POLITECNICO DI TORINO

Master course in Chemical and Sustainable Processes Engineering

Master thesis

Recovery of antioxidants molecules from bio-food beverage waste

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Summary

Italian summary

Introduzione

L' industria chimica moderna concentra gran parte delle sue risorse nella gestione dei residui che derivano dai diversi processi produttivi. L'obiettivo principale è gestire questi residui nel rispetto dell'ambiente sfruttando la loro potenzialità, valorizzandone quindi il contenuto. L'intento è quello di ottenere dagli scarti di produzione ottenuti lungo tutto il processo dei composti ad alto valore biologico, che possano essere successivamente utilizzati come additivi in differenti campi, quali l'industria alimentare, farmaceutica o cosmetica. Il conseguimento di questo obiettivo avrebbe riscontro positivo su due fronti: da un lato, permetterebbe di mitigare la pericolosità dei residui industriali che, se rilasciati nell'ambiente senza i necessari controlli, provocano la contaminazione di suolo e acque; dall'altro, preziosi composti di origine naturale potrebbero essere estratti da questi residui e utilizzati in sostituzione ai composti artificiali nei suddetti settori industriali.

Il presente documento si propone come riassunto del lavoro sperimentale realizzato all' Universitat Politècnica de Catalunya (UPC-EEBE); gli esperimenti effettuati e i risultati ottenuti concorreranno al completamento di un più ampio lavoro di ricerca. Il lavoro è sostenuto dal progetto Waste2Product (CTM2014-57302-R) e dal progetto R2MIT (CTM2017-85346-R), finanziato dal Ministero di Economia e competitività spagnolo (MINECO) e dal governo catalano (ref. 2017-SGR-312).

Il presente lavoro sperimentale mira al recupero di un tipo specifico di composti, i polifenoli. Le matrici industriali dalle quali si tenta di recuperare questi composti sono alcuni dei residui che si formano durante il processo produttivo dell'olio di oliva e del vino. In particolare, tra le varie tipologie di residui che si producono, per entrambe i processi la scelta è ricaduta su uno di matrice solida, rispettivamente la sansa di oliva e le fecce di vino. Questi due residui in particolare sono stati oggetto di studio durante l'intera sperimentazione, selezionati nelle fasi preliminari della ricerca. L'obiettivo finale del progetto di ricerca è l'identificazione dei polifenoli contenuti nelle matrici selezionate, la loro estrazione tramite tecniche specifiche e la loro successiva separazione tramite l'utilizzo di membrane, attraverso le quali si mira a purificare specifici polifenoli per il loro successivo utilizzo in campo industriale.

Nel presente lavoro, due tecniche di estrazione sono state studiate: l'estrazione accelerata con solvente (pressurized-liquid extraction, PLE) e l'estrazione assistita da ultrasuoni (ultrasound-assisted extraction, UAE). Per entrambe vengono individuate le condizioni operative ottime che permettono la più alta resa di estrazione. Parallelamente, una volta nota la composizione degli estratti in termini di polifenoli, due miscele sintetiche sono state preparate per mimare la composizione degli estratti. Con queste miscele, una per residuo scelto, sono state testate diverse membrane.

La sperimentazione si divide in due parti, parallele: da una parte si implementano le due tecniche estrattive, con l'obiettivo di identificare le condizioni operative che permettono la resa estrattiva più alta. Questa fase estrattiva prevede l'identificazione delle condizioni operative ottimali per le due tecniche di estrazione sopracitate e il successivo confronto per valutare quale tecnica risulti più efficace. Per valutare la resa estrattiva si utilizza la cromatografia liquida ad alta prestazione (HPLC) per identificare e quantificare i polifenoli contenuti all'interno delle matrici. Si analizza inoltre il contenuto polisaccaridico degli estratti ottenuti attraverso la spettrofotometria ultravioletta/visibile (UV-Vis). Parallelamente, tramite l'utilizzo di miscele sintetiche di polifenoli standard, si testano diverse membrane a scala di laboratorio, processando bassi volumi del campione. Lo scopo è valutare l'affinità delle membrane verso i polifenoli selezionati, analizzando il contenuto del volume di permeato e del volume di retentato. In questa fase si utilizzano membrane di microfiltrazione in formato disco, filtrazione per centrifugazione e nanofiltrazione.

1. Antiossidanti: classificazione, caratteristiche e utilizzi in ambito industriale

L'interesse crescente per componenti bioattivi di origine naturale ha spostato l'investigazione verso tecniche e soluzioni che possano permetterne il recupero da fonti che altrimenti andrebbero perse, e di cui è necessario prevedere lo smaltimento. Gli antiossidanti sono sostanze chimiche che possono essere usate in numerose applicazioni: tra le più note e vicine alla sensibilità del pubblico, vi è il loro impiego in determinati alimenti per rallentare i processi ossidativi che ne provocano la degradazione rendendoli non più edibili. Numerosi antiossidanti artificiali, quali l'idrossianisolo butilato (BHA), idrossitoluene butilato (BHT), propil gallato (PG) e butilidrochinone terziario (TBHQ), vengono ancora utilizzati per migliorare la qualità della carne e posporre i processi ossidativi a carico dei fosfolipidi endogeni. Tuttavia, numerosi studi ne hanno comprovato la tossicità, da qui la necessità di sostituirli con composti che abbiano la stessa funzione e ridotta, se non nulla, dannosità (Jiang and Xiong, 2016).

Gli antiossidanti permettono di contrastare l'ossidazione a carico dei lipidi: questo fenomeno si sviluppa in tre fasi, iniziazione, propagazione e terminazione. A seconda del meccanismo di iniziazione e formazione di radicali si parla di autossidazione, fotossidazione e ossidazione enzimatica. Questo tipo di reazione diminuisce la conservabilità e la freschezza dei cibi, rendendo necessaria l'aggiunta di determinate sostanze che possano bloccarle o ritardarle, per evitare lo sviluppo di sapori indesiderati o sostanze potenzialmente tossiche (Yang *et al.*, 2018).

Gli antiossidanti vengono classificati secondo struttura o meccanismo di azione; Comunemente si suddividono in tre classi, vitamine, carotenoidi e polifenoli. Quest'ultima classe è in particolare argomento di studio del presente lavoro.

I polifenoli sono metaboliti secondari delle piante: derivano dalla risposta delle stesse all'ambiente. Più di 8000 diversi composti sono noti alla comunità scientifica (Oroian and Escriche, 2015). Essi non sono dei nutrienti, tuttavia la loro importanza nella dieta diaria è riconosciuta, e numerosi studi dimostrano l'azione benefica di questi composti sulle malattie croniche (Acosta *et al.*, 2014).

Dato l'elevato numero di composti possibili, una classificazione sistematica risulta necessaria: la più comune prevede la distinzione dei diversi composti a seconda del numero degli anelli fenolici presenti nella struttura chimica. Secondo questo principio, si delineano 5 classi: acidi fenolici, flavonoidi, stilbeni, lignani e tannini (Cutrim and Cortez 2018; Brglez Mojzer *et al*. 2016). La figura 1 presenta uno schema completo delle classi e sottoclassi della famiglia dei polifenoli.

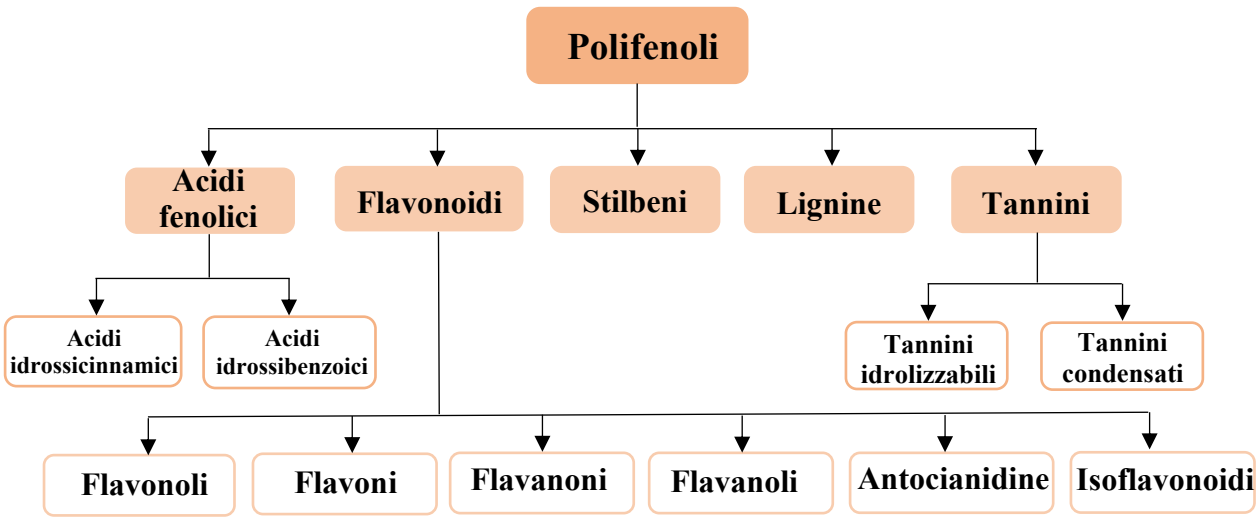

Figura 1 *- Classificazione dei polifenoli*

Grazie alle loro proprietà antiossidanti, i potenziali effetti benefici sulla salute di questi composti che ne derivano hanno suscitato grande interesse, rendendo questa classe di antiossidanti oggetto di numerosi studi (Santhakumar *et al.* 2018; Cory *et al.* 2018; Khoo *et al.*, 2017; Niedzwiecki *et al.* 2016). I polifenoli sono noti per essere anticancerogeni, poiché agiscono sia sul sistema immunitario, in maniera indiretta, sia sulla crescita incontrollata delle cellule cancerose (Niedzwiecki *et al.*, 2016). Inoltre, essi rivestono un ruolo importante nella prevenzione e nel trattamento di diverse patologie, quali obesità, arteriosclerosi e malattie neurodegenerative (Cory *et al.*, 2018).

Le proprietà di questa famiglia di antiossidanti, e gli effetti benefici che ne derivano, motivano gli studi condotti per rendere possibile in maniera efficace la loro applicazione in diversi campi industriali.

Nell'industria alimentare odierna una delle principali sfide è ottenere alimenti che possano mantenere a lungo la loro freschezza e qualità: gli antiossidanti naturalmente presenti negli alimenti non sono sufficienti per mantenere a lungo termine la qualità del prodotto; ciò ha reso necessaria l'aggiunta di additivi artificiali per ridurre lo stress ossidativo negli alimenti e con esso lo sviluppo di sostanze pericolose e indesiderate (Aguiar *et al.* 2016). Nonostante questi composti sintetici siano tuttora ampiamente utilizzati, la possibilità di recuperare composti antiossidanti di origine naturale per poterli utilizzare con la medesima funzione ha suscitato grande interesse in questo campo. Il loro impiego permetterebbe la riduzione del rischio di tossicità e degli effetti nocivi degli additivi artificiali. Tuttavia, questi composti di origine naturale sono suscettibili di degradazione da agenti esterni: ciò rende la loro introduzione all'interno degli alimenti complessa, fonte di numerosi studi per ovviare al problema e permettere una somministrazione efficace e duratura. La microincapsulazione e le nanotecnologie sono state indicate in diversi studi come possibili tecniche risolutive: l'utilizzo di queste tecnologie permette di regolare il tempo di rilascio, aumentare la loro biodisponibilità anche a basse concentrazioni, riducendo così il rischio di alterazione del sapore originario (Aguiar *et al.* 2016; Pathakoti *et al.* 2017).

I polifenoli sono stati impiegati inoltre in prodotti cosmetici: lo stress ossidativo è stato indicato come una delle principali cause dell'invecchiamento della pelle e la loro azione antiossidante permette di prevenire e mitigare gli effetti di questo problema (Zillich *et al.*, 2015).

2. Residui derivanti dalla produzione di olio di oliva e vino

Nei processi produttivi di olio di oliva e vino vengono generati diversi residui di matrice solida e liquida. A seconda delle tecniche utilizzate e delle fasi produttive implementate, i residui possono variare in tipologia, quantità e caratteristiche fisico-chimiche.

2.1 Produzione di olio di oliva

La produzione di olio di oliva può essere effettuata in tre diversi modi: a seconda del metodo selezionato variano le quantità e le proprietà dei residui prodotti, e con esse le modalità di gestione degli stessi (Nunes *et al.*, 2016). L'elevato contenuto organico e la fitotossicità di questi residui costituiscono il problema fondamentale del loro smaltimento, per cui sono necessari impianti specifici. L'elevato contenuto fenolico provoca l'inquinamento del suolo e delle acque, influenzando lo sviluppo delle popolazioni microbiche; inoltre, le emissioni di anidride solforosa e fenolo provocano l'inquinamento dell'aria (Dermeche *et al.*, 2013). Durante le diverse fasi della produzione di olio di oliva si generano diversi residui: la sansa di oliva è un residuo solido oggetto del presente lavoro, le cui proprietà sono descritte nel seguente paragrafo.

2.1.1 Sansa di oliva

Si stima che più del 98% dei composti fenolici rimanga all'interno dei sottoprodotti dell'olio di oliva (Araújo *et al.*, 2015). La sansa di oliva è un sottoprodotto eterogeneo, di matrice solido-liquida con elevato contenuto di umidità e olio; contiene parti di buccia, nocciolo e polpa dell'oliva. È oggetto di studio per il recupero di polifenoli dato l'elevato contenuto di oleuropeina, acido caffeico, vanillico e cumarico e idrossitirosolo (Ruiz *et al.* 2017; Nunes *et al.* 2016). La possibilità di recuperare questi composti insieme ad altri – è importante sottolineare che la composizione dipende dai diversi fattori che influenzano le diverse fasi di produzione dell'olio – rende questo particolare residuo un valido soggetto per il recupero di composti fenolici.

La tabella 1 presenta la composizione chimica di diversi residui derivanti dalla produzione di olio di oliva.

Tabella 1 *- Composizione fenolica di residui derivanti dalla produzione di olio di oliva*

Residuo	Determinazione	Polifenoli	Riferimento
Acque reflue di frantoio	HPLC	3,4-DHPEA-EDA, H-tirosolo.	(La Scalia *et al.*, 2017)
Foglie di olivo	HPLC-DAD	Oleuropeina	(Aissa *et al.*, 2017)
Acque reflue di frantoio		Tirosolo, Idrossitirosolo	
Acque reflue di frantoio	HPLC-DAD	Idrossitirosolo	(Ioannou-Ttofa *et al.*, 2017)
Sansa di oliva Foglie di olivo Olio di sansa	HPLC-UV-VIS	Luteolina-7-rutinoside, Rutina, Diidro-quercetina, 10-idrossi-oleuropeina, Luteolin-7-glucoside, Verbascoside, Apigenin-7-glucoside, Chrysoeriol-7-O-glucoside, Oleuropein glucoside, Oleuropeina, Oleoside, Apigenina	(Abdel-Razek *et al.* 2017)
Sansa di oliva	HPLC-DAD	Idrossitirosolo, Luteolina (Acido Caffeico, Acido trans-Ferulico, Apigenina, Tirosolo, Rutina Idrata)	(Fernández *et al.*, 2018)
Sansa di oliva Foglie di olivo	HPLC-DAD	Idrossitirosolo-4-glucoside, idrossitirosolo, forma dialdeidica dell'acido decarbossimetil-elencoolico collegato all'idrossitirosolo (HyEDA) (verbascoside, tirosol, salidroside)	(Ruiz *et al.*, 2017)

Come si deduce dalla tabella 1, le composizioni in termini di polifenoli variano: ciò dipende dall'origine delle olive, dai metodi di coltivazione e dal processo produttivo impiegato.

Araujo e collaboratori (2015) indicano l'idrossitirosolo come il polifenolo presente in maggior quantità nei principali sottoprodotti di questo processo produttivo. Questo composto grazie alle sue proprietà è stato utilizzato come ingrediente nella carne per migliorarne la qualità e la conservazione (Martínez *et al.* 2018); inoltre, uno studio ha valutato la possibilità di sostituire l'anidride solforosa con questo stesso composto nell'industria vinicola (Ruiz *et al.* 2017). L'idrossitirosolo e l'oleuropeina possiedono inoltre effetti positivi sulla pelle (Rodrigues *et al.* 2015).

2.2 Produzione di vino

Insieme alla produzione di olio di oliva, l'industria vinicola è una tra le più importanti nel campo dell'agricoltura. Anche in questo caso le modalità produttive, anche a seconda del prodotto finale desiderato, influenzano le caratteristiche finali dei sottoprodotti (Beres *et al.*, 2017). I sottoprodotti sono di varia matrice, solida e liquida; oggetto di questo lavoro sono le fecce di vino, le cui caratteristiche sono presentate nel seguente paragrafo.

2.2.1 Fecce di vino

Le fecce di vino sono residui bifasici generati durante la fase di fermentazione del vino. Possono essere utilizzate come fonte di etanolo e di polifenoli (Pérez-Bibbins *et al.* 2015), ma la loro disposizione resta una sfida importante nel panorama dell'industria vinicola.
La tabella 2 presenta la composizione dei vari residui in termini di composti fenolici.

Tabella 2 *- Composizione fenolica di residui derivanti dalla produzione di vino*

Residuo	Determinazione	Polifenoli	Riferimento
Buccia d'uva	HPLC-DAD	malvidin-3-glucoside, Quercetina Rutina, Catechina, Epicatechina	(Caldas *et al.*, 2018)
Vinaccia	HPLC-DAD-ESI-MS/MS	malvidin-3-O-glucoside, malvidin-3-O- (6 '× -p-cumumil) glucoside, (glucoside caffeoilato e acetilato di malvidina) (delfinidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, petunidin-3-O- (6'-acetil) glucoside, delphinidin-3-O- (6'-p -coumaroyl) glucoside, peonidin-3-O- (6'-acetyl) glucoside, peonidin-3-O- (6'-caffeoyl) glucoside, petunidin-3-O- (6'-p-coumaroyl) glucoside e peonidin -3-O- (6'-p-cumaroil) glucoside) Quercetina, Miricetina	(Drosou *et al.*, 2015)
Vinaccia	HPLC-MWD	Catechina, Acido Siringico, Epicatechina, Quercetina, Malvidin 3-O- glucoside	(Antoniolli *et al.*, 2015)
Fecce di vino	HPLC-DAD	Quercetina, Acido Ellagico, Acido p-Cumarico, Acido Gallico, Acido Caffeico, Acido Clorogenico, Kaempferol	(Jurcevic *et al.*, 2017)
Fecce di vino	HPLC-DAD	Acido Gallico, Catechina, Epicatechina, Procianidine B1 e B2, Miricetina e Quercetina, Acido trans-Caftarico, trans-Cutarico, Caffeico, p-Cumarico e Ferulico	(Kopsahelis *et al.* 2017)
Vinaccia	HPLC-DAD	Acido Gallico, Catechina, Epicatechina, Quercetina	(Díaz-Reinoso *et al.*, 2017)

Come si può dedurre dalla tabella precedente, uno stesso tipo di residuo può avere composizione differente: ciò dipende da vari fattori, quali il tipo di uva, la vendemmia e le modalità di produzione.

Come già approfondito, le applicazioni dei polifenoli estratti possono essere varie in diversi campi industriali: prodotti cosmetici contenenti polifenoli estratti dalla vinaccia sono stati commercializzati con successo. Il resveratrolo ottenuto da residui vinicoli è inoltre stato utilizzato come complemento alimentare (Beres *et al.* 2017).

3. Recupero di polifenoli: tecniche di estrazione

L'obiettivo in un processo di estrazione è ottenere una resa elevata del prodotto target senza ridurne la qualità o inficiarne le caratteristiche. Negli ultimi anni inoltre la necessità di sviluppare tecniche sicure, ragionevolmente economiche e ecologiche ha indirizzato la comunità scientifica verso metodi non convenzionali che possano soddisfare questi principi e sostituire i metodi classici (Oroian and Escriche 2015). Nel definire i parametri operativi delle varie tecniche, è necessario in generale tenere in considerazione specifici fattori, quali il solvente utilizzato, il tempo di estrazione, la temperatura e il numero di cicli, se previsto. La scelta del solvente è la più critica, dovuto alle potenziali conseguenze che esso può avere sul prodotto finito a seconda delle sue proprietà. Oltre le caratteristiche da valutare quali la viscosità, la selettività e il costo, è importante valutarne la tossicità e quali siano le concentrazioni accettabili nel prodotto finale: per esempio, in ambito alimentare, a prescindere dalle altre proprietà solo determinati solventi possono essere utilizzati, ovvero quelli riconosciuti come sicuri, i cosiddetti GRAS (Generally-Recognized-As-Safe). L'etanolo (solvente GRAS), nonostante risulti più caro del metanolo, è il solvente d'elezione in questo tipo di applicazioni (Tiwari 2015; Oroian and Escriche 2015; Medina-Torres *et al.* 2017).

L'approccio generale nella scelta della tecnica di estrazione più opportuna, qui utilizzato con un numero ristretto di tecniche, è individuare le condizioni operative ottime per ogni tecnica estrattiva per poterle paragonare e identificare la più efficace per la matrice studiata.

Le tecniche convenzionali prevedono l'utilizzo di una grande quantità di solvente e tempi di estrazione lunghi: risulta necessaria la loro sostituzione con tecniche più all'avanguardia e con un approccio più sensibile alle tematiche ambientali. Tra queste le più importanti sono l'estrazione assistita da microonde (MAE), l'estrazione assistita da ultrasuoni (UAE), l'estrazione accelerata con solvente (PLE) e con fluidi supercritici.

Nel presente lavoro, per mancanza di tempo e dati, solamente due tecniche tra queste sono state studiate: l'estrazione assistita da ultrasuoni e l'estrazione accelerata con solvente.

L'estrazione assistita da ultrasuoni è una tra le alternative più promettenti per l'estrazione di polifenoli, grazie alla sua versatilità e alla possibile implementazione a scala industriale. Si caratterizza per la riduzione della quantità di solvente utilizzato e del tempo di estrazione (Tiwari 2015). Gli ultrasuoni applicati permettono al solvente di estrarre determinati composti ad esso affini da una matrice selezionata: il principio su cui si basa questa tecnica è la cavitazione acustica, il fenomeno che avviene quando un mezzo viene sottoposto a onde ultrasoniche. La serie di compressioni e decompressioni che si generano durante la propagazione di queste onde provoca la formazione di bolle nel mezzo liquido; la loro implosione causa cambiamenti in temperatura e pressione e genera turbolenza, ma soprattutto provoca la rottura delle cellule. Un conseguente aumento dei coefficienti di trasferimento di massa e una maggiore penetrazione del solvente all'interno del campione da estrarre permettono una elevata resa di estrazione (Medina-Torres *et al.* 2017; Tiwari 2015).

L'estrazione accelerata con solvente è un processo automatico in cui si possono selezionare diversi parametri, quali la temperatura del solvente, il numero di cicli di estrazione e la durata degli stessi. L'estrazione si effettua con il solvente scelto in fase liquida ad alta temperatura: è necessario perciò impostare una pressione elevata per mantenerlo allo stato liquido (Araújo *et al.* 2015).

Numerosi studi hanno provato l'efficacia di queste e delle altre tecniche menzionate nell'estrazione di polifenoli da residui derivanti dalla produzione di olio di oliva e vino. Nonostante tecniche più classiche – con elevati consumi di solvente e lunghi tempi di estrazione - vengano ancora ampiamente studiate, l'interesse scientifico si è spostato verso le tecniche sopramenzionate. In particolare, l'intento di numerosi trattati di recente pubblicazione è di dimostrare una maggiore efficacia di queste tecniche non convenzionali quando paragonate alle tecniche più dispendiose in termini di tempo e costi e meno ecologiche (Abdel-Razek *et al.* 2017;

Fernández *et al*. 2018; Tan *et al.* 2017; Lozano-Sánchez *et al.* 2014; Caldas *et al.* 2018; Drosou *et al.* 2015; Da Porto *et al.* 2015; Poveda *et al.* 2018; Tao *et al.* 2014).

4. Tecniche di separazione e purificazione

L'utilizzo di membrane per separare composti di interesse, con l'intento finale di purificarli e ottenerli in determinate concentrazioni, è molto diffuso, specialmente nell'industria alimentare. I vantaggi di questo tipo di tecnologia sono numerosi: le condizioni di lavoro permettono di evitare la degradazione di questi composti, generalmente sensibili a determinate condizioni di temperatura e pressione, e l'assenza del solvente permette di eliminare i dispendiosi processi di rimozione dello stesso dal prodotto finale, così come il suo smaltimento. Questa tipologia di processo prevede inoltre un basso consumo energetico e la possibilità di ottenere gli stessi risultati a scala più elevata. La possibilità di integrare membrane di diverso tipo inoltre permette di frazionare ulteriormente ciò che ci si propone di separare, aumentando così il grado di purificazione e con esso la resa del processo di separazione (Castro-Muñoz *et al.* 2016).

La separazione di polifenoli tramite utilizzo di membrane è un processo complesso, oggetto di numerosi studi. La resa di queste tecniche si basa sull'interazione dei composti con la membrana stessa: la ritenzione è direttamente collegata alla grandezza della molecola presente nell'alimentazione. Molecole più grandi dei pori delle membrane verranno trattenute, mentre quelle più piccole permeeranno. È fondamentale, dato il meccanismo di separazione, considerare le possibili interazioni delle molecole target sia con la membrana che con altri composti. I polifenoli possono per esempio interagire tra loro o con le proteine, creando composti di grandezza più elevata (Cassano *et al.* 2017; Bazinet and Doyen 2017).

I processi a membrana generalmente si distinguono per la grandezza dei pori e per la pressione trans-membrana che è necessario applicare per implementare la separazione. In questo lavoro sono state utilizzate la microfiltrazione e la nanofiltrazione.

La microfiltrazione è un processo di filtrazione fisica, per cui le particelle presenti nell'alimentazione vengono separate a seconda della loro dimensione. La dimensione dei pori della membrana va da 0.1 a 5 μm, e le pressioni di lavoro possono passare da 1 a 10 bar, a seconda della resistenza intrinseca della membrana e della resistenza data da ciò che si deposita durante il processo di separazione.

La nanofiltrazione differisce dalla precedente per dimensione dei pori, 0,5 – 10 nm, e per la pressione applicata, 10 – 30 bar. In entrambe i casi, l'obiettivo è valutare l'efficienza di ritenzione per i composti di interesse, per comprendere la selettività della membrana. In questo lavoro si applica la formula seguente per ogni polifenolo considerato, per comprendere quale membrana permetta di ottenere il miglior grado di separazione (1).

$$R = 1 - \frac{C_P}{C_A} \qquad (1)$$

In (1), C_P e C_A si riferiscono alla concentrazione del composto nel permeato e nell'alimentazione (kg/m^3), rispettivamente.

Negli ultimi anni, diversi studi hanno dimostrato l'efficacia dei processi a membrana nel recupero di composti di interesse da residui industriali. Generalmente, diverse tecniche vengono applicate in processi sequenziali, che permettono di separare molecole di diversa grandezza in diverse fasi. La microfiltrazione viene spesso utilizzata per l'eliminazione di impurità in una prima fase, mentre l'ultrafiltrazione e la nanofiltrazione vengono utilizzate in fasi successive per la separazione più mirata tra i vari composti. In uno studio del 2017, diverse tecniche a membrana sono state implementate e combinate tra loro, con obiettivo il recupero di polifenoli contenuti nelle fecce di vino, e la loro separazione dai polisaccaridi. L'ultrafiltrazione è risultata efficace nella separazione dei composti polifenolici dagli zuccheri, mentre la nanofiltrazione successiva ha permesso la purificazione di una specifica classe di polifenoli, le antocianidine, da quelli presenti (Giacobbo *et al.* 2017). Nel trattamento dei residui della produzione di olio di oliva, l'approccio è

il medesimo: Sygouni e collaboratori (2019) applicano diverse tecniche in maniera sequenziale con l'obiettivo di recuperare polifenoli dagli estratti ottenuti dalle acque reflue derivanti dalla produzione di olio.

Le tabelle 3 e 4 presentano studi relativi al recupero di polifenoli con membrana da residui della produzione di olio di oliva e vino, rispettivamente.

Tabella 3 *- Tecniche a membrana per il trattamento di residui derivanti dalla produzione di olio di oliva*

Residuo	Tipologia di membrana	Riferimento
Acque reflue di frantoio	UF (0,04 μm)+ NF (MWCO 150-300 Da)	(Ioannou-Ttofa *et al.*, 2017)
	UF (100 nm) + NF (MWCO 800 Da) + RO (99% ritenzione del NaCl)	(Sygouni *et al.*, 2019)
	UF (100 nm) + NF (470 Da) + RO (99% ritenzione del NaCl)	(Zagklis *et al.*, 2015)
	UF + NF	(Cassano *et al.* 2013)

Tabella 4 *- Tecniche a membrana per il trattamento di residui derivanti dalla produzione di vino*

Residuo	Tipologia di membrana	Riferimento
Vinacce (vino bianco)	MF (0,5 μm)+ NF + UF (per ridurre l'impatto ambientale del volume ritenuto nella NF)	(Díaz-Reinoso *et al.*, 2017)
Fecce di vino rosso	MF	(Giacobbo *et al.*, 2015)
	MF + UF + NF	(Giacobbo *et al.*, 2017)
	MF + UF + NF	(Giacobbo, Bernardes and de Pinho, 2017)
Winery sludge	UF	(Galanakis *et al.* 2013)
Acque reflue	RO (l'obiettivo è la purificazione delle acque reflue, piuttosto che il recupero dei polifenoli)	(Ioannou *et al.*, 2013)

5. Materiale e metodologia

Con l'obiettivo finale di recuperare i polifenoli dalle matrici di derivazione industriale precedentemente indicate, il presente lavoro si sviluppa in due fasi parallele, ottimizzazione delle tecniche di estrazione e identificazione di membrane che posseggano elevata selettività nei confronti di specifici polifenoli. Gli esperimenti sono stati condotti all' Università di Barcellona (UB) e all' Universitat Politècnica de Catalunya (UPC-EEBE). La sansa di oliva è stata fornita dalle industrie Borges, le fecce di vino sono state fornite dalle cantine Torres.

5.1 Esperimenti di estrazione

Due tecniche sono state studiate: l'estrazione accelerata con solvente e l'estrazione assistita da ultrasuoni. Gli esperimenti sono stati condotti in parallelo per poter condurre entrambe le tipologie di estrazione allo stesso tempo e comparare i risultati.

5.1.1 Estrazione con solvente pressurizzata

Per eseguire l'estrazione, 1 grammo di campione di ogni residuo è stato pesato e successivamente mescolato a 2 grammi di terra diatomea. Questo adsorbente viene aggiunto per migliorare l'estrazione, rendendo omogenea la texture della miscela solida. La miscela ottenuta è stata poi posta all'interno dell'apposita camera di estrazione in acciaio inossidabile. Sono state condotte tre repliche per ciascun esperimento. Il solvente utilizzato è una soluzione di etanolo e acqua in diverse percentuali: i test sono stati condotti a diverse temperature (80, 100, 120 °C) e a diversa percentuale di etanolo (40, 60, 80%). Inizialmente è stato svolto un ciclo di estrazione della durata di cinque minuti; successivamente, una volta individuate la temperatura e composizione del solvente ottimali, è stato aumentato il numero di cicli, 1, 2 o 3, e la durata dell'estrazione, 5, 10 e 15 minuti. L'estrazione è stata effettuata in un apparato Dionex ASE 350 a una pressione di 10 bar; una volta ottenuto l'estratto, esso è stato centrifugato per 15 minuti a 3500 rpm e successivamente filtrato con l'ausilio di una siringa e due diversi filtri: un filtro in poliammide da 0,45 μm e uno in Nylon da 0,22 μm, in maniera da rimuovere le particelle residue che potrebbero inficiare l'analisi dei risultati e il corretto funzionamento dell'HPLC.

5.1.2 Estrazione assistita da ultrasuoni

Nel caso di questa tecnica estrattiva, le condizioni ottimali di estrazione erano già state individuate nelle fasi precedenti del progetto di ricerca in cui si inserisce questo lavoro. Anche in questo caso, 1 grammo di campione è stato pesato per ogni residuo e ad esso sono stati aggiunti 20 mL di solvente, la cui composizione era già stabilita per entrambi i residui, EtOH/H2O/HCl 80/19,5/0,5 (*v/v*) per le fecce di vino e EtOH/H2O/HCl 60/39/1 (*v/v*) per la sansa di oliva. Sono state condotte tre repliche per ciascun esperimento in un bagno ad ultrasuoni – Branson 5510 - con una durata di trenta minuti e una frequenza degli ultrasuoni di 40 kHz. I processi di purificazione e stabilizzazione a valle dell'estrazione sono i medesimi descritti nel paragrafo precedente.

5.2 Esperimenti di separazione con membrane

Diverse tipologie di membrane sono state testate per individuare quale tra queste possedesse maggiore affinità con i polifenoli studiati. I test sono stati condotti con miscele sintetiche di polifenoli: l'obiettivo di queste miscele è di mimare la composizione degli estratti effettivi, per

porre le basi alla successiva fase di sperimentazione, dove gli estratti vengono trattati attraverso le membrane selezionate in questa fase.

Le due miscele sintetiche, una per ogni tipo di residuo, sono state ottenute nota la composizione dei vari estratti: la composizione di ogni miscela è presentata nelle tabelle 5 e 6.

***Tabella 5** - Composizione iniziale della miscela sintetica (sansa di oliva)*

Polifenoli standard	Concentrazione (mg/L)
Acido Omogentisico	9
2- (3,4 diidrossifenil) etil alcol	17
Acido Caffeico	12
Acido *p*-Cumarico	4
Oleuropeina	44
Luteolina	15

***Tabella 6** - Composizione iniziale della miscela sintetica (fecce di vino)*

Polifenoli standard	Concentrazione (mg/L)
Acido Gallico	5
Acido 3,4 diidrossibenzoico	8
Catechina	12
Acido 4-idrossibenzoico	14
Acido Siringico	7
Etil Gallato	25
Esperidina	7
Resveratrolo	3
Quercetina	127

Per valutare la resa e i risultati delle diverse tecniche, si è valutata per ogni esperimento la concentrazione nell'alimentazione e nel permeato, in modo da calcolare per ogni polifenolo l'efficienza di ritenzione come da (1).

Tre diverse tecniche sono state testate: microfiltrazione con dischi, filtrazione per centrifugazione e nanofiltrazione. Per ogni membrana/tubo sono state effettuate due ripetizioni.

5.2.1 Filtrazione mediante centrifugazione

Per ogni miscela sono stati prelevati 4 mL da processare all'interno dei tubi di centrifugazione (Amicon ® Ultra – 4, Merck Millipore). L'operazione di centrifugazione è stata condotta a 2600 rpm e bloccata una volta ottenuto l'intero volume per filtrazione.

5.2.2 Microfiltrazione

Tre diversi filtri sono stati testati, differenti per dimensione dei pori, rispettivamente di 0.1, 0.45 µm (Sartorius Stedim Biotech SA) e 0.22 µm, in nylon (FILTER-LAB). Per operare la filtrazione, nella parte inferiore del dispositivo (in vetro, capacità 25 mL) è stato creato il vuoto, in modo tale da permettere la filtrazione di 5 mL di miscela inseriti dall'alto. È stato poi prelevato il volume permeato per poterne analizzare la composizione.

5.2.3 Nanofiltrazione

Cinque diverse membrane sono state testate: ritagliate da fogli più grandi, sono state ricavate con un'area di circa 7 cm^2 e immerse in acqua Milli-Q per rimuovere residui formatisi durante il periodo di conservazione. Le membrane utilizzate sono la NF270 (DOW Chemical), NF90 (DOW Chemical), TFCS (Koch Membrane Systems), TFC-HR (Koch Membrane Systems) e DURACID (Suez Environment). Inizialmente è stata utilizzata aria pressurizzata a 7 bar; tuttavia, data l'elevata resistenza dimostrata da alcune membrane al passaggio di liquido, è stato successivamente utilizzato azoto, con una pressione che andava dagli 8 agli 11.5 bar. 3 mL di miscela sono stati processati e il volume di permeato è stato utilizzato per le successive analisi.

Una volta effettuati i test, i campioni ottenuti sono stati analizzati in termini di composizione tramite HPLC, valutando la concentrazione finale di ogni polifenolo per determinare la selettività delle diverse membrane nei confronti di questi composti.

5.3 Analisi dei risultati tramite HPLC

La cromatografia liquida ad alta prestazione è stata utilizzata per ottenere i risultati dei processi di estrazione e di separazione. L'apparecchio é un cromatografo Agilent Series 1100 (Agilent Technologies, Palo Alto, California, USA) accoppiato a una colonna cromatografica che lavora a fase inversa Kinetex C18 (100 mm x 4,6 mm, 2,6 μm). La fase mobile è formata da una fase acquosa con lo 0.1% di acido formico e da acetonitrile. I gradienti utilizzati sono diversi a seconda che si analizzino i risultati dei processi di estrazione o di separazione. I cromatogrammi vengono ottenuti a 3 diverse lunghezze d'onda, 280,310 e 370 nm. Ogni picco individuato corrisponde a un composto specifico, l'area sottesa al picco è proporzionale alla sua concentrazione. Per questa ragione, data la sensibilità dell'apparecchiatura nei confronti dei vari polifenoli, viene costruita una retta di calibrazione per ciascuno di essi per poter valutare i risultati ottenuti.

5.4 Analisi del contenuto di zuccheri

Oltre alla composizione in termini di polifenoli, è stata valutata la quantità di zuccheri presenti nei vari estratti. Si utilizza un metodo colorimetrico, il metodo Dubois: esso prevede di valutare la colorazione ottenuta dai vari estratti in seguito a una specifica reazione tramite spettrofotometro.

Inizialmente, soluzioni di glucosio sono state preparate a diverse concentrazioni per costruire una retta di calibrazione. Successivamente, è stato preparato il fenolo necessario per la reazione: è importante che un quantitativo sufficiente venga preparato e che venga utilizzato il medesimo per l'intero procedimento, poiché questa specifica preparazione è la maggior fonte di errori nelle fasi successive. Per ogni campione, soluzioni di glucosio e estratti, è stato prelevato 1 mL a cui è stato aggiunto 1 mL di fenolo e 5 mL di acido solforico e la miscela risultante è stata agitata dolcemente. Dopo 10 minuti, le provette sono state agitate con ausilio di un vortex e sono state inserite in un bagno a 30°C per 20 minuti. Per misurare l'assorbanza a una lunghezza d'onda di 490 nm è stato utilizzato uno spettrofotometro SPECORD 200 PLUS Diode-Array (Analytik Jena AG). Sei diversi estratti sono stati analizzati, come riportato nella tabella 7.

***Tabella 7** - Estratti analizzati*

PLE Fecce di vino 100°C, 60 % EtOH, 1 ciclo, 5 minuti
UAE Fecce di vino 60 % EtOH, 0,1% HCl, 30 minuti
PLE Sansa di oliva 100°C, 60 % EtOH, 1 ciclo, 5 minuti
UAE Sansa di oliva 60 % EtOH, 0,1% HCl, 30 minuti

PLE Fecce di vino 100°C, 60 % EtOH, 2 cicli, 5 minuti
UAE Fecce di vino 60 % EtOH, 0,1% HCl, 30 minuti

Per ogni estratto è stato valutato un fattore di diluizione opportuno, per evitare che il colore naturale dell'estratto coprisse il colore derivante dalla reazione inficiando la misurazione finale.

6. Risultati e discussione

6.1 Risultati dei processi di estrazione

Entrambe le tecniche di estrazione sono state testate per le due tipologie di residui. Le condizioni operative ottimali per l'estrazione assistita da ultrasuoni (UAE) erano già state determinate nelle fasi di sperimentazione precedenti; per l'estrazione assistita da solvente (PLE) è stato necessario eseguire diversi esperimenti variando temperatura e percentuale di etanolo. I risultati ottenuti sono mostrati nelle tabelle 8 e 9.

***Tabella 8** - Risultati PLE (sansa di oliva)*

$g_{estratto(GAE)}/ kg_{residuo}$ SANSA DI OLIVA (Borges)			
Temperatura (°C)	**80**	**100**	**120**
40 % EtOH	4,19 ± 0,38	4,74 ± 0,36	4,59 ± 0,39
60 % EtOH	4,54 ± 0,22	4,69 ± 0,27	4,67 ± 0,01
80 % EtOH	2,87 ± 0,49	2,32 ± 0,40	3,68 ± 0,73

***Tabella 9** - Risultati PLE (fecce di vino)*

$g_{estratto(GAE)}/ kg_{residuo}$ FECCE DI VINO (Bodega Torres)			
Temperatura (°C)	**80**	**100**	**120**
40 % EtOH	1,08 ± 0,09	1,30 ± 0,22	1,12 ± 0,09
60 % EtOH	1,82 ± 0,04	1,93 ± 0,10	1,75 ± 0,04
80 % EtOH	1,36 ± 0,29	1,74 ± 0,29	2,17 ± 0,10

Come si può vedere dalle due tabelle precedenti è stato possibile individuare le condizioni ottime di estrazione, nonostante la differenza tra i valori ottenuti sia in alcuni casi minima. Date queste minime discrepanze è possibile valutare in maniera critica i risultati per individuare la condizione ottimale che permetta di ottenere un compromesso tra resa estrattiva, consumo di solvente e consumi energetici in termini di temperatura. Nel caso della sansa di oliva, La concentrazione di estratto maggiore si ottiene lavorando a 100°C con una soluzione al 40 % di etanolo. La figura 2 mostra l'andamento dell'estrazione con temperatura e percentuale di etanolo.

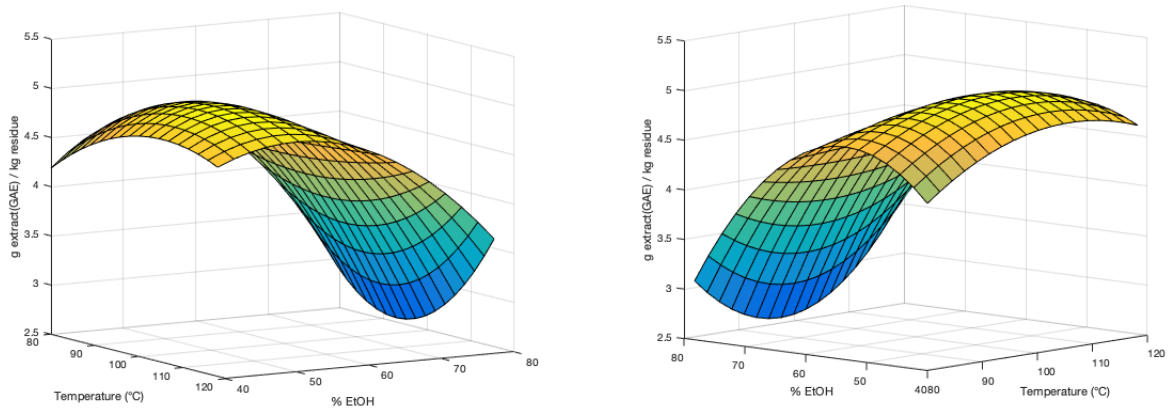

***Figura 2** - Rappresentazione 3D dei risultati del PLE per la sansa di oliva*

Come si può vedere dalla figura 2, la composizione del solvente è il fattore che influenza maggiormente la resa estrattiva, con valori minimi ottenuti quando si opera con una percentuale pari all' 80%. La concentrazione maggiore si ottiene lavorando a 100°C con una soluzione al 40% di etanolo. È possibile quindi lavorare con la percentuale minima di solvente, riducendo i costi del solvente stesso e delle eventuali operazioni successive per la sua rimozione dal prodotto finale.
La figura 3 invece mostra i risultati dell'estrazione delle fecce di vino.

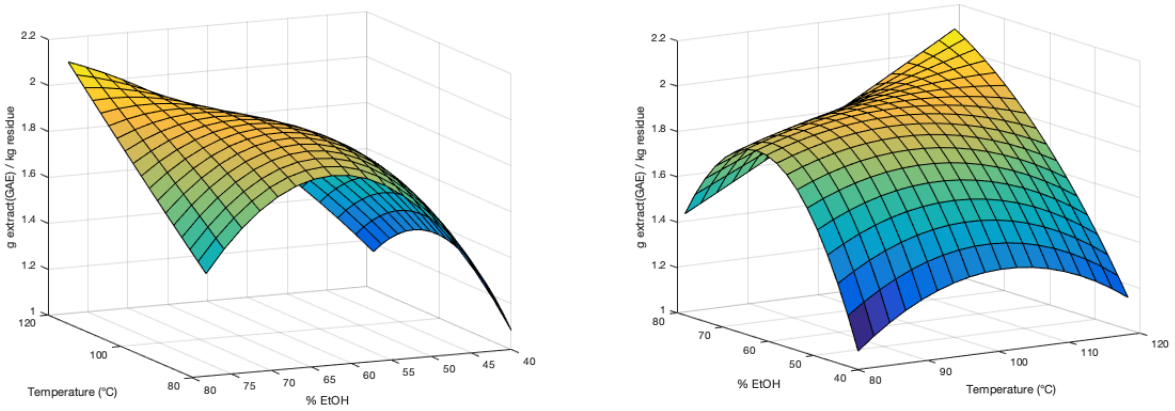

Figura 3 *- Rappresentazione 3D dei risultati del PLE per le fecce di vino*

Dalla figura 3 si può notare come a 120 °C e 80% di etanolo si registra un insolito incremento nella resa di estrazione. La formazione di nuovi picchi e la scomparsa di altri nel cromatogramma hanno però portato a scartare questi dati, poiché questi cambiamenti si possono addurre alla degradazione di alcuni composti fenolici presenti nelle matrici da trattare. La concentrazione più elevata si ottiene a 100 °C con una percentuale di etanolo pari al 60%. In questo caso, diminuendo la temperatura è comunque possibile ottenere una buona resa estrattiva, riducendo i costi energetici.

Una volta ottenute le condizioni ottimali per un ciclo della durata di 5 minuti, il numero dei cicli e la durata degli stessi sono stati incrementati. Nel caso della sansa di oliva, le condizioni ottimali di estrazione si sono confermate operando con un unico ciclo di 5 minuti; diversamente, per l'estrazione delle fecce di vino, incrementare il numero di cicli a 2 ha migliorato la resa di estrazione, mantenendone la durata a 5 minuti. Incrementando il numero di cicli si è ottenuta una concentrazione pari a 2±0,23 $g_{estratto(GAE)}$/ $kg_{residuo}$. La tabella 10 riassume le condizioni operative determinate in questa fase per entrambe le tecniche di estrazione.

Tabella 10 *- Condizioni di estrazione ottime per PLE e UAE*

Matrice	Tecnica	Condizioni operative ottime
Sansa di oliva	PLE	EtOH/H_2O 40/60 *(v/v)* 100°C 1 ciclo 5 minuti
	UAE	EtOH/H_2O/HCl 60/39,9/0,1 *(v/v/v)* 30 minuti
Fecce di vino	PLE	EtOH/H_2O 60/40 *(v/v)* 100°C 2 cicli 5 minuti
	UAE	EtOH/H_2O/HCl 80/19,5/0,5*(v/v/v)* 30 minuti

In seguito alla determinazione delle condizioni operative, è necessario determinare per ogni residuo quale tecnica permette la migliore estrazione. La figura 4 mostra il confronto tra le due tecniche per la sansa di oliva.

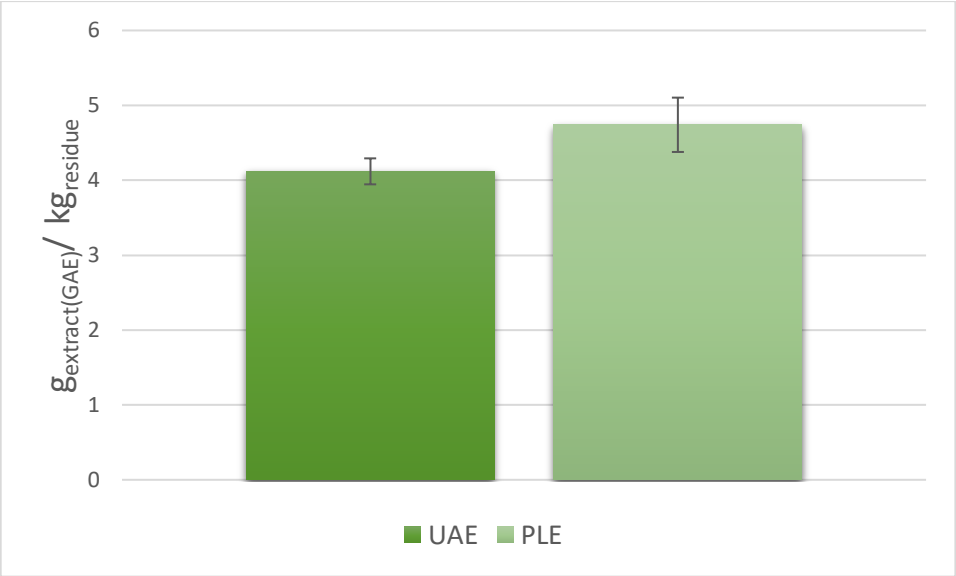

Figura 4 *- Confronto delle tecniche estrattive (sansa di oliva)*

L'estrazione pressurizzata con solvente permette una resa estrattiva maggiore, con una concentrazione finale di 4,74±0,36 g di estratto per chilogrammo di residuo, paragonata all'estrazione assistita da ultrasuoni con la quale si ottiene una concentrazione inferiore di 4,12±0,17g di polifenoli estratti per chilogrammo di residuo. Con le stesse modalità, la figura 5 mostra il confronto fra le due tecniche per l'estrazione di fecce di vino.

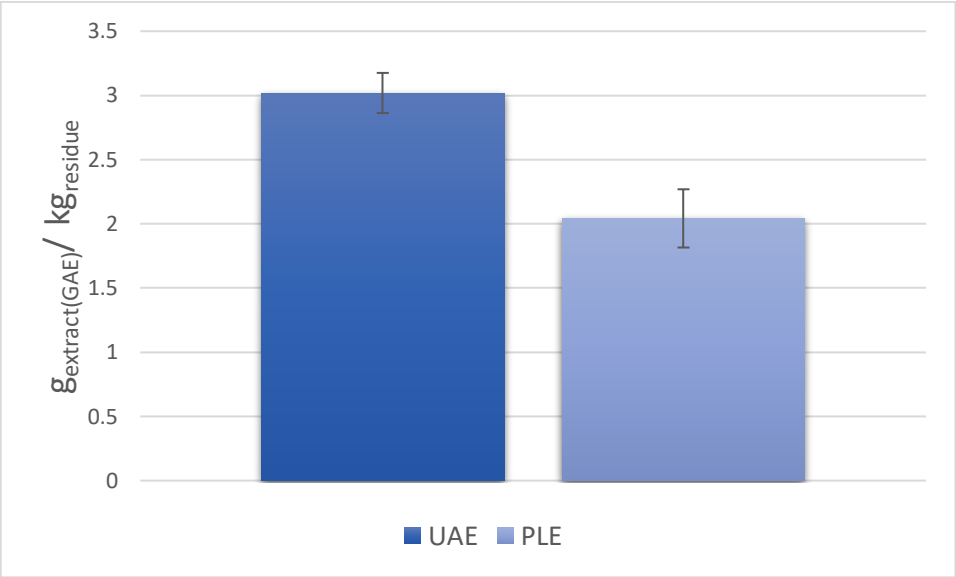

Figura 5 *- Confronto tra le tecniche estrattive (fecce di vino)*

Diversamente dal caso precedente, l'estrazione assistita da ultrasuoni permette una resa estrattiva più elevata delle fecce di vino, con una concentrazione di 3±0,16 grammi di polifenoli estratti per chilogrammo di feccia di vino. In uno studio simile, Tao e collaboratori (2014) hanno implementato la medesima tecnica su questo residuo specifico: in questo caso la composizione del solvente è risultata differente, ovvero EtOH/H2O 50/50 (*v/v*). Questa differenza può dipendere dal fatto che la composizione di ogni residuo può variare a seconda delle condizioni climatiche e altri fattori che influenzano la produzione stessa. Altri studi si sono concentrati sull'applicazione dell'estrazione pressurizzata con solvente: nonostante in questo caso risulti la meno efficace tra le

due tecniche studiate, la differenza in termini di resa non è elevata; è quindi interessante valutare la possibilità di implementare questa tecnica utilizzando come solvente l'acqua, sostituendo solventi più cari e potenzialmente tossici (Poveda *et al*. 2018).

Dai risultati ottenuti, la sansa di oliva risulta una fonte più ricca in polifenoli delle fecce di vino. L'estrazione assistita da ultrasuoni tuttavia permette applicazioni a scala industriale, diversamente dal PLE, e un minor uso di solvente.

6.2 Analisi degli zuccheri contenuti negli estratti

Per valutare in maniera completa la composizione degli estratti è stata effettuata un'analisi del contenuto di zuccheri all'interno degli estratti: in questo modo la composizione completa verrà utilizzata nelle successive fasi di sperimentazione. Nota la composizione iniziale, sarà possibile processare gli estratti e valutare la performance delle membrane.

Nel paragrafo 5.4 si descrivono i passaggi da seguire per eseguire questo metodo colorimetrico; l'assorbanza è stata ottenuta per le soluzioni di glucosio a diversa concentrazione per costruire la retta di calibrazione (in questo caso da 1 a 150 mg/L). Dai valori di assorbanza media ottenuti per ogni estratto, è stata valutata la concentrazione corrispondente. Noti i volumi di estratto e la quantità di residuo utilizzata è stata calcolata la quantità di zuccheri estratta per chilogrammo di residuo. La figura 6 mostra i risultati preliminari dei due tipi di estratti, con il PLE effettuato con un ciclo di 5 minuti.

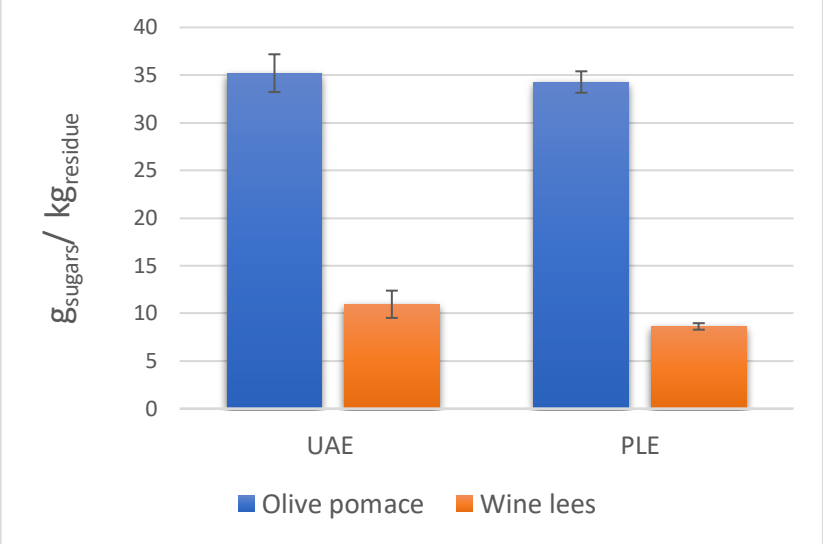

Figura 6 *- Contenuto in zuccheri degli estratti di sansa di oliva (olive pomace) e delle fecce di vino (wine lees)*

Come si può vedere dalla figura 6, entrambe le tecniche permettono di estrarre elevate quantità di zuccheri. Tuttavia, le concentrazioni risultano più elevate per la sansa di oliva: la differenza è minima tra le due tecniche, si ottengono 35,2±2 $g_{zuccheri}$/ $kg_{residuo}$ con l'UAE e 34,3±0,5 $g_{zuccheri}$/ $kg_{residuo}$ con l'PLE. A parità di zuccheri estratti, la tecnica del PLE permette di estrarre una maggiore quantità di polifenoli, secondo quanto evidenziato nel paragrafo precedente. Anche nel caso delle fecce di vino, non si nota una differenza evidente tra i due tipi di estratti: l'UAE permette di ottenere una concentrazione di 10,9±1,4 $g_{zuccheri}$/ $kg_{residuo}$, mentre il PLE una concentrazione leggermente inferiore pari a 8,6±0,34 $g_{zuccheri}$/ $kg_{residuo}$. Diversa è la situazione quando si confrontano l'UAE e il PLE effettuato con due cicli di 5 minuti, come si può vedere in figura 7.

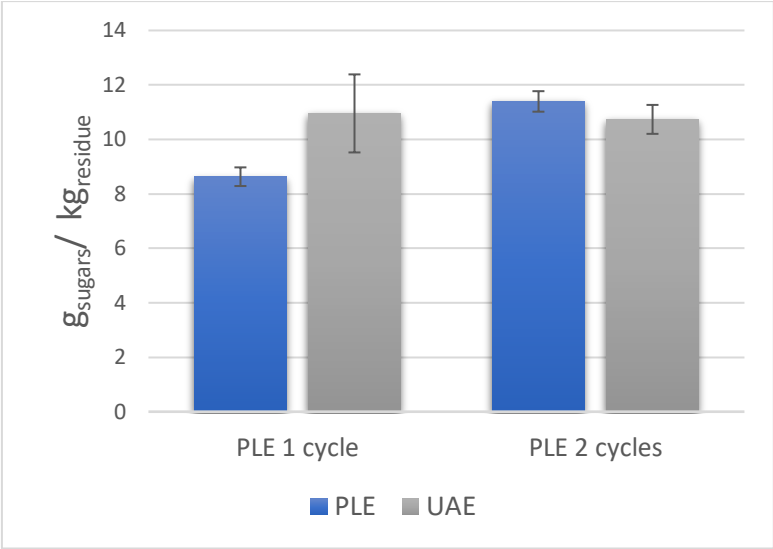

***Figura 7** - Contenuto in zuccheri negli estratti di fecce di vino all'incrementare del numero di cicli di estrazione*

Incrementando il numero di cicli, la differenza tra le rese estrattive in termini di zuccheri risulta più netta, la quantità di zuccheri estratti risulta maggiore quando si lavora con il PLE. Implementando un ciclo estrattivo in più la concentrazione passa da 8,6±0,34 $g_{zuccheri}/ kg_{residuo}$ a 11,4±0,38 $g_{zuccheri}/ kg_{residuo}$.

6.3 Separazione tramite membrana

Le tabelle 5 e 6 del paragrafo 5.2 indicano la reale composizione delle due miscele sintetiche utilizzate per valutare il comportamento delle diverse membrane nei confronti dei polifenoli selezionati. Per valutare il rendimento di ogni membrana è stata calcolata l'efficienza di ritenzione secondo l'equazione 1. L'obiettivo di ritenzione è stato fissato al 70%.

6.3.1 Filtrazione per centrifugazione

Per entrambe le miscele, questa tipologia di filtrazione è risultata inefficace. Nel caso della miscela delle fecce di vino, la ritenzione è risultata nulla per ogni polifenolo. Per quanto riguarda la seconda miscela, alcuni polifenoli hanno mostrato un valore di ritenzione diverso da zero, tuttavia valori troppo bassi per poter essere presi in considerazione per una separazione efficace. Per esempio, per l'acido omogentisico si è registrato un valore di ritenzione del 8%, tra i valori più alti della miscela, ma troppo basso perché questo composto si separi in maniera significativa.

6.3.2 Microfiltrazione: fecce di vino

La miscela corrispondente agli estratti di fecce di vino è stata la prima a essere processata: come previsto, a pori di dimensione maggiore corrispondono tempi di filtrazione inferiore. In questo caso solo due composti hanno mostrato una ritenzione diversa da zero, l'acido gallico e la quercetina. La figura 8 mostra i valori di ritenzione calcolati per questi due composti per i tre filtri (0,1, 0,22 e 0,45 μm).

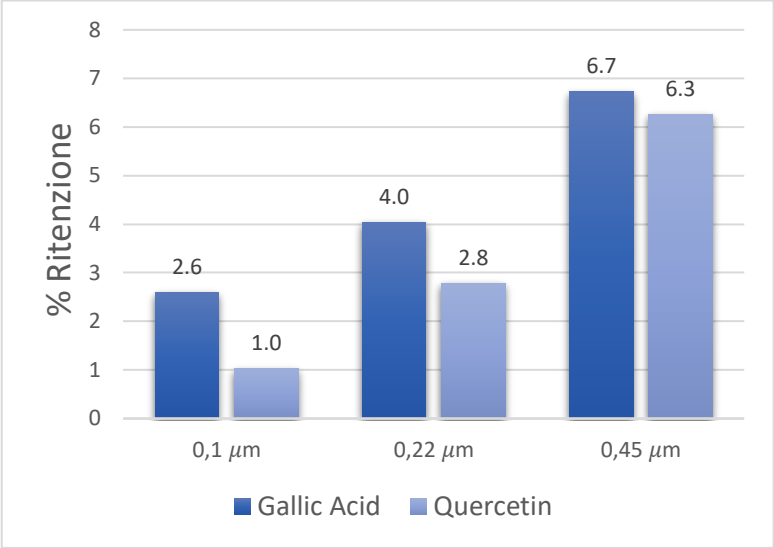

Figura 8 *- Risultati della microfiltrazione per l'acido gallico e la quercetina*

Come si può dedurre dalla figura 8, per entrambi i polifenoli il valore di ritenzione cresce all'aumentare della dimensione dei pori. Questo andamento si discosta da quello previsto: per il semplice fattore sterico, a dimensione maggiore si prevede una ritenzione minore. Tuttavia, è fondamentale considerare le possibili interazioni tra i polifenoli e la possibilità che altri meccanismi di separazione possano intervenire, tra i quali possibili effetti elettrici. Analizzando i dati, si può notare che la differenza percentuale tra i differenti valori di ritenzione è solo del 2%. Procedendo con la valutazione dell'errore di natura sperimentale in termini di deviazione standard, si può notare che la differenza tra i valori di ritenzione dei tre filtri è minima, come da figura 9.

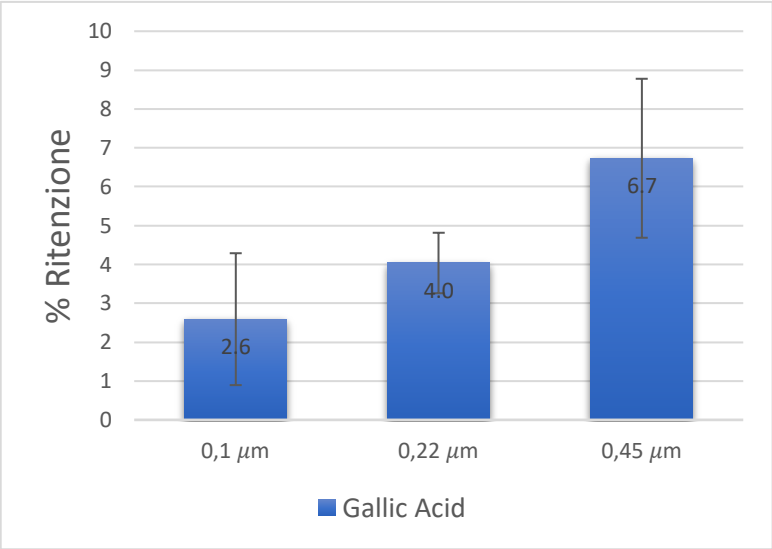

Figura 9 *- Analisi dei risultati di microfiltrazione per l'acido gallico*

Come si può notare dalla figura precedente, data la minima differenza tra le prestazioni dei tre filtri sarebbe necessario procedere con uno studio statistico per confermare l'effettiva variazione della ritenzione. In ogni caso, i valori di ritenzione ottenuti sono troppo bassi per ritenere la separazione attuata efficace. La microfiltrazione è stata oggetto di studio nella separazione dei polifenoli: Giacobbo e collaboratori (2015) hanno provato l'efficienza di questa tecnica, separando il 21% dei polifenoli nel permeato. Questa discrepanza dei risultati si può addurre alle possibili

interazioni tra i diversi polifenoli; inoltre nel sopramenzionato studio gli effluenti vengono processati direttamente, mentre qui si lavora con miscele sintetiche. Date queste considerazioni, è evidente la necessità di procedere con ulteriori studi, data la complessità di questo tipo di interazioni.

6.3.3 Microfiltrazione: sansa di oliva

Dopo un'accurata pulizia, la seconda miscela corrispondente agli estratti di sansa di oliva è stata processata per microfiltrazione. L'andamento riscontrato è differente dal precedente: con il filtro da 0.45 μm nessun polifenolo ha mostrato ritenzione, mentre gli altri due filtri hanno permesso la separazione di alcuni polifenoli, ma con valori troppo bassi per essere ritenuti validi allo scopo prefissato. Anche in questo caso, la microfiltrazione si è dimostrata inefficace.

6.3.4 Nanofiltrazione: fecce di vino

Cinque diverse membrane sono state testate: nel caso specifico di questa miscela è stato necessario scartare una membrana, la TFC-HR, poiché i risultati di una ripetizione non hanno trovato corrispondenza nell'altra, rendendo inservibili i valori riscontrati. Tra i polifenoli contenuti nella miscela, la quercetina ha dimostrato un'elevata ritenzione con tutte le membrane, intorno al 100%. La figura 10 mostra i valori di ritenzione per i polifenoli che hanno mostrato elevata affinità con la membrana, oltre la quercetina.

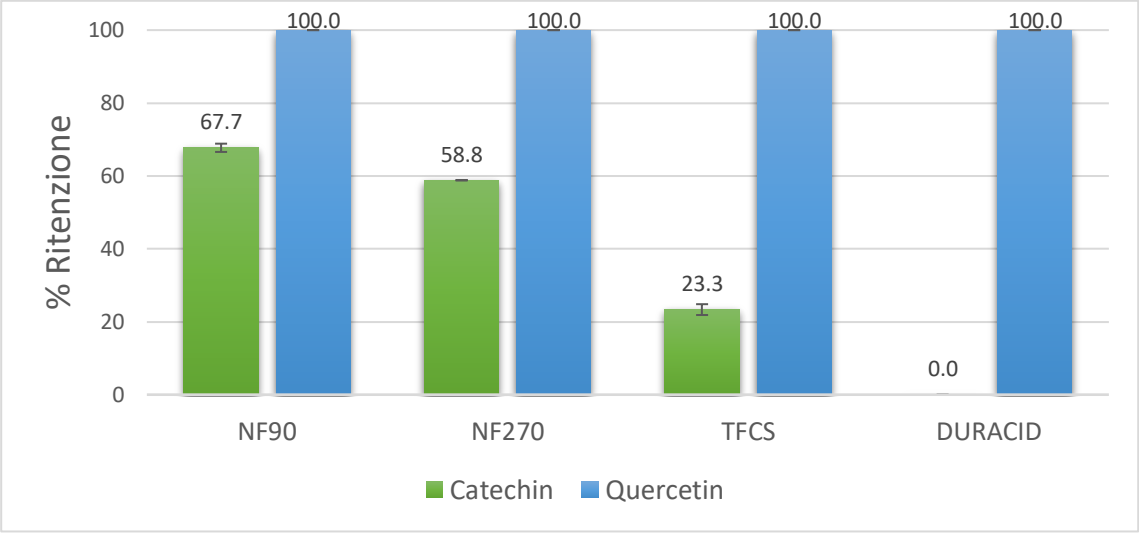

Figura 10 *- Risultati della nanofiltrazione per la catechina e la quercetina*

Come si può notare dalla figura precedente, gli unici valori di ritenzione competitivi con quelli della quercetina sono quelli relativi alla catechina, nonostante siano essi inferiori all'obiettivo stabilito del 70%. La membrana DURACID ha mostrato una elevata selettività per la quercetina, per cui è possibile ottenere una completa separazione dagli altri composti presenti. Per questo motivo, questa specifica membrana è un'ottima candidata per implementare la separazione di questo composto.

La figura 11 è uno schema della membrana stessa, dove si può notare il grado di separazione nei due volumi generati.

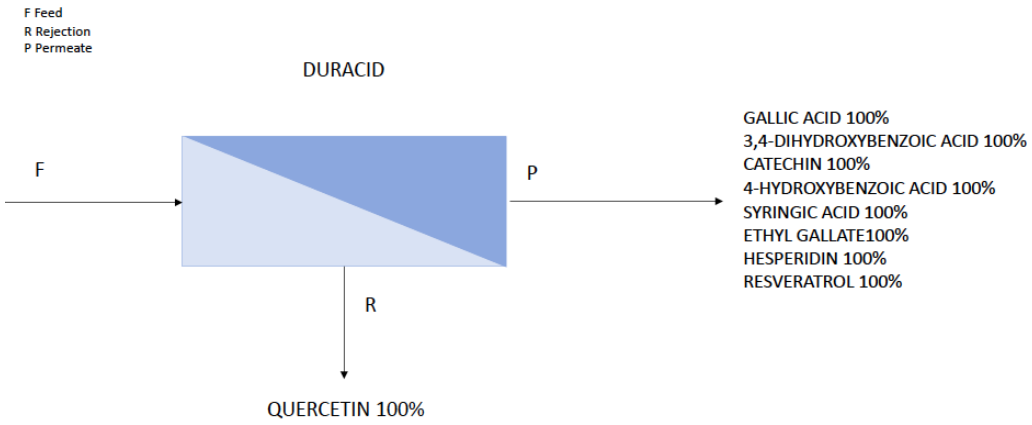

Figura 11 *- Schema DURACID (F, feed: alimentazione, R, rejection:ritenzione, P, permeate: permeato)*

La quercetina è un flavonoide anticancerogeno, antivirale e antinfiammatorio, nonché un agente antiobesità (Maalik *et al.* 2014). Dato l'elevato interesse di questo composto e la sua bassa biodisponibilità, la quercetina è oggetto di numerosi studi che tentano di risolvere il problema legato alla sua somministrazione. Conte e collaboratori (2016) riportano nel loro studio la possibilità di utilizzare nanoparticelle solide lipidiche per la sua somministrazione.

6.3.5 Nanofiltrazione: sansa di oliva

Successivamente, la seconda miscela è stata processata: in questo caso la prestazione di tutte le membrane è stata valutata con successo. Tuttavia, non è stato possibile valutarla per uno specifico composto, l'acido omogentisico, poiché non è stato possibile individuarlo nel cromatogramma corrispondente alla miscela iniziale. Il lasso di tempo intercorso tra gli esperimenti di microfiltrazione e nanofiltrazione può aver permesso l'innesco di meccanismi di degradazione a carico di questo composto. I risultati relativi ai composti che hanno mostrato alti valori di ritenzione sono mostrati in figura 12.

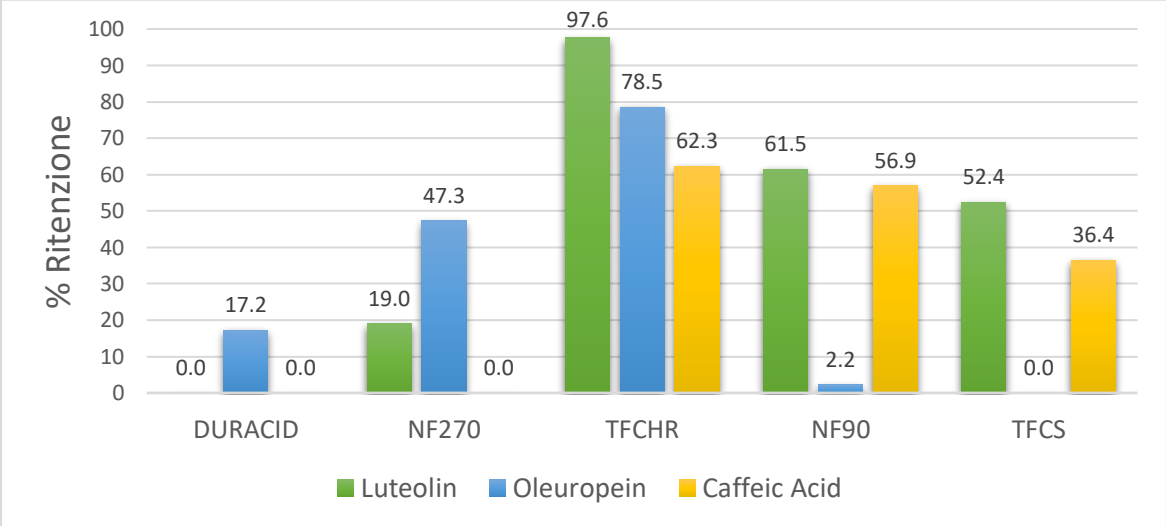

Figura 12 *- Risultati della nanofiltrazione per la luteolina, oleuropeina e l'acido caffeico*

I polifenoli per cui si sono registrati valori significativi di ritenzione sono la luteolina, l'oleuropeina, rispettivamente del 97,6 e 78,5% utilizzando la membrana TFC-HR. Per l'acido caffeico, presente in figura 12, è stata registrata una ritenzione del 62,3%, elevata, ma al di sotto della soglia prestabilita. Un quadro più completo si può evincere dalla figura 13, dove quest' ultimo composto viene riportato nella corrente di permeato.

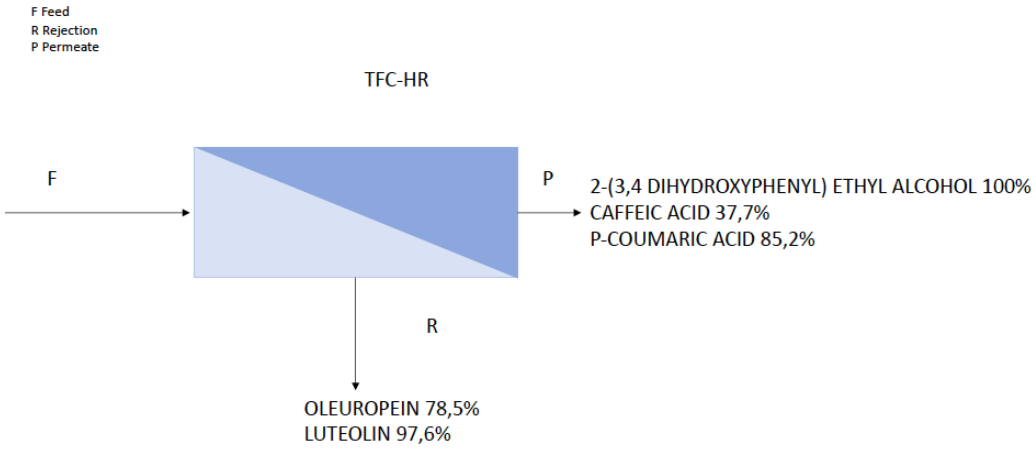

Figura 13 *- Schema TFC-HR (F, feed: alimentazione, R, rejection: ritenzione, P, permeate: permeato)*

Dalla figura precedente si può notare la possibilità di sfruttare entrambe i volumi derivanti dalla filtrazione, rendendo questa membrana la più efficiente tra le cinque testate. Tra i polifenoli già menzionati, troviamo l'idrossitrisolo e la oleuropeina, le cui proprietà sono indicate nel paragrafo 2.1.1 e la cui separazione è di elevato interesse tecnologico.

Conclusioni

Le proprietà dei composti fenolici rendono questa classe di antiossidanti soggetto di innumerevoli studi e accrescono l'interesse nel loro recupero da residui industriali. Le tecniche di estrazione non convenzionali sono state investigate per poter individuare le migliori condizioni estrattive, in termini di resa, costi e caratteristiche del solvente. Un approccio più innovativo consiste nell'accoppiare il processo di estrazione con processi di separazione a membrana, per ottimizzare la separazione e purificazione dei polifenoli.

Il presente lavoro è stato sviluppato all' Universitat Politècnica de Catalunya (UPC-EEBE), sostenuto dal progetto Waste2Product (CTM2014-57302-R) e dal progetto R2MIT (CTM2017-85346-R), finanziato dal Ministero di Economia e competitività spagnolo (MINECO) e dal governo catalano (ref. 2017-SGR-312). Questa ricerca di dottorato si propone di individuare le tecniche più efficaci per il recupero di polifenoli da due tipologie di residui industriali, della produzione del vino e dell'olio di oliva. L' HPLC è stata ampiamente investigata per ottimizzare l'analisi degli estratti e dei risultati ottenuti. In questa fase qui presentata, l'obiettivo era individuare le condizioni ottime di estrazione di due tecniche selezionate e le membrane con maggiore selettività verso polifenoli standard. Le fasi successive di sperimentazione prevedono di collegare le due parti, ovvero sfruttare gli estratti ottenuti e processarli attraverso le membrane identificate.

Due tecniche di estrazione sono state studiate: PLE e UAE. Per entrambi i residui è stata individuata la tecnica di estrazione più efficace; per la sansa di oliva è risultata la PLE, con una concentrazione ottenuta di 4,74±0,36 $g_{estratto(GAE)}/kg_{residuo}$ alle seguenti condizioni: EtOH/H2O 40/60 (*v/v*) operando con un ciclo di 5 minuti a una temperatura di 100 °C. Per le fecce di vino, è risultata più efficace la UAE, con una concentrazione ottenuta di $3±0,16 g_{estratto(GAE)}/kg_{residuo}$ operando alle seguenti condizioni: EtOH/H2O/HCl 80/19,5/0,5 (*v/v*).

Gli estratti sono inoltre stati analizzati per determinarne il contenuto in zuccheri: gli estratti di sansa di oliva (PLE: 34,3±0,5 $g_{zuccheri}/ kg_{residuo}$ UAE: 35,2±2 $g_{zuccheri}/ kg_{residuo}$) sono risultati più ricchi in zuccheri di quelli delle fecce di vino (PLE: 8,6±0,34 $g_{zuccheri}/ kg_{residuo}$ UAE: 10,9±1,4 $g_{zuccheri}/ kg_{residuo}$). La differenza in termini di concentrazione tra le due tecniche è minima nel caso della sansa di oliva, leggermente maggiore per le fecce di vino, dove l'UAE permette di estrarre una maggior quantità di zuccheri. Questa differenza si annulla quando si aumenta il numero di cicli estrattivi a due nel PLE, dove la quantità di zuccheri estratta risulta ora superiore a quella dell'UAE, con una concentrazione analizzata di 11,4±0,38 $g_{zuccheri}/ kg_{residuo}$.

Gli esperimenti effettuati con le diverse tecniche a membrana hanno permesso di individuare due membrane ad alta selettività verso specifici polifenoli. La membrana DURACID ha permesso di separare la quercetina con un'efficienza di ritenzione intorno al 100%, mentre la membrana TFC-HR ha permesso di separare in entrambe i volumi generati dell'operazione di diversi polifenoli. L'idrossitirosolo è permeato per il 100% mentre è possibile recuperare la luteolina nel volume ritenuto per il 97,6%.

Gli esperimenti di separazione tramite membrana sopramenzionati sono stati svolti su miscele sintetiche di polifenoli scelti, individuati durante l'analisi della composizione degli estratti. Le fasi di sperimentazione future prevedono di sfruttare le membrane selezionate per processare gli estratti e completare il ciclo di recupero/purificazione dei polifenoli contenuti nelle matrici solide studiate.

Acknowledgments

This research was supported by the Waste2Product project (CTM2014-57302-R) and the R2MIT project (CTM2017-85346-R) financed by the Spanish Ministry of Economy and Competitiveness (MINECO) and the Catalan Government (ref. 2017-SGR-312).

First of all, I would like to thank the people that permitted me to carry out this project; to Professor José Luis Cortina, a special thank you for offering me this possibility in the first place, and to Xanel Vecino Bello and Mònica Reig Amat another sincere thanks to you both for dedicating your time for the completion of this work. You managed to help me live the positive and advantageous experience I was hoping for when I first thought of doing this experience abroad. A special thanks to Professor Mauro Banchero who helped me complete the work once I came back to Politecnico di Torino.

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Then, the most important and sincere thanks go to my family and my friends: even if they were not here with me, they gave me unfailing support when I decided to spend this period abroad, as they always did in my past years of study. I will never find a proper way to completely thank them; even if they had some worries and doubts, they always gave me their complete support.

List of abbreviations

ACN: acetonitrile

MeOH: Methanol

EtOH: Ethanol

CO_2: Carbon dioxide

HCl: Hydrochloric acid

PLE: Pressurized liquid extraction

MAE: Microwave-assisted extraction

SFMAE: Solvent-free microwave-assisted extraction

UAE: Ultrasound-assisted extraction

SFE: Supercritical fluid extraction

MF: Microfiltration

UF: Ultrafiltration

NF: Nanofiltration

HPLC: High-performance liquid chromatography

MS: Mass spectrometry

DAD: Diode-array detector

MWD: Multi-Wavelength detector

UV: Ultraviolet

OP: Olive pomace

WL: Wine lees

OMWW: Olive mill wastewater

Introduction

There is an increasing trend within the chemical industry to focus on the recovery of compounds of interest from various kinds of residuals. The aim of the industries is to valorize the residues that derive from the production process as much as possible by using conventional technologies and investigating non-conventional ones. The attempt is to exploit production waste and obtain valuable compounds, such as polyphenols, that can be reused in different fields; moreover, it would allow these wastes to be managed, since they could be hazardous for the environment and have to be treated properly for each single case. Hence, the importance of investigating green alternative methods for this treatment.

The present work was realized at the Universitat Politècnica de Catalunya (UPC-EEBE) in Barcelona. It was supported by the Waste2Product project (CTM2014-57302-R) and the R2MIT project (CTM2017-85346-R) financed by the Spanish Ministry of Economy and Competitiveness (MINECO) and the Catalan Government (ref. 2017-SGR-312).

In addition to my courses at university, I performed these experiments by collecting data and performing an analysis. This work was a part of a doctoral project, however, due to a lack of time some of the intended experimental steps could not be further developed: for instance, microwave-assisted extraction, a noteworthy extraction technique, could not be carried out effectively, therefore the corresponding results have not been included. Microwave-assisted extraction appears a promising technique and would benefit from further investigation.

This work focuses on the recovery of polyphenols from olive pomace and wine lees. Firstly, best operative conditions were assessed for two different extraction techniques: pressurized-liquid extraction (PLE) and ultrasound-assisted extraction (UAE). Then, different membrane techniques were employed on synthetic mixtures of polyphenols.

This specific work starts with the implementation of the two extraction techniques, pressurized liquid extraction (PLE) and ultrasound-assisted extraction (UAE), in order to find the optimal conditions of each one, and later identify the most efficient among them. Once identified, different measurements and tests were carried out on samples of olive pomace and wine lees. Employing high-performance liquid chromatography (HPLC) coupled with mass spectrophotometry, the principal polyphenols contained within wine and olive residues were identified, to confirm HPLC results coupled with UV-VIS detector.

While dealing with extraction procedures, different membranes were tested using synthetic mixtures of polyphenols containing those previously identified within the respective matrices. The behavior of microfiltration filters in disk format, filtration by centrifugation and nanofiltration membranes was studied, as well as their rejection and permeate conditions. Lab-scale equipment was used, with low volumes of sample, to identify the components that will or will not pass through the different tested membranes. In parallel, sugar content analysis of the extracts from wine and olive oil residues was made through a colorimetric method, using Ultraviolet – Visible (UV-VIS) spectrophotometry.

1. Brief introduction on antioxidants

Over the past few years, there is growing interest in the recovery of new natural bioactive components, this is due to the necessity of dealing with the waste itself and the possibility of exploiting it to extract compounds of interest that could substitute the synthetic ones. Traditionally, synthetic compounds have been used for different applications: among the best-known antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary buthylhydroquinone (TBHQ) can be accounted, all proven by different studies to have toxic effects. These compounds are widely used to improve the quality of meat: the nature of meat, referring to the presence of endogenous phospholipids, and the addition of salt, both lead to an increase in the susceptibility to oxidative reactions. To avoid deterioration due to lipid oxidation, the addition of synthetic antioxidants results necessary to guarantee good quality and a proper shelf-life of this type of product (Jiang and Xiong 2016).
Lipid oxidation can occur by three different mechanisms, autoxidation, photooxidation and enzyme-involved oxidation, all divided in the three phases of initiation, propagation and termination as shown in Figure 1 (Yang *et al.* 2018).

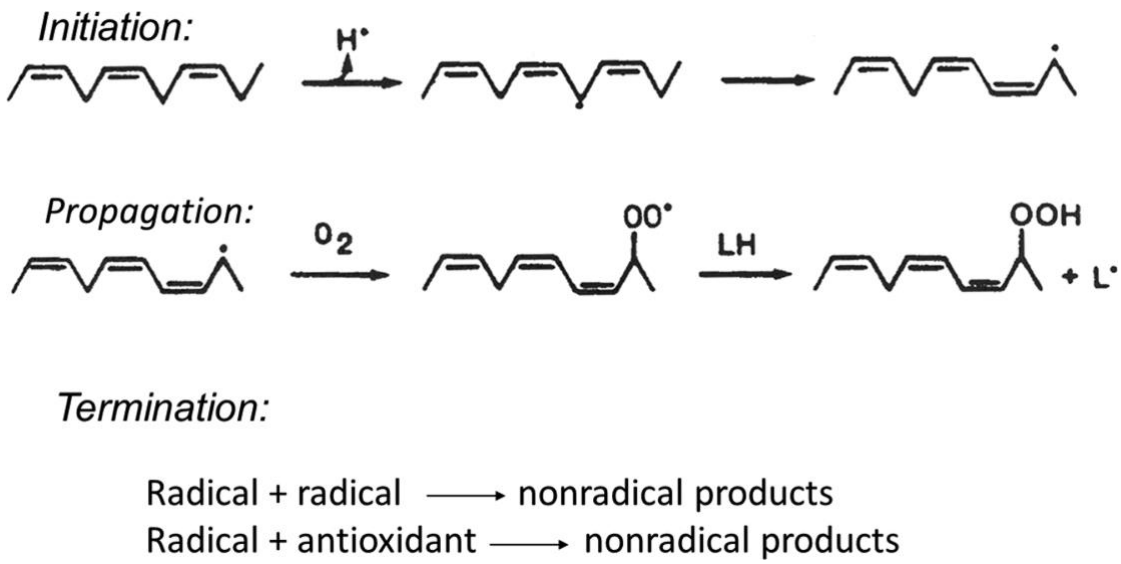


Figure 1 – *The three stages of lipid peroxidation: initiation, propagation, termination* (Yang *et al*. 2018)

Initiation reaction can be catalyzed by heat, light and transition metals. It is the phase in which a radical is produced. Due to its instability, this radical perpetuates the reaction in the propagation phase: it reacts with molecular oxygen and forms a new radical. This species is also unstable, it can react with another molecule and lead to the formation of another radical. The reaction occurs in a cycle and only stops when a radical reacts with another radical or in the presence of a neutralizing molecule, in this case an antioxidant, as shown in Figure 1.

Commonly, the species that have to be addressed and that perpetuate these reactions are the reactive oxygen species (ROS), which are produced during oxygen metabolism, playing a key role in food degradation and are proven to participate in many disease processes. For instance, they are responsible for oxidative stress, which is associated with different diseases, such cancer, coronary heart disease (CHD) and osteoporosis. In general, lipid oxidation is the most destructive type of oxidation (Nimse and Pal 2015). The three most important species are superoxide, hydrogen peroxide and hydroxyl radical which, in addition to others, are shown in Table 1:

Table 1 *- List of ROS* (Nimse and Pal 2015)

O_2	**Singlet Oxygen**
O_2c-	Superoxide anion radical
cOH	Hydroxyl radical
ROc	Alkoxyl radical
ROOc	Peroxyl radical
H_2O_2	Hydrogen Peroxide
LOOH	Lipid hydroxiperoxide

Lipid oxidation and other oxidative reactions lead to deterioration of foods, resulting in a decrease in freshness and diminished shelf life of the product. These modes of reaction are to be blocked to guarantee the possibility of storage and distribution of food for a certain period, avoiding generation of undesirable flavors or potential toxic substances (Yang *et al.* 2018). These considerations not only prove that it is necessary to use antioxidants, but also to replace the synthetic toxic ones in parallel. This lead to focus the interest on the seek for natural antioxidants, interest that is growing in pharmaceutical and food domains, since these compounds can be used not only as ingredients in food for quality preservation, but as important components of drug and as nutraceuticals as well (Kumar *et al.* 2017). These molecules can be found in different types of food, mostly fruits and vegetables, this is dependent on how they are cultivated and the weather they are exposed to (Bazinet and Doyen 2017). For example, polyphenols, one of the most important classes of antioxidants is produced as secondary metabolites from plants. The organism reacts to aggression by pathogens and/or to ultraviolet radiation, therefore the quantity produced depends on the occurrence of these situations (Acosta *et al.* 2014).

Various studies report descriptions of different classes of antioxidants, classified either by their structure or their mechanism of action. It is important to underline that their effectiveness in terms of antioxidative action depends on their chemical structure or their physical location within the food considered (Oroian and Escriche 2015).

1.1 Classification of polyphenols

The major classes of antioxidants are vitamins, carotenoids and polyphenols. However, only polyphenols are reported in this project.

Polyphenols, which are the objective of this work, are phytochemicals that derive directly from the metabolism of plants; more precisely they are secondary metabolites, deriving from the plant's response to the environment. Phytochemicals are not actually nutrient compounds, but their importance is well known, especially in the case of polyphenols, whose ability to act on chronical diseases has been well established (Acosta *et al.* 2014). Even if polyphenols don't bring an effective nutritional intake in a diet, they are essential to human health due to their antioxidant and antiradical qualities, both within the organism and in food, to preserve quality and freshness (Hajji *et al*. 2018). In addition to antioxidative properties, polyphenols also show anti-microbial activity, anti-proliferation and anti-inflammatory activity and are also shown to be antidiabetic (Oroian and Escriche 2015).

Polyphenols consist in a wide and diverse group of substances: more than 8000 polyphenolic compounds are now known (Oroian and Escriche 2015). Due to this huge amount of possibilities, a division in different classes is necessary to better understand their function which is directly connected to the configuration and structure they assume (Brglez Mojzer *et al.* 2016). They are normally divided into flavonoids or no-flavonoids, but due to the complexity and diversity a further division into subclasses is always given. The possibility of the presence of sugar within the molecular structure should be noted, with the occurrence of polyphenols in conjugated forms, the so-called glycosides with those who don't present this specific called aglycones instead. (Santhakumar *et al.*

2018). The different potential classifications are all customarily based on structural features. Typically, the classification is based on the number of phenolic rings present within the chemical structure. Cutrim and Cortez (2018) report the most common classification of polyphenols in 4 classes: phenolic acids, flavonoids, stilbenes and lignans. In addition, Brglez Mojzer and coworkers (2016) proposed a fifth class, the tannins.
In Figure 2 it is possible to see a scheme presenting classes and relative subclasses of these molecules.

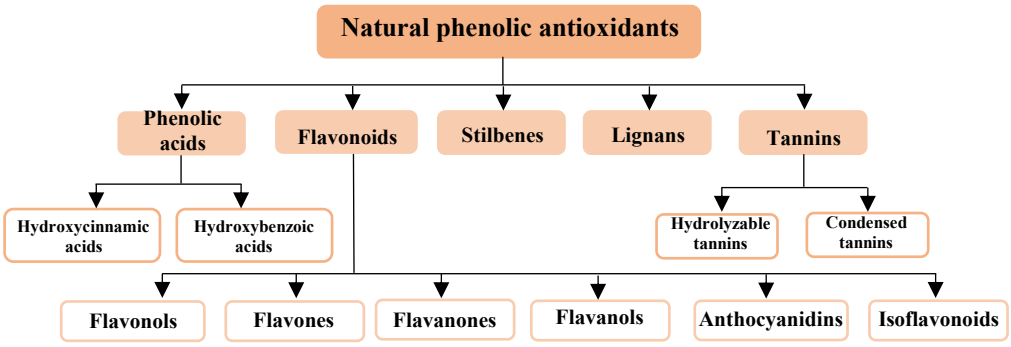


Figure 2 *- Classification of phenolic antioxidants*

1.1.1 Phenolic acids

These non-flavonoid polyphenolic compounds have the most elementary structure among the others; they possess the basic structure from which more complex molecules derive theirs (Cutrim and Cortez 2018). Depending on the source, they can be divided into two types: hydroxycinnamic acids and hydroxybenzoic acids. (Abbas *et al.* 2017). General structures are shown in Figures 3 and 4, while in Tables 2 and 3 the typical substituents are shown.

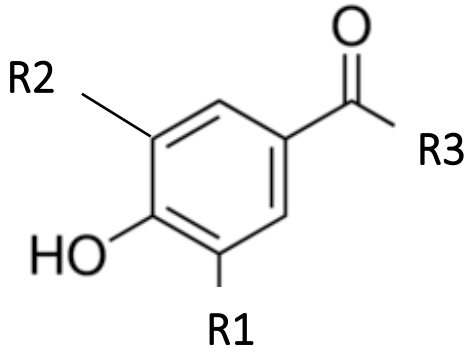


Figure 3 *- General structure of hydroxybenzoic acids*

Table 2 *– Hydroxybenzoic acid substituents*

HYDROXYBENZOIC ACID	**R_1**	**R_2**	**R_3**
***p*-Hydroxibenzoic acid**	H	H	OH
Protocatechuic acid	OH	H	OH
Gallic acid	OH	OH	OH
Vanillic acid	OCH_3	H	OH
Syringic acid	OCH_3	OCH_3	OH
Methyl *p*-hydroxybenzoate	H	H	OCH_3
Methyl gallate	OH	OH	OCH_3
Ethyl protocatechuate	OH	H	OCH_2CH_3

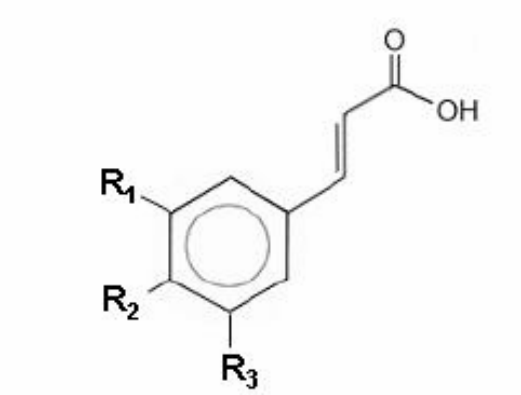


Figure 4 *- General structure of hydroxycinnamic acids*

Table 3 *- Hydroxycinnamic acid substituents*

HYDROXYCINNAMIC ACID	R_1	R_2	R_3
***p*-Coumaric acid**	H	OH	H
Caffeic acid	H	OH	OH
Ferulic acid	H	OH	OCH_3
Sinapic acid	OCH_3	OH	OCH_3

It is evident that depending on the substituent we can have different acids with different peculiar characteristics and behaviors; in addition to this difference, it is important to emphasize the possibility of finding them in free or bound form. From Table 2 it is possible to see the most common hydroxybenzoic acids, that are actually more likely to be found in the glycosylated form in foods. In Table 3 the most important hydroxycinnamic acids are listed; in general, phenolic acids are more likely to be found in food in bound form, in form of esters or in combination with more compounds.

Due to their peculiar chemical structure, as with all polyphenols, they behave as antioxidants: even if their structure is one of the simplest, the hydroxyl group present on the aromatic ring (phenol) permits the antioxidant activity to be performed, releasing a hydrogen to block the radical activity, that if not neutralized would perpetuate the oxidative reaction. Because of their effective action, they play an important role in daily diet, being also consumed as medical preparations (Shahidi and Ambigaipalan 2015).

1.1.2 Flavonoids

Among the other classes, flavonoids represent a big part of polyphenols, constituting around 60% of these bioactive compounds (Brglez Mojzer *et al.* 2016). As with other types of polyphenols they show specific positive characteristics: first of all they present antioxidative properties, and moreover, they have been studied for their anti-carcinogenic, anti-inflammatory and anti-mutagenic properties (Panche *et al.* 2016). They can be divided, as shown in Figure 2 in different subclasses such as flavonols, flavononols, flavones, flavanols, anthocyanidins and isoflavonoids, depending on the substituents present in the common structure (Oroian and Escriche 2015). From a more general point of view, flavonoids, which are low molecular weight compounds, are all characterized by the same structure: two benzene rings joined by a 3-carbon linear bridge bound as part of a further oxygenated heterocyclic ring (Figure 5) (Cassano *et al.* 2017). Moreover, differentiation depends on the site of the attachment of B ring and the characteristic of group C, as shown in Figure 5.

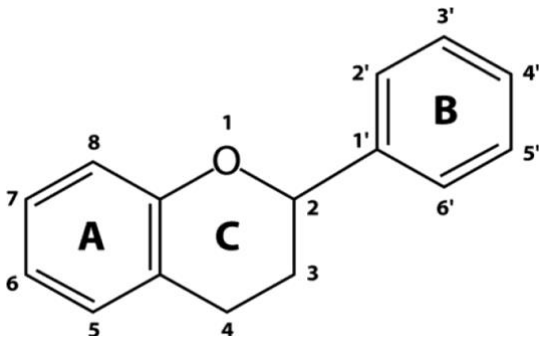


Figure 5 *- General structure of flavonoids*

According to the nature of the substituents, flavonoids can be further subdivided in the classes mentioned before and here presented in dedicated paragraphs.

1.1.2.1 Flavonols

Differences among the subclasses depend on structural features and position of specific groups that characterize each subclass. In this case, flavonols are characterized by the presence of a hydroxyl group on C ring in position 3 and a ketonic group in position 4 (Figure 6). Figure 6 shows the basic structure of flavonols, with its characteristic OH group and a series of numbers indicating possible positions for specific substituents.

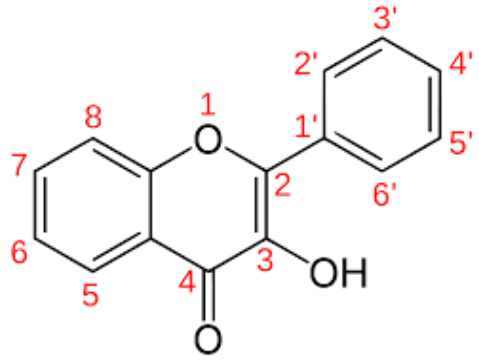


Figure 6 *- General structure of flavonols*

These compounds can be found in several fruits and vegetables, such as fennels or onions, or in beverages such as wine (Oroian and Escriche 2015). Among all these compounds, it is important to consider the quercetin, the most present in diary diet, that can be found in big quantities in onions (Shahidi and Ambigaipalan 2015).

1.1.2.2 Flavones

If this class is compared with the previous one, the hydroxyl group on C3 of the C ring is lacking, as shown in Figure 7:

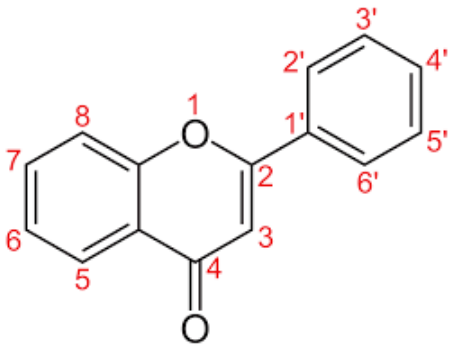


Figure 7 *- Generic structure of flavones*

Flavones can be found in many different natural matrices, such as celery, parsley, mint (Panche *et al.* 2016), onions and citrus peel (Oroian and Escriche 2015). Their properties differ minimally in respect to the general behavior assumed by flavonoids. It has been shown that they possess significant anti-microbial and anti-ulcer activity (Oroian and Escriche 2015).

1.1.2.3 Flavanones

This class of compounds displays the same structure as flavones (Figure 7). They can be found in plants in form of glycosides. Citrus fruits are typical sources (Oroian and Escriche 2015).

1.1.2.4 Flavanols

This class' structure differs from the basic one for the presence of a hydroxyl group on the C ring, as shown in Figure 8:

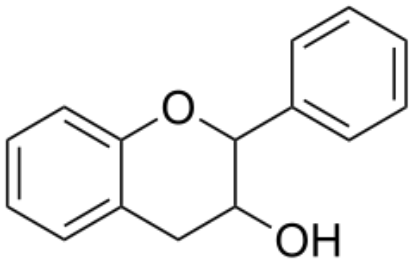


Figure 8 *- Structure of flavanols*

Due to the emblematic positioning of the OH group on carbon 3 they are normally referred as flavan-3-ols. In this case the vulnerability of this class of compounds, in specific conditions of temperature, pH or in presence of oxidative species should be observed (Oroian and Escriche 2015).

1.1.2.5 Anthocyanidins

Anthocyanidins are one of the most significant pigments in nature. They are known as aglycones. When a sugar is present within their structure they are called anthocyanins, that are in form of glycosides (Khoo *et al.* 2017). They differ from each other due to the nature of their possible substituents. Figure 9 shows their general structure.

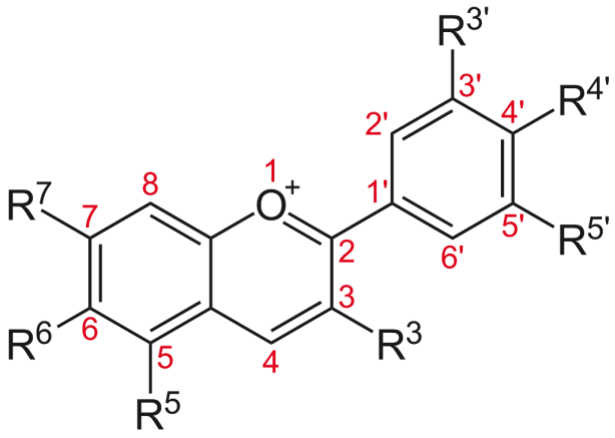


Figure 9 *- General structure of anthocyanidins*

Presently, the use of additives is a theme of high concern, but since these compounds are natural and show low to no toxicity, they could be easily used to substitute synthetic ones. Moreover, as they belong to the polyphenols' family, they also show antioxidant properties, which gives them a higher nutritive value (Khoo *et al.* 2017).

1.1.2.6 Isoflavones

This last subclass of flavonoids belongs to the family of phytoestrogens, see structure shown in Figure 10:

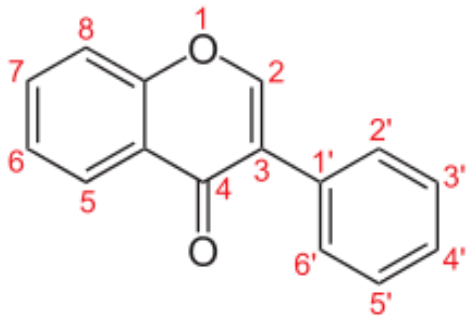


Figure 10 *- General structure of isoflavones*

In this case, it is clear to see that the difference within the structure lies on the site of attachment of the B ring.

On a related note, soy beans or legumes are an important source of this molecule (Oroian and Escriche 2015). Routinely, isoflavones can be found within plants in the form of glycosides, showing all the common properties of flavonoids. In addition to their antioxidative and anti-microbial properties, they are capable of pseudo hormonal activity, so they bind to estrogen receptors. Hence they can be classified within the family of phytoestrogens (Yu *et al.* 2016).

1.1.3 Stilbenes

Stilbenes are another class of polyphenols that can act as antioxidants at lower concentrations. Due to their particular structure they can exist in two isomeric forms, the cis- and trans- isomers. They are constituted by two aromatic rings linked by an unsaturated carbon chain. Figure 11 shows a generic structure of the two possible forms in which stilbenes may exist:

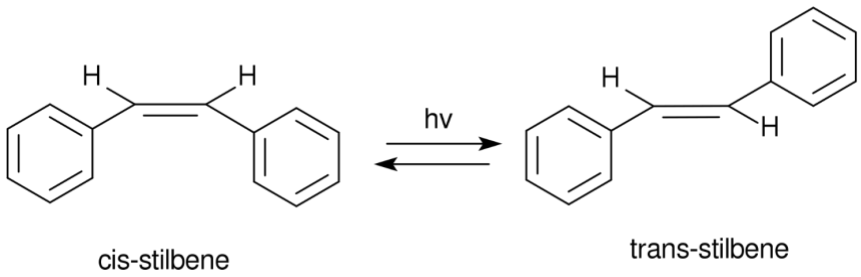


Figure 11 *- Two isomeric forms of stilbenes*

Moreover, they can also exist in monomeric or oligomeric form (Oroian and Escriche 2015).

1.1.4 Lignans

Lignans are polyphenolic substances, whose structure is derived directly from another phenolic compound, the hydroxycinnamic acids and specifically from their single units. Figure 12 presents the typical C6-C3 unit:

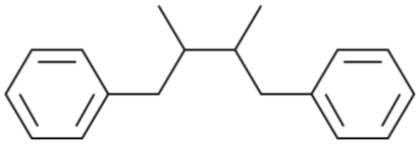


Figure 12 *- General structure of lignans*

They can be found in various foods, including roots, leaves and seeds of different plants. Their role in inhibiting (and delaying) the growth of experimental mammary cancer should be pointed out (Oroian and Escriche 2015).

1.1.5 Tannins

Tannins are the last class of polyphenols here presented. They are characterized by a wide range of molecular weight, characteristic which allows them to be divided into two classes according to their mass: the hydrolysable tannins (between 500 to 5000 Da) and the condensed tannins (up to 30000 Da). Due to the complexity of these molecules, only a monomeric unit of generic condensed tannin is presented in Figure 13.

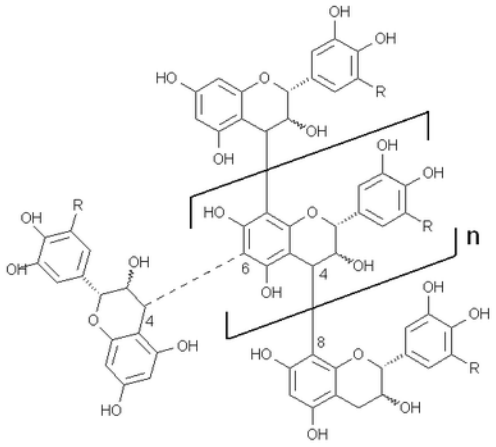


Figure 13 *- General structure of condensed tannins*

Among all other properties, tannins are also told to have anti-thrombotic and antiviral properties; sources are strawberries, pomegranates and green coffee beans. Particularly wine wastes are a good source of these molecules (Oroian and Escriche 2015).

1.2 Mechanism of action of polyphenolic antioxidants

As previously stated, the oxidation mechanism is commonly constituted by three phases, initiation, propagation and termination. Antioxidants can either inhibit or retard this phenomenon, depending on their own nature and (since they are proven to be more effective at lower concentrations) the concentration at which they are present. (Bazinet and Doyen 2017). According to this same review by Bazinet and Doyen (2017), antioxidants can be divided into primary and secondary antioxidants depending on how they act on the oxidation reaction.

Polyphenols can be considered as primary antioxidants because of their peculiar mechanism of action towards oxidative reactions. Due to their specific structure, they present at least a phenolic group and are able to inhibit ROS by exchanging a proton, subsequently stopping propagation reactions. Since different polyphenols have a divergent number of hydroxyl groups and arrangements, this results in a variable antioxidative potential. The main reaction that may take place is the following, whereby the polyphenolic agent is indicated as AH (1):

$$AH + R \cdot\therefore RO \cdot\therefore ROO \cdot \rightarrow A \cdot + RH \therefore ROH \therefore ROOH \qquad (1)$$

In addition to this basic reaction, further combinations can occur, as shown in (2) and (3):

$$A \cdot + RO \cdot\therefore ROO \cdot \rightarrow ROA \therefore ROOA \qquad (2)$$

$$ROO \cdot + RH \rightarrow ROOH + R \cdot \qquad (3)$$

The radical species that results from these reactions, which is denoted as A• possesses a structural stability, despite its radical nature, and would not propagate an oxidative reaction. The structural stability of the radical relies on the presence of different resonance structures. The compounds with a higher number of "limit structures" generally result in higher stability. The resonance stabilization of a phenolic radical (Shahidi and Ambigaipalan 2015) is presented in Figure 14.

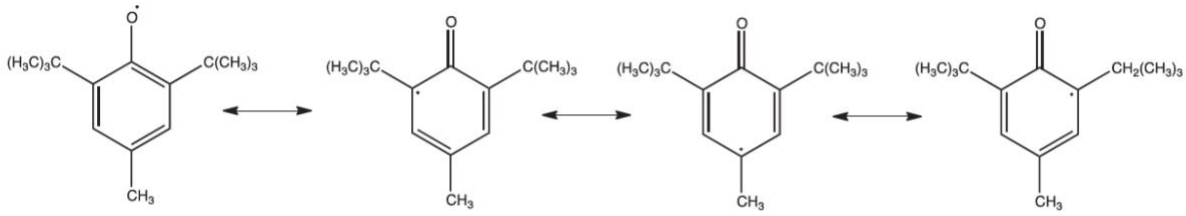


Figure 14 *- Resonance stabilization of a phenolic radical*

1.3 Positive effects of polyphenols on human health

Many studies have proven polyphenols to have positive effects on health (Santhakumar *et al.* 2018; Cory *et al.* 2018; Khoo *et al.* 2017;Niedzwiecki *et al.* 2016). Hence the pervasive attempts to enhance their recovery from food wastes and to utilize them in food and other products. Their effect strictly depends upon the way they are metabolized in our organism, and their bioavailability should be assessed in order to find an optimal way of administration.

Their ability to prevent diseases has been linked most significantly to their antioxidative properties and their mechanism of action is the subject of numerous studies. These investigations endeavor to reveal how they can actually permit diseases to be prevented or propagated, so impacting also on the recovery process (Brglez Mojzer *et al.* 2016). The main guideline is based on the idea of a potential synergistic effect. For example, their anti-cancer efficacy increases when they can be combined, allowing use of lower concentration of each one, so reducing risk of toxicity (Niedzwiecki *et al.* 2016). Since the mechanism of development of a cancer is highly complex, polyphenols can actually act at different stages: indirectly they can support the immunitary system and directly they can mitigate the uncontrolled growth and proliferation of cancer cells (Niedzwiecki *et al.* 2016).

Besides anticarcinogenic properties, polyphenols play an important role in the prevention or treatment of other diseases, such as neurodegenerative diseases, obesity and a potential interaction with the gut microbiota, in addition to type 2 diabetes and cardiovascular diseases (Cory *et al.* 2018).

Santhakumar and coworkers (2018) explain widely in their review the different mechanisms by which polyphenols can act on atherosclerosis.

Due to the copious effects polyphenols can have on health, industrial applications are increasing as a response to the ever-growing health related concerns of consumers.

1.4 Current applications of polyphenols in industry

Extracting polyphenols from food waste would not be the only method by which to handle these wastes, but also provides an opportunity to extract natural bioactive compounds, such as polyphenols, to use them in food, cosmetic or pharmaceutical industries. These natural antioxidants are of growing interest, due to the possible substitution of potentially toxic and synthetic ones.

1.4.1 Food industry applications

Food additives became necessary in modern society, to guarantee a longer shelf-life of different products, to permit good preservation, avoid lipid oxidation and preserve their color and aspect. Due to the wide use in modern industries of these additives, consumers are becoming progressively concerned about their actual safety. In fact, many of these compounds and specifically the synthetic ones, have been proven to have toxic, carcinogenic or mutagenic effects (Carocho *et al.* 2014). For this reason, health-concerned customers are looking for functional foods containing intentionally-added natural antioxidants. In fact, antioxidants naturally present in foods are not sufficient in effectively reducing the oxidative stress, hence the necessity to fortify food with the addition of these compounds (Aguiar *et al.* 2016).

However, introducing natural antioxidants in food is not easy, this is due to the possible degradation of these compounds by external agents; for this reason, in order to be effective, they should be added in large quantities, resulting in higher costs and changes in organoleptic properties. The aim is therefore to decrease the need for high doses of these compounds (Aguiar *et al.* 2016). Many studies present different solutions to avoid a wide use of polyphenols, in order to optimize their utilization and make it effective at lower concentrations as well. The attempt is to create coatings that would allow either the release of the antioxidants or the scavenging of prooxidants from the product (Roman *et al.* 2016). Aguiar and coworkers (2016) report in their review the possibility of microencapsulating antioxidants extracted from coffee, which diminishes degradation and solves potential concerns related to unpleasant taste or a scarce bioavailability of the target compound. Microencapsulation permits the molecules of interest to be surrounded with specific coatings, so allowing the release time to be controlled, which would result in using a lower quantity of compound (Aguiar *et al.* 2016).

Among other widely studied techniques, nanotechnologies are being applied in many different sectors. Extracted antioxidants could be used within these nanostructures for many applications, such as active packaging, that allows a target antioxidant to be released in a controlled way (Pathakoti *et al.* 2017). In this study, Pathakoti and coworkers (2017) describe all possibilities of application; one of the most interesting is the application of microemulsions combined with the release of hydrophilic and lipophilic antioxidants.

All the above-cited techniques could be used with natural antioxidants recovered from wastes, when proper and cost-effective methods of extraction and separation of antioxidants are implemented.

1.4.2 Cosmetic industry applications

Oxidative stress has also been found to be a cause of skin aging and, in the worst cases, skin cancer (Zillich *et al.* 2015). This is not the only cause of the problem, UV irradiation also contributes to the development of these diseases, and the synergic effect of the two increases their incidence. Antioxidants, specifically polyphenols, have been indicated as active substances with the capability

to prevent or mitigate the effects of such problems. Their action is multiple: (i) support the endogenous antioxidant system of the skin, which is weakened by the UV irradiations, and also (ii) inhibit the proteinase, an enzyme responsible for the degradation of collagen and elastin. Furthermore, polyphenols show their own activity as photoprotectors, which would also indirectly help the endogenous antioxidant system of human skin. Observably, to guarantee the effectiveness of the action of these molecules on the skin a proper formulation of the composition of the product should be outlined, to guarantee the component's stability (Zillich *et al.* 2015).

2. Olive oil and wine production wastes

To allow for the wide use of polyphenols in industry, the implementation of new techniques or optimization of traditional ones it is necessary to extract these bioactive compounds in such concentrations that they could be used in the applications cited above. Extraction from industrial wastes permits a solution for the management of the wastes to be found and their rich composition in polyphenols to be exploited. The following text will present the matrices used in this work, their potentiality and the possible techniques that can be used to extract polyphenols in an efficient and green way.

2.1 Olive oil production

Olive oil production process includes different steps, each one producing waste that may or may not be used within the process itself. Apart from the first phases of olive recollection, cleaning and pressing, the most interesting part, in terms of possible waste recovery, is extraction. Extraction can be performed in three different ways, and depending on the choice, waste treatment could become more challenging, due to the number of phases. Except for the mechanical one, which typically only used by small producers, the other two ways include a centrifugation stage, which can be in two or three phases, respectively. The latter essentially differs from the former for a further addition of water, which is not performed in the two phases of the centrifugation system. For this reason, this last one is replacing the other because of its higher sustainability, due to the reduction of waste water (Nunes *et al.* 2016).

Oil production wastes, due to their high organic content and phytotoxicity, are potentially problematic for the environment as they require a specific plant for their treatment. There are some conventional disposal methods, such as burning or spreading in fields. However, they result in being both costly and dangerous for the environment (Ruiz *et al.* 2017). The implementation of a cost-effective and sustainable recovery process is gaining an increasing amount of interest in this field; it would permit the problem of waste treatment and its exploitation to be solved, making it possible to recover bioactive compounds that could be reused in different applications (Nunes *et al.* 2016).

In the last decade the volumes of oil production have risen by more than 40% worldwide (Mateo and Maicas 2015). The discovery of the beneficial effects of the Mediterranean diet has led to a wide demand for this product, with a production of 3 million tons in 2016 (IOC, International Olive Council 2018). Leading producers are Mediterranean countries, such as Italy, Spain, Portugal and Greece, which produce around 98% of world's olive oil (Dermeche *et al.* 2013).

2.1.1 Olive production derived-residues

Olive oil industry generates a high amount of waste, both in the liquid and solid phase, and their impact on the environment is highly negative and needs to be handled properly. Due to the high toxic organic content and low pH, olive mill waste water is a particularly serious threat for the microenvironment (Dermeche *et al.* 2013). Its negative action hits soil, water and air, thus generating an array of problems. Its high phenolic content leads to soil pollution and in consequence these compounds can inhibit plant growth; since they have antimicrobial activity, it can also affect the development of soil microbial populations. This waste water cannot be discharged into fresh water, because it would affect the marine ecosystem development, so reducing the fresh water's oxygen availability. A less direct effect, which is also very important, is the air pollution generated by stored waste waters, with emissions of sulfur dioxide and phenol to air (Dermeche *et al.* 2013).

Therefore, negative effects of waste deriving from olive oil production is widely recognized and finding proper solutions to manage them or exploit their potential is of high interest.

2.1.1.1 Olive pomace (OP)

This heterogeneous by-product of olive oil production is a liquid-solid substance that contains high moisture and oil. The content in water depends on the selected extraction system. The content in oil, 2% of its total weight, can be still exploited to produce a further quantity of oil by solvent extraction – usually using hexane – which will have to be further processed to attain edible oil (Ruiz *et al*. 2017).

Olive pomace contains parts of olive skin, stone and pulp. Apart from the possibility of using it to produce edible olive oil, it can be used in combustion, which does not really recover its valuable contents, but allows to manage its disposal. This by-product is known to be rich, apart from in sugars and some proteins, in polyphenols such as hydroxytyrosol, oleuropein, caffeic, vanillic and p-coumaric acid and tyrosol (Ruiz *et al.* 2017; Nunes *et al.* 2016).

In general, it is estimated that more than 98% of phenolic compounds remain in olive oil by-products (Araújo *et al.* 2015). In this case, the content of antioxidants is higher in the pomace than in the produced olive oil itself, therefore its exploitation through recovery of high added value compounds, rather than using it as an energy source, is receiving attention from numerous industries, such as food and pharmaceuticals (Nunes *et al.* 2016).

2.1.1.2 Olive mill waste water (OMWW)

Depending on the implemented process of extraction, olive mill waste water can be generated in different quantities and with different characteristics. In some cases, olives can be washed before the extraction, so generating another fraction of waste water usually presents a less organic load (Ruiz *et al.* 2017). Anyhow, olive mill waste water usually refers to water generated within the discontinuous pressing extraction process, or the three phase-centrifugation system, even if it generates in different quantities of waste. It presents a pH between 3 and 5,9 and it is characterized by a red-to-black color (Cassano *et al.* 2016).

The high content in polyphenols, which is dangerous for the environment but beneficial for health, attracted the attention on the possibility to recover these compounds (Nunes *et al.* 2016).

2.1.2 Chemical composition of olive oil production wastes

In the previous section the content of the main olive oil by-products has been briefly described, however, as stated by Dermeche and coworkers (2013), it is important to understand that a general composition of these residues cannot be outlined in detail because it can vary according to various aspects: the climate conditions and cultivation practices of olives, as much as following extraction process, can interfere with the variety of valuable compounds contained in the generated wastes. Table 4 shows the identified polyphenols present within the different matrices.

Table 4 *- Polyphenolic content in olive oil wastes*

Sample	Determination	Polyphenols	Reference
OMWW	HPLC	3,4-DHPEA-EDA, H-tyrosol.	(La Scalia *et al.* 2017)
Olive leaves	HPLC-DAD	Oleuropein	(Aissa *et al.* 2017)
OMWW		Tyrosol, Hydroxytyrosol	
OMWW	HPLC-DAD	Hydroxytyrosol	(Ioannou-Ttofa *et al.* 2017)
Olive pomace Olive leaves Pomace olive oil	HPLC-UV-VIS	Tyrosol, Luteolin-7-rutinoside, Rutin, Dihydro-quercetin, 10-hydroxy-oleuropein, Luteolin-7-glucoside, Verbascoside, Apigenin-7-glucoside, Chrysoeriol-7-O-glucoside, Oleuropein glucoside, Oleuropein, Oleoside, Apigenin	(Abdel-Razek *et al.* 2017)

Olive cake	HPLC-DAD	Hydroxytyrosol, Luteolin (Caffeic Acid, trans-Ferulic Acid, Apigenin, Tyrosol, Rutin Hydrate)	(Fernández *et al.* 2018)
Olive pomace Olive leaves	HPLC-DAD	Hydroxytyrosol-4-glucoside, Hydroxytyrosol, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) (verbascoside, tyrosol, salidroside)	(Ruiz *et al.* 2017)

The most widespread one is hydroxytyrosol, which corresponds to 70% of the olive mill waste water's content and to high concentrations in olive pomace (Araújo *et al.* 2015; El-Abbassi *et al.* 2012).

2.1.3 Applications

The advantages of exploiting these wastes have been widely discussed, and their recovery has been accounted as the optimal solution for their disposal. The application possibilities of the most present polyphenols in these specific matrices will be briefly outlined to fully understand their potential. Certainly, hydroxytyrosol is found in high quantities in both olive pomace and olive mill waste waters (Araújo *et al.* 2015).
Hydroxytyrosol (HT), 2-(3,4-dyhydroxyphenyl) ethanol, is a phenolic alcohol with a molecular weight of 154,16, amphipathic, whose structure is shown in Figure 15.

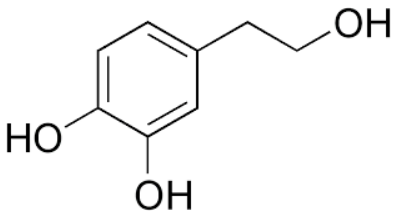


Figure 15 *– Structure of Hydroxytyrosol*

Due to its particular structure it possesses both water and fat-solubility, it can penetrate cell membranes, so enabling it to perform positive effects on the organism. It has been described as able to prevent cardiovascular diseases, it protects cells from oxidative damage and inhibits platelet aggregation, which would lead to the formation of a thrombus (Vilaplana-Pérez *et al.* 2014). In addition to these positive effects, HT has been used as a functional ingredient in meat in different concentrations, to improve its quality and preservation, due to the HT's antimicrobial and antioxidative activity (Martínez *et al.* 2018). Its antimicrobial activity has also been investigated to substitute sulfur dioxide, which is widely used in wine industry (Ruiz *et al.* 2017).

Polyphenols extracted from olive by-products can also be used in different cosmetic application, since they are able to cross membranes due to their amphipathic behavior. Hydroxytyrosol and oleuropein have positive effects on skin, making it softer or acting on its aging process (Rodrigues *et al. 2015)*. In particular, as Galanakis and coworkers (2018) report in their work, compounds derived from olive oil by-products can effectively be used as a UV booster in cosmetic products.

2.2 Wine production

Indisputably, wine production is one of the key activities in the agricultural field. Data related to the worldwide extension of vineyards show that 7.5 million hectares are dedicated to wine production, with 5 countries holding 50% of these territories. In descending order, these countries are Spain with 13% of world's vineyards, China with 11%, France with 10%, Italy with 9% and Turkey with 7%, respectively. In 2017, grape production reached values of 73 million tons on the world stage, with China as the leading country. The same order is not reflected in wine production, with an estimated 279 million hectoliters produced worldwide, with Italy as the leading country (OIV 2018). This can

be explained by the fact that approximately 75-80% of grapes are destined for wine production (Zhu *et al.* 2015).

The wine making process starts with grape harvest, which is performed during specific periods of the year and greatly depends on the type of grape. Also, the presence of the stem influences the wine's properties, it can therefore be maintained or removed. In the same way, pomace can be present or not depending on the type of wine; in red wine it is fermented with the juice, while in white wine fermentation pomace is removed. Therefore, grapes are pressed, and while in the first case pomace (mostly skin) participates at the fermentation providing anthocyanins that will confer the color to the wine, in the second case skin and other residues are removed before fermentation (Beres *et al.* 2017). Fermentation is then followed by sedimentation, maturation, clarification and stabilization (Barba *et al.* 2016).

2.2.1 Wine production derived-residues

As for the olive oil by-products, also the disposal of wine industry wastes is one of the most challenging issue to deal with, as solid waste and waste water is higher during the harvest period (Oliveira and Duarte 2014). Beres and coworkers (2017) report in their review four critical points related to the effects that wine production has on the environment. Among these the most impactful is transport, which comprises 41% of the entire environmental impact. Anyhow, even if the percentage related to wastes was lower, enhancing its recovery is of high importance due to the high content of high added value compounds.

2.2.1.1 Grape pomace

Grape pomace is the solid part derived from the pressing of grapes and is formed by the skin, pulp and seeds. It can be used in different ways: as fertilizer, animal feed or, in this case, as a source of polyphenolic compounds (Beres *et al.* 2017). As stated by Teixeira and coworkers (2014) , the annual production of grape pomace was equal to 9 million tons. Here, a difference can be outlined between white and red grape pomace, since only in the latter case the solid parts participate in the fermentation process. Red grape pomace has a lower carbohydrate composition, while white grape pomace maintains its original levels of sugar. This does not significatively affect the content of valuable compounds, therefore they are both good candidates for polyphenol recovery (Beres *et al.* 2017).

The components of grape pomace are present in different percentages (skin for example constitutes the 65% of the total), where the moisture percentage lies between 50 and 70%. Its varied composition makes it suitable also for different production processes, such as the production of citric acid, methanol and ethanol (Teixeira *et al.* 2014).

2.2.1.2 Wine lees (WL)

Wine lees are biphasic wastes derived from wine production and they are generated in the fermentation phase. They comprise of a liquid and a solid part; the solid part is formed by the dead yeast that performs the alcoholic fermentation, but also by bacteria, insoluble carbohydrates and polyphenols. The liquid part, known as vinasses, instead is the fermentation broth in which the yeast grew during the fermentation, therefore it contains ethanol and organic acids. Due to the high phenolic and organic content, their disposal is challenging, both from an economic and environmental point of view. They can also be used as a source of ethanol and polyphenols (Pérez-Bibbins *et al.* 2015).

2.2.1.3 Wine waste water

One of the foremost issues surrounding the management of wine wastewater is its high quantity. The quantity of winery wastewater produced depends on the production (Greece 650,000 m_3, Spain

18,000,000 m3); waste results in approximately 5 tons per hectare of land per year (Zacharof 2017). In fact, it is not produced in a single phase, but it comes from different production stages, so results in different compositions that lead to different impacts on the environment. Waste water is produced during the cleaning phases throughout the whole process, before and after fermentation, maturation, clarification and bottling (Zacharof 2017). Apart from the high organic load which confers them high acidity and phytotoxicity, solvents or detergents can also be added for the purpose of cleaning, consequently increasing the content of substances with an elevated impact on the environment. The release of these waters to soil would lead to irreversible changes within its physical and chemical properties and subsequent damage to its ecosystem. The same effect would be registered within the aquatic ecosystem, due to a reduction in oxygen content (Zacharof 2017).

2.2.2 Chemical composition of wine production wastes

All the previously described wine production wastes, as well as containing different organic compounds such as sugars and organic acid, are rich in polyphenols. Depending on the considered waste and on aspects such as grape type, conditions of grape harvesting and wine production, polyphenolic content and composition will be different. As already discussed, polyphenolic compounds can be divided into five different classes: anthocyanins, hydroxycinnamic and hydroxybenzoic acids, flavan-3-ols, flavonols and stilbenes. Anthocyanins can be found in grape skin; they are pigments that confer the characteristic color to the wine. Also present in the skin, but with a higher concentration in the seed are the flavan-3-ols. Table 5 illustrates the main compounds present and indicates the specific wine waste in which they have been found.

Table 5 *- Polyphenolic content in wine wastes*

Sample	Determination	Polyphenols	Reference
Grape skin	HPLC-DAD	malvidin-3-glucoside, Quercetin Rutin, Catechin, Epicatechin	(Caldas *et al.* 2018)
Grape pomace	HPLC-DAD-ESI-MS/MS	malvidin-3-O-glucoside, malvidin-3-O-(6'×-p-coumaroyl) glucoside, (caffeoylated and acetylated glucoside of malvidin) (delphinidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, petunidin-3-O-(6'-acetyl)glucoside, delphinidin-3-O-(6'-p-coumaroyl) glucoside, peonidin-3-O-(6'-acetyl) glucoside, peonidin-3-O-(6'-caffeoyl) glucoside,petunidin-3-O-(6'-p-coumaroyl) glucoside and peonidin-3-O-(6'-p-coumaroyl) glucoside) Quercetin, Myricetin	(Drosou *et al.* 2015)
Grape pomace	HPLC-MWD	Catechin, Syringic acid, Epicatechin, Quercetin, Malvidin 3-O- glucoside	(Antoniolli *et al.* 2015)
Wine lees	HPLC-DAD	Quercetin, Ellagic acid, p-Coumaric acid, Gallic acid, Caffeic acid, Chlorogenic acid, Kaempferol	(Jurcevic *et al.* 2017)
Wine lees	HPLC-DAD	Gallic acid, Catechin, Epicatechin, Procyanidins B1 and B2, Myricetin and Quercetin, trans-Caftaric, trans-Coutaric, Caffeic, p-Coumaric and Ferulic acid	(Kopsahelis *et al.* 2018)
Wine vinasses	HPLC-DAD	Gallic acid, Catechin, Epicatechin, Quercetin	(Díaz-Reinoso *et al.* 2017)

2.2.3 Applications

The high variety of polyphenols, that can be obtained through the application of different extraction techniques on wine wastes, allows their utilization in different fields of applications, ranging from the food industry to the pharmaceutical and cosmetic ones.

For cosmetic purposes, Wittenauer and coworkers (2015) propose the possibility of using polyphenols as inhibitors of the enzymatic actions performed by enzymes such as collagenase and

elastase which are normally involved in the skin aging process. This is one of the many examples in which polyphenols are used in cosmetic industries: creams or serum containing grape-pomace-derived polyphenols have already been commercialized, and the resveratrol that has been obtained from wine waste extraction, despite many problems related to its instability and low bioavailability, has already been used as food supplement (Beres *et al.* 2017).

3. Polyphenols recovery

Due to the variability in the polyphenolic content within the different matrices, the technology and the conditions that are employed to extract these compounds from them is another important point to be considered. The need to develop green, safe and affordable solutions is evident; however, it is important that high yields of the target product are obtained without reducing its properties and quality. Many factors can influence the yield of extraction, including temperature, time of extraction, number of cycles and, most importantly, the selected solvent (Oroian and Escriche 2015). The choice of the solvent has to be based on different aspects, which include the properties required for the implementation of the selected extraction process and the use of the extracted polyphenols. In food industry, for example, there are many restrictions regarding the quantities of specific substances allowed within a product, therefore the selection of the most appropriate solvent is fundamental to avoid possible toxic effects (Tiwari 2015). Furthermore, as observed by Drosou and coworkers (2015), the preservation of natural matrices which, in this specific case referred to grape pomace, is essential in order to avoid undesired chemical reactions, and to inhibit the growth of microorganisms. They compared different extraction techniques, using matrices treated with two different drying treatments to see how they affect the following extraction steps.

The general approach, when dealing with a specific matrix, is to find the best technique by comparing different methods of extraction. Many traditional extraction processes have been implemented in the past years, however, due to the large volumes of solvent used in these approaches, the attention has been moved to newer, greener technologies that would reduce the use of solvents and shorten the extraction time (Brglez Mojzer *et al.* 2016). Solvent is an important issue when dealing with these methods: it should be selective, cheap, non-toxic, with low viscosity to allow for ease of transport and chemical stability. Obviously, obtaining all these features within a unique solvent is not always possible, since each extraction method implemented requires specific characteristics to obtain the final product. For example, in food industry, even if methanol would be cheaper, it has often been replaced with the more expensive ethanol (EtOH), which is safer for customers (Oroian and Escriche 2015). Here a short review of some of the possible extraction techniques to be implemented is reported, starting from the most traditional ones and up to the newest ones.

3.1 Conventional extraction methods

Conventional methods involve the use of high quantities of solvents and, despite their efficiency, have to be replaced by new technologies that would permit a greener approach. A lower amount of solvent would also permit the removal of those stages that are needed to eliminate solvent traces in the product. However, conventional techniques are still widely used and worthy of a brief description.

Extraction can be performed in both solid and liquid phase, which can be referred as solid-liquid and liquid-liquid extraction, respectively. The common principle rests with the transfer of compounds from the solid or liquid source to the solvent. Selectivity will depend on the compounds' solubility in the chosen solvent (Brglez Mojzer *et al.* 2016).

On an industrial scale, liquid-liquid extraction is one of the earliest implemented techniques and still plays an important role within the extraction processes. It is applied to aqueous sample, exploiting the immiscibility between this phase and a specific organic solvent. Those compounds that have higher solubility in the organic solvent are extracted by passing from one phase to another (Daso and Okonkwo 2015).

3.2 Non-conventional extraction methods

3.2.1 Microwave-assisted extraction (MAE)

Microwave-assisted extraction is one of the techniques that have been attracting attention in the past few years for the extraction of compounds of interest from natural matrices. The interest depends on the ease of adaption to different volumes, which allows for both laboratory and industrial application, and to the higher yields and lower extraction time when compared to the more traditional techniques. Moreover, it can be accounted as a green technique, since it allows the quantity of solvent used to be decreased, thus reducing waste and human exposure (Destandau *et al.* 2013).

Many factors can influence its efficiency and its overall yield; among them, the choice of the solvent, along with the solvent to feed ratio. The solvent has to be able to absorb the microwaves and to be selective, towards the target compounds that have to be soluble in it. Others important factor are temperature, pressure, number of cycles, the size of sample particles and time of extraction (Tan *et al.* 2017).

Microwave-assisted extraction bases how it functions on the formation of electromagnetic waves with a frequency ranging from 300 MHz to 30 GHz. As energy vectors, they act directly on the material and can absorb part of this energy and convert it into heat. This heat promotes the release of substances contained within the solid matrix, with their flow going from the solid to the liquid phase (Quiroz *et al.* 2019). According to the mechanism the technique is based on, it is evident that selecting a solvent able to absorb these waves is of high importance.

Temperature results homogeneous within the whole sample due to the lack of thermal inertia and due to the heating being volumetric, which differs from conventional heating where parts in contact with the heating surface are the ones whose temperature increases first (Destandau *et al.* 2013).

The mechanism of action of this technique lays on the fact that the cellular and membrane disruption is provoked through moisture evaporation, so facilitating the diffusion of those second metabolites to the solvent (Destandau *et al.* 2013).

MAE is one of the most studied techniques among the choice of greener solutions. Drosou and coworkers (2015) compare this technique with more traditional methods and ultrasound-assisted extraction, which is another green method. MAE showed higher yield compared to solvent extraction when operating for 60 minutes; this proves its higher efficiency, since extraction time is reduced. Quiroz and coworkers (2019) implement this method, focusing their attention on the assessment of the antioxidant and antimicrobial activity maintained throughout the whole operation. An interesting study tries to perform a solvent-free MAE (SFMAE) for the extraction of polyphenols from olive leaves (Tan *et al.* 2017). The implementation of this method without solvent would solve all the environmental issues connected with the use of the extracted compounds, since they would not be contaminated with traces of solvent and they would not need further treatments to remove them.

3.2.2 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction is a promising alternative for the extraction of phenolic compounds. Also, the interest lies in the possibility of reducing the amount of the employed solvent as well as to reduce the extraction time. Selectivity, of course, is an important parameter, hence the necessity to minimize the extraction of undesired compounds. Among all the advantages, the versatility and low costs of this technique are attractive, especially as far as industrial applications are concerned. Sound waves are employed, with frequencies higher than the audible ones but lower than the microwave frequencies (Tiwari 2015).

While MAE was based on the heat derived from electromagnetic energy, UAE is based on the employment of ultrasonic waves with a frequency range of 20-100 kHz. The driving force that permits its application in processes such as extraction is acoustic cavitation. Acoustic cavitation is the phenomenon occurring within a medium submitted to ultrasonic waves. During propagation, these

waves generate a series of compression and decompression that lead to the formation of bubbles in the liquid medium. These bubbles end up collapsing, this causes changes in temperature and pressure within the liquid medium (Medina-Torres *et al.* 2017). The implosion of these cavitation bubbles, and its side effects, lead to cell disruption, so allowing the solvent to better penetrate the solid matrix and improve extraction. The turbulence created meanwhile leads to increased mass transfer coefficients. The extraction can be promoted through different phenomena, a higher penetration of the solvent within the matrix, which is made possible by the disruption of cells, and a further extraction that is facilitated by a higher surface contact between the liquid and solid phase (Tiwari 2015).

Important factors to be considered are similar to MAE's. An important role is played by the pre-treatment of the matrix, but other aspects that have to be considered and carefully monitored too, they are temperature, extraction time and the choice of the solvent (Medina-Torres *et al.* 2017).

Temperature plays an important role; while it increases due to acoustic cavitation and its effects, it improves the extraction, so resulting in higher mass transfer coefficients and lower solvent viscosity. However, it is of high importance not to reach temperatures that would lead to the degradation of target compounds (Medina-Torres *et al.* 2017).

The choice of the solvent has to be made among solvents in which target compounds are highly soluble, to improve selectivity; then, when products are meant to be applied in such fields that don't allow the presence of residual toxic solvents, the choice is forced towards those solvents that would not need costly and time-consuming purification steps. GRAS (Generally-Recognized-As-Safe) status, ethanol is the most used solvent, since it is able to solve many polyphenolic compounds and can be used in food industry (Medina-Torres *et al.* 2017;Tiwari 2015).

UAE can be performed within an ultrasonic bath or by means of a probe as shown in Figures 16 and 17.

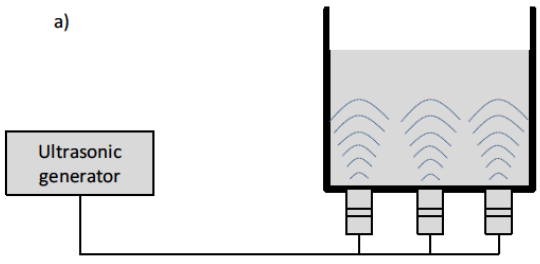


Figure 16 *- Scheme of ultrasonic bath* (Tiwari 2015)

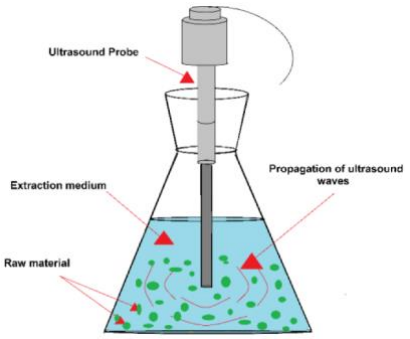


Figure 17 *- Ultraosund system coupled to a probe* (Medina-Torres *et al.* 2017)

Using the former possibility, Xu and coworkers (2017) aim to find the optimal extraction conditions to obtain antioxidants from the flower of Limonium Sinuatum. The results confirm a higher yield obtained with the implementation of UAE with respect to conventional techniques and enlist the optimal conditions of extraction.

3.2.3 Pressurized-liquid extraction (PLE)

Pressurized liquid extraction is an automated process, in which high temperatures are reached: to maintain the liquid state of the solvent, pressure is set at a higher value. Other parameters to be selected are the extraction time and number of cycles to be performed. The sample to be extracted is placed within a stainless-steel chamber; this chamber is heated by an oven for a fixed period of time to reach the set temperature and the solvent is later pumped through the chamber to perform the extraction as shown in Figure 18 (Araújo *et al.* 2015).

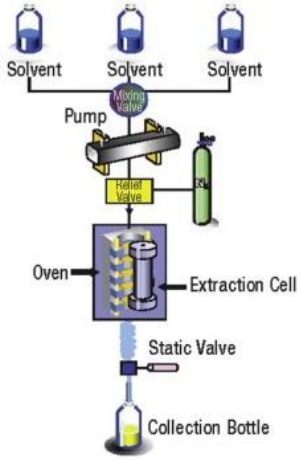


Figure 18 *- PLE scheme* (Picó 2017)

It can be performed in three different modes: (i) static, (ii) dynamic and (iii) static-dynamic (Araújo *et al.* 2015).

Machado and coworkers (2015) evaluated the behavior of the system, by considering the yield and the resulting total phenolic content as a function of various parameters, such as the type of solvent, its composition and temperature. Mixture of solvents were used, with addition of acids, meant to be effective for the enhancement of solubility. Results showed an increase in total phenolic content at higher temperatures, this was due to enhanced solubility and desorption kinetics, highlighting there was improved performance compared to conventional methods. This makes PLE a promising technique of extraction.

3.2.4 Supercritical fluid extraction (SFE)

A supercritical fluid is any substance at a pressure and temperature higher than the critical ones, as shown in the general phase diagram reported in Figure 19.

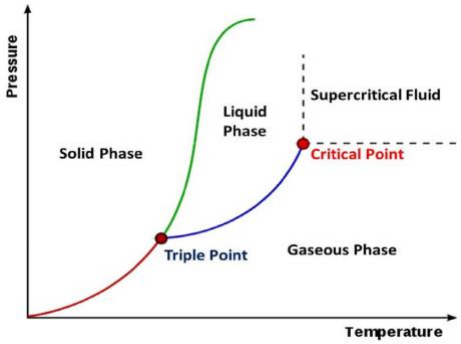


Figure 19 *- Phase diagram* (Gupta *et al.* 2014)

At these conditions, all fluids assume properties with a liquid-like solvent-power and a gas-like diffusivity. This technique has been accounted among the newest green technologies since non-toxic, highly available and less costly fluids effectively replace organic solvent, so solving all problems related to their disposal (Roselló-Soto *et al.* 2015).

CO_2 is considered the most suitable fluid for this purpose, due to its properties, it has a low critical temperature of 31.1 °C and a critical pressure of 7.38 MPa. Moreover, supercritical carbon dioxide possesses properties that lead to an enhancement in mass transfer, therefore making it the best candidate to be used in this type of process. Eventually, selectivity can be easily modulated by simply varying density with pressure (Roselló-Soto *et al.* 2015).

Figure 20 reports a general scheme; as it can be noticed, in normal applications, CO_2 is not used as the only solvent, but it is coupled with a more polar co-solvent whose aim is to enhance the solubility of the targeted compounds. This is due to the fact that CO_2 does not possess a polar character (Brglez Mojzer *et al.* 2016).

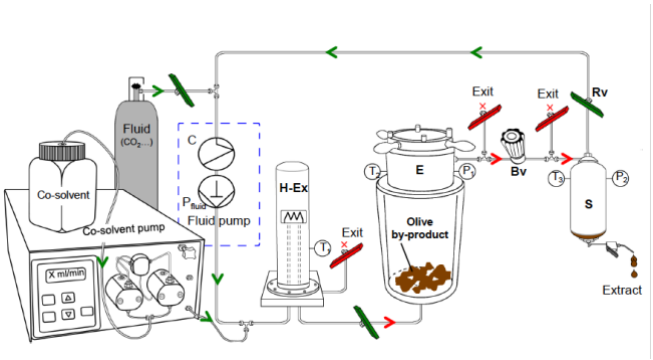


Figure 20 *- Schematic lab-scale set-up of supercritical fluid extraction* (Roselló-Soto *et al.* 2015)

Furthermore, both CO_2 and the employed co-solvent, normally ethanol, can be easily eliminated diminishing pressure and through evaporation (Barba *et al.* 2016).

This method requires minimum quantities of organic solvents, around 5 to 10 mL, but also low extraction time (from 10 to 60 minutes) (Oroian and Escriche 2015).

Many other technologies have been studied as promising alternatives to conventional methods. Barba and coworkers (2016) enlist a series of extraction techniques referring especially to wine waste recovery, among which Pulsed Electric Field treatment and high voltage electrical discharges technology can be mentioned, but whose description is omitted for sake of brevity.

3.3 Polyphenols extraction from wine and oil wastes

Table 6 shows a list of different studies conducted to extract polyphenols from oil wastes. In addition, the types of extraction technique with a specification on type of solvent, temperature and time of operation are reported.

Table 6 *– Extraction methods implemented to extract polyphenols from olive oil production wastes*

Sample	Extraction technique	Solvent	T(°C)	t(min)	Reference
Olive pomace	UAE	Isopropanol:H_2O 1:1 (*v/v*)	25	40	(Abdel-Razek *et al.* 2017)
Olive leaves	UAE	Isopropanol: H_2O 1:1 (*v/v*)	25	40	(Abdel-Razek *et al.* 2017)
Pomace olive oil	UAE	Hexane	25	40	(Abdel-Razek *et al.* 2017)
Olive cake	UAE	Natural Deep Eutectic Solvents (NADES):	40	60	(Fernández *et al.* 2018)

		LGH(lactic acid:glucose 5:1) CGH(citric acid:glucose 1:1) FCH(fructose:citric acid 1:1)			
OMWW	Liquid-liquid extraction	Ethyl acetate	25	20	(Elkacmi *et al.* 2017)
Olive cake	Soxhlet extraction	EtOH	80	720	(Leouifoudi *et al.* 2015)
OMWW	Liquid-liquid extraction	Ethyl acetate (1:1 *v/v*)	25	-	(Leouifoudi *et al.* 2015)
Olive pomace Dry olive mill residue	Solid-liquid extraction	MeOH: H_2O 8:2 *v/v*, H_2O	25	40	(Ramos *et al.* 2013)
Olive leaves, olive pomace	SFE	CO_2, co-solvent: EtOH at 60% (1:3 *w/v*)	50	60	(Caballero *et al.* 2019)
Olive leaves	SFMAE	none	-	2-3	(Tan *et al.* 2017)
Olive leaves	Solid-liquid extraction	MeOH: H_2O 80:20 *v/v*	25	192	(Aissa *et al.* 2017)
OMWW	Liquid-liquid extraction	Ethyl acetate	25	-	(Aissa *et al.* 2017)
Olive cake	PLE	EtOH: H_2O 50:50 *v/v*	120	-	(Lozano-Sánchez *et al.* 2014)

As can be seen in Table 6, the non-conventional methods have been widely studied in the past few years: UAE, for example, have been implemented with different solvents, showing a growing interest in finding the most suitable solvent type and composition. However, even if the attention has moved towards these greener techniques, conventional methods still play an important role and have been implemented extensively in recent years. Techniques such as solid-liquid extraction and Soxhlet extraction are more time-consuming, if compared to non-conventional methods. For example, while for UAE extraction the time is approximately 40 minutes, a solid-liquid extraction can take up to 192 minutes.

On the other hand, Table 7 shows a list of different studies conducted to extract polyphenols from wine wastes. In addition, the types of extraction technique with a specification on type of solvent, temperature and time of operation are reported.

***Table 7** - Extraction methods implemented to extract polyphenols from wine production wastes*

Sample	Extraction technique	Solvent	T(°C)	t(min)	Reference
Grape skin	Solid-liquid extraction, UAE,MAE	EtOH:H_2O (8-20-50-80-92 %) Solid-liquid ratio 1.3-1.17	30	-	(Caldas *et al.* 2018)
Grape pomace	Soxhlet extraction	EtOH, H_2O	-	EtOH 120-180 H_2O 300-360	(Drosou *et al.* 2015)
Grape pomace	MAE	H_2O, H_2O - EtOH 1:1 *v/v*	50	60	(Drosou *et al.* 2015)
Grape pomace	UAE	H_2O, H_2O - EtOH 1:1 *v/v*	20	60	(Drosou *et al.* 2015)
Grape pomace	Solid-liquid extraction	EtOH:H_2O 50:50 *v/v*	60	120	(Antoniolli *et al.* 2015)

Wine lees	Solid-liquid extraction	MeOH, 2% HCl 95:5 *v/v*	-	60	(Jurcevic *et al.* 2017)
Wine lees	Solid-liquid extraction	EtOH:H_2O 70:30 *v/v*	25	-	(Kopsahelis *et al.* 2018)
Grape marc	UAE	EtOH:H_2O	20-50-80	4-7-10	(Da Porto *et al.* 2015)
Grape marc	SFE	CO_2, co-solvent: EtOH(10%):H_2O	40	30	(Da Porto *et al.* 2015)
Grape pomace	PLE	H_2O	120	2 cycles - 10	(Poveda *et al.* 2018)
Grape pomace	UAE	EtOH 44%	<50	3	(Poveda *et al.* 2018)
Wine lees	UAE	EtOH:H_2O 50:50 *v/v*	40	15	(Tao *et al.* 2014)
Grape pomace	Solid-liquid extraction	6 different extracting solutions Best results: ethyl acetate	25	360	(Pintać *et al.* 2018)

Many different techniques have been studied in recent years for both olive waste (Table 6) and wine waste extraction. As highlighted in Table 7, conventional methods are still widely investigated: solid-liquid extraction has been studied at different temperatures, solvent type and composition. Greener technologies have also been studied for this type of waste, resulting in lower extraction time and in some cases the possibility of using water as solvent.

4. Separation and purification techniques

Membrane separation processes are frequently investigated as they possess several advantages. Currently, the employment of membranes in the food industry makes it the second biggest market, with an assessed volume of money of about 800 million dollars (Lipnizki 2010). Apart from the application in the dairy and beverage industries, the use of different membrane-based technologies would permit the recuperation, separation and purification of the above-cited valuable compounds from industrial wastes (Castro-Muñoz *et al.* 2016). Moreover, the focus is moving to the possibility of integrating different membrane processes with different pore sizes, in order to completely fractionate the residues. The main advantage is the non-destructiveness towards compounds of interest, as well as lower energy consumption, milder operation conditions and easier scale-up (Cassano *et al.* 2016). Another important advantage that would overcome the problems related to both conventional and non-conventional extraction techniques is the absence of an extractive solvent, which would solve all problems related to its removal.

A new persepective would see an approach where both extraction and membrane processes are integrated, so exploiting the advantages of both processes (Cassano *et al.* 2017). As far as polyphenols are concerned, Cassano and coworkers (2017) focus on the interaction between them and different membranes, since this aspect plays an important role within the performance of the membrane itself and its fouling, which vastly affects operation conditions and operation time. Moreover, since retention is directly related to the size of the particles within the feed, which may or may not pass according to the size of the pores – higher particles will be retained and smaller will pass through – it is of high importance to focus on the interactions between the molecules in the target solution. In fact, polyphenols can interact within themselves, or with proteins, leading to the formation of compounds with a size comparable to colloidal compounds (Bazinet and Doyen 2017).

Following is a brief review of the main pressure-driven membrane processes, divided by pore size and transmembrane pressure.

4.1 Pressure-driven membrane processes

4.1.1 Microfiltration (MF)

Microfiltration is a physical filtration process that employs porous membranes where the pore size ranges from 0,1 to 5 μm while transmembrane pressure range goes from 1 to 10 bar (Bazinet and Doyen 2017). It is used in liquid sterilization, clarification, as well as colloids, yeast and bacteria elimination. Microfiltration can be performed in two configurations: in-line filtration (or dead-end filtration) and cross-flow filtration. In the first case, the flow passes perpendicularly through the membrane, causing the formation of a cake and in many cases requiring different washing cycles in order to reach the desired quantities. Many are the advantages in the second case, where the fluid passes tangentially with respect to the membrane: the formation of the cake is reduced and the permeate flux is higher (Figure 21). However, the initial investment is higher, which is compensated by lower operational costs.

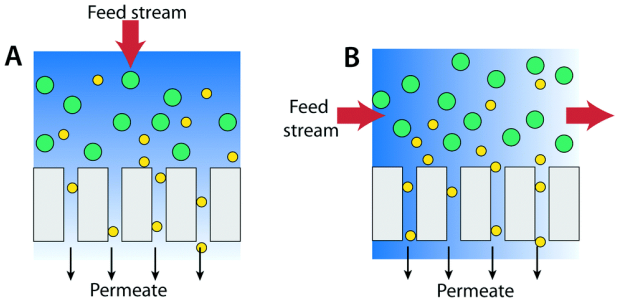


Figure 21 *- Scheme of dead-end filtration (A) and cross-flow filtration (B)*

Microfiltration equation is based on Darcy's law, as shown in Equation 4:

$$J = \frac{1}{A}\frac{dV}{dt} = \frac{\Delta P}{\mu(R_m + R_c)} \quad (4)$$

Where ΔP is the transmembrane pressure, R_m and R_c are the membrane and cake resistance (m^{-1}), respectively and μ refers to permeate viscosity (Pa s). As the thickness of the cake increases, its resistance increases. R_c can be expressed as follows (5):

$$R_c = \frac{\alpha C_F V}{A} \quad (5)$$

Where α is the specific cake resistance expressed in m/kg, C_F is the feed concentration (kg/m^3), V is the permeate volume (m^3) and A is the membrane area (m^2).
An operation can be conducted either at constant flux or constant pressure. In the former, case pressure is increased to maintain the flux as the thickness of the cake on the membrane increases, which increases the total resistance. In the latter, the flux decreases as a function of time (Seader J.D. *et al.* 2011).
Upon reaching a threshold value operation is stopped to perform washing operations. Through these operations, which can be both physical and chemical and depend on the fouling degree, the reversible component of the resistance is removed, and the irreversible component remains: this implies a lower flux when restarting operation. Membrane fouling refers to the accumulation of material on the membrane surface, normally colloids, soluble organic compounds and microorganism (Pulido 2016).

Microfiltration is in many cases used as a pretreatment when employed for the recovery of valuable compounds and is followed by ultrafiltration, nanofiltration and reverse osmosis, which depends on the application (Díaz-Reinoso *et al.* 2017; Giacobbo *et al.* 2017). Targeted compounds can either remain in the rejection part because they do not manage to pass through the membrane, or they can permeate. To evaluate rejection, the following formula can be applied, which is valid also for the methods reported in the following paragraphs (6).

$$R = 1 - \frac{C_P}{C_F} \quad (6)$$

Where C_P and C_F are permeate and feed concentration (kg/m^3), respectively.

4.1.2 Ultrafiltration (UF)

Ultrafiltration is another physical filtration process that employs porous membranes, it differs from microfiltration for pore size, while the transmembrane pressure needed to perform the filtration remains the same. Pore size ranges from 1 to 100 nm (Bazinet and Doyen 2017). Also in this case, both dead-end and cross-flow filtrations are possible. The ultrafiltration membranes are defined by the molecular weight cut-off (MWCO), so they retain the 90 % of molecules with the same size as the MWCO's (Seader J.D. *et al.* 2011).

Fouling occurs also in this case, the two following models are normally employed to explain this phenomenon, which in both cases leads to a decreased permeation flux.

4.1.2.1 Gel-polarization model

This model permits the effect of concentration polarization to be described, assuming that after a certain threshold, even though pressure is increased, the permeation rate gets limited by the gel layer formed on the membrane, whose thickness increases with time (Thiess *et al.* 2017).

In this model, C_{gel} is assumed to be constant in time, while thickness and porosity are variable. Moreover, in this model the effect of osmotic pressure is completely neglected, altough it has been shown to play an important role. The scheme of the described situation is shown in Figure 22:

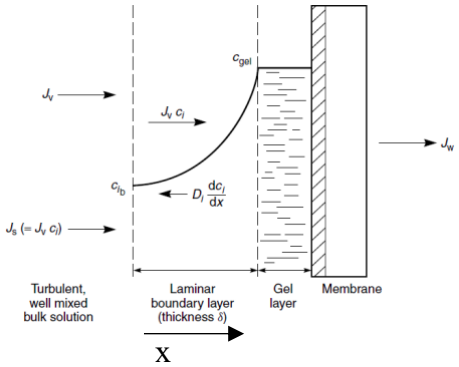


Figure 22 *– Schematic representation of gel-layer model*

According to this assumption, the gel layer can be assumed as the limiting resistance, therefore the calculation of the solvent flux can be based on the back flux of the retained molecules from the layer over the membrane back to the bulk. In such conditions, the flux is independent from the difference of pressure: the layer thickness will reach a steady state where the convective flux of the retained species due to the solvent flux is equal to the diffusive back flux (Sablani *et al.* 2001). At steady state (7):

$$J_v C_i = D_i \frac{dc_i}{dx} \qquad (7)$$

The previous equation can be solved since boundary conditions are known; being δ the boundary layer thickness, at x=0 concentration is equal to the bulk concentration, while at x = δ, on the membrane surface, concentration is equal to C_{gel} (8).

$$J_v = \frac{D_i}{\delta} ln \frac{C_{gel}}{C_{i\,bulk}} \qquad (8)$$

Therefore, the equation shows that the flux wholly depends on the solute characteristic and the thickness of the boundary layer. Thus, increasing pressure will not lead to an increase in flux, but it is necessary to manage the flow in order to decrease δ, therefore increasing the mass transfer. It can be defined a mass transfer coefficient K as (9):

$$K = \frac{D_i}{\delta} \qquad (9)$$

And (8) becomes as follows (10).

$$J_v = K ln \frac{C_{gel}}{C_{i\,bulk}} \qquad (10)$$

The mass transfer coefficient can be evaluated in both laminar and turbulent flow conditions by exploiting mass transfer correlations that helps describe the fluid dynamic of the system (Sablani *et al.* 2001).

4.1.2.2 Osmotic pressure model

Since the concentration at the membrane surface reaches higher values when compared to bulk concentration, its related osmotic pressure can not be neglected in this case, and the flux is corrected by a specific term incorporated within the Darcy's law (11).

$$J = \frac{\Delta P - \Delta \pi}{\mu R_m} \qquad (11)$$

Thus, the flux is not a linear function of pressure. The applied transmembrane pressure is diminished by the osmotic term. Therefore it is necessary to apply such a pressure that permits to contrast osmotic pressure so reaching the desired total transmembrane pressure. In other words, osmotic pressure difference implies a reduced driving force, and a reduced flux as a consequence (Figure 23).

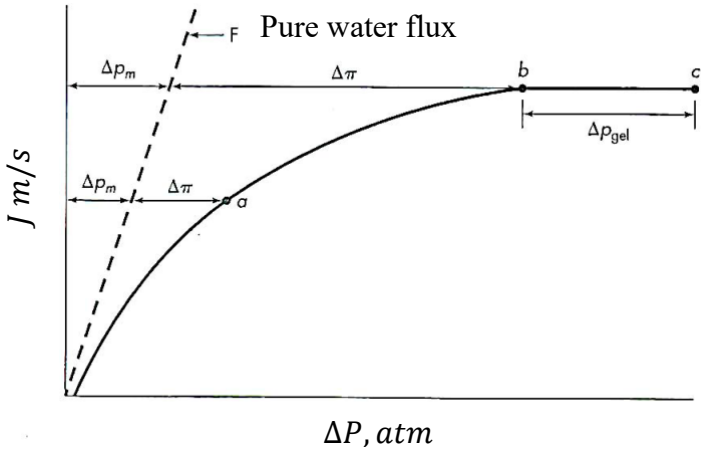


Figure 23 *– Flux vs applied pressure*

Osmotic pressure can be calculated in various ways, also depending on the type of solute. (12), (13) and (14) show three different possibilities, where M refers to concentration at membrane surface expressed as molarity.

$$\pi = MRT \qquad (12)$$

If the species is an electrolyte the previous expression is multiplied by the Van't Hoff coefficient i.

$$\pi = aC^n \qquad (13)$$

$$\pi = A_1 C + A_2 C^2 + A_3 C^3 \qquad (14)$$

Where C is gel concentration and A_i are virial coefficients depending on the solute.

Substituting, for example in equation (13), the gel concentration expressed through equation (10) the flux can be obtained as a function of transmembrane pressure and solute concentration (15).

$$J = \frac{\Delta P - a C_{bulk}^n \exp\left(\frac{nJ}{K}\right)}{\mu R_m} \qquad (15)$$

The same can be done using another expression for the osmotic pressure (Thiess *et al.* 2017).

4.1.3 Nanofiltration (NF)

Nanofiltration is a pressure driven process, where pore size diminishes with respect to the previous membranes, and the transmembrane pressure needed to perform the process increases. Pore size ranges from 0,5 to 10 nm and transmembrane pressure from 10 to 30 bar (Bazinet and Doyen 2017). In polyphenols' recovery it is customarily used for the final separation and purification (Cassano *et al.* 2016). The modeling of this technique is the same as reverse osmosis, which is presented in the following paragraph.

4.1.4 Reverse osmosis (RO)

Reverse osmosis is a purification technology that employs dense semipermeable membranes, with a separation range that goes from 1 to 10 Å. It differs from the other filtration processes since separation does not occur through molecular filtration, but through solution-diffusion, where high pressures need to be applied, which range from 30 up to 100 bar. Basically, the separation mechanism is based on the size, charge and interaction of the molecule with the membrane.

To describe this model, it is necessary to make some hypothesis (Figure 24).

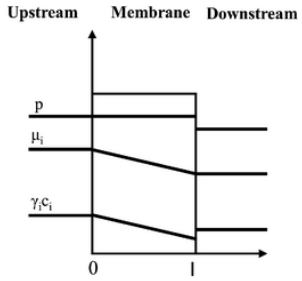


Figure 24 *- Solution-diffusion model* (Vandezande, Gevers, and Vankelecom 2008)

Pressure within the membrane is equal to the higher value, which assumes that the membrane behaves like a liquid and the gradient of the chemical potential is continuous, which indicates that at the interface the fluid is in equilibrium with the membrane (Vandezande *et al.* 2008). According to these considerations, the flux of solvent can be expressed as equation (16):

$$J = A\ (\Delta P - \Delta\pi) \qquad (16)$$

Where A is the membrane permeability. The flux of the solute does not depend on pressure, but on the difference between its concentration at the two sides of the membrane (17).

$$J_S = B\ (C_0 - C_l) \qquad (17)$$

Where B is the solute permeability coefficient (Scott 2007).

4.2 Polyphenols extraction from wine and oil wastes employing membranes

As previously stated, different membrane processes have been described. Many studies prove the possibility of applying membranes for polyphenols recovery.

Giacobbo and coworkers (2017) implement a sequential process, integrating and combining different membrane techniques such as microfiltration, ultrafiltration and nanofiltration. The aim is to fractionate polyphenols and separate them from polysaccharides contained in wine lees. In this study, ultrafiltration has proved effective in separating polyphenols from polysaccharides,

subsequently improving purity, while nanofiltration has been found to succeed in concentrating specific classes of polyphenols, in this case the anthocyanidins. Two configurations were implemented, both preceded by a microfiltration stage to remove the colloidal material, as a first stage of purification. In the first configuration, a microfiltration stage was followed by a nanofiltration one; in the second one, ultrafiltration was added as an intermediate stage between the two previous ones.

In another study Giacobbo and coworkers (2015) employ microfiltration to recover polyphenols. Dilutions and vacuum filtration were used as pre-treatment stages to remove solid particles, in order to fully optimize the following stage. They tested different combinations, as shown in Figure 25.

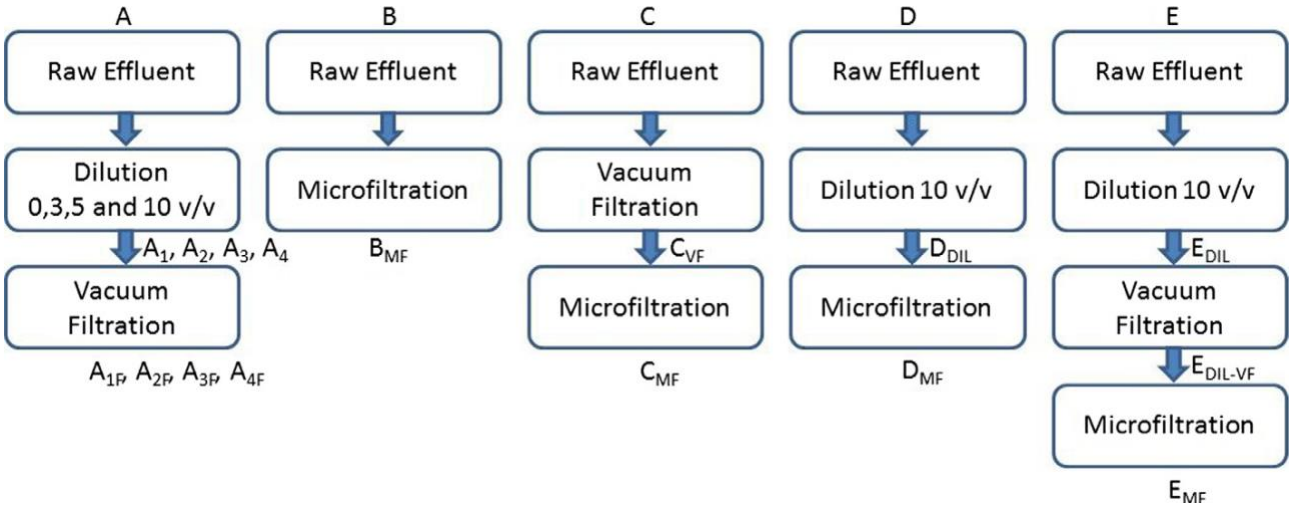


***Figure 25**- Flow chart of the experiments (Giacobbo et al. 2015)*

Sygouni and coworkers (2019) worked with olive mill wastewater. In this work, after implementing extraction processes to optimize as much as possible the yield in polyphenols, different membranes were used in order to separate and concentrate the extracted compounds. The extract was sent to a system of ultrafiltration, nanofiltration and reverse osmosis. The first filtration was used to remove solid particles, fat and lipids; then in NF and RO the concentration of the total phenolic content was performed. The total phenolic content was equal to 225 mg/L in the concentrate stream at the outlet of the RO unit, while only 10 mg/L were left at the permeate, so proving a high factor of concentration.

Tables 8 and 9 report a list of different studies conducted to the implementation of separate membrane techniques for the treatment of olive oil and wine production waste, respectively.

***Table 8** - Membrane techniques employed to treat olive oil production waste*

Sample	Membrane technique	Reference
OMWW	UF (0,04 μm) + NF (MWCO 150-300 Da)	(Ioannou-Ttofa *et al.* 2017)
	UF (100 nm) + NF (MWCO 800 Da) + RO (99% rejection of NaCl)	(Sygouni *et al.* 2019)
	UF (100 nm) + NF (470 Da) + RO (99% rejection of NaCl)	(Zagklis *et al.* 2015)
	UF + NF	(Cassano *et al.* 2013)

***Table 9** - Membrane techniques employed to treat wine production waste*

Sample	Membrane technique	Reference
White wine vinasses	MF (0,5 μm)+ NF + UF (to reduce the NF retentate environmental impact)	(Díaz-Reinoso *et al.* 2017)
Red wine lees	MF	(Giacobbo *et al.* 2015)
	MF + UF + NF	(Giacobbo, Meneguzzi, *et al.* 2017)
	MF + UF + NF	(Giacobbo, Bernardes, and de Pinho 2017)
Winery sludge	UF	(Galanakis *et al.* 2013)
Winery wastewater	RO (here the aim is the purification of the wastewater, rather than the recovery of polyphenols)	(Ioannou *et al.* 2013)

In the case of wine production waste, multiple residues have been treated with membrane processes, in the case of olive oil production waste only studies where olive mill wastewater had been treated with this type of separation technique were found. The aim of this work was the recovery of polyphenols trough membrane separation starting from olive pomace's extract and wine less. Olive pomace extract could give an innovative footprint on this topic.

5. Material and methodology

The present work is based on the experimental methodology presented in Figure 26.

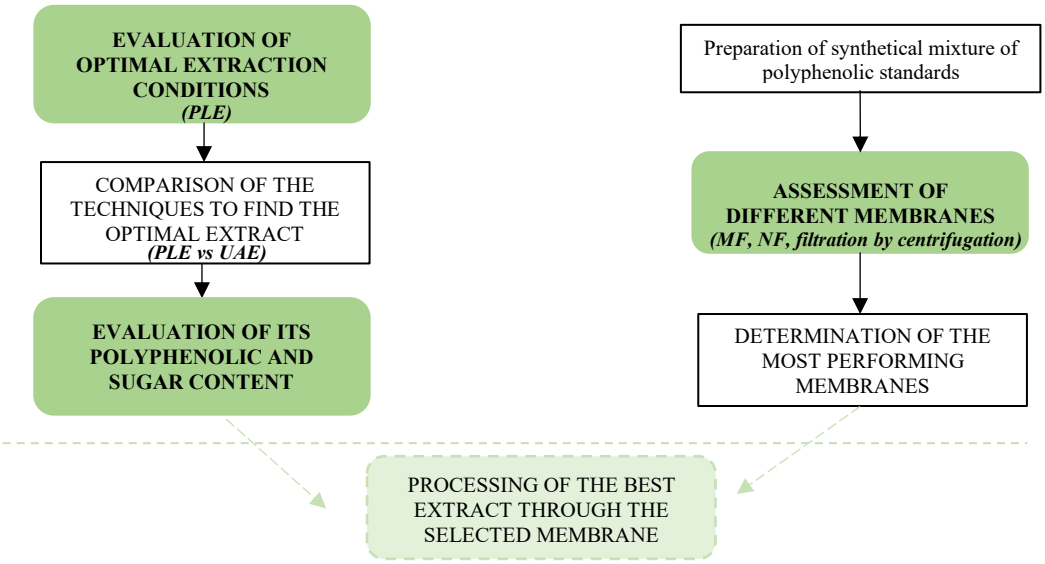


Figure 26 *- General scheme of experimental methodology*

The idea was to work in parallel on both extraction and membrane techniques. On one hand, evaluation of best extraction conditions of PLE was carried out to later compare its performance with UAE. Composition of these extracts was assessed by evaluating polyphenolic content by HPLC and sugar content by a spectrophotometric method. On the other hand, different membranes (microfiltration (MF), nanofiltration (NF) and filtration by centrifugation) were assessed, passing synthetic mixtures that could mimic extracts' composition, in order to determine the best performing one. In future, the idea is to exploit these results and instigate the processing of the best extract encountered through the most performing membrane. Part of this work also concerned an environmental and economic analysis, that can be found in appendix A and B, respectively.

Experiments were held in both, Universitat de Barcelona (UB) and Universitat Politècnica de Catalunya (UPC-EEBE). In fact, membranes experiments were held at the EEBE, while extractions and polyphenols concentration measurements were held at the UB.

5.1 Olive oil and wine residues

Olive oil residues were provided by Borges industries; recollection was performed during the period between November and February. For the present work, olive pomace is treated as an example among the other residues.

Wine residues were provided by Bodegas Torres; in general, the recollection of these residues is made when they are generated. In this case, all residues but wine lees are recollected, in the period between August and October. Wine lees can be obtained along the whole productive process. For the present work, wine lees are treated as an example among the other residues.

Olive pomace and wine lees samples were stored in a refrigerator in tubes (-20°C), as shown in Figure 27.

Figure 27 *- Samples of wine lees and olive pomace*

5.2 Extraction experiments

Previously, different extraction techniques have been explored; there are many non-conventional methods, each one with advantages and specific procedural needs. In this work, however, only pressurized liquid extraction and ultrasound assisted extraction have been studied. Pressurized liquid extraction (PLE) and ultrasound assisted extraction (UAE) were performed each time in parallel. This would guarantee that the organic matrices were at the same conditions, since they are likely to suffer from degradation that leads to changes in composition. In this way, the comparison between the two techniques was based on the same starting conditions (samples were refrigerated, and the quantity needed was taken and weighed at room temperature).

5.2.1 Pressurized liquid extraction (PLE)

PLE experiments were performed on both types of residues: 1 gram of sample was weighed with a calibrated balance and then mixed with 2 grams of adsorbent, diatomaceous earth (Thermo Scientific), to obtain a homogenous texture and improve the extraction. Then, the mixture was placed in a stainless-steel chamber, as shown in Figures 28 and 29. This procedure was done in order to improve the extraction through texture improvement.

Figure 28 *- Homogenization of the mixture sample-diatomaceous earth and parts of the stainless-steel chamber*

Figure 29 *- Placement of the mixture within the stainless-steel chamber*

The chamber is formed in two parts, one at the bottom where a fiberglass filter is preventively placed, and one at the top to close it at the end. These experiments were carried out in triplicate. The solvent selected is a solution of ethanol at different percentages. Hydrochloric acid was used in the first stages of the experimentation, but showed no influence on the extraction, hence it was removed in order to perform extraction with only ethanol. It should be noted that HCl was also harmful for the equipment used. Ethanol (EtOH) (99,8%) was provided by Honeywell. Tests were conducted at different temperatures (80, 100, 120 °C) and ethanol percentages (40, 60, 80%), in one cycle lasting 5 minutes. Then, the number of cycle and the duration of the extraction were increased. 1, 2 and 3 cycles were tested with a duration of 5, 10 and 15 minutes. Pressure was maintained at 10 bar using nitrogen. Extraction was performed in a Dionex ASE 350 equipment (Figure 30).

Figure 30 *- Equipment for pressurized liquid extraction -Dionex ASE 350*

After a first rinsing of the equipment and setting the operative conditions, each chamber was preventively heated through an oven at the desired temperature, and once this was reached, the extraction was performed. Finally, the extracted samples with a volume of 15 mL were recollected in test tubes and subsequently centrifuged in a Labofuge 400, at 3500 rpm for 15 minutes, with particular care on balancing the weights within the centrifuge.
After centrifugation, filtration was performed by a syringe (NORM-JECT, Henke-Sass Wolf). Samples were initially filtered in a 12 mL vial with a 0.45 μm polyamide filter (Chromafil® Xtra PA-45/25, MACHEREY-NAGEL); then, they were filtered in a 1.5 mL vial suitable for chromatography, with a 0.22 μm Nylon filter (FILTER-LAB), in order to remove the particles that were left as they could damage the HPLC equipment used to analyze the extracts. Figure 31 and 32 show the equipment used and how filtration was performed in practice.

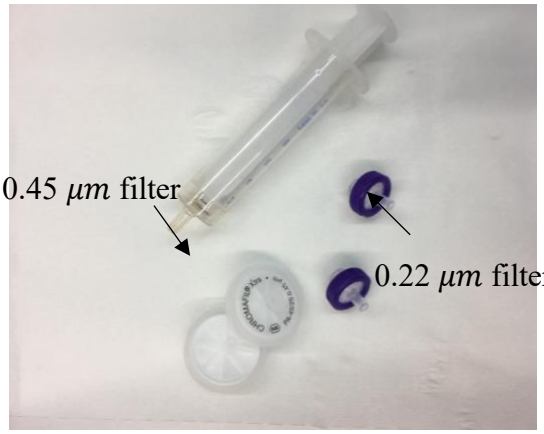


Figure 31 *- Syringe and the two types of filters*

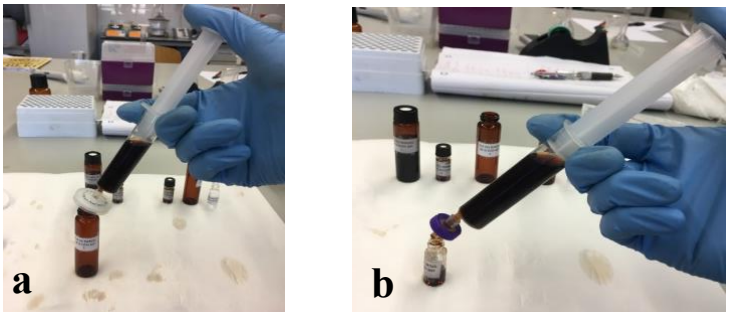


***Figure 32** - a) Filtration with a 0.45 µm filter b) Filtration with a 0.22 µm to proceed with HPLC analysis*

5.2.2 Ultrasound-assisted extraction (UAE)

UAE experiments were performed on both residues. Again, 1 gram of sample was weighed and 20 mL of a selected solvent (EtOH) were added. Optimal composition of this solvent was already established by previous experiments of this research group: the compositions of the solvents for the two matrixes are reported in Table 10. HCl (32%) was provided by EMSURE.

***Table 10** – Optimal extraction solvent composition for each residue*

Olive oil residues	**%**
Ethanol	60.0
Water	39.9
HCl	0.1
Wine lees	**%**
Ethanol	80.0
Water	19.5
HCl	0.5

Experiments were carried out in triplicate in a distilled water ultrasonic bath in a Branson 5510 (Figure 33) for a duration of 30 minutes.

***Figure 33** - UAE equipment, BRANSON 5510*

Once the extraction process was over, samples were centrifugated at 3500 rpm for 15 minutes, and then filtered following the same procedure as explained for the PLE experiments. Extracts were then analyzed by HPLC.

5.3 Separation and purification by membranes

Different membranes were evaluated to identify which one could be used to separate and later concentrate specific polyphenols: the ideal membrane would separate one or two polyphenols either in the permeate or the rejected volume, leaving the others in the other side. Tests were performed with synthetic mixtures for those polyphenols identified within the extracts by mass spectrometry. This was completed to mimic extracts and see how each membrane behaved. 100 mL of each mixture was prepared in order to perform repetitions for each membrane, polyphenols were dissolved in 60% ethanol with 0.1 % of HCl. This composition was selected once the best extracting conditions were identified, in order to perfectly mimic the extracts' composition.

Centrifugation tubes, microfiltration and nanofiltration membranes were tested. In each case, the rejection of each compound was calculated following Equation 18. Feed and permeate concentrations were obtained

$$R(\%) = \left(1 - \frac{C_P}{C_F}\right) x100 \qquad (18)$$

where C_P refers to the concentration of each polyphenol in the permeate and C_F to the one in the feed stream.

Careful cleaning of the equipment was performed when changing the type of mixture, to avoid any type of contamination between the two. First tap water was used, then each part was rinsed with distilled and milli-Q water.

5.3.1 Filtration by centrifugation

Separation tests were performed with centrifugation tubes (Amicon ® Ultra – 4, Merck Millipore); the experiments were carried out in duplicate. 4 mL of extracted samples were processed by a centrifuge at 2600 rpm. Operation was stopped once the entire volume passed through the filter. Figure 34 shows an image of the filter placed inside the centrifugation tube.

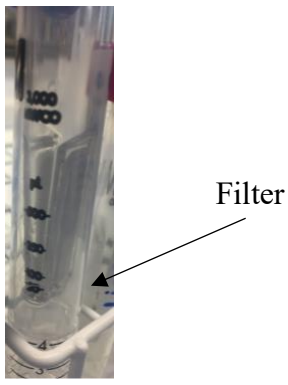
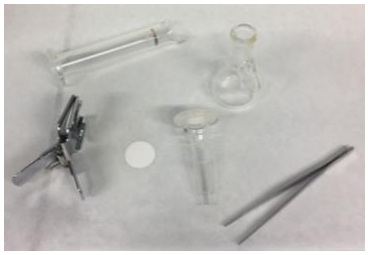


Figure 34 *- Centrifugation tube*

5.3.2 Microfiltration (MF)

Three membranes were tested: the difference between them laid within pore size, respectively of 0.1, 0.45 μm (Sartorius Stedim Biotech SA) and 0.22 μm, in nylon (FILTER-LAB).

Experiments were carried out in duplicate, in the equipment shown in Figure 35, placing the active side on the top of the filtration system (in this case both sides are active) and the sample just above the filter. Then, vacuum was created in order to let the liquid pass through the membrane from the up side of the membrane to the erlenmeyer (placed under the membrane).



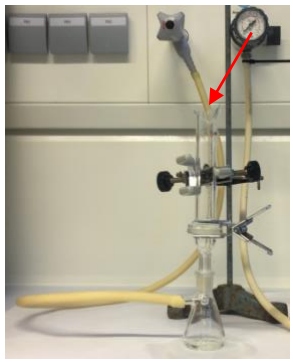


***Figure 35** - Microfiltration equipment*

Although the equipment had a volume of 25 mL, only 5 mL of mixture were processed from the top side of the system (red arrow) to the bottom part, where permeate was recollected, after the filtration process.

5.3.3 Nanofiltration (NF)

Five different membranes were tested; each one was cut in a round shape, with an area of approximately 7 cm^2 to make it fit in the equipment. They were then submerged in Milli-Q water overnight, to remove possible particles that may have remained on the surface due to conservation products.
Experiments were carried out in duplicate. Table 11 displays each membrane used and the corresponding provider.

***Table 11** - Nanofiltration membranes*

Membrane	Company
NF270	DOW Chemical
NF90	DOW Chemical
TFCS	Koch Membrane Systems
TFC-HR	Koch Membrane Systems
DURACID	Suez Environment

The duration of each experiment was dependent on the membrane: at the beginning pressurized air was used, reaching values of pressure around 7 bars. However, to fasten the process, and due to some membranes having a higher resistance to the passage of liquid, the second membrane nitrogen was used, with pressure going from 8 up to 11.5 bar (only the NF270 was used with pressurized air, then nitrogen was used for the others). Figure 36 shows the equipment used.

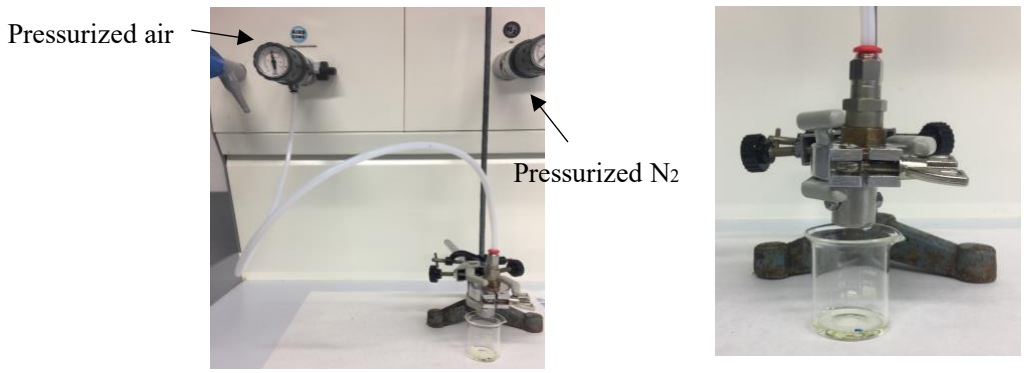


***Figure 36** - Equipment and final configuration*

Volumes of 3 mL of mixture were processed; each operation was stopped once 1 mL was obtained, to fasten the recollection. Permeate was then passed into a tube.

Once all tests were concluded, each repetition was diluted in acetonitrile by a dilution factor of 6 to make sure concentration of each polyphenols would lay within the detection range of HPLC. Then, permeate samples from each membrane filtration test were characterized by HPLC, to analyze the polyphenolic content and be able to calculate its rejection.

5.4 Determination of phenolic composition by HPLC

Quantification of antioxidants can be understood from two different perspectives: specific components or families of components can be identified, or total phenolic content can be determined. To do so, different techniques were employed: in the former case, high-performance liquid chromatography (HPLC) permits identification and quantification of polyphenols, while for the latter a spectrophotometric assay can be employed by the Folin-Ciocalteu method. In this work, quantification was performed through the first-mentioned method (HPLC) in order to determine specific components and the total phenolic content.

Analyzing the phenolic content of each extract permits the identification of optimal operational configuration in PLE, also allowing for a comparison with UAE technique, in order to ascertain which is most effective as an extraction method. Moreover, HPLC has also been employed to characterize the performance of each membrane tested by analyzing the phenolic content after being treated by different membranes. A HPLC Agilent Series 1100 (Agilent Technologies, Palo Alto, California, USA) chromatograph was used: it is equipped with a quaternary pump, an automatic injection system and a diode array detector. To process data and control the instruments, the software AgilentChemStation was used. The column used was a Kinetex C18 (100 mm x 4,6 mm, 2,6 μm): polar compounds elute faster than non-polar ones that are retained for longer (Figure 37).

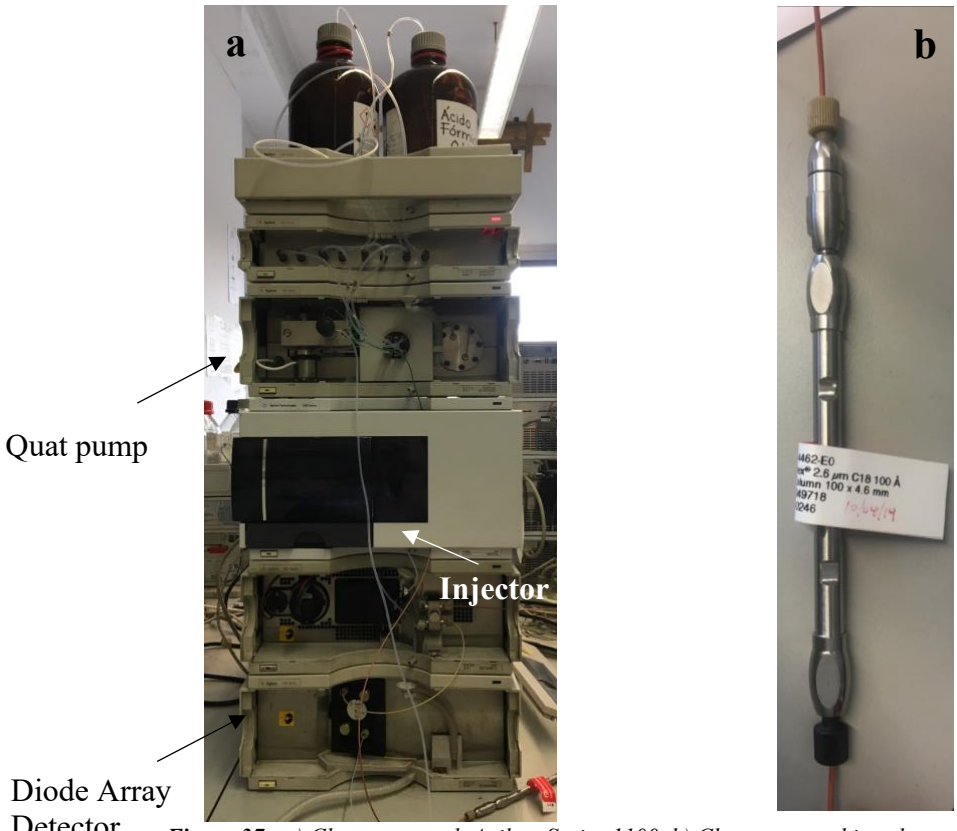


***Figure 37** - a) Chromatograph Agilent Series 1100, b) Chromatographic column*

Mobile phase consisted in two phases: an aqueous phase with a 0,1% of formic acid (for analysis EMSURE, 98-100%, solvent A) and acetonitrile (Fisher Chemical, solvent B).

Pressure limits were 0 and 400 bars. Flux was set at 0,4 mL/min, and the volume withdrawn by the injector from each vial was set at 5 μL. For each run, it was necessary to wait for a specific time in order to stabilize the internal pressure and equipment, ordinarily around 80 bar. Chromatograms were registered and analyzed at three different wavelengths, 280, 310 and 370 nm, those at which polyphenols can be detected. Each peak corresponds at a specific compound, characterized by its retention time at that operative flux and a specific area, proportional to its concentration.

Analysis was performed with different gradients for extracts and membrane tests results.

The standards of polyphenols' used are enlisted in Table 12, with the corresponding retention times depending on the elution method used (paragraphs 5.4.1, 5.4.2).

***Table 12** - Standards of polyphenols used with the corresponding retention times*

		RETENTION TIME	
Standard	**NM**	**POLIACETONITRILO.M**	**MET_12/10**
Gallic Acid	**280**, 310	5.85	6.34
Homogentisic Acid	**280**, 310	7.70	8.15
2-(3,4 Dihydroxyphenyl) Ethyl Alcohol	**280**	8.47	8.86
3,4 Dihydroxybenzoic Acid	**280**, 310	9.16	9.70
Catechin	**280**	11.49	12.13
3,4 Dihydroxybenzaldehyde	**280**, 310	11.60	11.67
Ac. 4 Hidroxibenzoico	**280**	11.79	12.49
Clorogenic Acid	**310**, 280, 370	11.80	11.88
Syringic Acid	**280**, 310	12.77	13.50
Caffeic Acid	**310**, 280, 370	12.78	13.39
Vanillic Acid	**280**, 310	13.20	13.30
Epicatechin	**280**	13.30	13.47
Ethyl Gallate	**280**, 310	14.86	15.54
P-Coumaric Acid	**310**, 280, 370	15.49	16.31
Hesperidin	**280**, 310, 370	16.64	17.39
Ferulic Acid	**310**, 280, 370	16.80	16.90
Oleuropein	**280**	18.18	18.60
Resveratrol	**370**, 310, 280	20.26	21.19
Luteolin	**370**, 310, 280	21.71	22.37
Quercetin	**370**, 310, 280	21.76	21.00
Kaempferol	**370**, 310, 280	25.50	25.51

5.4.1 HPLC analysis for residue extracts

Two different gradients were employed in this case, MET_12 and MET_10 (as named in the equipment), respectively for the olive pomace extracts and for the wine lees extracts. Tables 13 and 14 display the two different composition in function of time.

Table 13 *- HPLC gradient for olive pomace extracts*

	Solvent composition (%)	
Time (minutes)	A	B
0.0	95	5
38.0	60	35
40.0	10	90
42.0	10	90
42.2	95	5
50.0	95	5

Table 14 *- HPLC gradient for wine lees extracts*

	Solvent composition (%)	
Time (minutes)	A	B
0.0	95	5
38.0	50	45
40.0	10	90
42.0	10	90
42.2	95	5
50.0	95	5

The elution gradients were found as the most suitable for the analysis of each matrix in another stage of the project, previous to the present work. The need for a different elution method for each type of extract derived from a poor separation between peaks when using the same one for both extracts, especially in olive pomace extracts.

To evaluate the extraction performance, the total area at 280 nm was calculated: at this wavelength the signal is the highest.

Once the total area of the chromatograms of the extracts was obtained, total phenolic content was evaluated and PLE and UAE techniques were compared to determine which one was the most efficient for polyphenol extraction from olive pomace and wine lees residues.

5.4.2 HPLC analysis for polyphenol separation and purification by membranes

Synthetic mixtures of polyphenols were prepared, one for each type of residue: olive pomace and wine lees. Concentration of each polyphenols was selected according to the HPLC sensibility. Mixtures were prepared in vials at 0,5, 1, 2, 5, 7 and 10 ppm of each polyphenol, to build a calibration curve and be able to evaluate the concentration of the polyphenols within the permeate after the membrane treatment. A calibration line was built for the following polyphenols: homogentisic acid, 2-(3,4 dihydroxyphenyl) ethyl alcohol, caffeic acid, p-coumaric acid, oleuropein, luteolin, gallic acid, 3,4 dihydroxybenzoic acid, catechin, 4-hydroxybenzoic acid, syringic acid, ethyl gallate, hesperidin, resveratrol and quercetin. Table 15 reports the HPLC sensibility towards each polyphenol.

Table 15 *- HPLC sensibility towards polyphenols' standards*

Standard	Minimum detected concentration (ppm)
Homogentisic Acid	1
2-(3,4 Dihydroxyphenyl) Ethyl Alcohol	1
Caffeic Acid	1
***P*-Coumaric Acid**	1
Oleuropein	5
Luteolin	1
Gallic Acid	1
3,4 Dihydroxybenzoic Acid	1
Catechin	2
4-Hydroxybenzoic Acid	1
Syringic Acid	1
Ethyl Gallate	1
Hesperidin	1
Resveratrol	1
Quercetin	5

To analyze membrane permeates in terms of polyphenolic content a different gradient was used, named POLI.ACETONITRILO.M. Solvent composition used in function of time is displayed in Table 16:

Table 16 *- HPLC gradient*

	Solvent composition (%)	
Time (minutes)	A	B
0.0	95	5
25.0	50	50
27.0	10	90
29.0	95	5
29.2	95	5
39.0	95	5

5.5 Sugar content quantification

While analyzing the phenolic content of different extracts by HPLC, quantification of their sugar content was carried out in parallel. A colorimetric method was used, called the Dubois method (Dubois *et al.*, 1956). First of all, solutions of glucose at different concentrations were prepared in order to generate a calibration curve. For that, 0,1 g of glucose anhydrous (Scharlab, S.L.) was weighed, and dissolved in Milli-Q water in a 100 mL flask, to obtain a solution with a concentration of 1000 ppm. From this one, all the others were prepared, following the scheme shown in Figure 38:

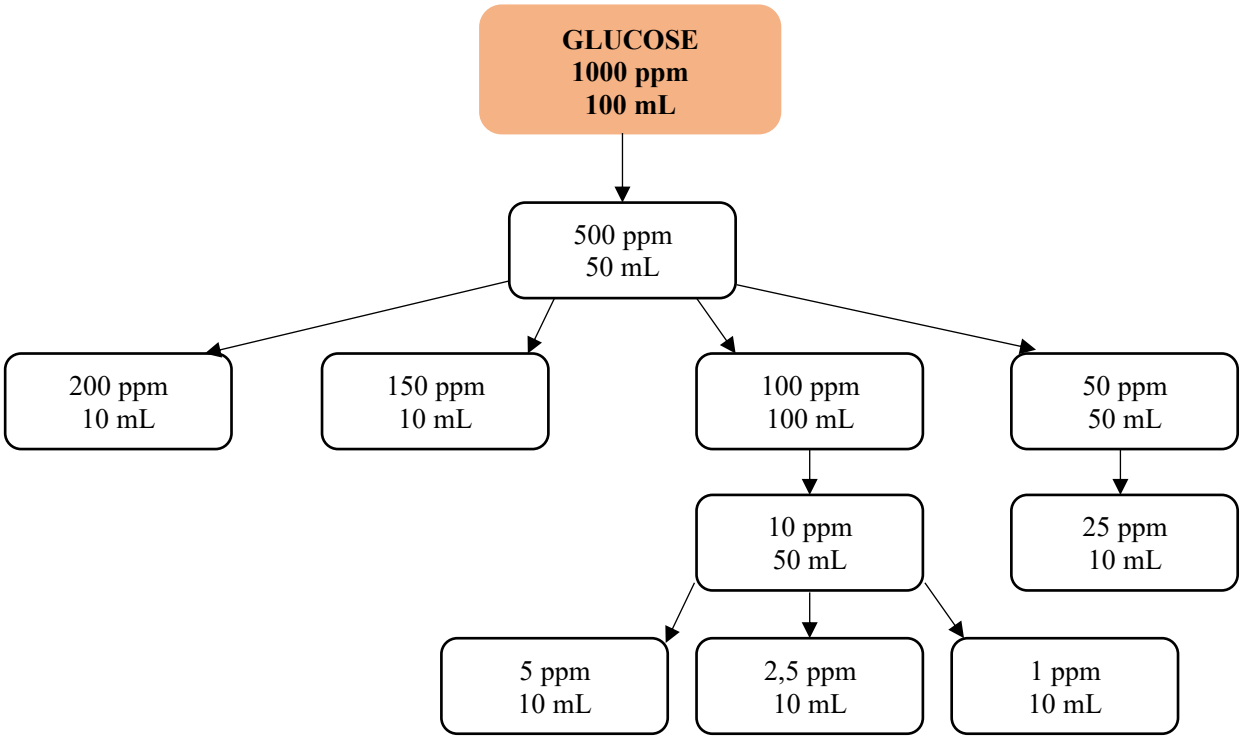


Figure 38 *- Preparation of glucose calibration line*

To perform the reaction that will give some color to the sample in order to apply the colorimetric method, it was also necessary to prepare a solution of phenol (Sigma-Aldrich), since one mL (5% volume) was necessary for each assay, each one carried out in triplicate. Therefore, 100 mL were prepared, by weighing 5 grams of phenol and diluting it in Milli-Q water in a volumetric flask. After that, by mean of an automatic pipette, 1 mL of the sample and 1 mL of phenol were measured and put in assay tubes. Two blanks were also prepared, using Milli-Q water. After the explained procedure, 5 mL of sulfuric acid (Scharlab, S.L.) was added in each tube, sealed by a plastic cap and slowly agitated. After 10 minutes, samples were vortexed and put in a bath at 30°C for 20 minutes. It is important when performing this type of analysis that the calibration line and the samples are analyzed on the same day, using the same solution of phenol, since many errors derive from its preparation process. The resulting colorimetric scale is shown in Figure 39. At higher concentration, a darker coloration appeared.

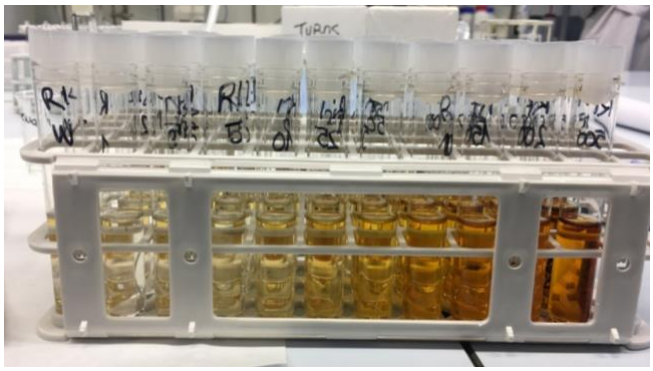


Figure 39 *- Test tubes after performing reaction*

A SPECORD 200 PLUS Diode-Array spectrophotometer (Analytik Jena AG) was used to measure the absorbance at each concentration at a wavelength of 490 nm. Two glass cuvettes were used, one

used as a reference (blank sample) and the other used for the different samples. Once all results were recollected, a calibration line, concentration vs absorbance, was evaluated.
Exploiting this calibration line, six different samples were analyzed to determine the sugar content (Table 17):

***Table 17** - Extracts analyzed in terms of sugar content*

PLE Wine lees 100°C, 60 % EtOH, 1 cycle, 5 minutes
UAE Wine lees 60 % EtOH, 0,1% HCl, 30 minutes
PLE Olive pomace 100°C, 60 % EtOH, 1 cycle, 5 minutes
UAE Olive pomace 60 % EtOH, 0,1% HCl, 30 minutes
PLE Wine lees 100°C, 60 % EtOH, 2 cycles, 5 minutes
UAE Wine lees 60 % EtOH, 0,1% HCl, 30 minutes

UAE of wine lees was analyzed twice because each analysis was performed the same day as the corresponding PLE. In this way, a comparison between the two techniques was made possible. Different dilution factors were used for each sample depending on each singular case to make sure that the measured absorbance would fit in the concentration range of the calibration line. In this case, dilution is an important factor, since the aim of the colorimetric method is to avoid that the natural color of the sample covers the one derived from reaction, thus affecting the result.

6. Results and discussion

6.1 Extraction processes results

PLE and UAE were performed at the same time for wine lees and olive pomace, respectively. To compare the performance of both extraction techniques, the total area of 280 nm was evaluated by using HPLC. This area is proportional to the total phenolic concentration. To evaluate this concentration, gallic acid calibration curve was used: concentration of total phenolic compounds was expressed in g gallic acid equivalents (GAE) per kilogram of dry residue.

6.1.1 PLE performance results to obtain the best operational conditions

UAE optimal operative conditions were already assessed by the research group; further tests were made for PLE, instead. Starting from 1 cycle of extraction of 5 minutes, all combinations of solvent (ethanol) percentage (40, 60, 80 %) and temperature (80, 100, 120 ºC) were tested for the two matrices (olive pomace and wine lees). Each sample extraction lasted 13 minutes, although only 5 minutes were of actual extraction, since the remaining time was dedicated to the heating of the cell to reach the desired temperature in each case.

Olive pomace and wine lees extraction results by using PLE technique are displayed in Tables 18 and 19, respectively.

Table 18 *- Olive pomace PLE results*

$g_{extract(GAE)}/kg_{residue}$ **OLIVE POMACE (Borges)**			
Temperature (°C)	**80**	**100**	**120**
40 % EtOH	4,19 ± 0.38	4,74 ± 0.36	4,59 ± 0.39
60 % EtOH	4,54 ± 0.22	4,69 ± 0.27	4,67 ± 0.01
80 % EtOH	2,87 ± 0.49	2,32 ± 0.40	3,68 ± 0.73

Table 19 *- Wine lees PLE results*

$g_{extract(GAE)}/kg_{residue}$ **WINE LEES (Bodega Torres)**			
Temperatura (°C)	**80**	**100**	**120**
40 % EtOH	1,08 ± 0.09	1,30 ± 0.22	1,12 ± 0.09
60 % EtOH	1,82 ± 0.04	1,93 ± 0.10	1,75 ± 0.04
80 % EtOH	1,36 ± 0.29	1,74 ± 0.29	2,17 ± 0.10

As observed in table 18 and 19, both cases reveal that best extraction conditions were found to be at 100°C with a solvent composition of 40% of ethanol for olive pomace and 60% of ethanol for wine lees. In the case of wine lees extraction, using 80% of ethanol at 120°C gave higher yields in terms of total area and later of concentration; however, when comparing the different chromatograms, some peaks would disappear, leading to a change in the chromatogram: this can be due to degradation of polyphenols and other compounds that could suffer at a higher temperature. For this reason, the specific value obtained at 120ºC did not prove reliable, and best conditions were considered to be at 100°C using a solvent with 60 % of ethanol.

Moreover, 3D surface response graphics of PLE extraction results exhibited the overall yield of the extraction at different conditions. Figure 40 shows the results of the olive pomace extraction as function of temperature and solvent percentage.

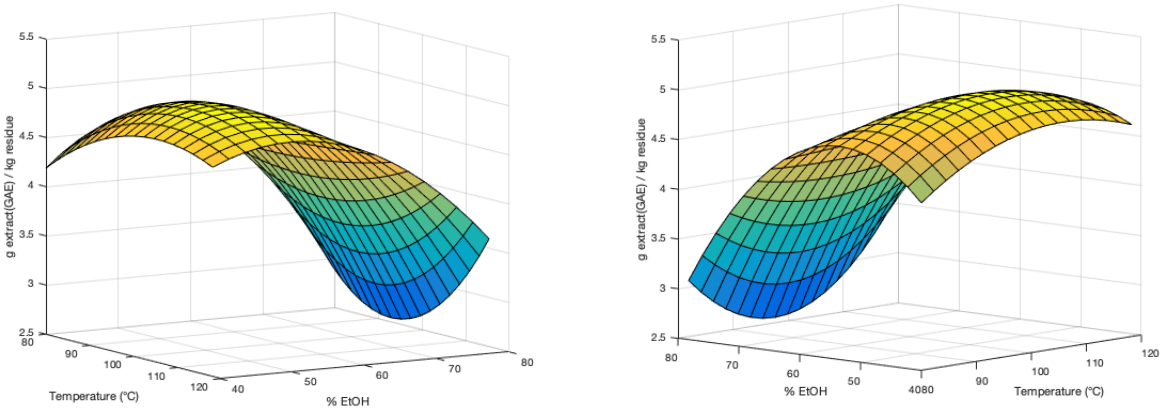


Figure 40 *- 3D surface representation of olive pomace PLE results*

Figure 40 is related to the grams of gallic acid equivalents (GAE) per gram of dry residue calculated from the area of each corresponding chromatogram at the different parameters studied for PLE extraction. By means of the 3D surface graph, it is possible to see how the quantity of extracted compounds decrease significantly when operating with a higher percentage of ethanol, while the difference is not so obvious with the change in temperature. In fact, apart from the high decrease when using an ethanol percentage equal to 80%, it is evident that the extraction needs to be operated with 40% of ethanol to obtain the highest concentration. Using this composition results cost effective by using the lowest quantity of ethanol possible. In an abovementioned work, Lozano-Sánchez and coworkers (2014) found the best extraction conditions to be with a solvent composition of EtOH/H_2O 50/50 (*v/v*)., working at 120°C. The differences in temperature and composition from the present results can be due to the different residual matrixes having various compositions depending on the conditions at which they have been obtained.

Figure 41 shows the corresponding chromatogram of the sample extracted at 100 ºC using 40% ethanol:

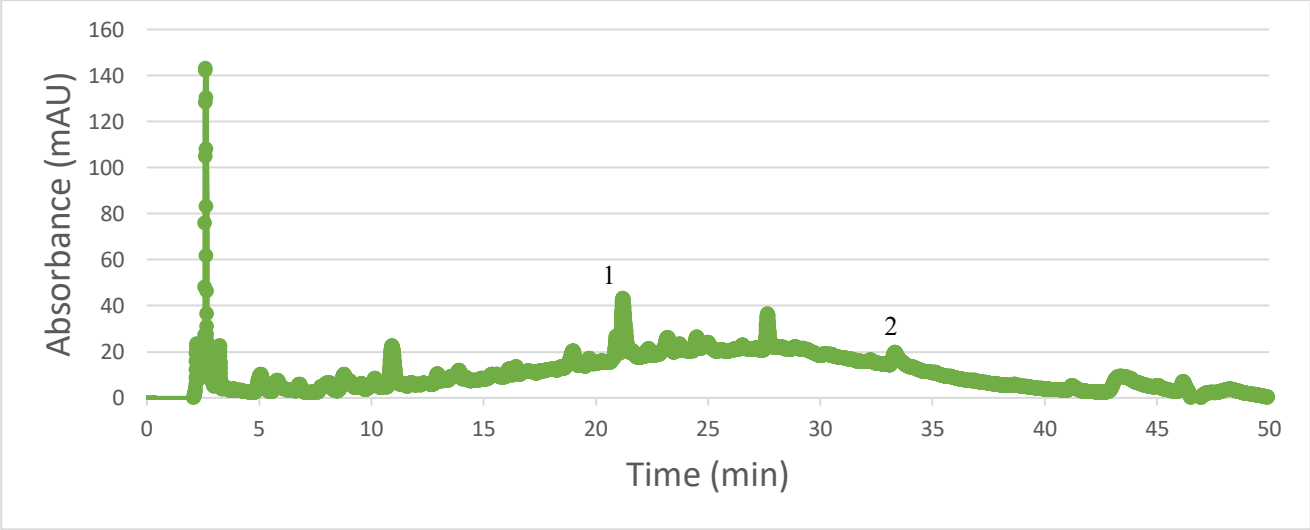


Figure 41 *- PLE Olive pomace chromatogram (370 nm) working at 100°C, 40 % EtOH, 1 cycle during 5 minutes*

From the chromatogram showed in Figure 41, it was possible to identify two polyphenols, luteolin (1) and apigenin (2), because its retention time.

On the other hand, 3D surface response graphics were also obtained for wine lees extracts (Figure 42).

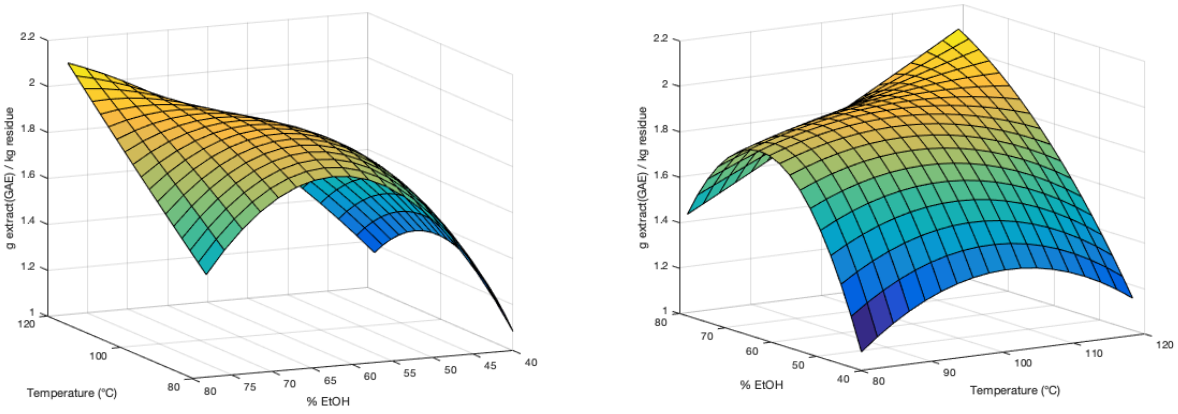


Figure 42 *- 3D surface representation of wine lees PLE results*

The unusual increment of area when operating at 120°C can be easily noticed in Figure 42. Moreover, the 3D surface graph shows the yields of extraction as a function of EtOH percentage. While the calculated area did not present high variations with temperature, the extraction yield highly depended on the composition of the solvent. Maximum yield of extraction was obtained when the composition was EtOH/H_2O 60/40 (*v/v*).

Again, Figure 43 shows the corresponding chromatogram of the sample extracted at 100 ºC using 60 % ethanol:

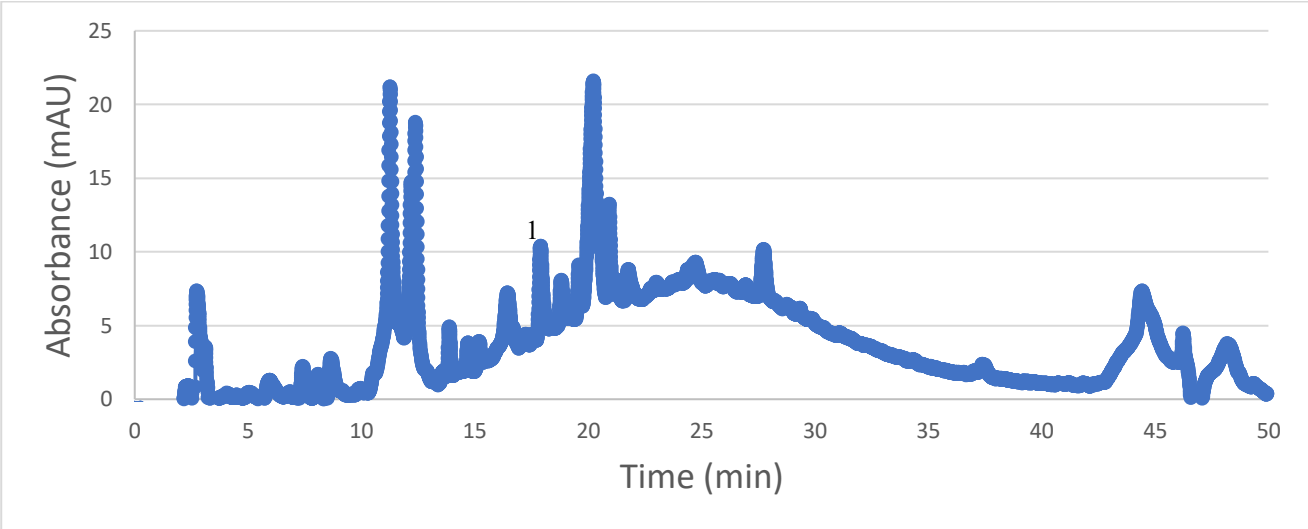


Figure 43 *- PLE Wine lees chromatogram (280 nm) working at 100°C, 60% EtOH, 1 cycle during 5 minutes*

In this case, as it can be seen in Figure 43, the only polyphenol that could be identified was the rutin (1). Other peaks were identified, but further analysis is necessary to see the compound they correspond with.

Once the optimal % ethanol and temperature extraction conditions were obtained, the influence of extraction time and number of cycles was also studied. In this case, tests were held with durations of 5, 10 and 15 minutes and with a number of cycles going from 1 up to 3. While for olive pomace extraction the best conditions were the same as previously encountered (5 minutes and 1 cycle), wine lees extraction showed higher yield when the number of cycles was increased at two, with a duration of five minutes. The mean total area in this case resulted to be 9804 ± 1089 mAU, with a corresponding concentration of 2±0.23 $g_{extract(GAE)}/kg_{residue}$. However, difference is minimum, operating with one cycle would lead to consume less solvent, decreasing its cost.

Figure 44 shows the corresponding chromatogram for wine less extraction at 100°C, using 60 % ethanol for 5 minutes and 2 operation cycles:

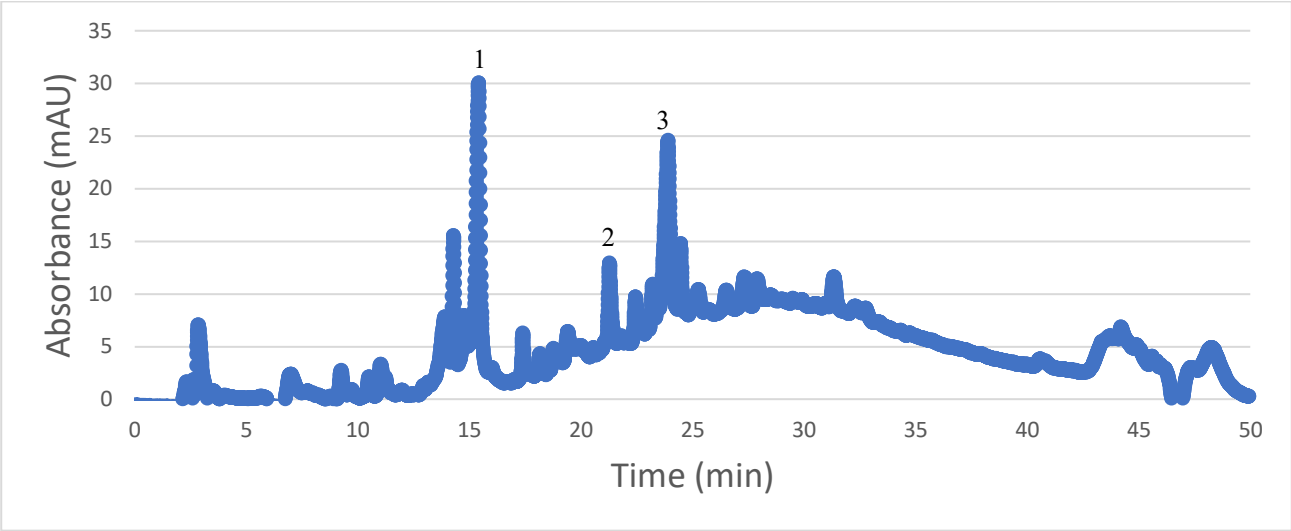


***Figure 44** - PLE Wine lees 100°C, 60% EtOH, 2 cycles during 5 minutes*

As can be observed in Figure 44, caffeic acid (1), rutin (2) and hesperidin (3) were individuated by means of the HPLC chromatograph of the extracted sample at the optimal conditions tested.

6.1.2 Comparison of PLE and UAE performances

As commented, UAE tests were held in parallel, to compare the two performances. Thus, Table 20 displays the corresponding results while extracting polyphenols from olive pomace and wine lees.

***Table 20** - UAE results (working conditions from Table 10)*

	$g_{extract(GAE)}/ kg_{residue}$
UAE Olive pomace	4.12 ± 0.17
UAE Wine lees	3 ± 0.16

Finally, a summary of best conditions for both matrixes and the two extraction techniques used is displayed in Table 21.

***Table 21** - Optimal extraction conditions for PLE and UAE*

Matrix	Technique	Optimal conditions
Olive pomace	PLE	EtOH/H_2O 40/60 *(v/v)* 100°C 1 cycle 5 minutes
	UAE	EtOH/H_2O/HCl 60/39,9/0,1 *(v/v/v)* 30 minutes
Wine lees	PLE	EtOH/H_2O 60/40 *(v/v)* 100°C 2 cycles 5 minutes
	UAE	EtOH/H_2O/HCl 80/19,5/0,5*(v/v/v)* 30 minutes

As presented in Table 21, best conditions were found for the extraction of each matrix. For wine lees PLE, the solvent composition to be used resulted EtOH/H_2O 60/40 *(v/v);* for olive pomace, the composition resulted EtOH/H_2O 40/60 *(v/v)*.

Furthermore, to find the most effective extraction technique, performances of UAE and PLE were compared, in order to realize which gave the best yield, also considering aspects like reproducibility, use of solvent, easiness of implementation and possibility to scale up at an industrial scale. Figure 45

shows the comparison between the two techniques for olive pomace extracts which were both performed at optimum conditions (see Table 21).

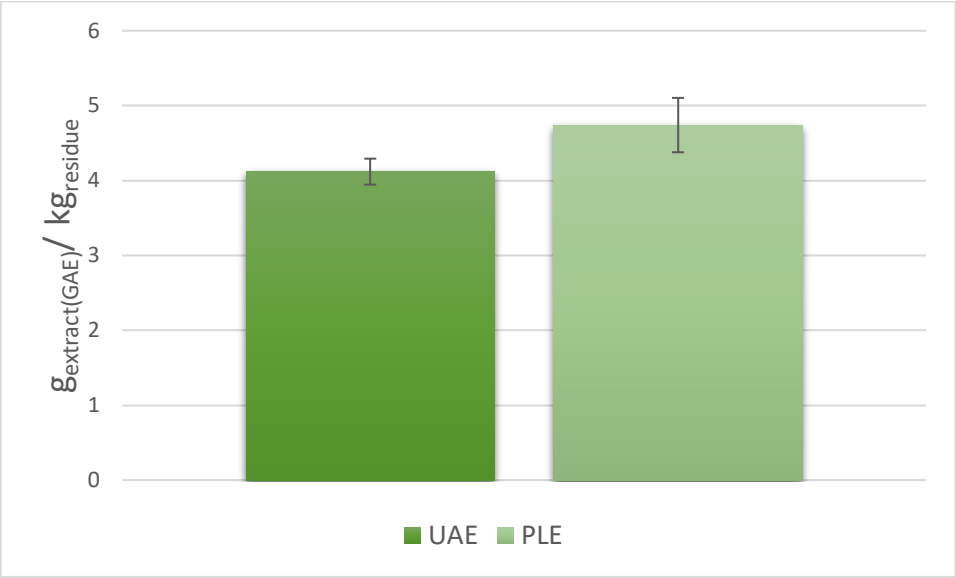


Figure 45 *- PLE and UAE of olive pomace working at the optimal conditions*

As demonstrated in Figure 45, PLE is the technique that gave a higher yield, with a HPLC chromatogram area of 22524 ± 828 mAU, corresponding to a concentration of 4.74±0.36 $g_{extract(GAE)}$/ $kg_{residue}$, compared to a lower area obtained using UAE, 14832 ± 625 mAU corresponding to 4.12±0.17 $g_{extract(GAE)}$/ $kg_{residue}$. PLE/UAE (%) is equal to 150.
A comparison was made also between wine lees extracts using both techniques, as shown in Figure 46.

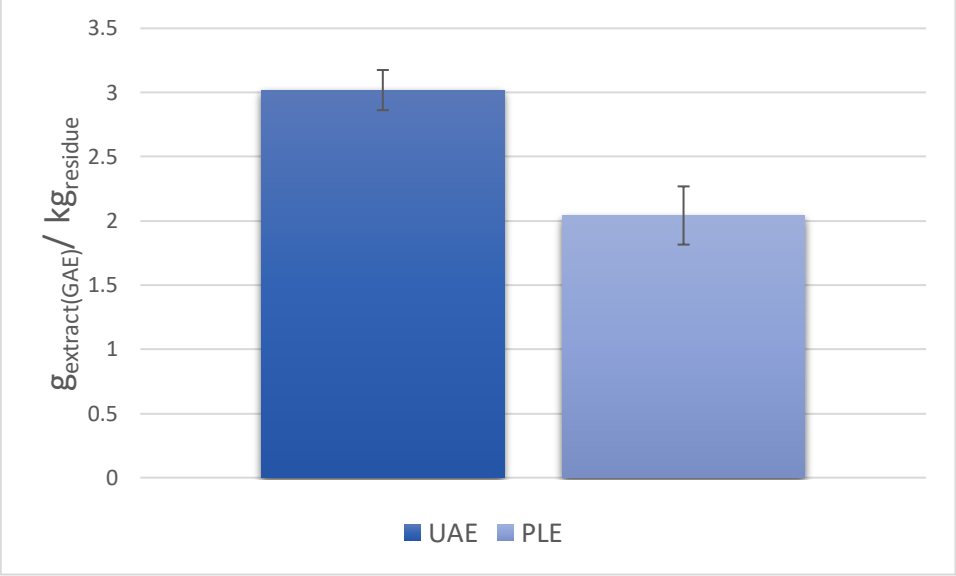


Figure 46 *- PLE and UAE of wine lees working at the optimal conditions*

In this case, as can be observed in Figure 46, UAE permitted a higher extraction (PLE/UAE (%) was equal to 84). Tao and coworkers (2014) also implemented this technique on this specific matrix and in their study they also used ethanol as a solvent. The optimal solvent composition EtOH/H_2O 50/50 (*v/v*), was revealed to be very different from the one used in the present study. This difference can depend on the fact that the total polyphenolic content can vary drastically and depends on the residue

itself. Different productive conditions, climatic conditions and time of recollection can explain the difference in yield. About PLE, this technique was performed on another solid residue, grape pomace, using water by Poveda and coworkers (2018): even if in the present case it did not result the most performing one, it would be interesting to compare UAE performance with the one of PLE when implemented with water. The advantages would lay in the use of a non-toxic, cheaper solvent. This opens new perspectives for the implementation of these extractive techniques on wine lees.

If comparing the extraction results of the two matrixes, it seems that olive pomace results a more promising source of polyphenols.

UAE is an easier technique to be implemented, compared to PLE. In fact, UAE is one of the most promising alternatives for the extraction of phenolic compounds, since reduction of solvent could be achieved. For that, UAE could be a feasible option for industrial applications.

6.2 Extracts analysis

Once the extraction process was completed, sugar content and polyphenols identification of obtained extracts were carried out.

6.2.1 Sugar content evaluation within the extracts

Once the extracts were obtained at optimal operative conditions, evaluation of sugar content was performed.

Absorbance at different glucose concentration was measured: the calibration line was extrapolated from values of concentration going from 1 up to 150 mg/L. Low standard deviation values proved a good reproducibility. An r_2 of 0,9992 proved that the linear model properly fitted the data.

The obtained calibration line was then used to evaluate sugar content of the above-mentioned samples, for which it was necessary to make dilutions. Figure 47 shows resulting samples after reaction with phenol and sulfuric acid.

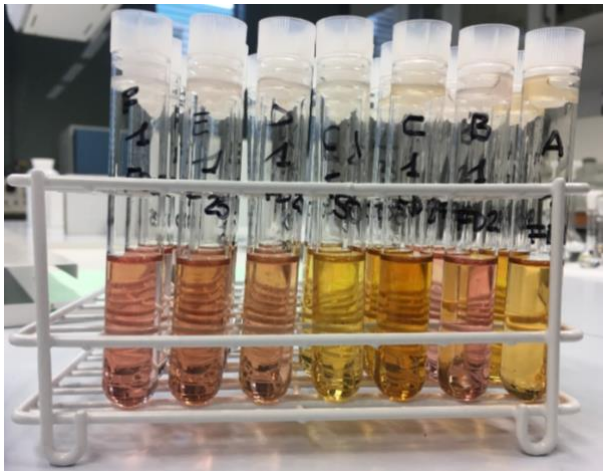


***Figure 47** - Samples after reaction*

For each repetition absorbance was measured: reproducibility in each measurement was proved by a relative low value of standard deviation. Then, sugar concentration could be calculated by means of the calibration line. Figure 48 shows the sugar content in g of sugars per kilogram of residue of wine lees and olive pomace extracts when using UAE (at optimal conditions from Table 10) and PLE (1 cycle, 5 minutes).

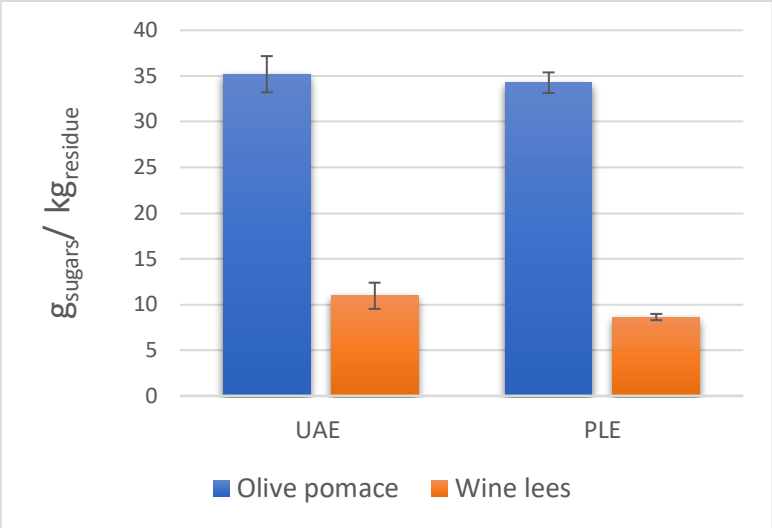


Figure 48 *- Sugar content in olive pomace and wine lees extracts*

As be observed in Figure 48, both extraction techniques allowed a high extraction of sugars. PLE olive pomace extracts had a concentration of 34,3±0,5 g_{sugars}/ $kg_{residue}$, while sugar concentration in the corresponding UAE extracts resulted 35,2±2 g_{sugars}/ $kg_{residue}$. On the contrary, there was a slightly higher difference between the wine lees extracts when PLE was performed in one cycle of five minutes: concentration in PLE resulted 8,6±0,34 g_{sugars}/ $kg_{residue}$, and it resulted slightly higher in UAE, 10,9±1,4 g_{sugars}/ $kg_{residue}$. Finally, it can be reported that the sugar content resulted much higher in olive pomace's extracts.

Notably, PLE extraction was also performed in two cycles of five minutes. In fact, these extraction conditions were found to be the optimum for wine lees extraction (Table 21). In this case, a higher sugar extraction yield for wine lees was obtained. Sugar concentration of this extract assumed slightly higher values, confirming a higher extraction capacity deducted from the HPLC analysis. Resulting concentration was 11,4±0,4 g_{sugars}/ $kg_{residue}$.

Figure 49 shows the change in concentration when operative conditions in PLE were changed (1 or 2 cycles), along with the comparison with UAE extract's concentration for wine lees extraction.

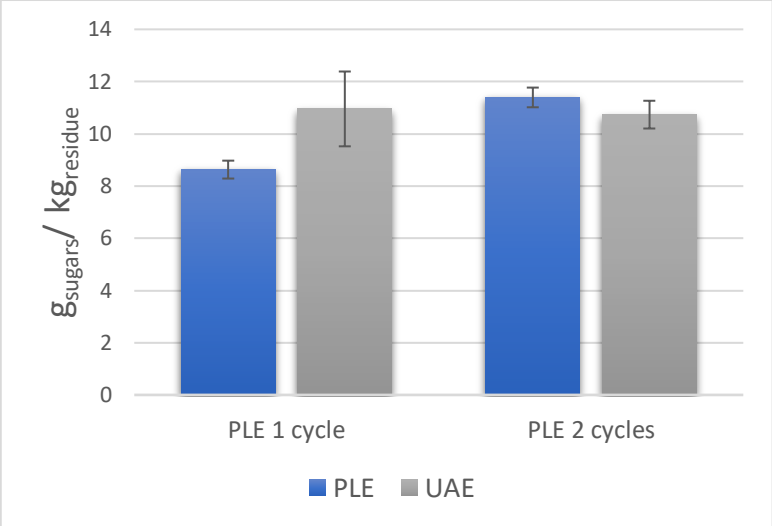


Figure 49 *- Changes in wine lees extracts' sugar content in the extract when the number of cycles in PLE is increased*

As can be seen in Figure 49, an increase in the number of cycles resulted in a higher sugar concentration in the extract; when implementing PLE with two cycles, a higher sugar extraction was obtained, consequentially there was a higher difference in the two technique performances.

The following steps will consist of an attempt at separating and purifying the extracts themselves by membrane techniques. This preliminary analysis will permit the comparison of sugar contents between extracts and membrane permeates, to see whether this separation step could be implemented not only to separate target polyphenols, but also to purify them from polysaccharides.

6.2.2 Identification of polyphenols present within the extracts

To be able to process polyphenols' mixtures by micro and nanofiltration, and to mimic real extracts, it was firstly necessary to individuate those polyphenols present within the extracts and determine its concentration. To do so, analysis by HPLC-DAD was not sufficient, since it wouldn't allow a precise analysis. Therefore, HPLC coupled with mass spectrometry (HPLC-MS) was used to identify each polyphenolic compound and its concentration. Tables 22 and 23 display the composition of each synthetic mixture (olive pomace and wine lees, respectively), with the corresponding extract technique and the maximum concentration at which they were found.

Table 22 *- Composition of the synthetic mixture miming olive pomace extract*

OLIVE POMACE	Retention time	Extraction type	Maximum concentration (ppm)
Homogentisic Acid	7.90	MAE (120°C)	2.89
2-(3,4 Dihydroxyphenyl) Ethyl Alcohol	8.75	MAE (90°C)	2.83
Caffeic Acid	13.15	UAE	1.13
***p*-Coumaric Acid**	15.83	UAE	0.78
Oleuropein	18.33	MAE (90°C)	2.70
Luteolin	22.18	MAE (90°C)	4.94

Table 23 *- Composition of the synthetic mixture miming wine lees extract*

WINE LEES	Retention time	Extraction type	Maximum concentration (ppm)
Gallic Acid	6.25	MAE (60°C)	1.45
3,4 Dihydroxybenzoic Acid	9.50	MAE (120°C)	2.54
Catechin	11.88	UAE	0.56
4-Hydroxybenzoic Acid	12.22	PLE (120°C, 40% EtOH)	0.66
Syringic Acid	13.22	MAE (120°C)	7.10
Ethyl Gallate	15.37	MAE (120°C)	2.15
Hesperidin	17.15	PLE (80°C, 40% EtOH)	10.60
Resveratrol	20.89	UAE	0.19
Quercetin	22.79	UAE	4.30

As it can be noticed in Tables 22 and 23, two mixtures could be outlined to mimic the extracts' composition. These polyphenols were found at the indicated concentrations when implementing the respective technique, evident in the extraction type column. In this way, a broad spectrum of polyphenols belonging to different families such as phenolic acids, flavanols, stilbenes and flavanones has been analyzed.

6.3 Polyphenol separation results by membranes

With the two synthetic mixtures obtained in accordance with the previous analysis (paragraph 6.2.2, Tables 22 and 23), membrane filtration tests were performed. To assess the performance of each separation technique, rejection was evaluated for each polyphenol following Equation 18. The fixed rejection target was 70%.

First of all, initial mixtures were analyzed by HPLC-DAD to evaluate the effective initial concentration. Tables 24 and 25 display the actual initial compositions for olive pomace and wine lees synthetic mixtures, respectively.

***Table 24** - Actual composition of polyphenols present in the initial mixture (OP)*

Olive pomace Standards	Real concentration (mg/L)
Homogentisic Acid	9
2-(3,4 dihydroxyphenyl) ethyl alcohol	17
Caffeic Acid	12
***p*-Coumaric acid**	4
Oleuropein	44
Luteolin	15

***Table 25** - Actual composition of polyphenols present in the initial mixture (WL)*

Wine lees Standards	Real concentration (mg/L)
Gallic acid	5
3,4 dihydroxybenzoic acid	8
Catechin	12
4-hydroxybenzoic acid	14
Syringic Acid	7
Ethyl Gallate	25
Hesperidin	7
Resveratrol	3
Quercetin	127

First, wine lees mixture was processed, followed by olive pomace mixture.

6.3.1 Membrane filtration results by centrifugation: wine lees and olive pomace

Centrifugation membrane filtration tests were performed for both mixtures at the same time. Experiments were carried out in duplicate.

On both mixtures, this type of filtration proved ineffective. Rejection was evaluated for all polyphenols: for the wine lees mixture each of them gave a rejection of 0%, indicating that all of them can pass through the membrane. For olive pomace mixture instead, some polyphenols showed rejection different from zero, but still very low. For example, homogentisic acid had the highest rejection percentage and was rejected by 8%. For this reason, this separation process did not lead to an effective separation nor a purification of any polyphenol.

6.3.2 Microfiltration results for wine lees extracts

Microfiltration was first performed on the wine lees mixture. Each repetition did not last more than 5 minutes; as expected, the higher the pore size the faster was the filtration. The only compounds that showed a rejection different from zero were gallic acid and quercetin. Figure 50 shows the

rejection of these two polyphenols, evaluated by the three microfiltration membranes (0.10, 0.22 and 0.45 µm pore size).

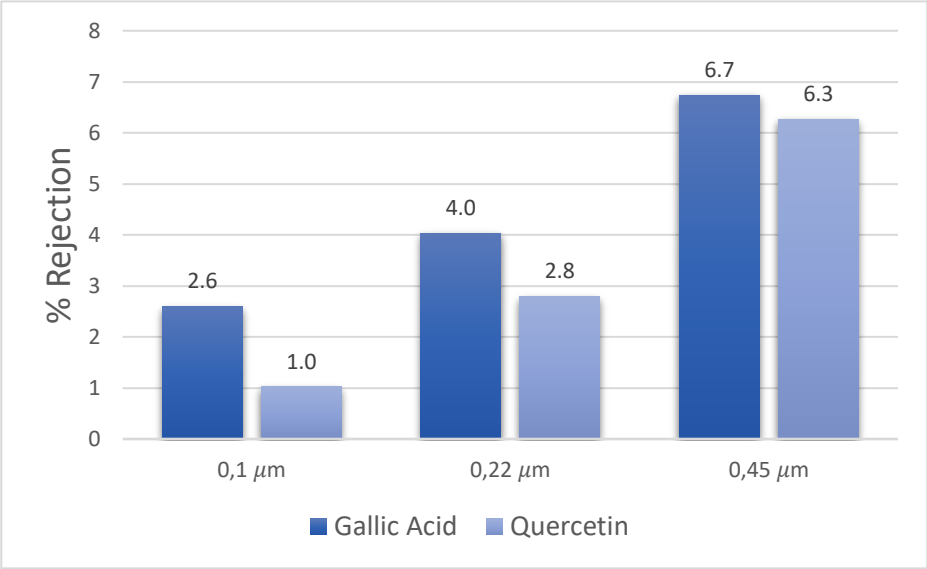


***Figure 50** - Microfiltration results for gallic acid and quercetin*

As observed in Figure 50, both polyphenols followed the same path; however, the trend was not the one expected: at a higher pore size, where rejection is expected to be lower, rejection principally increased. This fact can be due to various reasons: for example for gallic acid, rejection resulted 2,6 % with the 0,1 μm MF filter while it had a higher value for 4 % with the 0,22 μm MF filter and even higher (6.7 %) with the 0.45 μm filter. Transportation through MF membranes is mainly based on steric effects, however, other mechanisms by which molecules are transported have to be taken into account, such as electrical effects or interactions between different molecules. A deeper analysis of the results revealed that these differences between rejections were minimum, around the 2%. Drawing a simple graph of the rejection values showing the respective errors in terms of standard deviation allows to see that differences between the membranes are minimum (Figure 51). Hence, it can be concluded from these data they did not permit to perform any kind of separation with the target of 70 % of polyphenol rejection.

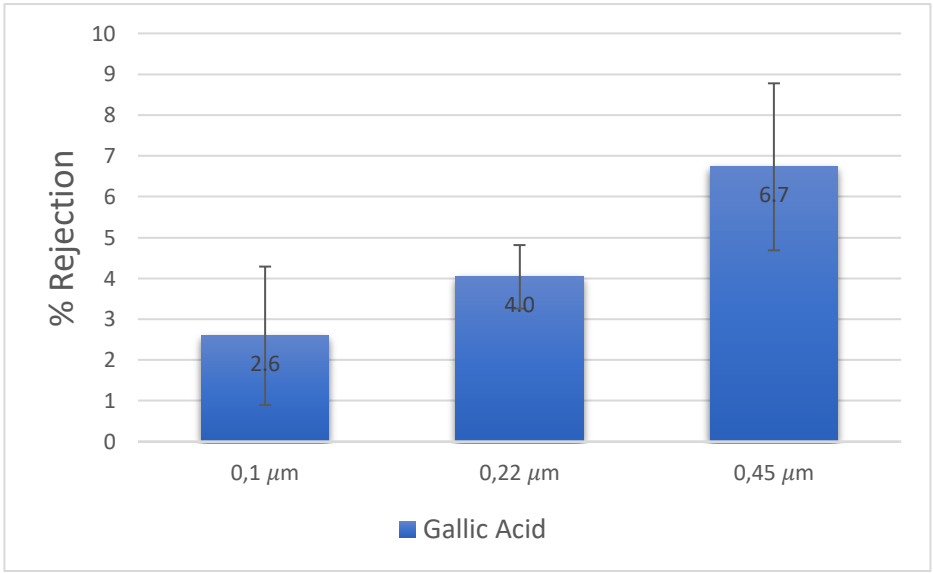


***Figure 51** - Analysis of microfiltration results for gallic acid*

As seen in Figure 51, the rejection values obtained by MF would not permit a conclusive outcome for the purposes of the present work, since it would not allow for isolation of a specific compound from the others. Therefore, microfiltration is not a feasible option for the isolation of this group of polyphenols. Giacobbo and coworkers (2015) worked using microfiltration; in their case, it was possible to separate 21% of polyphenols in the permeate, making this technique a suitable option for polyphenols recuperation. This discrepancy in the results could be due to the possible interactions that can occur among the different molecules: in this case, pure standards were used, while in the above-mentioned work raw effluent was directly processed (or indirectly, after a vacuum filtration/dilution step). With these considerations, it is evident that further studies are necessary to fully understand these interaction mechanisms and see how they can affect the performance of this technique.

6.3.3 Microfiltration results for olive pomace extracts

After processing the previous mixture, the equipment was fully cleaned to avoid any possible contamination. The duration of each repetition was the same as before (less than 5 minutes). None of the polyphenols showed rejection when filtered with the 0,45 μm filter. The other two membranes would reject some of the polyphenols, but, in this case, rejection did not show a specific trend as before. Homogentisic acid for example would permeate completely in both 0,1 and 0,45 μm filters, while be rejected by a 10% in the 0,22 μm. In the same way, 2-(3,4 dihydroxyphenyl) ethyl alcohol showed a 12% rejection with 0,1 μm filter, while total permeation was obtained with the others.
These rejection values were too low to obtain an effective isolation of the compounds; as before, microfiltration proved ineffective, hence it was not a feasible option for the separation and purification of the polyphenols present in the olive pomace extract.

6.3.4 Nanofiltration results for wine less extracts

Nanofiltration was performed by 5 different membranes (NF270, NF90, TFCS, TFC-HR and DURACID), whose performances were compared. The TFC-HR membrane test had to be repeated, since results between the two repetitions differed: peaks detected by HPLC were not the same for both permeate sample of the duplicate, making impossible a comparison between them. Despite further repetitions being made, reliable results were not obtained. Again, the two repetitions were not comparable in terms of polyphenols' concentrations. For this reason, TFC-HR membrane was discarded.

Quercetin showed high rejection in all tested membranes (around 100 %). However, other compounds showed distinct behaviors depending on the membrane used. Among all compounds in the wine lees extract, only catechin showed values of rejection. Only in one case (NF90), the rejection value resulted competitive with the one of quercetin and close to the target rejection selected. Results are shown in Figure 52:

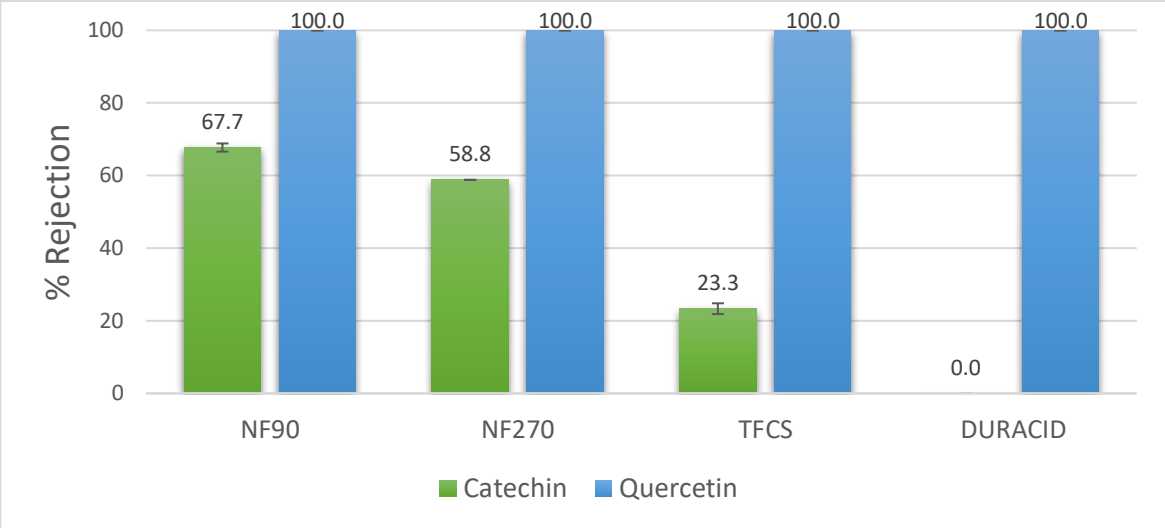


Figure 52 *–Rejection percentages for catechin and quercetin by nanofiltration*

As it can be noticed in Figure 52, a separation of quercetin from the other compounds can be obtained with all membranes in the rejected stream, a part with the NF90, where catechin shows a high rejection value (almost 70 %). However, only with the membrane DURACID, a complete separation of both polyphenols can be achieved: this makes this membrane the best candidate to perform an effective separation of quercetin compound.
A scheme of DURACID membrane performance is shown in Figure 53:

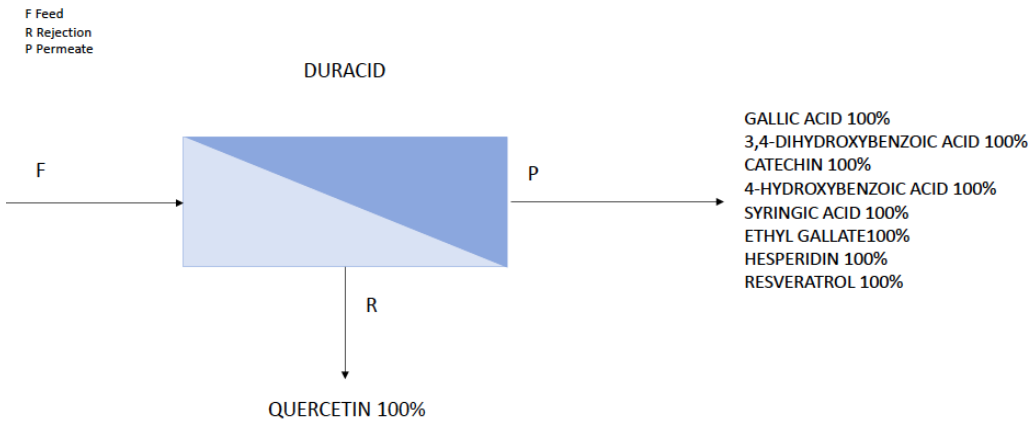


Figure 53 *- Scheme of the membrane system performance for DURACID membrane and corresponding result*

Quercetin is a polyphenol that belongs to flavonoids; it is an anticancer, antiviral, anti-inflammatory and anti-obesity agent (Maalik *et al.* 2014). One of the major challenges related to the administration of this compound is its bioavailability once administered: Conte R. and coworkers (2016) reported in their work the possibility of using solid lipid nanoparticles to increase oral availability. In another work, Souza and coworkers (2014), used quercetin for food applications.
Figure 54 shows the schemes of the other membrane systems used.

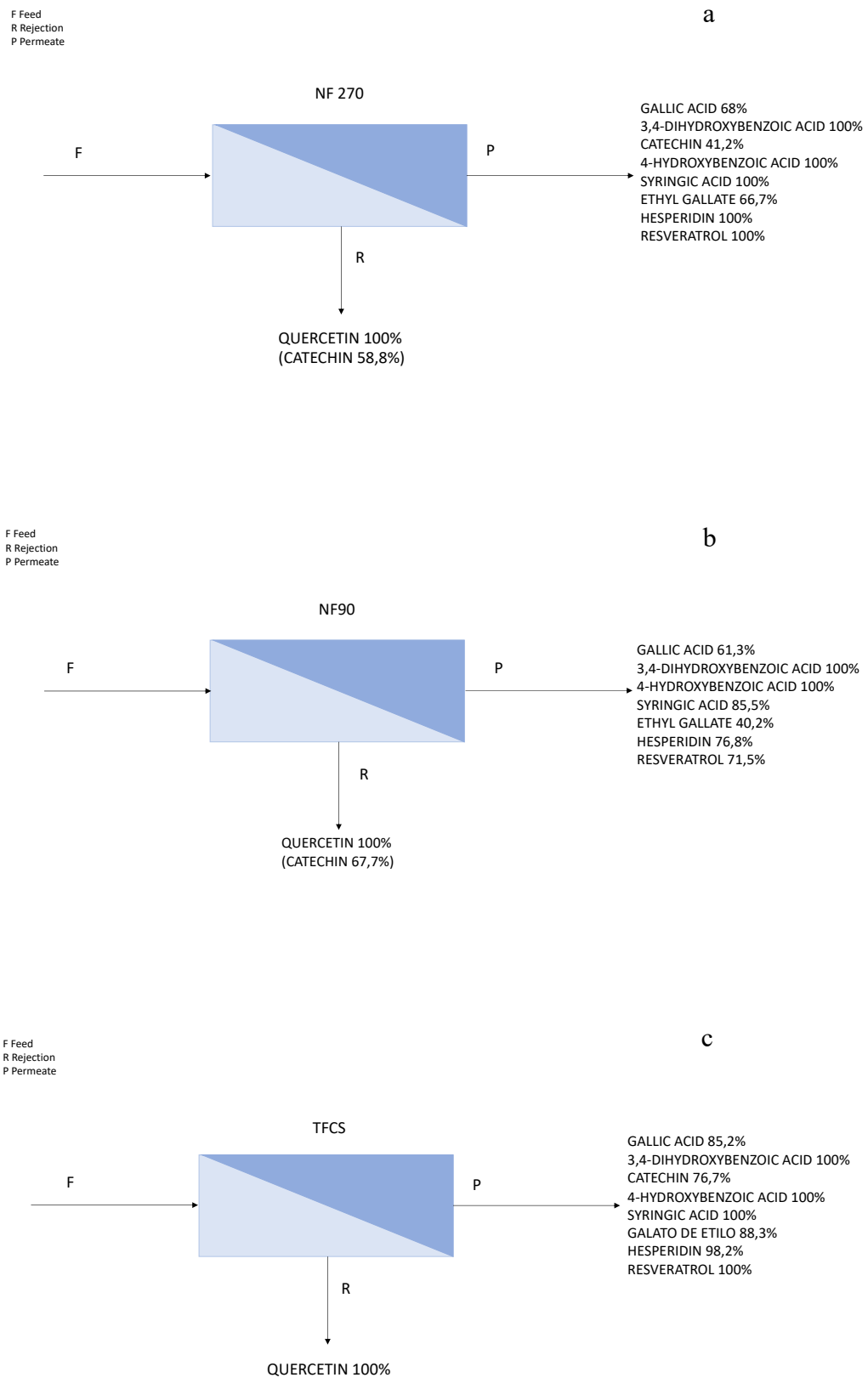


Figure 54 *- Nanofiltration membranes schemes a) NF270, b) NF90, c) TFCS*

As can be noticed by Figure 54, the performances of these membranes resulted as lower when compared to DURACID. All of them permit the quercetin to be rejected by a 100% rejection; however, in each case an impurity represented by the rejected catechin was present. Only DURACID permitted the quercetin to be obtained at the highest purity. Giacobbo and coworkers (2017) focused their attention in different studies on the recovery of polyphenols from wine lees by means of different membrane techniques. In their work, combined systems of MF, UF and NF were implemented: NF proved effective for the concentration of polyphenols in the retentate. They used diluted wine lees. The present results could be used as a basis for further studies, with the idea to combine different membranes, once assessed the performances of the outlined membrane with the real extracts.

6.3.5 Nanofiltration results for olive pomace extracts

Nanofiltration was then performed using the olive pomace synthetic mixture using the same membranes. With the change of tested sample, duration of the different filtrations also changed, supposedly due to variances between the two mixtures.
For these experiments, homogentisic acid was not detected by the HPLC. This could be due to degradation of this specific compound while preserving it, so this compound was not considered when analyzing nanofiltration results.
Results for nanofiltration tests are shown in Figure 55, where only compounds with higher rejection (luteolin, oleuropein and caffeic acid) were considered.

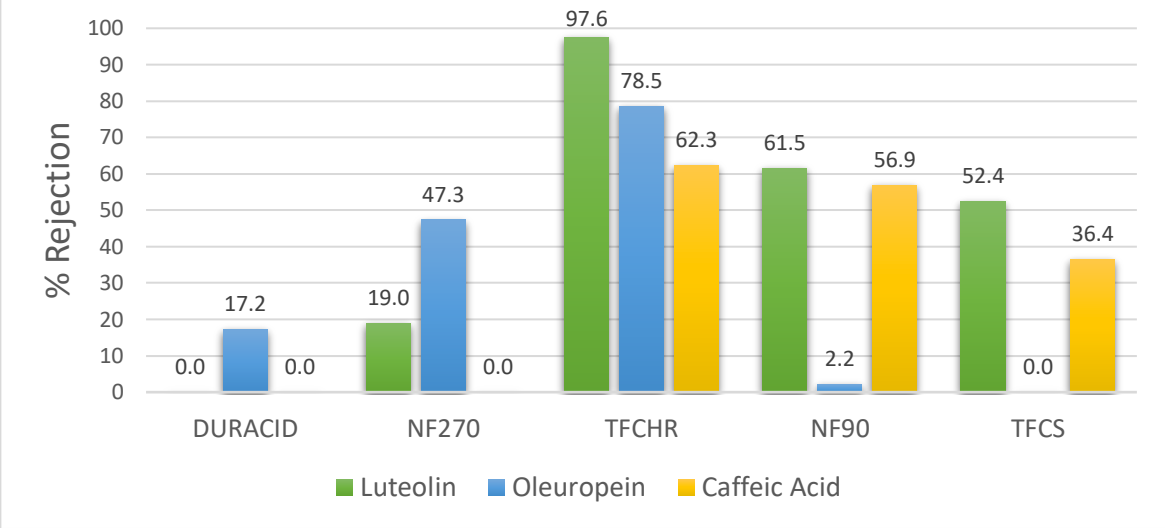


Figure 55 *- Nanofiltration results for luteolin, oleuropein and caffeic acid (% rejection)*

As observed in Figure 55, the polyphenol that showed the highest rejection was luteolin, when using the membrane TFC-HR (97.6 %), followed by oleuropein (78.5 %) and caffeic acid (62.3 %) with the same membrane.
A representative scheme when using TFC-HR membrane is shown in Figure 56.

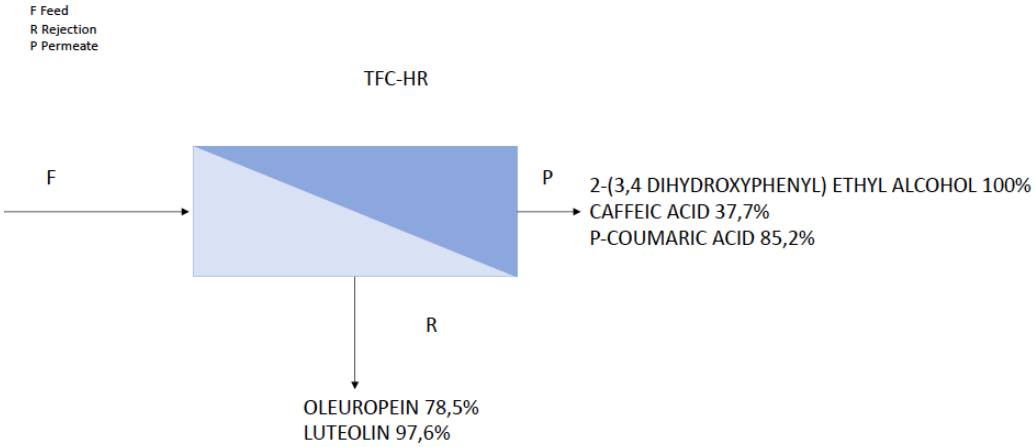
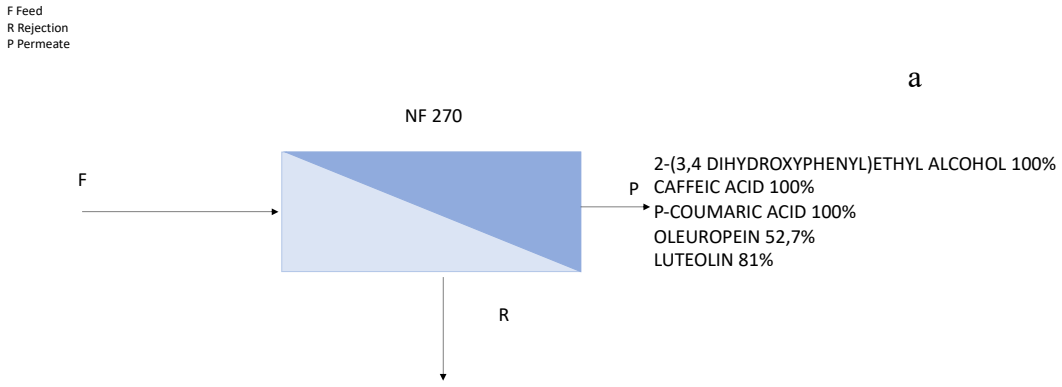


Figure 56 *- Scheme of the TFC-HR membrane system performance and corresponding result*

Figure 56 displays the composition of both product streams: concentrate and permeate. In this case, high 2-(3,4 dihydroxyphenyl) ethyl alcohol recovery was obtained (100% passage), along with the p-coumaric acid (85.2 % passage) in the permeate stream.
As previously observed, 2-(3,4 dihydroxyphenyl) ethyl alcohol (commonly named hydroxytyrosol) is one of the polyphenols found in higher quantities in olive oil residues. Thus, implementing its separation would be a great achievement. Irrefutably, TFC-HR nanofiltration membrane resulted to be the best candidate to perform an effective separation as it exploited both the permeate and the rejection streams.
On the other hand, oleuropein is well-known for having a blood pressure lowering effect among others, such as an anticancer, anti-inflammatory and antioxidant effect (Omar 2010).
Finally, luteolin is one of the most common flavonoids; it has been applied in form of micelles to treat gliomas; its hydrophobicity and its low bioavailability by oral administration are one of the biggest challenges for its application in this field. This study by Zheng and coworkers (2017) proved these micelles to have the potential to be applied in glioma chemotherapy.
Figure 57 shows the schemes of the other membrane systems used.



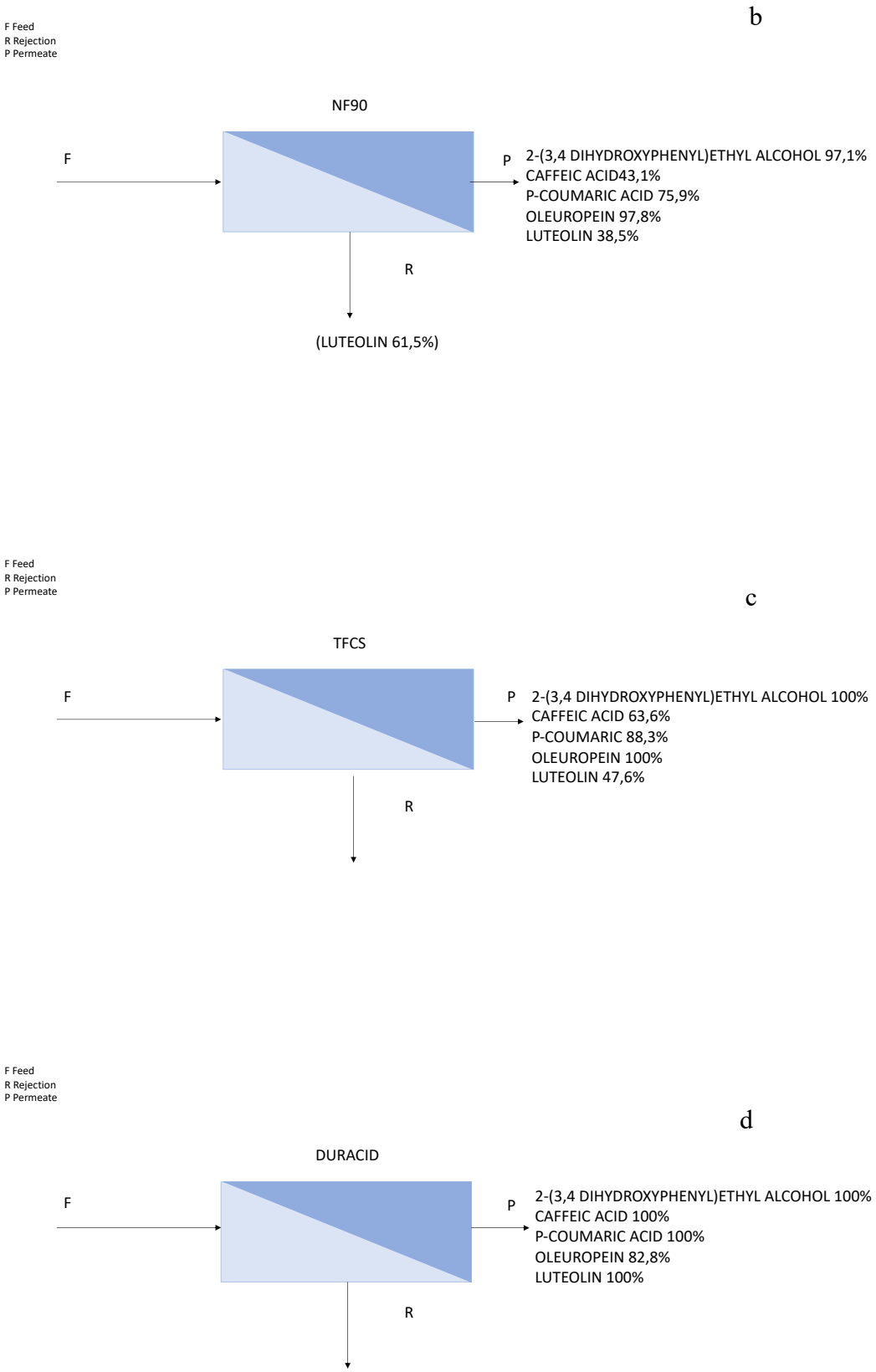


***Figure 57** - Nanofiltration membranes schemes a) NF270, b) NF90, c) TFCS, d) DURACID*

As perceived in Figure 57, three of the four membranes did not show significant rejection values. Only for membrane NF90 a rejection value of about 62% was outlined for luteolin; however, this value resulted lower than the threshold limit (set at 70%), so the membrane proved ineffective. This confirms that among the membrane tested only TFC-HR proved permitted significant rejection values to be obtained.

Conclusion

Polyphenols are currently being studied for their protective effects against different pathologies: exploiting food waste would allow not only their recovery, but also the management of these wastes. For these reasons, the investigation of extraction and separation techniques from agro-industries residues is nowadays playing an important role in the scientific community.

As previously mentioned, this work was developed at the Universitat Politècnica de Catalunya (UPC-EEBE) in Barcelona, supported by the Waste2Product project (CTM2014-57302-R) and the R2MIT project (CTM2017-85346-R) financed by the Spanish Ministry of Economy and Competitiveness (MINECO) and the Catalan Government (ref. 2017-SGR-312). This research was part of a bigger project, a doctoral thesis that aims at the recovery of certain polyphenols from two types of industrial waste, deriving from olive oil and wine production processes. It began with the identification of the polyphenols present within these two types of waste to understand the effectiveness of the extraction processes tested; HPLC has been exhaustively studied, as it is the selected method for the characterization of these extracts, leading to the methods used in the present research. In this phase, also different membranes started to be tested to find out the role they could play in the separation and purification of polyphenols. Once identified, the future aim is to use these membranes with real extracts.

In this work, PLE and UAE methods were studied for polyphenol extraction from olive pomace and wine lees. Best conditions were found for both extraction methods: the recommended techniques are PLE for the extraction of olive pomace and UAE for the extraction of wine lees. PLE for olive pomace permitted to obtain a concentration of 4,74±0,36 $g_{extract(GAE)}/kg_{residue}$ at the following conditions: EtOH/H_2O 40/60 (*v/v*) operating in one cycle of five minutes with a temperature of 100°C. On the other hand, UAE for wine lees gave a concentration of 3±0,16 $g_{extract(GAE)}/kg_{residue}$, at the following conditions EtOH/H_2O/HCl 80/19,5/0,5 (*v/v*) for 30 minutes. UAE is a technique that can be scaled up easily and could be used at industrial scale for the recovery of polyphenols from these matrixes.

Sugar concentration was higher in olive pomace's extracts (34,3±0,5 $g_{sugars}/ kg_{residue}$ UAE: 35,2±2 $g_{sugars}/ kg_{residue}$) than in those of wine lees (PLE: 8,6±0,34 $g_{sugars}/ kg_{residue}$ UAE: 10,9±1,4 $g_{sugars}/ kg_{residue}$). In the first case (olive pomace), between the two extraction techniques there was not a noticeable difference in terms of sugar extraction. In the second case (wine lees) the difference was slightly higher, with a higher extraction obtained when working with UAE. However, when increasing the number of PLE extractive cycles up to two, the difference reversed, and PLE extract resulted in a higher concentration, equal to 11,4±0,38 $g_{sugars}/ kg_{residue}$.

Membrane experiments permitted to select two membranes for the separation of specific polyphenols: DURACID permitted the quercetin to be separated in the rejection stream with a rejection of 100%, while TFC-HR allowed different polyphenols to be separated in both the permeate and the rejection volume. 2-(3,4 dihydroxyphenyl) ethyl alcohol permeated by a 100%, while luteolin could be effectively recovered in the retentate, with a 97.6 % of rejection.

Membrane experiments were performed using synthetic mixtures of selected polyphenols: exploiting this data would permit a correct membrane to be selected for future separation of real extracts, that would then be analyzed for both polyphenols and sugar content. This gives further perspectives for future work with the aim to purify polyphenols present within the optimum extracts.

Appendix

A. Environmental impact analysis

Due to the nature of the work, whose aim is to recover food residues to valorize them and find a solution for their disposal, the focus on the environmental impact analysis would be more focused on the positive effect of this work.

To perform the lab-scale experiments of this work, solid residues were created, such as pipette tips, gloves, syringes, filters: all these wastes were unavoidable in the process of performing each experiment, but these residues were properly recycled afterwards. The only liquid residues produced were obtained during the sugar content analysis and their disposal was made according to laboratory regulations, in the dedicated bin for acid solutions.

The impact that the two considered industries (olive and wine) have on the environment is well-known, and its reduction is one of the biggest challenges in recent years. Christ and Burritt (2013) in their review analyzed all the possible areas of environmental concern related to wine production, focusing also on the organic solid waste. Moldes and coworkers (2008) studied the effects of discharging wine lees on soils: they evaluated their chemical content and other properties such as pH. This residue contains many nutrients that could prove advantageous for plant growth and theoretically used as components for plant growing media. However, biological tests were carried out to assess their phytotoxicity: no germination was observed. In fact, recovery of industrial waste such as wine lees, is one of the key topics is playing an important role in modern industry: proper management of these wastes would permit an effective recovery, thus solving issues pertaining to disposal.

In the case of olive oil production waste, many works outline the phytotoxicity of polyphenols contained within solid and liquid wastes. In 2003, DellaGreca and coworkers proved the inhibition carried out by polyphenols on bacterial and algal growth. In a more recent work, Rusan and coworkers (2015) compared the phytotoxicity of olive mill waste water untreated and treated to reduce its polyphenolic content. The different treatments proved effective in reducing phytotoxicity. In some cases, they used membrane systems, therefore a recovery could be approached in this case, helping to reduce the environmental impact of this specific residue.

B. Economic analysis

A brief economic evaluation is provided in this section. Two different subsections are considered: experimental cost and personnel cost.

Experimental cost

Experimental cost comprises of both reactants and equipment costs. While the reactants costs depend on the quantities used, in the case of the equipment it is necessary to evaluate the initial cost, the effective time of use and the actual lifespan in order to calculate the equipment cost.
Table 26 displays the equipment costs in these terms.

Table 26 *- Equipment costs*

Equipment	Equipment cost (€)	Time of use (year)	Actual life (year)	Cost (€)
UAE	815	0.25	10	20.4
PLE	24000	0.25	10	600.0
HPLC	49600	0.33	10	1636.8
Spectrophotometer	8400	0.13	10	105.0
Balance	376	0.25	10	9.4
Filtration equipment	200	0.13	5	5.0

As can be seen in Table 26, the total equipment cost was 2894 €.

Then, it is necessary to evaluate cost of reactants, according to the quantities used, and cost of the lab material used. Tables 27 and 28 resume these costs, respectively.

Table 27 *- Reactants costs* (Fisher Scientific 2019)(Honeywell,2019)

Reactants	Quantity	Price (€)	Price (€/kg-L)	Quantity used	Final price (€)
Acetonitrile	1.00 L	25.50	25.50	1.00 L	25.50
Ethanol	2.50 L	85.82	34.33	1.00 L	34.33
Hydrochloric acid	1.00 L	25.50	25.50	0.02 L	0.51
Diatomaceous earth	500.00 g	119.00	0.24	110.00 g	26.18
Glucose	1.00 kg	62.72	62.72	0.002 kg	0.13
Phenol	1.00 kg	76.50	76.50	0.005 kg	0.38
MILLI Q Water	1.00 L	1.00	1.00	2.00 L	2.00
Solforic acid	2.50 L	77.56	31.02	0.27 L	8.38

Table 28 *- Material cost* (Fisher Scientific 2019)

Lab material	Units	Price (€)	Price (€/u)	Used units	Final price (€)
0,45 μm syringe filter	100.00	50.00	0.50	150.00	75.00
0,22 μm syringe filter	100.00	62.00	0.62	150.00	93.00
12 mL vials	100.00	50.00	0.50	150.00	75.00
2 mL vials	1000.00	150.00	0.15	200.00	30.00
Glass tubes	1.00	0.19	0.19	100.00	19.00
FALCON tubes 15 mL	500.00	160.00	0.32	60.00	19.20

FALCON tubes 25 mL	500.00	105.00	0.21	60.00	12.60
Syringes	100.00	12.95	0.13	50.00	6.48
Flask 5 mL	2.00	25.50	12.75	2.00	25.50
Flask 10 mL	2.00	25.75	12.88	6.00	77.25
Flask 50 mL	2.00	28.70	14.35	3.00	43.05
Flask 100 mL	2.00	33.80	16.90	2.00	33.80
Glass pipette 25 mL	6.00	25.75	12.88	1.00	13.59
Glass pipette 20 mL	6.00	52.85	8.81	1.00	8.81
Glass pipette 10 mL	6.00	46.35	7.73	1.00	7.73
Glass pipette 5 mL	6.00	45.50	7.58	5.00	37.92
Glass pipette 1 mL	12.00	60.45	5.04	1.00	5.04
Automatic pipette 10-1000 *µL*	1.00	119.79	119.79	1.00	119.79
Automatic pipette 0,5-5 mL	1.00	94.74	94.74	1.00	94.74
Beaker 1 L	10.00	121.00	12.10	1.00	12.10
Beaker 500 mL	10.00	77.00	7.70	2.00	15.40
Beaker 50 mL	10.00	46.95	4.70	1.00	4.70
Pipette tips	100.00	9.98	0.10	200.00	19.96

As observed in Tables 27 and 28, reactants total cost was equal to 97,40 € while lab material total cost was 888,54 €.
Moreover, membrane costs have to be added, displayed in Table 29.

Table 29 *- Membranes costs* (Fisher Scientific 2019)

Membranes	Price (€)	Price (€/u)	Units (u)	Cost (€)
NF270	103.44	20.68	1	20.68
NF90	131.35	26.27	1	26.27
DURACID	131.35	26.27	1	26.27
TFCS	150	30	1	30.00
TF-HR	150	30	1	30.00
Filter 0,1 µm	-	1.33	4	5.32
Filter 0,22 µm	-	1.62	4	6.48
Filter 0,22 µm	-	1,9	4	7.60
Centrifugation filters	77.80	9.73	4.00	38.90

As represented in Table 29, total cost for membranes was equal to 191.52 €.
Total experimental cost was 4071.47 €.

Personnel cost

The person in charge of carrying out experiments is one that coordinates and executes different tasks, reporting to a supervisor. Relative cost is displayed in Table 30.

***Table 30** - Author cost*

Author	Time (hours)	Price(€/h)	Price (€)
Bibliographic research	60	12	720
Experimentation	300	12	3600
Results	100	12	1200
Work redaction	80	12	960
Total	540		6480

At the author cost it is necessary to add the costs related to the director of the project. Price per hour would be approximately 25 €/h, since it is a role that implicates higher responsibilities and knowledge (Table 31).

***Table 31** - Director cost*

Director	Time (hours)	Price (€/h)	Price (€)
Project direction	90	25	2250

Personnel cost is in total 8730€.

Total Cost

The following graph in Figure 58 gives an overall view of total cost.

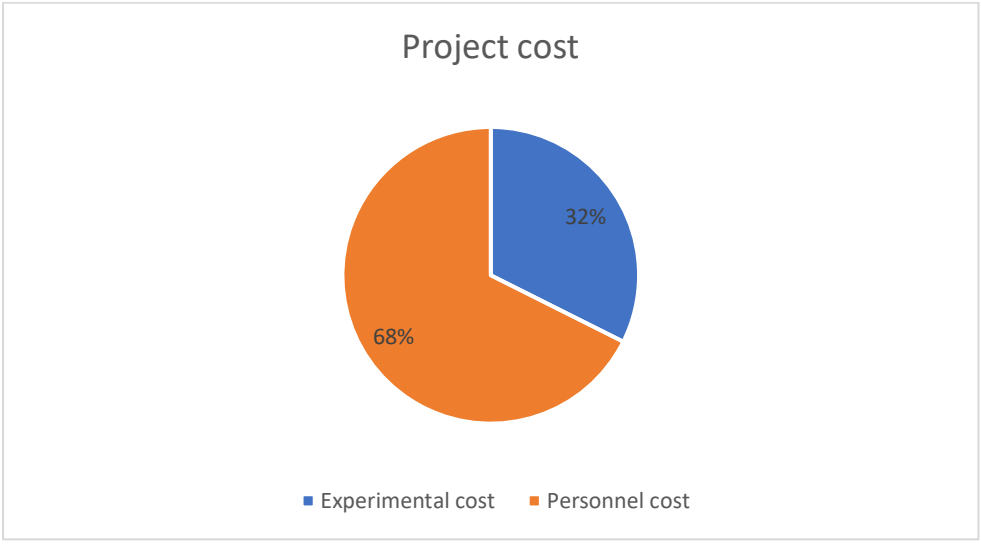


***Figure 58** - Summary of total project cost*

Notably, engineering costs are the largest contributor to the total cost of the project. Finally, the total cost of the project was around 12800 €.

References

Abbas M., Saeed F., Anjum F.M., Afzaal M., Tufail T., Muhammad S. B., Ishtiaq A., Hussain S. and Hafiz A. R. S. 2017. "Natural Polyphenols: An Overview." *International Journal of Food Properties* 20 (8): 1689–1699. https://doi.org/10.1080/10942912.2016.1220393.

Abdel-Razek A., Badr A. and Shehata M. 2017. "Characterization of Olive Oil By-Products: Antioxidant Activity, Its Ability to Reduce Aflatoxigenic Fungi Hazard and Its Aflatoxins." *Annual Research & Review in Biology* 14 (5): 1–14. https://doi.org/10.9734/arrb/2017/35065.

Acosta O., Vaillant F., Pérez A. M. and Dornier M. 2014. "Potential of Ultrafiltration for Separation and Purification of Ellagitannins in Blackberry (*Rubus Adenotrichus* Schltdl.) Juice." *Separation and Purification Technology* 125: 120–125. https://doi.org/10.1016/j.seppur.2014.01.037.

Aguiar J., Estevinho B. N. and Santos L. 2016. "Microencapsulation of Natural Antioxidants for Food Application – The Specific Case of Coffee Antioxidants – A Review." *Trends in Food Science and Technology* 58: 21–39. https://doi.org/10.1016/j.tifs.2016.10.012.

Aissa I., Kharrat N., Aloui F., Sellami M., Bouaziz M. and Gargouri Y. 2017. "Valorization of Antioxidants Extracted from Olive Mill Wastewater." *Biotechnology and Applied Biochemistry* 64 (4): 579–589. https://doi.org/10.1002/bab.1509.

Antoniolli A., Fontana A. R., Piccoli P. and Bottini R. 2015. "Characterization of Polyphenols and Evaluation of Antioxidant Capacity in Grape Pomace of the Cv. Malbec." *Food Chemistry* 178: 172–178. https://doi.org/10.1016/j.foodchem.2015.01.082.

Araújo M., Pimentel F. B., Alves R. C. and Oliveira M. B. P. P. 2015. "Phenolic Compounds from Olive Mill Wastes: Health Effects, Analytical Approach and Application as Food Antioxidants." *Trends in Food Science and Technology* 45 (2): 200–211. https://doi.org/10.1016/j.tifs.2015.06.010.

Barba F. J., Zhu Z., Koubaa M., Anderson S. S. and Vibeke O. 2016. "Green Alternative Methods for the Extraction of Antioxidant Bioactive Compounds from Winery Wastes and By-Products: A Review." *Trends in Food Science and Technology* 49: 96–109. https://doi.org/10.1016/j.tifs.2016.01.006.

Bazinet L. and Doyen A. 2017. "Antioxidants, Mechanisms, and Recovery by Membrane Processes." *Critical Reviews in Food Science and Nutrition* 57 (4): 677–700. https://doi.org/10.1080/10408398.2014.912609.

Beres C., Costa G. N. S., Cabezudo I., da Silva-James N. K., Teles A. S. C., Cruz A. P. G., Mellinger-Silva C., Tonon R. V., Cabral L. M. C. and Freitas S. P. 2017. "Towards Integral Utilization of Grape Pomace from Winemaking Process: A Review." *Waste Management* 68: 581–594. https://doi.org/10.1016/j.wasman.2017.07.017.

Brglez Mojzer E., Knez Hrnčič M., Škerget M., Knez Ž. and Bren U. 2016. "Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects." *Molecules* 21 (7): 1–38. https://doi.org/10.3390/molecules21070901.

Caballero A. S., Romero-García J. M., Castro E. and Cardona C. A. 2019. "Supercritical Fluid Extraction for Enhancing Polyphenolic Compounds Production from Olive Waste Extracts." *Journal of Chemical Technology and Biotechnology* 1–22. https://doi.org/10.1002/jctb.5907.

Caldas T. W., Mazza K. E. L., Teles A. S. C., Mattos G. N., Brígida A. I. S., Conte-Junior C. A., Borguini R. G., Godoy R. L. O., Cabral L. M. C. and Tonon R. V. 2018. "Phenolic Compounds Recovery from Grape Skin Using Conventional and Non-Conventional Extraction Methods." *Industrial Crops and Products* 111: 86–91. https://doi.org/10.1016/j.indcrop.2017.10.012.

Carocho M., Barreiro M. F., Morales P. and Ferreira I. C. F. R. 2014. "Adding Molecules to Food, Pros and Cons: A Review on Synthetic and Natural Food Additives." *Comprehensive Reviews in Food Science and Food Safety* 13 (4): 377–399. https://doi.org/10.1111/1541-4337.12065.

Cassano A., Conidi C., Galanakis C. M. and Castro-Muñoz R. 2016. "Recovery of Polyphenols

from Olive Mill Wastewaters by Membrane Operations." *Membrane Technologies for Biorefining*, 163–187. https://doi.org/10.1016/B978-0-08-100451-7.00007-4.
Cassano A., Conidi C., Giorno L. and Drioli E. 2013. "Fractionation of Olive Mill Wastewaters by Membrane Separation Techniques." *Journal of Hazardous Materials* 248–249 (1): 185–193. https://doi.org/10.1016/j.jhazmat.2013.01.006.
Cassano A., De Luca G., Conidi C. and Drioli E. 2017. "Effect of Polyphenols-Membrane Interactions on the Performance of Membrane-Based Processes. A Review." *Coordination Chemistry Reviews* 351: 45–75. https://doi.org/10.1016/j.ccr.2017.06.013.
Castro-Muñoz R., Yáñez-Fernández J. and Fíla V. 2016. "Phenolic Compounds Recovered from Agro-Food by-Products Using Membrane Technologies: An Overview." *Food Chemistry* 213: 753–762. https://doi.org/10.1016/j.foodchem.2016.07.030.
Christ K. L. and Burritt R. L. 2013. "Critical Environmental Concerns in Wine Production: An Integrative Review." *Journal of Cleaner Production* 53: 232–242. https://doi.org/10.1016/j.jclepro.2013.04.007.
Conte R., Calarco A., Napoletano A., Valentino A., Margarucci S., Di Cristo F., Di Salle A. and Peluso G. 2016. "Polyphenols Nanoencapsulation for Therapeutic Applications." *Journal of Biomolecular Research & Therapeutics* 5 (2): 1-13. https://doi.org/10.4172/2167-7956.1000139.
Cory H., Passarelli S., Szeto J., Tamez M. and Mattei J. 2018. "The Role of Polyphenols in Human Health and Food Systems: A Mini-Review." *Frontiers in Nutrition* 5: 1–9. https://doi.org/10.3389/fnut.2018.00087.
Cutrim C. S. and Sloboda Cortez M. A. 2018. "A Review on Polyphenols: Classification, Beneficial Effects and Their Application in Dairy Products." *International Journal of Dairy Technology* 71 (3): 564–578. https://doi.org/10.1111/1471-0307.12515.
Da Porto C., Natolino A. and Decorti D. 2015. "The Combined Extraction of Polyphenols from Grape Marc: Ultrasound Assisted Extraction Followed by Supercritical CO_2 Extraction of Ultrasound-Raffinate." *LWT - Food Science and Technology* 61 (1): 98–104. https://doi.org/10.1016/j.lwt.2014.11.027.
Daso A. P. and Okonkwo O. J. 2015. "Conventional Extraction Techniques: Soxhlet and Liquid-Liquid Extractions and Evaporation." *Analytical Separation Science* 5: 1437–1468. https://doi.org/10.1002/9783527678129.assep053.
DellaGreca M., Monaco P., Pinto G., Pollio A., Previtera L. and Temussi F. 2003. "Phytotoxicity of Low-Molecular-Weight Phenols from Olive Mill Waste Waters." *Bulletin of Environmental Contamination and Toxicology* 67 (3): 352–359. https://doi.org/10.1007/s001280132.
Dermeche S., Nadour M., Larroche C., Moulti-Mati F. and Michaud P. 2013. "Olive Mill Wastes: Biochemical Characterizations and Valorization Strategies." *Process Biochemistry* 48 (10): 1532–1552. https://doi.org/10.1016/j.procbio.2013.07.010.
Destandau E., Michel T. and Elfakir C. 2013. "Microwave-Assisted Extraction" in "Natural Product Extraction: Principles and Applications" *Royal Society of Chemistry* https://doi.org/10.1039/9781849737579-00113.
Díaz-Reinoso B., Moure A., González J. and Domínguez H. 2017. "A Membrane Process for the Recovery of a Concentrated Phenolic Product from White Vinasses." *Chemical Engineering Journal* 327: 210–217. https://doi.org/10.1016/j.cej.2017.06.088.
Drosou C., Kyriakopoulou K., Bimpilas A., Tsimogiannis D. and Krokida M. 2015. "A Comparative Study on Different Extraction Techniques to Recover Red Grape Pomace Polyphenols from Vinification Byproducts." *Industrial Crops and Products* 75: 141–149. https://doi.org/10.1016/j.indcrop.2015.05.063.
Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A. and Smith F. 1956 "Colorimetric Method for Determination of Sugars and Related Substances." *Analytical Chemistry* 28 (3): 350–356.
El-Abbassi A., Kiai H. and Hafidi A. 2012. "Phenolic Profile and Antioxidant Activities of Olive Mill Wastewater." *Food Chemistry* 132 (1): 406–412.

https://doi.org/10.1016/j.foodchem.2011.11.013.
Elkacmi R., Kamil N. and Bennajah M. 2017. “Separation and Purification of High Purity Products from Three Different Olive Mill Wastewater Samples.” *Journal of Environmental Chemical Engineering* 5 (1): 829–37. https://doi.org/10.1016/j.jece.2017.01.005.
Fernández M. L. A., Espino M., Gomez F. J. V. and Silva M. F. 2018. “Novel Approaches Mediated by Tailor-Made Green Solvents for the Extraction of Phenolic Compounds from Agro-Food Industrial by-Products.” *Food Chemistry* 239: 671–678. https://doi.org/10.1016/j.foodchem.2017.06.150.
Fisher Scientific. 2019. “Fisher Scientific: Lab Equipment and Supplies.” https://www.fishersci.ca/ca/en/home.html.
Galanakis C. M., Markouli E. and Gekas V. 2013. “Recovery and Fractionation of Different Phenolic Classes from Winery Sludge Using Ultrafiltration.” *Separation and Purification Technology* 107: 245–251. https://doi.org/10.1016/j.seppur.2013.01.034.
Galanakis C. M., Tsatalas P. and Galanakis I. M. 2018. “Implementation of Phenols Recovered from Olive Mill Wastewater as UV Booster in Cosmetics.” *Industrial Crops and Products* 111: 30–37. https://doi.org/10.1016/j.indcrop.2017.09.058.
Giacobbo A., Bernardes A. M. and De Pinho M. N. 2017. “Sequential Pressure-Driven Membrane Operations to Recover and Fractionate Polyphenols and Polysaccharides from Second Racking Wine Lees.” *Separation and Purification Technology* 173: 49–54. https://doi.org/10.1016/j.seppur.2016.09.007.
Giacobbo A., Meneguzzi A., Bernardes A. M. and De Pinho M. N. 2017. “Pressure-Driven Membrane Processes for the Recovery of Antioxidant Compounds from Winery Effluents.” *Journal of Cleaner Production* 155: 172–178. https://doi.org/10.1016/j.jclepro.2016.07.033.
Giacobbo A., Matos Do Prado J., Meneguzzi A., Bernardes A. M. and De Pinho M. N. 2015. “Microfiltration for the Recovery of Polyphenols from Winery Effluents.” *Separation and Purification Technology* 143: 12–18. https://doi.org/10.1016/j.seppur.2015.01.019.
Gupta R., Mishra A. K. and Pathak A. K. 2014. “Supercritical Fluid Technology a Boon for Pharmaceutical Particle Manufacturing. ” https://doi.org/10.13140/RG.2.1.3632.7286.
Hajji T., Mansouri S., Vecino-Bello X., Cruz-Freire J. M., Rezgui S. and Ferchichi A. 2018. “Identification and Characterization of Phenolic Compounds Extracted from Barley Husks by LC-MS and Antioxidant Activity in Vitro.” *Journal of Cereal Science* 81: 83–90. https://doi.org/10.1016/j.jcs.2018.03.008.
“Honeywell.” https://shop-lab-honeywell.com/products/chemicals
Ioannou-Ttofa L., Michael-Kordatou I., Fattas S. C., Eusebio A., Ribeiro B., Rusan M., Amer A. R. B., Zuraiqi S., Waismand M., Linder C., Wiesman Z., Gilron J. and Fatta-Kassinos D. 2017. “Treatment Efficiency and Economic Feasibility of Biological Oxidation, Membrane Filtration and Separation Processes, and Advanced Oxidation for the Purification and Valorization of Olive Mill Wastewater.” *Water Research* 114: 1–13. https://doi.org/10.1016/j.watres.2017.02.020.
Ioannou L. A., Michael C., Vakondios N., Drosou K., Xekoukoulotakis N. P., Diamadopoulos E. and Fatta-Kassinos D. 2013. “Winery Wastewater Purification by Reverse Osmosis and Oxidation of the Concentrate by Solar Photo-Fenton.” *Separation and Purification Technology* 118: 659–669. https://doi.org/10.1016/j.seppur.2013.07.049.
IOC (International Olive Council). 2018. “Olive Oil Production and Consumption up by 1 Million Tonnes in the Last 25 Years - Olive Oil Production and Consumption up by 1 Million Tonnes in the Last 25 Years - International Olive Council.” *Olive Oil Production and Consumption up by 1 Million Tonnes in the Last 25 Years*. http://www.internationaloliveoil.org/news/view/698-year-2018-news/1071-olive-oil-production-and-consumption-up-by-1-million-tonnes-in-the-last-25-years.
Jiang J. and Xiong Y. L. 2016. “Natural Antioxidants as Food and Feed Additives to Promote Health Benefits and Quality of Meat Products: A Review.” *Meat Science* 120: 107–117.

https://doi.org/10.1016/j.meatsci.2016.04.005.
Jurcevic I. L., Dora M., Guberovic I., Petras M., Rimac Brncic S. and Dikic D. 2017. "Polyphenols from Wine Lees as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidaemia." *Food Technology and Biotechnology* 55 (1): 109–116. https://doi.org/10.17113/ft b.55.01.17.4894.
Khoo H. E., Azlan A., Teng Tang S. and Meng Lim S. 2017. "Anthocyanidins and Anthocyanins: Colored Pigments as Food, Pharmaceutical Ingredients, and the Potential Health Benefits." *Food & Nutrition Research* 61 (1): 1–21. https://doi.org/10.1080/16546628.2017.1361779.
Kopsahelis N., Dimou C., Papadaki A., Xenopoulos E., Kyraleou M., Kallithraka S., Kotseridis Y., Papanikolaou S. and Koutinas A. A. 2017 "Refining of Wine Lees and Cheese Whey for the Production of Microbial Oil, Polyphenol-rich Extracts and Value-added Co-products. " *Journal of Chemical Technology and Biotechnology*, 93(1): 257–268, https://doi.org/10.1002/jctb.5348 .
Kumar K., Ajar N. Y., Vinod K., Pritesh V. and Harcharan S. D. 2017. "Food Waste: A Potential Bioresource for Extraction of Nutraceuticals and Bioactive Compounds." *Bioresources and Bioprocessing* 4 (18): 1–14. https://doi.org/10.1186/s40643-017-0148-6.
La Scalia G., Micale R., Cannizzaro L. and Marra F. P. 2017. "A Sustainable Phenolic Compound Extraction System from Olive Oil Mill Wastewater." *Journal of Cleaner Production* 142: 3782–3788. https://doi.org/10.1016/j.jclepro.2016.10.086.
Leouifoudi I., Harnafi H. and Zyad A. 2015. "Olive Mill Waste Extracts: Polyphenols Content, Antioxidant, and Antimicrobial Activities." *Advances in Pharmacological Sciences* 2015: 1–11. https://doi.org/10.1155/2015/714138.
Lipnizki F. 2010. "Cross-Flow Membrane Applications in the Food Industry" in "Membrane Technology, Vol. 3: Membranes for Food Applications. " *Wiley-VCH Verlag GmbH & Co KGa* Edited by Peinemann K. V., Nunes S. P., Giorno L.
Lozano-Sánchez J., Castro-Puyana M., Mendiola J. A., Segura-Carretero A., Cifuentes A. and Ibáñez E. 2014. "Recovering Bioactive Compounds from Olive Oil Filter Cake by Advanced Extraction Techniques." *International Journal of Molecular Sciences* 15 (9): 16270–16283. https://doi.org/10.3390/ijms150916270.
Maalik A., Khan F. A., Mumtaz A., Mehmood A., Azhar S., Atif M., Karim S., Altaf Y. and Tariq I. 2014. "Pharmacological Applications of Quercetin and Its Derivatives: A Short Review." *Tropical Journal of Pharmaceutical Research* 13 (9): 1561–1566. https://doi.org/10.4314/tjpr.v13i9.26.
Machado A. P. D. F., Pasquel-Reátegui J. L., Fernández Barbero G. and Martínez J. 2015. "Pressurized Liquid Extraction of Bioactive Compounds from Blackberry (Rubus Fruticosus L.) Residues: A Comparison with Conventional Methods." *Food Research International* 77: 675–683. https://doi.org/10.1016/j.foodres.2014.12.042.
Martínez L., Ros G. and Nieto G. 2018. "Hydroxytyrosol: Health Benefits and Use as Functional Ingredient in Meat." *Medicines* 5: 1-13. https://doi.org/10.3390/medicines5010013.
Mateo J. J. and Maicas S. 2015. "Valorization of Winery and Oil Mill Wastes by Microbial Technologies." *Food Research International* 73: 13–25. https://doi.org/10.1016/j.foodres.2015.03.007.
Medina-Torres N., Ayora-Talavera T., Espinosa-Andrews H., Sánchez-Contreras A. and Pacheco N. 2017. "Ultrasound Assisted Extraction for the Recovery of Phenolic Compounds from Vegetable Sources." *Agronomy* 7 (3): 47. https://doi.org/10.3390/agronomy7030047.
Moldes A. B., Vázquez M., Domínguez J. M., Díaz-Fierros F. and Barral M. T. 2008. "Negative Effect of Discharging Vinification Lees on Soils." *Bioresource Technology* 99 (13): 5991–5996. https://doi.org/10.1016/j.biortech.2007.10.004.
Niedzwiecki A., Mohd W. R., Kalinovsky T. and Rath M. 2016. "Anticancer Efficacy of Polyphenols and Their Combinations." *Nutrients* 8 (9): 1–17. https://doi.org/10.3390/nu8090552.

Nimse S. B. and Dilipkumar P. 2015. “Free Radicals, Natural Antioxidants, and Their Reaction Mechanisms.” *RSC Advances* 5 (35): 27986–28006. https://doi.org/10.1039/c4ra13315c.

Nunes M. A., Pimentel F. B., Costa A. S. G, Alves R. C. and Oliveira M. B. P . P. 2016. “Olive By-Products for Functional and Food Applications: Challenging Opportunities to Face Environmental Constraints.” *Innovative Food Science and Emerging Technologies* 35: 139–148. https://doi.org/10.1016/j.ifset.2016.04.016.

OIV. 2018. “OIV Statistical Report on World Vitiviniculture.” *International Organisation of Vine and Wine*, 27. https://doi.org/64/19/6835 [pii]\n10.1158/0008-5472.CAN-04-1678.

Oliveira M. and Duarte E. 2016. “Integrated Approach to Winery Waste: Waste Generation and Data Consolidation.” *Frontiers of Environmental Science and Engineering* 10 (1): 168–176. https://doi.org/10.1007/s11783-014-0693-6.

Omar S. H. 2010. “Oleuropein in Olive and Its Pharmacological Effects.” *Scientia Pharmaceutica* 78 (2): 133–154. https://doi.org/10.3797/scipharm.0912-18.

Oroian M. and Escriche I. 2015. “Antioxidants: Characterization, Natural Sources, Extraction and Analysis.” *Food Research International* 74: 10–36. https://doi.org/10.1016/j.foodres.2015.04.018.

Panche A. N., Diwan A. D. and Chandra S. R. 2016. “Flavonoids: An Overview.” *Journal of Nutritional Science* 5: 1–15. https://doi.org/10.1017/jns.2016.41.

Pathakoti K., Manubolu M. and Huey M. H. 2017. “Nanostructures: Current Uses and Future Applications in Food Science.” *Journal of Food and Drug Analysis* 25 (2): 245–253. https://doi.org/10.1016/j.jfda.2017.02.004.

Pérez-Bibbins B., Torrado-Agrasar A., Salgado J. M., Pinheiro de Souza Oliveira R. and Domínguez J. M. 2015. “Potential of Lees from Wine, Beer and Cider Manufacturing as a Source of Economic Nutrients: An Overview.” *Waste Management* 40: 72–81. https://doi.org/10.1016/j.wasman.2015.03.009.

Picó Y. 2017. “Pressurized Liquid Extraction of Organic Contaminants in Environmental and Food Samples.” *Comprehensive Analytical Chemistry* 76: 83–110. https://doi.org/10.1016/bs.coac.2017.03.004.

Pintać D., Majkić T., Torović L., Orčić D., Beara I., Simin N., Mimica–Dukić N. and Lesjak M. 2018. “Solvent Selection for Efficient Extraction of Bioactive Compounds from Grape Pomace.” *Industrial Crops and Products* 111: 379–390. https://doi.org/10.1016/j.indcrop.2017.10.038.

Poveda J. M., Loarce L., Alarcón M., Díaz-Maroto M. C. and Alañón M. E. 2018. “Revalorization of Winery By-Products as Source of Natural Preservatives Obtained by Means of Green Extraction Techniques.” *Industrial Crops and Products* 112: 617–625. https://doi.org/10.1016/j.indcrop.2017.12.063.

Pulido J.M.O. 2016. “A Review on the Use of Membrane Technology and Fouling Control for Olive Mill Wastewater Treatment.” *Science of the Total Environment* 563–564: 664–675. https://doi.org/10.1016/j.scitotenv.2015.09.151.

Quiroz J. Q. , Torres A. C., Ramirez L. M., Garcia M. S., Gomez G. C. and Rojas J. 2019. “Optimization of the Microwave-Assisted Extraction Process of Bioactive Compounds from Annatto Seeds (Bixa Orellana L.).” *Antioxidants* 8 (2): 1–11. https://doi.org/10.3390/antiox8020037.

Ramos P., Santos S. A. O., Guerra Â. R., Guerreiro O., Felício L., Jerónimo E., Silvestre A. J. D., Pascoal Neto C. and Duarte M. 2013. “Valorization of Olive Mill Residues: Antioxidant and Breast Cancer Antiproliferative Activities of Hydroxytyrosol-Rich Extracts Derived from Olive Oil by-Products.” *Industrial Crops and Products* 46: 359–368. https://doi.org/10.1016/j.indcrop.2013.02.020.

Rodrigues F., Pimentel F. B. and Oliveira M. B. P. P. 2015. “Olive By-Products: Challenge Application in Cosmetic Industry.” *Industrial Crops and Products* 70: 116–124. https://doi.org/10.1016/j.indcrop.2015.03.027.

Roman M.J., Decker E.A. and Goddard J.M. 2016. “Biomimetic Polyphenol Coatings for Antioxidant Active Packaging Applications.” *Colloids and Interface Science Communications* 13: 10–13. https://doi.org/10.1016/j.colcom.2016.06.002.

Roselló-Soto E., Koubaa M., Moubarik A., Lopes R. P., Saraiva J. A., Boussetta N., Grimi N. and Barba F. J. 2015. “Emerging Opportunities for the Effective Valorization of Wastes and By-Products Generated during Olive Oil Production Process: Non-Conventional Methods for the Recovery of High-Added Value Compounds.” *Trends in Food Science and Technology* 45 (2): 296–310. https://doi.org/10.1016/j.tifs.2015.07.003.

Ruiz E., Romero-García J. M., Romero I., Manzanares P., Negro M. J. and Castro E. 2017. “Olive-Derived Biomass as a Source of Energy and Chemicals.” *Biofuels, Bioproducts and Biorefining* 11 (6): 1077–1094. https://doi.org/10.1002/bbb.1812.

Rusan M. J. M., Albalasmeh A. A., Zuraiqi S. and Bashabsheh M. 2015. “Evaluation of Phytotoxicity Effect of Olive Mill Wastewater Treated by Different Technologies on Seed Germination of Barley (Hordeum Vulgare L.).” *Environmental Science and Pollution Research* 22 (12): 9127–9135. https://doi.org/10.1007/s11356-014-4004-3.

Sablani S., Goosen M., Al-Belushi R. and Wilf M. 2001. “Concentration Polarization in Ultrafiltration and Reverse Osmosis: A Critical Review.” *Desalination* 141 (3): 269–289. https://doi.org/10.1016/S0011-9164(01)85005-0.

Santhakumar A. B., Battino M. and Alvarez-Suarez J. M. 2018. “Dietary Polyphenols: Structures, Bioavailability and Protective Effects against Atherosclerosis.” *Food and Chemical Toxicology* 113: 49–65. https://doi.org/10.1016/j.fct.2018.01.022.

Scott K. 1998. “Introduction To Membrane Separations.” in "Handbook of Industrial Membranes." 2nd edition Elsevier B.V https://doi.org/10.1016/B978-1-85617-233-2.X5000-4

Seader J. D., Henley E. J. and Roper D. K. 2011 “Membrane Separations.” in "Separation Process Principles-Chemical and Biochemical Operations" 3rd edition *John Wiley and sons, Inc*

Shahidi F. and Ambigaipalan P. 2015. “Phenolics and Polyphenolics in Foods, Beverages and Spices: Antioxidant Activity and Health Effects - A Review.” *Journal of Functional Foods* 18: 820–897. https://doi.org/10.1016/j.jff.2015.06.018.

Souza M. P., Vaz A. F. M., Correia M .T. S., Cerqueira M. A., Vicente A. A. and Carneiro-da-Cunha M. G. 2014. “Quercetin-Loaded Lecithin/Chitosan Nanoparticles for Functional Food Applications.” *Food and Bioprocess Technology* 7 (4): 1149–1159. https://doi.org/10.1007/s11947-013-1160-2.

Sygouni V., Pantziaros A. G., Iakovides I. C., Sfetsa E., Bogdou P. I., Christoforou E. A. and Paraskeva C. A. 2019. “Treatment of Two-Phase Olive Mill Wastewater and Recovery of Phenolic Compounds Using Membrane Technology.” *Membranes* 9 (2): 1–16. https://doi.org/10.3390/membranes9020027.

Tan A. S. B., Chemat F., Şahin S., Barba F. J., Samli R., Lorenzo J. M. and Cravotto G. 2017. “Solvent-Free Microwave-Assisted Extraction of Polyphenols from Olive Tree Leaves: Antioxidant and Antimicrobial Properties.” *Molecules* 22 (7): 1–13 https://doi.org/10.3390/molecules22071056.

Tao Y., Wu D., Qing A. Z. and Da W. S. 2014. “Ultrasound-Assisted Extraction of Phenolics from Wine Lees: Modeling, Optimization and Stability of Extracts during Storage.” *Ultrasonics Sonochemistry* 21 (2): 706–715. https://doi.org/10.1016/j.ultsonch.2013.09.005.

Teixeira A., Baenas N., Dominguez-Perles R., Barros A., Rosa E., Moreno D. A. and Garcia-Viguera C. 2014. “Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review.” *International Journal of Molecular Sciences* 15 (9): 15638–15678. https://doi.org/10.3390/ijms150915638.

Thiess H., Leuthold M., Grummert U. and Strube J. 2017. “Module Design for Ultrafiltration in Biotechnology: Hydraulic Analysis and Statistical Modeling.” *Journal of Membrane Science* 540: 440–453. https://doi.org/10.1016/j.memsci.2017.06.038.

Tiwari B. K. 2015. “Ultrasound: A Clean, Green Extraction Technology.” *Trends in Analytical*

Chemistry 71: 100–109. https://doi.org/10.1016/j.trac.2015.04.013.
Vandezande P., Gevers L. E. M. and Vankelecom I. F. J. 2008. "Solvent Resistant Nanofiltration: Separating on a Molecular Level." *Chemical Society Reviews* 37 (2): 365–405. https://doi.org/10.1039/b610848m.
Vilaplana-Pérez C., Auñón D., García-Flores L. A. and Gil-Izquierdo A. 2014. "Hydroxytyrosol and Potential Uses in Cardiovascular Diseases, Cancer, and AIDS." *Frontiers in Nutrition* 1: 1–11. https://doi.org/10.3389/fnut.2014.00018.
Wittenauer J., MäcKle S., Sußmann D., Schweiggert-Weisz U. and Carle R. 2015. "Inhibitory Effects of Polyphenols from Grape Pomace Extract on Collagenase and Elastase Activity." *Fitoterapia* 101: 179–187. https://doi.org/10.1016/j.fitote.2015.01.005.
Xu D. P., Zheng J., Zhou Y., Li Y., Li S. and Hua B. L. 2017. "Ultrasound-Assisted Extraction of Natural Antioxidants from the Flower of Limonium Sinuatum: Optimization and Comparison with Conventional Methods." *Food Chemistry* 217: 552–559. https://doi.org/10.1016/j.foodchem.2016.09.013.
Yang C. S., Zhang K., Lim J., Zhang J., Wan X. and Chi-Tang H. 2018. "Antioxidants: Differing Meanings in Food Science and Health Science." *Journal of Agricultural and Food Chemistry* 66 (12): 3063–3068. https://doi.org/10.1021/acs.jafc.7b05830.
Yu J., Bi X., Yu B. and Chen D. 2016. "Isoflavones: Anti-Inflammatory Benefit and Possible Caveats." *Nutrients* 8 (6): 1–16. https://doi.org/10.3390/nu8060361.
Zacharof M. P. 2017. "Grape Winery Waste as Feedstock for Bioconversions: Applying the Biorefinery Concept." *Waste and Biomass Valorization* 8 (4): 1011–1025. https://doi.org/10.1007/s12649-016-9674-2.
Zagklis D. P., Vavouraki A. I., Kornaros M. E. and Paraskeva C. A. 2015. "Purification of Olive Mill Wastewater Phenols through Membrane Filtration and Resin Adsorption/Desorption." *Journal of Hazardous Materials* 285: 69–76. https://doi.org/10.1016/j.jhazmat.2014.11.038.
Zheng S., Cheng Y., Teng Y., Liu X., Yu T., Wang Y., Liu J., Hu Y., Wu C., Wang X., Liu Y., You C., Gao X. and Wei Y. 2017. "Application of Luteolin Nanomicelles Anti-Glioma Effect with Improvement *in Vitro* and *in Vivo*." *Oncotarget* 8 (37): 61146–61162. https://doi.org/10.18632/oncotarget.18019.
Zhu F., Du B., Zheng L. and Li J. 2015. "Advance on the Bioactivity and Potential Applications of Dietary Fibre from Grape Pomace." *Food Chemistry* 186: 207–212. https://doi.org/10.1016/j.foodchem.2014.07.057.
Zillich O. V., Schweiggert-Weisz U., Eisner P. and Kerscher M. 2015. "Polyphenols as Active Ingredients for Cosmetic Products. " *International Journal of Cosmetic Science*. 37(5): 455–464. https://doi.org/10.1111/ics.12218.