be fully paid in 5 years. In the first year of operation (2016) it was assumed that only the half of the calculated amounts of products would be produced because of the preparation time (6 months) for the factory to start the functions. The results of this economic study showed that after the sixth year of operation, the profits would be almost 743,000 €. Due to limited information regarding equipment, since the research made only by internet and not interviewing the suppliers, this study only contained general information for engineering and equipment specifications; thus, many other specifications can be added to this project enabling the economic prediction more accurately.

5. Conclusion and future work

The obtained results on the energy value and chemical composition of GP provided guidance for developing innovative industrial applications. Regarding the chemical composition of the two GP varieties, the results indicate a high content of oil (16.4% and 15.7% w/w DM in Vinhão and Loureiro, respectively) and fibers (31.0% and 29.0% w/w DM in Vinhão and Loureiro, respectively) in the grape-seed. In the grape skin it was found high amount of protein (13.18% and 11.95% w/w DM in Vinhão and Loureiro, respectively), but also of minerals and carbohydrates. These findings enables grape skin and seed to be used for human nutrition, as it can be a substitute of wheat flour for bakery or a source of high value vegetable oil. The high content of total phenolic compounds (TPC) makes them excellent candidates as food additives which provide food commodities with high antioxidant activity and natural colorants. With the identification and quantification of some polyphenols in GP, resulted that the skin and the seed have higher content in flava-3-ols (catechin, epicatechin) and the stalks from white Loureiro GP appeared to contain a high content of trans-resveratrol, which opens horizons for the use in the pharmaceutical and cosmetic industries. Finally, from the data obtained, it was concluded that the extracted GP still has potential as a high value product because it is rich in minerals and other nutrients to be used as fertilizer, animal feed or a biofuel, because it has a high heating value.

Considering the amounts of GP that are produced annually in Minho region, and from the chemical properties of GP, it is evident that this sub-product from the winery industry provides new perspectives on potential profitability with alternative industrial applications. However this exploitation is only possible if the bioactive products are firstly removed from the GP and to the remaining is applied for human nutrition and cosmetology. Also, the remaining stalks could be used for the common uses of today, as fertilizers, animal feed and pellet. Thus, recycling of winery by-products constitutes an opportunity for providing valuable materials to pharmaceutical, cosmetic, nutraceuticals, and food industries, contributing to reduce costs and environmental impact linked to the disposal of these by-products in the production areas.

Concluding, it can be said that grape pomace cannot be considered anymore as a winery waste, but as a by-product, which could extend the grape's commercial life cycle.

Several aspects of this study, however, are worthy further studies.

- A future study should be done in the determination of other compounds in grape pomace:
  -the content of dietary fibers,
  -the characterization of fatty acids
  -the quantification of anthocyanins
- Development of a more environmentally friendly and efficient extraction method
- Development of a separation method for the extracted polyphenols;
- Finally, it could be developed an industrial pilot plant to implement the mentioned processes with modern technologies to evaluate the real economic benefits from the reported applications.

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Annex 1 Data and equation of calibration curves

Table A Data for the calculations of CH, TPC, DPPH and COD content

Parameter	Work range	Regression equation	R^2	DL (mg/L)	QL (mg/L)
Carbohydrates (%)	0.082-1.50	y = 0.2278x - 0.026	0.9998	0.027	0.082
Total phenolic content (mg GAE/g DM)	0.28-30	y =0.0021x + 0.0062	0.9996	0.095	0.28
Antioxidant Activity (mg AAE/g DM)	0.036-175	y =2.2254x + 22.476	0.9998	0.012	0.036
COD (mg O_2/g)		y = 0.0004x + 0.0025	0.9997		

Table B Data for calculation the minerals content

Element	Work range	Regression equation	R^2	DL (mg/L)	QL (mg/L)
Potassium	5.2 - 1.400	y =0.2064x – 0.0051	0.9993	1.72	5.2
Calcium	5.3 - 5.000	y =0.0306x + 0.0002	0.9998	1.76	5.3
Phosphorus	0.37 - 0.500	y =0.3597x + 0.0016	0.9997	0.12	0.37
Magnesium	0.24 - 0.400	y =0.5513x + 0.0067	0.9999	0.082	0.24
Sodium	1.6 - 2.400	y =0.571 x – 0.0049	0.9999	0.52	1.6
Iron	1.7 - 3.000	y =0.0935x + 0.0009	0.9999	0.57	1.7
Copper	1.1 - 2.000	y =0.1497x + 0.0006	1	0.37	1.1
Zinc	0.81 - 0.500	y =0.406 x + 0.0194	0.9993	0.27	0.81
Manganese	4.0 - 3.000	y =0.202 x + 0.0052	0.9994	1.33	4.0
Chromium	2.9 - 2.000	y =0.052x + 0.0024	0.9997	0.97	2.9
Nickel	2.3 - 1.000	y =0.0625x + 0.022	0.9981	0.78	2.3
Lead	4.8 - 4.000	y =0.057x + 0.0172	0.9996	1.6	4.8
Cadmium	1.5 - 1.000	y =0.3442x + 0.008	0.9997	0.50	1.5

Table C Data for the calculation of the phenolic compounds in examined samples by HPLC

Phenolic compound	Wavelength (nm)	Regression equation			Work range	DL (mg/L)	QL (mg/L)
		Lower range	Higher range	Global range			
Gallic acid	280	y =48.356x + 2.6334 R^2 = 0.9988	y =54.274x + 33.126 R^2 = 0.9998	y =54.387x + 12.727 R^2 = 0.9998	0.0016-257	0.00052	0.0016
(+)Catechin	280	y =14.011x – 2.857 R^2 = 0.9995	y =13.855x + 28.697 R^2 = 0.9994	y =13.934x + 12.021 R^2 = 0.9994	0.0012-300	0.00041	0.0012
(-) Epicatechin	280	y =16.951x – 3.3516 R^2 = 0,9967	y =15.675x + 31,585 R^2 = 0.9997	y =15.734x + 14.504 R^2 = 0.9997	0.0042-412	0.0014	0.0042
Syringic acid	280	y =32.2x + 0,.864 R^2 = 0.9968	y =35.857x + 22.711 R^2 = 0.9996	y =35.951x + 9.3596 R^2 = 0.9997	0.0021-203	0.00071	0.0021
Caffeic acid	320	-	-	y =103.31x – 0.9287 R^2 = 0.9992	0.0009-203	0.00030	0.0009
p-Coumaric acid	320	-	-	y =129.68x + 0.675 R^2 = 0.9996	0.005 - 148	0.00016	0.0005
Ferrulic acid	320	-	-	y =102.94x + 0.6481 R^2 = 0.9999	0.0003-158	0.00011	0.0003
trans-Resveratrol	320	-	-	y =149.42x + 0.7783 R^2 = 0.9999	0.0004-204	0.00012	0.0004
Quercetin	370	-	-	y =69.4x – 2.0975 R^2 = 0.9993	0.0022-199	0.00015	0.0022

Annex 2 Economic analysis

IAPMEI
Parceiros para o Crescimento

Empresa: Oil & Bioenergy, Lda

Pressupostos Gerais

Valide os pressupostos aqui indicados e ajuste-os de acordo com o seu projecto

Unidade monetária	Euros	
1º Ano actividade	2016	
Prazo médio de Recebimento (dias) / (meses)	30	1,0
Prazo médio de Pagamento (dias) / (meses)	30	1,0
Prazo médio de Stockagem (dias) / (meses)	180	6,0
Taxa de IVA - Vendas	23%	
Taxa de IVA - Prestação Serviços	23%	
Taxa de IVA - CMVMC	6%	
Taxa de IVA - FSE	23%	
Taxa de IVA - Investimento	23%	
Taxa de Segurança Social - entidade - órgãos sociais	23,75%	
Taxa de Segurança Social - entidade - colaboradores	23,75%	
Taxa de Segurança Social - pessoal - órgãos sociais	11,00%	
Taxa de Segurança Social - pessoal - colaboradores	11,00%	
Taxa média de IRS	20,00%	
Taxa de IRC	21,00%	
Taxa de Aplicações Financeiras Curto Prazo	1,00%	
Taxa de juro de empréstimo Curto Prazo	5,00%	
Taxa de juro de empréstimo ML Prazo	6,00%	
Taxa de juro de activos sem risco - Rf	1,00%	NOTA: Quando não se aplica *Beta*, colocar:
Prémio de risco de mercado - (Rm-Rf)* ou pº	10,00%	- O prémio de risco (pº) adequado ao projecto
Beta empresas equivalentes	100,00%	- Beta = 100%
Taxa de crescimento dos cash flows na perpetuidade	0,00	==> R(Tx actualização) = Rf + pº

* Rendimento esperado de mercado

Métodos de avaliação considerados:

Free Cash Flow to Firm

Em linhas gerais, o método dos fluxos de caixa descontados consiste em estimar-se os fluxos de caixa futuros da empresa e trazê-los a valor presente por uma determinada taxa de desconto (WACC). Em outras palavras, o valor de uma empresa pode ser expresso como o valor presente do fluxo FCFF (fluxo de caixa líquido para a firma, do inglês Free Cash Flow to Firm).

Free Cash Flow to Equity

No método de avaliação pelo desconto de fluxos de caixa líquido do acionista (FCFE – do inglês Free Cashflow to Equity), o objetivo é avaliar directamente o património líquido da empresa.

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Empresa: & Bioenergy, Lda

Euros

Vendas + Prestações de Serviços

	2016	2017	2018	2019	2020	2021
Taxa de variação dos preços		0,00%	0,00%	10,00%	0,00%	0,00%

VENDAS - MERCADO NACIONAL	2016	2017	2018	2019	2020	2021
Farinha semglúten	65.850	148.163	170.387	215.539	247.870	285.051
Quantidades vendidas	21.950	49.388	56.796	65.315	75.112	86.379
Taxa de crescimento das unidades vendidas		125,00%	15,00%	15,00%	15,00%	15,00%
Preço Unitário	3,00	3,00	3,00	3,30	3,30	3,30
Azeite de semente de uva "grape seed oil"	18.195	40.939	47.080	59.556	68.489	78.762
Quantidades vendidas	6.065	13.646	15.693	18.047	20.754	23.867
Taxa de crescimento das unidades vendidas		125,00%	15,00%	15,00%	15,00%	15,00%
Preço Unitário	3,00	3,00	3,00	3,30	3,30	3,30
	0	0	0	0	0	0
Quantidades vendidas		0	0	0	0	0
Taxa de crescimento das unidades vendidas						
Preço Unitário		0,00	0,00	0,00	0,00	0,00
Produto D*	0	0	0	0	0	0
Quantidades vendidas		0	0	0	0	0
Taxa de crescimento das unidades vendidas						
Preço Unitário		0,00	0,00	0,00	0,00	0,00
TOTAL	84.045	189.101	217.466	275.095	316.359	363.813

VENDAS - EXPORTAÇÃO	2016	2017	2018	2019	2020	2021
Polifenóis	293.188	659.672	758.623	959.658	1.103.606	1.269.147
Quantidades vendidas	2.346	5.277	6.069	6.979	8.026	9.230
Taxa de crescimento das unidades vendidas		125,00%	15,00%	15,00%	15,00%	15,00%
Preço Unitário	125,00	125,00	125,00	137,50	137,50	137,50
Farinha semglúten	197.550	444.488	511.161	646.618	743.611	855.153
Quantidades vendidas	65.850	148.163	170.387	195.945	225.337	259.137
Taxa de crescimento das unidades vendidas		125,00%	15,00%	15,00%	15,00%	15,00%
Preço Unitário	3,00	3,00	3,00	3,30	3,30	3,30
TOTAL	490.738	1.104.159	1.269.783	1.606.276	1.847.217	2.124.300

*Produtos/Famílias de Produtos/Mercadorias

NOTA: Caso não tenha conhecimento das quantidades, colocar o valor das vendas na linha das "Quantidades Vendidas" e o valor 1 na linha do "Preço Unitário".

PRESTAÇÕES DE SERVIÇOS - MERCADO NACIONAL	2016	2017	2018	2019	2020	2021
Serviço A		0	0	0	0	0
Taxa de crescimento						
Serviço B		0	0	0	0	0
Taxa de crescimento						
Serviço C		0	0	0	0	0
Taxa de crescimento						
Serviço D		0	0	0	0	0
Taxa de crescimento						
TOTAL	0	0	0	0	0	0

PRESTAÇÕES DE SERVIÇOS - EXPORTAÇÕES	2016	2017	2018	2019	2020	2021
Serviço A		0	0	0	0	0
Taxa de crescimento						
Serviço B		0	0	0	0	0
Taxa de crescimento						
Serviço C		0	0	0	0	0
Taxa de crescimento						
Serviço D		0	0	0	0	0
Taxa de crescimento						
TOTAL	0	0	0	0	0	0

		2016	2017	2018	2019	2020	2021
TOTAL VENDAS - MERCADO NACIONAL		84.045	189.101	217.466	275.095	316.359	363.813
TOTAL VENDAS - EXPORTAÇÕES		490.738	1.104.159	1.269.783	1.606.276	1.847.217	2.124.300
TOTAL VENDAS		574.783	1.293.261	1.487.250	1.881.371	2.163.577	2.488.113
IVA VENDAS	23%	19.330	43.493	50.017	63.272	72.763	83.677
TOTAL PRESTAÇÕES DE SERVIÇOS - MERCADO NACIONAL		0	0	0	0	0	0
TOTAL PRESTAÇÕES DE SERVIÇOS - EXPORTAÇÕES		0	0	0	0	0	0
TOTAL PRESTAÇÕES SERVIÇOS		0	0	0	0	0	0
IVA PRESTAÇÕES DE SERVIÇOS	23%	0	0	0	0	0	0
TOTAL VOLUME DE NEGÓCIOS		574.783	1.293.261	1.487.250	1.881.371	2.163.577	2.488.113
IVA		19.330	43.493	50.017	63.272	72.763	83.677
TOTAL VOLUME DE NEGÓCIOS + IVA		594.113	1.336.754	1.537.267	1.944.643	2.236.339	2.571.790
Perdas por imparidade	0%	0	0	0	0	0	0

EMPRESA: Bioenergy, Lda

Euros

CMVMC - Custo das Mercadorias Vendidas e Matérias Consumidas

CMVMC	Margem Bruta	2016	2017	2018	2019	2020	2021
MERCADO NACIONAL		**46.225**	**104.006**	**119.607**	**151.302**	**173.998**	**200.097**
Farinha sem glúten	45,00%	36.218	81.489	93.713	118.547	136.329	156.778
Azeite de semente de uva "grape seed oil"	45,00%	10.007	22.516	25.894	32.756	37.669	43.319
Produto D*							
MERCADO EXTERNO		269.906	607.288	698.381	883.452	1.015.969	1.168.365
Polifenóis	45,00%	161.253	362.820	417.242	527.812	606.983	698.031
Farinha sem glúten	45,00%	108.653	244.468	281.138	355.640	408.986	470.334
TOTAL CMVMC		**316.130**	**711.293**	**817.987**	**1.034.754**	**1.189.967**	**1.368.462**

IVA	6%	2.773	6.240	7.176	9.078	10.440	12.006
TOTAL CMVMC + IVA		**318.904**	**717.534**	**825.164**	**1.043.832**	**1.200.407**	**1.380.468**

NOTA: Mapa construído caso a caso:

a) Introduzir a Margem Bruta directamente, quando conhecida e passível de ser utilizada, ou efectuar a respectiva fórmula de cálculo;

b) Efectuar os cálculos auxiliares considerados necessários para alcançar a o nível de matéria-prima por unidade produzida e introduzir manualmente os valores;

c) Caso não seja possível alcançar o nível do consumo de matéria-prima por produto, introduzir o valor do custo total, após a realização dos respectivos cálculos auxiliares.

NOTA 2: Está disponível uma folha para cálculos auxiliares. Contém mapas para cálculo do CMVMC de projectos industriais.

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FSE - Fornecimentos e Serviços Externos

Empresa: OBoE, Oil & Bioe

Euros

	2016	2017	2018	2019	2020	2021
Nº Meses	8	12	12	12	12	12
Taxa de crescimento						

	Tx IVA	CF	CV	Valor Mensal	2016	2017	2018	2019	2020	2021
Subcontratos	23%	100%		200,00	1.600,00	2.400,00	2.400,00	2.400,00	2.400,00	2.400,00
Serviços especializados										
Trabalhos especializados	23%	100%		1.000,00	8.000,00	12.000,00	12.000,00	12.000,00	12.000,00	12.000,00
Publicidade e propaganda	23%	100%		4.000,00	32.000,00	48.000,00	48.000,00	48.000,00	48.000,00	48.000,00
Vigilância esegurança	23%	100%		1.700,00	13.600,00	20.400,00	20.400,00	20.400,00	20.400,00	20.400,00
Honorários	23%	100%		300,00	2.400,00	3.600,00	3.600,00	3.600,00	3.600,00	3.600,00
Comissões	23%	100%								
Conservação e reparação	23%	100%		500,00	4.000,00	6.000,00	6.000,00	6.000,00	6.000,00	6.000,00
Materiais										
Ferramentas e utensílios de desgaste rápido	23%	100%		800,00	6.400,00	9.600,00	9.600,00	9.600,00	9.600,00	9.600,00
Livros e documentação técnica	23%	100%		50,00	400,00	600,00	600,00	600,00	600,00	600,00
Material de escritório	23%	100%		200,00	1.600,00	2.400,00	2.400,00	2.400,00	2.400,00	2.400,00
Artigos para oferta	23%	100%								
Energia e fluidos										
Electricidade	23%	100%		2.500,00	20.000,00	30.000,00	30.000,00	30.000,00	30.000,00	30.000,00
Combustíveis	23%	100%		600,00	4.800,00	7.200,00	7.200,00	7.200,00	7.200,00	7.200,00
Água	6%	100%		500,00	4.000,00	6.000,00	6.000,00	6.000,00	6.000,00	6.000,00
Deslocações, estadas e transportes										
Deslocações eEstadas	23%	100%		400,00	3.200,00	4.800,00	4.800,00	4.800,00	4.800,00	4.800,00
Transportes de pessoal	23%	100%								
Transportes de mercadorias	23%	100%		650,00	5.200,00	7.800,00	7.800,00	7.800,00	7.800,00	7.800,00
Serviços diversos										
Rendas e alugueres		100%								
Comunicação	23%	100%		500,00	4.000,00	6.000,00	6.000,00	6.000,00	6.000,00	6.000,00
Seguros		100%		600,00	4.800,00	7.200,00	7.200,00	7.200,00	7.200,00	7.200,00
Royalties	23%	100%								
Contencioso e notariado		100%		100,00	800,00	1.200,00	1.200,00	1.200,00	1.200,00	1.200,00
Despesas de representação	23%	100%		600,00	4.800,00	7.200,00	7.200,00	7.200,00	7.200,00	7.200,00
Limpeza, higiene e conforto	23%	100%		800,00	6.400,00	9.600,00	9.600,00	9.600,00	9.600,00	9.600,00
Outros serviços	23%	100%								
TOTAL FSE					**128.000,00**	**192.000,00**	**192.000,00**	**192.000,00**	**192.000,00**	**192.000,00**
FSE - Custos Fixos					**#REF!**	**192.000,00**	**192.000,00**	**192.000,00**	**192.000,00**	**192.000,00**
FSE - Custos Variáveis					**#REF!**					
TOTAL FSE					**#REF!**	**192.000,00**	**192.000,00**	**192.000,00**	**192.000,00**	**192.000,00**
IVA					22.412,00	34.998,00	34.998,00	34.998,00	34.998,00	34.998,00
FSE + IVA					**150.412,00**	**226.998,00**	**226.998,00**	**226.998,00**	**226.998,00**	**226.998,00**

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Empresa: Bioenergy, Lda

Euros

Gastos com o Pessoal

	2016	2017	2018	2019	2020	2021
Nº Meses	7	14	14	14	14	14
Incremento Anual (Vencimentos + Sub. Almoço)						

Quadro de Pessoal	2016	2017	2018	2019	2020	2021
Administração / Direcção	1	1	1	1	1	1
Administrativa Financeira	1	1	1	1	1	1
Comercial / Marketing						
Produção / Operacional	2	2	2	2	2	2
Qualidade	1	1	1	1	1	1
Manutenção						1
Aprovisionamento	1	1	1	1	1	2
Investigação & Desenvolvimento	1	1	1	1	1	2
Outros						
TOTAL	**7**	**7**	**7**	**7**	**7**	**10**

Remuneração base mensal	2016	2017	2018	2019	2020	2021
Administração / Direcção	2.000	2.000	2.000	2.000	2.000	2.000
Administrativa Financeira	2.000	2.000	2.000	2.000	2.000	2.000
Comercial / Marketing	1.500	1.500	1.500	1.500	1.500	1.500
Produção / Operacional	800	800	800	800	800	800
Qualidade	1.000	1.000	1.000	1.000	1.000	1.000
Manutenção	1.000	1.000	1.000	1.000	1.000	1.000
Aprovisionamento	1.000	1.000	1.000	1.000	1.000	1.000
Investigação & Desenvolvimento	1.500	1.500	1.500	1.500	1.500	1.500
Outros						

Remuneração base anual - TOTAL Colaboradores	2016	2017	2018	2019	2020	2021
Administração / Direcção	14.000	28.000	28.000	28.000	28.000	28.000
Administrativa Financeira	14.000	28.000	28.000	28.000	28.000	28.000
Comercial / Marketing						
Produção / Operacional	11.200	22.400	22.400	22.400	22.400	22.400
Qualidade	7.000	14.000	14.000	14.000	14.000	14.000
Manutenção						14.000
Aprovisionamento	7.000	14.000	14.000	14.000	14.000	28.000
Investigação & Desenvolvimento	10.500	21.000	21.000	21.000	21.000	42.000
Outros						
TOTAL	**63.700**	**127.400**	**127.400**	**127.400**	**127.400**	**176.400**

Outros Gastos		2016	2017	2018	2019	2020	2021
Segurança Social							
Órgãos Sociais	23,75%	3.325	6.650	6.650	6.650	6.650	6.650
Pessoal	23,75%	11.804	23.608	23.608	23.608	23.608	35.245
Seguros Acidentes de Trabalho	2%	1.274	2.548	2.548	2.548	2.548	3.528
Subsídio Alimentação	130,46	10.045	10.045	10.045	10.045	10.045	14.351
Comissões & Prémios							
Órgãos Sociais							
Pessoal							
Formação							
Outros custos com pessoal		1.000	1.000	1.000	1.000	1.000	1.000
TOTAL OUTROS GASTOS		**27.448**	**43.851**	**43.851**	**43.851**	**43.851**	**60.774**

TOTAL GASTOS COM PESSOAL	91.148	171.251	171.251	171.251	171.251	237.174

QUADRO RESUMO	2016	2017	2018	2019	2020	2021
Remunerações						
Órgãos Sociais	14.000	28.000	28.000	28.000	28.000	28.000
Pessoal	49.700	99.400	99.400	99.400	99.400	148.400
Encargos sobre remunerações	15.129	30.258	30.258	30.258	30.258	41.895
Seguros Acidentes de Trabalho e doenças profissionais	1.274	2.548	2.548	2.548	2.548	3.528
Gastos de acção social	10.045	10.045	10.045	10.045	10.045	14.351
Outros gastos com pessoal	1.000	1.000	1.000	1.000	1.000	1.000
TOTAL GASTOS COM PESSOAL	**91.148**	**171.251**	**171.251**	**171.251**	**171.251**	**237.174**

Retenções Colaboradores		2016	2017	2018	2019	2020	2021
Retenção SS Colaborador							
Gerência / Administração	11,00%	1.540	3.080	3.080	3.080	3.080	3.080
Outro Pessoal	11,00%	5.467	10.934	10.934	10.934	10.934	16.324
Retenção IRS Colaborador	20,00%	12.740	25.480	25.480	25.480	25.480	35.280
TOTAL Retenções		**19.747**	**39.494**	**39.494**	**39.494**	**39.494**	**54.684**

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Parceiros para o Crescimento

Empresa: Bioenergy, Lda

Euros

Investimento em Fundo Maneio Necessário

	2016	2017	2018	2019	2020	2021
Necessidades Fundo Maneio						
Reserva Segurança Tesouraria	20.000	20.000	20.000	20.000	20.000	20.000
Clientes	49.509	111.396	128.106	162.054	186.362	214.316
Inventários	158.065	355.647	408.994	517.377	594.984	684.231
Estado	60.584					
*						
*						
TOTAL	**288.159**	**487.043**	**557.099**	**699.431**	**801.345**	**918.547**
Recursos Fundo Maneio						
Fornecedores	39.110	78.711	87.680	105.903	118.950	133.955
Estado		6.376	7.773	10.612	12.644	17.217
*						
TOTAL	**39.110**	**85.087**	**95.453**	**116.514**	**131.594**	**151.172**
Fundo Maneio Necessário	**249.049**	**401.955**	**461.646**	**582.916**	**669.751**	**767.375**
Investimento em Fundo de Maneio	**249.049**	**152.906**	**59.690**	**121.271**	**86.834**	**97.624**
ESTADO	**-60.584**	**6.376**	**7.773**	**10.612**	**12.644**	**17.217**
SS	2.766,97	3.689,29	3.689,29	3.689,29	3.689,29	5.108,25
IRS	1.592,50	2.123,33	2.123,33	2.123,33	2.123,33	2.940,00
IVA	-64.943,78	563,74	1.960,72	4.798,93	6.831,20	9.168,30

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Empresa: OBoE, Oil & Bioe

Euros

Investimento

Investimento por ano	2016	2017	2018	2019	2020	2021
Propriedades de investimento						
Terrenos e recursos naturais						
Edificios e Outras construções						
Outras propriedades de investimento						
Total propriedades de investimento						
Activos fixos tangíveis						
Terrenos e Recursos Naturais	142.500					
Edificios e Outras Construções	527.500					
Equipamento Básico	1.100.000					
Equipamento de Transporte	20.000					
Equipamento Administrativo	4.000					
Equipamentos biológicos						
Outros activos fixos tangiveis						
Total Activos Fixos Tangíveis	1.794.000					
Activos Intangíveis						
Goodwill						
Projectos de desenvolvimento						
Programas de computador	5.000					
Propriedade industrial						
Outros activos intangíveis						
Total Activos Intangíveis	5.000					
Total Investimento	1.799.000					
IVA 23%	253.920					

Valores Acumulados	2016	2017	2018	2019	2020	2021
Propriedades de investimento						
Terrenos e recursos naturais						
Edificios e Outras construções						
Outras propriedades de investimento						
Total propriedades de investimento						
Activos fixos tangíveis						
Terrenos e Recursos Naturais	142.500	142.500	142.500	142.500	142.500	142.500
Edificios e Outras Construções	527.500	527.500	527.500	527.500	527.500	527.500
Equipamento Básico	1.100.000	1.100.000	1.100.000	1.100.000	1.100.000	1.100.000
Equipamento de Transporte	20.000	20.000	20.000	20.000	20.000	20.000
Equipamento Administrativo	4.000	4.000	4.000	4.000	4.000	4.000
Equipamentos biológicos						
Outros activos fixos tangiveis						
Total Activos Fixos Tangíveis	1.794.000	1.794.000	1.794.000	1.794.000	1.794.000	1.794.000
Activos Intangíveis						
Goodwill						
Projectos de desenvolvimento						
Programas de computador	5.000	5.000	5.000	5.000	5.000	5.000
Propriedade industrial						
Outros activos intangíveis						
Total Activos Intangíveis	5.000	5.000	5.000	5.000	5.000	5.000
Total	1.799.000	1.799.000	1.799.000	1.799.000	1.799.000	1.799.000

Taxas de Depreciações e amortizações	
Propriedades de investimento	
Edificios e Outras construções	2,00%
Outras propriedades de investimento	10,00%
Activos fixos tangíveis	
Edificios e Outras Construções	4,00%
Equipamento Básico	12,50%
Equipamento de Transporte	25,00%
Equipamento Administrativo	20,00%
Equipamentos biológicos	25,00%
Outros activos fixos tangiveis	25,00%
Activos Intangíveis	
Projectos de desenvolvimento	33,333%
Programas de computador	33,333%
Propriedade industrial	33,333%
Outros activos intangíveis	33,333%

*nota: se a taxa a utilizar for 33,33%, colocar mais uma casa decimal, considerando 33,333%

Depreciações e amortizações	2016	2017	2018	2019	2020	2021
Total Depreciações & Amortizações	166.067	166.067	166.067	164.400	159.400	158.600

Depreciações & Amortizações acumuladas	2016	2017	2018	2019	2020	2021
Propriedades de investimento						
Activos fixos tangíveis	164.400	328.800	493.200	657.600	817.000	975.600
Activos Intangíveis	1.667	3.333	5.000	5.000	5.000	5.000
TOTAL	166.067	332.133	498.200	662.600	822.000	980.600

Valores Balanço	2016	2017	2018	2019	2020	2021
Propriedades de investimento						
Activos fixos tangíveis	1.629.600	1.465.200	1.300.800	1.136.400	977.000	818.400
Activos Intangíveis	3.333	1.667				
TOTAL	1.632.933	1.466.867	1.300.800	1.136.400	977.000	818.400

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Empresa: & Bioenergy, Lda

Euros

Financiamento

	2016	2017	2018	2019	2020	2021
Investimento	2.048.049	152.906	59.690	121.271	86.834	97.624
Margem de segurança	2%	2%	2%	2%	2%	2%
Necessidades de financiamento	**2.089.000**	**156.000**	**60.900**	**123.700**	**88.600**	**99.600**

Fontes de Financiamento	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**
Meios Libertos	66.082	207.660	276.623	416.383	515.657	578.783
Capital	1.000.000					
Outros instrumentos de capital						
Empréstimos de Sócios						
Financiamento bancário e outras Inst. Crédito	600.000					
Subsidios	629.650					
TOTAL	**2.295.732**	**207.660**	**276.623**	**416.383**	**515.657**	**578.783**

N.º de anos reembolso: 5
Taxa de juro associada: 6,00%

2016

Capital em dívida (início período)	600.000	600.000	480.000	360.000	240.000	120.000
Taxa de Juro	6%	6%	6%	6%	6%	6%
Juro Anual	24.000	36.000	28.800	21.600	14.400	7.200
Reembolso Anual		120.000	120.000	120.000	120.000	120.000
Imposto Selo (0,4%)	96	144	115	86	58	29
Serviço da dívida	24.096	156.144	148.915	141.686	134.458	127.229
Valor em dívida	600.000	480.000	360.000	240.000	120.000	

N.º de anos reembolso Taxa de juro associada: 6,00%

2017

Capital em dívida (início período)	24.000					
Taxa de Juro		6%	6%	6%	6%	6%
Juro Anual						
Reembolso Anual						
Imposto Selo (0,4%)						
Serviço da dívida						
Valor em dívida						

N.º de anos reembolso Taxa de juro associada: 6,00%

2018

Capital em dívida (início período)					
Taxa de Juro		6%	6%	6%	6%
Juro Anual					
Reembolso Anual					
Imposto Selo (0,4%)					
Serviço da dívida					
Valor em dívida					

N.º de anos reembolso Taxa de juro associada: 6,00%

2019

Capital em dívida (início período)				
Taxa de Juro	6%	6%	6%	
Juro Anual				
Reembolso Anual				
Imposto Selo (0,4%)				
Serviço da dívida				
Valor em dívida				

N.º de anos reembolso Taxa de juro associada: 6,00%

2020

Capital em dívida (início período)		
Taxa de Juro	6%	6%
Juro Anual		
Reembolso Anual		
Imposto Selo (0,4%)		
Serviço da dívida		
Valor em dívida		

N.º de anos reembolso Taxa de juro associada: 6,00%

2021

Capital em dívida (início período)	
Taxa de Juro	6%
Juro Anual	
Reembolso Anual	
Imposto Selo (0,4%)	
Serviço da dívida	
Valor em dívida	

Capital em dívida	600.000	480.000	360.000	240.000	120.000	

Juros pagos com Imposto Selo incluído	24.096	36.144	28.915	21.686	14.458	7.229
Reembolso		120.000	120.000	120.000	120.000	120.000

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Empresa: & Bioenergy, Lda

Euros

Ponto Crítico Operacional Previsional

	2016	2017	2018	2019	2020	2021
Vendas e serviços prestados	574.782,50	1.293.260,63	1.487.249,72	1.881.370,89	2.163.576,53	2.488.113,01
Variação nos inventários da produção						
CMVMC	316.130,38	711.293,34	817.987,35	1.034.753,99	1.189.967,09	1.368.462,15
FSE Variáveis	#REF!					
Margem Bruta de Contribuição	**#REF!**	**581.967,28**	**669.262,37**	**846.616,90**	**973.609,44**	**1.119.650,85**
Ponto Crítico	**#REF!**	**1.176.261,30**	**1.176.261,30**	**1.172.557,60**	**1.161.446,49**	**1.306.163,56**

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Parceiros para o Crescimento

Empresa: OBoE, Oil & Bioe

Demonstração de Resultados Previsional

	2016	2017	2018	2019	2020	2021
Vendas e serviços prestados	574.783	1.293.261	1.487.250	1.881.371	2.163.577	2.488.113
Subsídios à Exploração						
Ganhos/perdas imputados de subsidiárias, associadas e empreendimentos conjuntos						
Variação nos inventários da produção						
Trabalhos para a própria entidade						
CMVMC	316.130	711.293	817.987	1.034.754	1.189.967	1.368.462
Fornecimento e serviços externos	128.000	192.000	192.000	192.000	192.000	192.000
Gastos com o pessoal	91.148	171.251	171.251	171.251	171.251	237.174
Imparidade de inventários (perdas/reversões)						
Imparidade de dívidas a receber (perdas/reversões)						
Provisões (aumentos/reduções)						
Imparidade de investimentos não depreciáveis/amortizáveis (perdas/reversões)						
Aumentos/reduções de justo valor						
Outros rendimentos e ganhos						
Outros gastos e perdas						
EBITDA (Resultado antes de depreciações, gastos de financiamento e impostos)	**39.504**	**218.716**	**306.011**	**483.366**	**610.359**	**690.477**
Gastos/reversões de depreciação e amortização	166.067	166.067	166.067	164.400	159.400	158.600
Imparidade de activos depreciáveis/amortizáveis (perdas/reversões)						
EBIT (Resultado Operacional)	**-126.563**	**52.650**	**139.945**	**318.966**	**450.959**	**531.877**
Juros e rendimentos similares obtidos	2.549	4.092	8.451	14.559	22.889	32.499
Juros e gastos similares suportados	24.096	36.144	28.915	21.686	14.458	7.229
RESULTADO ANTES DE IMPOSTOS	**-148.110**	**20.597**	**119.480**	**311.839**	**459.390**	**557.148**
Imposto sobre o rendimento do período				63.799	96.472	117.001
RESULTADO LÍQUIDO DO PERÍODO	**-148.110**	**20.597**	**119.480**	**248.040**	**362.918**	**440.147**

Empresa: Oil & Bioenergy, Lda

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Parceiros para o Crescimento

Mapa de Cash Flows Operacionais

	2016	2017	2018	2019	2020	2021
Meios Libertos do Projecto						
Resultados Operacionais (EBIT) x (1-IRC)	-99.985	41.593	110.556	251.983	356.257	420.183
Depreciações e amortizações	166.067	166.067	166.067	164.400	159.400	158.600
Provisões do exercício						
	66.082	**207.660**	**276.623**	**416.383**	**515.657**	**578.783**
Investim./Desinvest. em Fundo Maneio						
Fundo de Maneio	-249.049	-152.906	-59.690	-121.271	-86.834	-97.624
CASH FLOW de Exploração	**-182.967**	**54.754**	**216.933**	**295.112**	**428.823**	**481.159**
Investim./Desinvest. em Capital Fixo						
Capital Fixo	-1.799.000					
CASH FLOW acumulado	**-1.981.967**	**-1.927.213**	**-1.710.281**	**-1.415.168**	**-986.345**	**-505.186**

Empresa: OBoE, Oil & Bioe

Euros

Plano de Financiamento

	2016	2017	2018	2019	2020	2021
ORIGENS DE FUNDOS						
Meios Libertos Brutos	39.504	218.716	306.011	483.366	610.359	690.477
Capital Social (entrada de fundos)	1.000.000					
Outros instrumentos de capital	629.650					
Empréstimos Obtidos	600.000					
Desinvest. em Capital Fixo						
Desinvest. em FMN						
Proveitos Financeiros	2.549	4.092	8.451	14.559	22.889	32.499
Total das Origens	2.271.702	222.808	314.462	497.925	633.247	722.977
APLICAÇÕES DE FUNDOS						
Inv. Capital Fixo	1.799.000					
Inv Fundo de Maneio	249.049	152.906	59.690	121.271	86.834	97.624
Imposto sobre os Lucros					63.799	96.472
Pagamento de Dividendos						
Reembolso de Empréstimos		120.000	120.000	120.000	120.000	120.000
Encargos Financeiros	24.096	36.144	28.915	21.686	14.458	7.229
Total das Aplicações	2.072.145	309.050	208.605	262.957	285.091	321.325
Saldo de Tesouraria Anual	199.557	-86.242	105.857	234.968	348.156	401.652
Saldo de Tesouraria Acumulado	199.557	113.315	219.172	454.140	802.296	1.203.948
Aplicações / Empréstimo Curto Prazo	254.851	409.152	845.070	1.455.945	2.288.862	3.249.930
Soma Controlo		-295.837	-625.898	-1.001.805	-1.486.566	-2.045.982

Acerto do modelo

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Parceiros para o Crescimento

Empresa: OBoE, Oil & Bioenerg

Euros

Balanço Previsional

	2016	2017	2018	2019	2020	2021
ACTIVO						
Activo Não Corrente	**1.632.933**	**1.466.867**	**1.300.800**	**1.136.400**	**977.000**	**818.400**
Activos fixos tangíveis	1.629.600	1.465.200	1.300.800	1.136.400	977.000	818.400
Propriedades de investimento						
Activos Intangíveis	3.333	1.667				
Investimentos financeiros						
Activo corrente	**543.010**	**896.195**	**1.402.169**	**2.155.376**	**3.090.207**	**4.168.477**
Inventários	158.065	355.647	408.994	517.377	594.984	684.231
Clientes	49.509	111.396	128.106	162.054	186.362	214.316
Estado e Outros Entes Públicos	60.584					
Accionistas/sócios						
Outras contas a receber						
Diferimentos						
Caixa e depósitos bancários	274.851	429.152	865.070	1.475.945	2.308.862	3.269.930
TOTAL ACTIVO	**2.175.943**	**2.363.061**	**2.702.969**	**3.291.776**	**4.067.207**	**4.986.877**
CAPITAL						
Capital realizado	1.000.000	1.000.000	1.000.000	1.000.000	1.000.000	1.000.000
Acções (quotas próprias)						
Outros instrumentos de capital próprio						
Reservas		-148.110	-127.513	-8.033	240.007	602.925
Excedentes de revalorização						
Outras variações no capital próprio	629.650	629.650	629.650	629.650	629.650	629.650
Resultado líquido do período	-148.110	20.597	119.480	248.040	362.918	440.147
TOTAL DO CAPITAL PRÓPRIO	**1.481.540**	**1.502.137**	**1.621.617**	**1.869.657**	**2.232.575**	**2.672.721**
PASSIVO						
Passivo não corrente	**600.000**	**480.000**	**360.000**	**240.000**	**120.000**	
Provisões						
Financiamentos obtidos	600.000	480.000	360.000	240.000	120.000	
Outras Contas a pagar						
Passivo corrente	**39.110**	**85.087**	**95.453**	**180.313**	**228.066**	**268.173**
Fornecedores	39.110	78.711	87.680	105.903	118.950	133.955
Estado e Outros Entes Públicos		6.376	7.773	74.411	109.116	134.218
Accionistas/sócios						
Financiamentos Obtidos						
Outras contas a pagar						
TOTAL PASSIVO	**639.110**	**565.087**	**455.453**	**420.313**	**348.066**	**268.173**
TOTAL PASSIVO + CAPITAIS PRÓPRIOS	**2.120.649**	**2.067.224**	**2.077.071**	**2.289.970**	**2.580.641**	**2.940.895**
	55.294	295.837	625.898	1.001.805	1.486.566	2.045.982

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Parceiros para o Crescimento

Empresa: OBoE, Oil & Bioenerg

Principais Indicadores

INDICADORES ECONÓMICOS	2016	2017	2018	2019	2020	2021
Taxa de Crescimento do Negócio		125%	15%	27%	15%	15%
Rentabilidade Líquida sobre o rédito	-26%	2%	8%	13%	17%	18%

INDICADORES ECONÓMICOS - FINANCEIROS	2016	2017	2018	2019	2020	2021
Return On Investment (ROI)	-7%	1%	4%	8%	9%	9%
Rendibilidade do Activo	-6%	2%	5%	10%	11%	11%
Rotação do Activo	26%	55%	55%	57%	53%	50%
Rendibilidade dos Capitais Próprios (ROE)	-10%	1%	7%	13%	16%	16%

INDICADORES FINANCEIROS	2016	2017	2018	2019	2020	2021
Autonomia Financeira	68%	64%	60%	57%	55%	54%
Solvabilidade Total	340%	418%	593%	783%	1169%	1860%
Cobertura dos encargos financeiros	-525%	146%	484%	1471%	3119%	7358%

INDICADORES DE LIQUIDEZ	2016	2017	2018	2019	2020	2021
Liquidez Corrente	13,88	10,53	14,69	11,95	13,55	15,54
Liquidez Reduzida	9,84	6,35	10,40	9,08	10,94	12,99

INDICADORES DE RISCO NEGÓCIO	2016	2017	2018	2019	2020	2021
Margem Bruta	130.652	389.967	477.262	654.617	781.609	927.651
Grau de Alavanca Operacional	-103%	741%	341%	205%	173%	174%
Grau de Alavanca Financeira	85%	256%	117%	102%	98%	95%

Empresa: Bioenergy, Lda

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Avaliação do Projecto / Empresa

Na perspectiva do Investidor	2016	2017	2018	2019	2020	2021	2022
Taxa de juro de activos sem risco	1,00%	1,00%	1,00%	1,10%	1,10%	1,10%	1,10%
Prémio de risco de mercado	10,00%	10,00%	10,00%	10,00%	10,00%	10,00%	10,00%
Taxa de Actualização	11,10%	11,10%	11,10%	11,21%	11,21%	11,21%	11,21%
Factor actualização	1	1,111	1,234	1,373	1,527	1,698	1,888
Fluxos Actualizados	**-1.406.063**	**-91.260**	**55.105**	**111.770**	**192.828**	**208.477**	**1.672.279**
	-1.406.063	**-1.497.324**	**-1.442.218**	**-1.330.448**	**-1.137.620**	**-929.143**	**743.136**

	CF	CF Acum	
Ano 0	-1.406.063	-1.406.063	FALSO
Ano 1	-91.260	-1.497.324	FALSO
Ano 2	55.105	-1.442.218	FALSO
Ano 3	111.770	-1.330.448	FALSO
Ano 4	192.828	-1.137.620	FALSO
Ano 5	208.477	-929.143	FALSO
Ano 6	1.672.279	743.136	5,3

Valor Actual Líquido (VAL) **743.136**

	#NÚM!	#NÚM!	-81%	-51%	-27%	-13%	20%

Taxa Interna de Rentibilidade **19,80%**

Pay Back period	**6**	**Anos**

Na perspectiva do Projecto	2016	2017	2018	2019	2020	2021	2022
Free Cash Flow to Firm	**-1.981.967**	**54.754**	**216.933**	**295.112**	**428.823**	**481.159**	**4.334.766**
WACC	9,47%	9,79%	10,19%	10,72%	11,10%	11,10%	11,10%
Factor de actualização	1	1,098	1,210	1,339	1,488	1,653	1,837
Fluxos actualizados	**-1.981.967**	**49.871**	**179.312**	**220.323**	**288.162**	**291.027**	**2.359.912**
	-1.981.967	**-1.932.096**	**-1.752.784**	**-1.532.461**	**-1.244.299**	**-953.273**	**1.406.639**

	CF	CF Acum	
Ano 0	-1.981.967	-1.981.967	FALSO
Ano 1	49.871	-1.932.096	FALSO
Ano 2	179.312	-1.752.784	FALSO
Ano 3	220.323	-1.532.461	FALSO
Ano 4	288.162	-1.244.299	FALSO
Ano 5	291.027	-953.273	FALSO

	#NÚM!	-97%	-66%	-39%	-19%	-8%	23%

Valor Actual Líquido (VAL) **1.406.639**

Taxa Interna de Rentibilidade **22,69%**

Pay Back period	**6**	**Anos**

Cálculo do WACC	2016	2017	2018	2019	2020	20
Passivo Remunerado	480.000	360.000	240.000	120.000	0	0
Capital Próprio	1.481.540	1.502.137	1.621.617	1.869.657	2.232.575	2.672.721
TOTAL	1.961.540	1.862.137	1.861.617	1.989.657	2.232.575	2.672.721
% Passivo remunerado	24,47%	19,33%	12,89%	6,03%	0,00%	0,00%
% Capital Próprio	75,53%	80,67%	87,11%	93,97%	100,00%	100,00%

Custo	2016	2017	2018	2019	2020	2021
Custo Financiamento	6,00%	6,00%	6,00%	6,00%	6,00%	6,00%
Custo financiamento com efeito fiscal	4,74%	4,74%	4,74%	4,74%	4,74%	4,74%
Custo Capital	11,00%	11,00%	11,00%	11,10%	11,10%	11,10%
Custo ponderado	0,094681422	9,79%	10,19%	10,72%	11,10%	11,10%

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Empresa: Oil&Bioenergy, Lda

Cálculos Auxiliares

Consumo de Unidades de Matérias-Primas por Unidade de Produto Acabado

Matérias Primas e Subsidiárias (descriminação)	Unidade de Medida	Produto A	Produto B	Produto C	Produto D

Produção (em Quantidades)

Unid

ades físicas

Produtos	2016	2017	2018	2019	2020	2021
TOTAL						

Consumo de Matérias Primas 1*

Unid

ades físicas

Matérias Primas e Subsidiárias	2016	2017	2018	2019	2020	2021

Preço das Matérias Primas e Subsidiárias

Matérias Primas e Subsidiárias	2016	2017	2018	2019	2020	2021

Valor do consumo 2*

Matérias Primas e Subsidiárias	2016	2017	2018	2019	2020	2021
TOTAL						

1 obtido da multiplicação da produção pelo consumo de matéria prima por unidade de produto acabado. 2* obtido da multiplicação do consumo das matérias-primas pelo preço.*