Bioactive Extracts of *Olea europaea* Waste Streams

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Abstract

The production of olive oil has seen an increase in recent years due to a broader understanding of the health benefits of the Mediterranean Aliment Culture. With this expanding industry we also see an increase in the waste products associated with olive oil production. Given the high polluting content of the waste streams and the economic costs associated with its removal and processing, waste remediation and disposal has become a significant point of interest for both producers and local bodies. In this project, wastes of the olive oil production industry are examined for their use as the raw material for a novel product used in the control of horticulturally important diseases, examining the effect of extraction protocols on the activity of the final product.

Active fractions of the olive oil wastes were identified from literature and protocols for their extraction and recovery developed; incorporating both standard solvent extraction and novel ultrasound-assisted extraction. Criteria for the analysis of extract quality were outlined and potential target applications identified.

The biophenolic compounds of olive wastes were identified as providing the majority of the active fraction, so protocols were developed for the recovery of these compounds. Standard solvent extraction and ultrasound-assisted extraction were examined for their effectiveness of biophenolic recovery and their effect on product quality. Certain horticulturally important diseases were identified as potential targets, and bioassays undertaken to determine the ability of a crude extract to inhibit and control these diseases.

It was found that the action of ultrasound during extraction provides a greater degree of recovery of biophenolic compounds, with minimal loss of product quality; as determined by bioassays and total biophenol determination. This increase in recovery is due primarily to the destruction of cellular material resulting in higher rates and absolute yields of recovery. This work provides evidence of the occurrence of some

interesting phenomenon in the recovery of biophenols from olive wastes that deserves further examination.

The crude olive leaf extract was shown to have an inhibitory effect on bacteria and effectively no inhibitory effect on fungal species in the total biophenol ranges tested. Erwinia amylovora and Staphylococcus aureus both showed a large susceptibility to the olive leaf extract. Results showed a higher degree of susceptibility of Gram positive bacteria and a potential resistance in soil microbes. For bacterial species, total biophenol concentrations of 0.15 to 3.50 mg GAE/ml provided inhibitory effects, while with the fungal species tested, no inhibitory effects were found at total biophenol concentrations of up to 2.50 mg GAE/ml.

Some evidence exists that there is an opportunity for the economic recovery of olive biophenols for use as a novel product, but more work is required to determine specific applications and/or targets of use, as well as optimisation of the extraction and purification protocol. A sample removed from interfering compounds will allow the examination of activity of particular compounds and hence a better understanding of the action of the olive waste extract.

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Chapter 1

Introduction

In recent years a trend has developed whereby consumers are demanding more responsibility from food producers to ensure a high level of safety of the food products that they manufacture. At the crux of the issue is the use of synthetic compounds in pest-management systems and as additives for the stability of food products and the effect such compounds can have on human and ecological systems with sustained exposure. In keeping with these demands, producers have moved to more natural inputs in both the primary cultivation and manufacture of food products, creating a demand for research into characterising and analysing natural compounds that show biological activities that may be of benefit in more sustainable and natural food production systems.

One such group of compounds to show promise are biophenols (BP), a major and prolific group of secondary plant metabolites that exhibit a wide range of biological activity. They have drawn much attention due to their contribution to the Mediterranean Aliment Culture (MAC), which is believed to contribute to lower rates of cardio- and cerebro-vascular disease, certain hormone-dependent cancers such as colon and breast cancer, and a generally longer life expectancy (Uccella and Saija, 2001). It is believed that the traditional diet of the Mediterranean region is responsible for such observations with its high quantity of polyunsaturated lipids and various minor components found in certain foods. BP's contribution is believed to be a function of their strong antioxidant activity, required to stabilise the highly unsaturated lipids in the products from lipo-oxidation. They are found predominantly in *Olea europaea*, the olive plant, which supplies large quantities of oil that is consumed in hedonistic quantities.

During the oil extraction process, these BP compounds are transferred to extra-virgin olive oil (EVOO) where they play a crucial role in the nutritional, organoleptic and technological qualities unique to olive oil. In addition to their strong antioxidant activity, olive BP's have been shown to have antimicrobial, antifungal, phytotoxic and insect repellent properties, showing promise for the use in organic pest-management and/or post-harvest contamination control systems.

Not all of the phenolic substances present in the plant material are extracted into the oil during EVOO production; a quantity is retained in the pomace (the fibrous husks remaining after malaxation), and a proportion is transferred into the aqueous phase during water-lipid separation, known as olive mill waste water (OMWW). In addition, a significant proportion of BP's are present in the leaves of the plant, with pruning wastes providing another possible source of raw material. Given the highly active nature of these chemicals and their relative abundance, these particular waste streams have been identified as highly polluting, and as such, disposal of these waste streams can present a substantial economic burden. Developing a natural novel product from such a polluting waste stream could present the possibility of turning this economic burden into an economic opportunity.

The extraction of BPs from pomace and leaves can be carried out using a number of solvent extraction systems incorporating acid- or alkali-, water or brine, and organic or alcohol solvents, all of which have been well documented. Each method provides different chemical profiles and yields, rates of extraction, and biological activity, compounded by differences in the cultivar, growing region, maturity and tissue component of the raw material. Interest in new and novel methods for secondary metabolite extraction from plant tissues has increased concurrently with the interest in their activities and possible application, ultrasound extraction being one such method. Ultrasound-assisted extraction could present the possibility of providing more effective, efficient, and environmentally sustainable extraction procedures.

The aims of this thesis were to examine the potential benefits for BP extraction from olive pomace and leaves using novel extraction technologies compared to conventional extraction methods with respect to the profile, rate of extraction, and biological activity of the extract achieved. With this information various applications

for the olive extract were proposed to be examined in terms of their economic, technological, and social feasibility.

The objective of this project can be summarised as follows:

- To examine the opportunity for the recovery of olive BP compounds from solid waste streams from the olive oil production industry for use in a novel product.
- To establish a protocol for the characterisation of olive extracts to determine the quality of the product.
- To determine the relative efficiency of various established and novel extraction technologies and examining their effect on product quality.
- To examine the bioactivity of a novel olive extract against various post-harvest, phyto-pathogenic and food-borne indicator microrganisms.

Chapter 2

Literature Review

2.1 Introduction

The study of olive phenolic species has become an intense point of interest for the olive industry in recent years as attempts are made to reduce the environmental impact of production, improve on methods of product characterisation, increase process efficiency as well as developing novel products from waste streams. As such, the peer-reviewed literature on the topic is expansive covering many areas of interest. This project deals specifically with the comparison of extraction methods for the recovery of secondary plant metabolites (specifically phenol compounds) from solid olive waste products, and the effect such extraction methods have on the activity of the resulting extract.

It is always important to have a broad understanding of the industry and production methods that are relevant to the project, the present case being the olive oil production industry; this helps in identifying areas of economic opportunity for the commercial application of results.

It is also fundamental to have an understanding of the composition, chemistry, and metabolic and physiochemical characteristics of the product of interest.

As expected, knowledge of the various extraction methods is critical; in this case they are simple solvent extraction and ultrasound-assisted solvent extraction.

Definitions of bioactivity need to be examined, including published results indicating specific areas of bioactivity and associated testing methodologies.

In addition, the various analytical techniques that will be important in shaping the results need to be examined for their suitability, where any analytical protocols requiring modification to be identified.

2.2 **Olive Oil Production**

The olive oil extraction process is a relatively uncommon process among fruit and seed oil extraction procedures. The extraction is achieved entirely through mechanical means, with no chemical additives except water. Without the harsh conditions of solvent extraction, the natural amphiphilic and lipophilic components of the fruit are transferred to the oil, contributing to its well regarded nutritional and organoleptic qualities.

Olive oil contributes about 4% of total vegetable oil worldwide with an annual production of 2 million tonnes per year with the Mediterranean contributing 95% of production (Visioli and Galli, 2002). With realisation of the nutritional benefits of the Mediterranean Aliment Culture, this annual production is rising considerably, and with it, the quantity of waste products.

2.2.1 **Olive Oil Extraction**

The extraction of olive oil can be achieved in a number of ways depending on tradition and scale, but generally involves the same nature of processing method. After an initial grading and debris removal step, the fruit is mixed, crushed and undergoes a malaxing process to form a paste. The next step is to extract the oil from the paste. This can be achieved by pressing, centrifugation, percolation, or a combination thereof. All of the mentioned procedures produce a lipid-water phase emulsion known as the *must*, with the lipid phase separated from the water phase in a centrifugation step. Generally the yield of olive oil is comparable for the different extraction protocols, with the water and energy requirements and waste stream composition being the points of distinction (Azbar *et al.*, 2004).

The oil extraction process yields olive oil (0.20 - 0.25 kg/kg olive), a semi-solid husk by-product of varying moisture content (0.300 - 0.400 kg/kg olive, moisture-free basis), and a liquid wastewater (0.60 - 1 *l*/kg olive) with variations between batch and

continuous operation due to differences in water input and degree of solids removal (Bradley and Baruchello, 1980; Azbar *et al.*, 2004). The more prevalent continuous process requires a greater input of water but achieves a greater degree of oil extraction with the benefits of continuous operation. As expected, the batch process produces a more concentrated product with more concentrated organic waste.

Taking data from literature on waste streams (Bradley and Baruchello, 1980; Vitolo *et al.*, 1999; Alburquerque *et al.*, 2004; Azbar *et al.*, 2004) and their typical biophenol (BP) content , we can construct a mass balance to determine relative quantities of BP's available for secondary extraction.

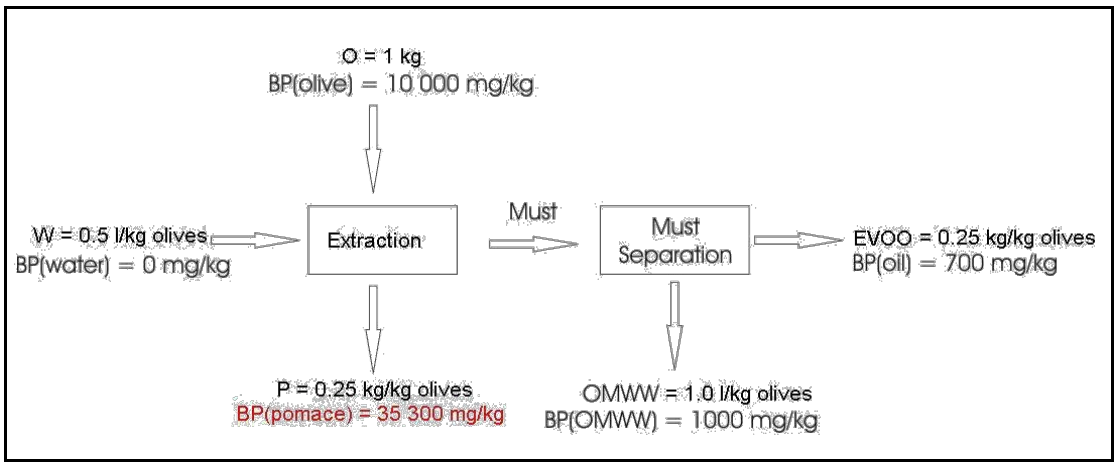


Figure 2-1 Mass balance showing BP concentrations in various oil production streams. Where O = olives, W = water, P = pomace, OMWW = olive mill wastewater, and EVOO = extra virgin olive oil.

It can be seen in Figure 2-1 that the olive pomace, the semi-solid waste left after extraction and separation, appears to be very rich in BP compounds. Such high concentrations remaining in the pomace is suggestive that they are not made available for removal during the oil extraction procedures or that equilibrium is not reached.

The BP's may not be made available for extraction as they are bound to various macromolecules as is suggested for bioavailability (Romeo *et al.*, 1997; Bianco and Uccella, 2000). As it is mainly the more complex glycosides that are involved in these macromolecular complexes, an increase in the smaller, simple substituted BP's should be seen in the OMWW and olive oil.

As noted, another explanation for the high quantity of BP's remaining in the husks after oil extraction could be due to a failure to reach equilibrium due to limited processing duration.

Olive mill wastewater (OMWW) is a bioactive waste product of olive oil production and contains both the process waters and vegetable waters from the olives. Due to the ampiphillic nature of some BP's, they can be present in both the oil and water phases after must separation. The phenolic compounds present in the OMWW present problems in disposal if not properly dealt with, having significant deleterious impacts on the environment. Progress has been made in the degradation and composting of olive oil waste products using various phenolic-metabolising organisms (Robles *et al.*, 2000; Robles *et al.*, 2000; Garrido Hoyos *et al.*, 2002), volume reduction in evaporation ponds and subsequent composting (Saez *et al.*, 1992), and its possible use as a fertilizer (Tomati and Galli, 1992; Ammar and Rouina, 1999).

Another waste stream produced from olive production, not necessarily from oil production, is the leaf removed during pruning of the plants. These leaves contain significant quantities of phenolic compounds that could be extracted for novel purposes. Considering that a significant quantity of the plant material may be excised from the plant (20%+), pruning may provide a large, albeit seasonal and sporadic, supply of raw material (Ferguson *et al.*, 1994). It has been shown that olive leaves can have a significant biophenolic (BP) fraction, with quantities of 60-90 mg/g of oleuropein alone (Le Tutour and Guedon, 1992).

Olive oil production has three waste streams that could pose an economic opportunity for the recovery of phenolic compounds for the use in novel products:

- The pomace left after must extraction
- The OMWW collected after must separation
- Leaf material from pruning

This project shall examine the potential of using the two solid waste products as a raw material for the recovery of active secondary BP metabolites.

2.2.2 **Problems to Face and Opportunities Exposed – Wastes and Recycle**

The waste products of the olive oil production industry have a significant quantity of highly active organic compounds that can impact heavily on ecosystems in 'unnatural' concentrations, in terms of soil quality, toxicity to plant life, and odour (Bradley and Baruchello, 1980; Sierra *et al.*, 2001; Azbar *et al.*, 2004). Therefore, the disposal of olive oil waste products poses a significant problem to the industry given the economic costs associated with such disposal.

Due to the active nature of the BP's and the significant interest from the scientific community, there may be opportunities where such a waste stream may be harnessed for use in a novel and ultimately profitable product; thereby turning a polluting waste stream into a product stream, and allowing the remaining wastes to be disposed of safely. Currently, the main trends in the novel disposal of solid wastes from the olive oil production industry are in agricultural/horticultural applications and as a processed solid fuel.

The main polluting factors that prevent application of olive wastes in agricultural industries are their heavy lipid, salt, and particularly, phenolic constituents (Azbar *et al.*, 2004). Remediation of the waste to first recover the oil, and then the phenolic species, may allow the remaining waste to be utilised for their potassium, phosphorous and other nutritionally important constituents; although it has been stated that the levels of macro and micronutrient content was lower in "alperujo" (an olive pomace material with a generally higher water content) than in most manures and other organic soil amendments (Alburquerque *et al.*, 2004), calling into question the commercial viability of such uses.

The use of solid by-products as a source of solid fuel has been suggested by a number of authors with studies looking at emission composition and heating values. Solid waste streams are treated with hexane to remove excess oil, to prevent putrefication, and then compressed into brisquettes for use as a solid fuel (Vitolo *et al.*, 1999; Azbar *et al.*, 2004). This can still be achieved after a step where commercially important components are recovered so they can be used in conjunction with a BP recovery process.

2.3 **Minor Components of *Olea europaea***

Phenolic compounds are ubiquitous in plants showing an incredibly diverse profile between various species, and between individual plant constituents, in terms of both structure and function. They play roles in maintaining plant structure, as in lignin (a polymeric phenolic compound); provide aesthetic pleasure and pollinator attraction as colour pigments, as anthocyanins and flavanoids; as well as antioxidants and providing for a wide range of biological functions (Walker, 1975).

The most basic phenol is a benzene ring with an alcohol, or hydroxyl, functional group (Figure 2-2). The activity of phenolic compounds derives from their highly reactive hydroxyl moieties, which can be found in a number of states depending on the physio-chemical environment.

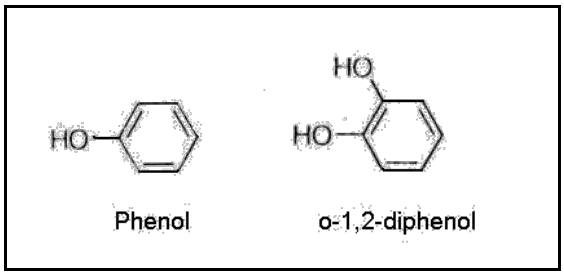


Figure 2-2 Structure of a simple phenol and 1,2-diphenol (Ryan *et al.*, 2002).

The uniqueness of this group of compounds comes from their water-soluble monomeric phenolic character. It is a misconception that these minor phenolic constituents of plants are commonly referred to as polyphenols, a term probably originating from studies of tannin and lignin compounds, before the extent of their functionalities were realised. This is due to the latter being described as compounds with molecular weights of 500-4000 Da, possessing some 12-16 hydroxyaromatic groups and 5-7 aromatic rings per 1000 relative molecular weight (Bastoni *et al.*, 2001). Olive BP's can be simple substituted phenolic structures of alkene, alcoholic and carboxylic functionalities of low molecular weight, and they can be complex compounds with glycosidic and monoterpenid moieties (Uccella, 2001), but in all cases the molecular weight will rarely exceed 600 Da. Olive BP's can also be distinguished by their ability to associate with primary metabolites, such as proteins and carbohydrates, giving rise to supramolecular moieties responsible for their

bioavailability and also affecting the technomolecular characteristics of the food product (Romeo *et al.*, 1997; Bianco and Uccella, 2000).

It is easy to be overwhelmed by the sheer complexity of olive phenolic composition due to the great variation in their structure and function and given the amount of interest shown in characterisation of the phenolic fraction. It is not the specific components that are of interest to this project, but rather the overall activity of a raw extract; it would add unnecessary complexity to analyse all olive BP's as individual functional entities.

Given this, it is still important to have an understanding of the structure of these compounds of interest. Therefore, the following section outlines the similarities and differences between the BP's that will be encountered.

2.3.1 **Structure of Major Biophenolic Constituents**

The simplest way of defining classes of phenolic compounds is by their general structure. In this respect we look at the hydroxyaromatic ring, any side chains, and/or glycosylation moieties. The phenols of interest can be 1) simple substituted phenols, 2) flavanoid compounds, or 3) secoiridoids.

In *Olea europaea*, the main phenolic component of interest, and the most abundant in all olive plant tissues, is oleuropein and its derivatives, which have proven antioxidant and antimicrobial functionality. Oleuropein is a member of the secoiridoid group of compounds and is an ester of the oleoside skeleton and 2'-(3',4'-dihydroxyphenyl)ethanol (hydroxytyrosol). The structure of the main secoiridoids in olives can be found in Figure 2-3.

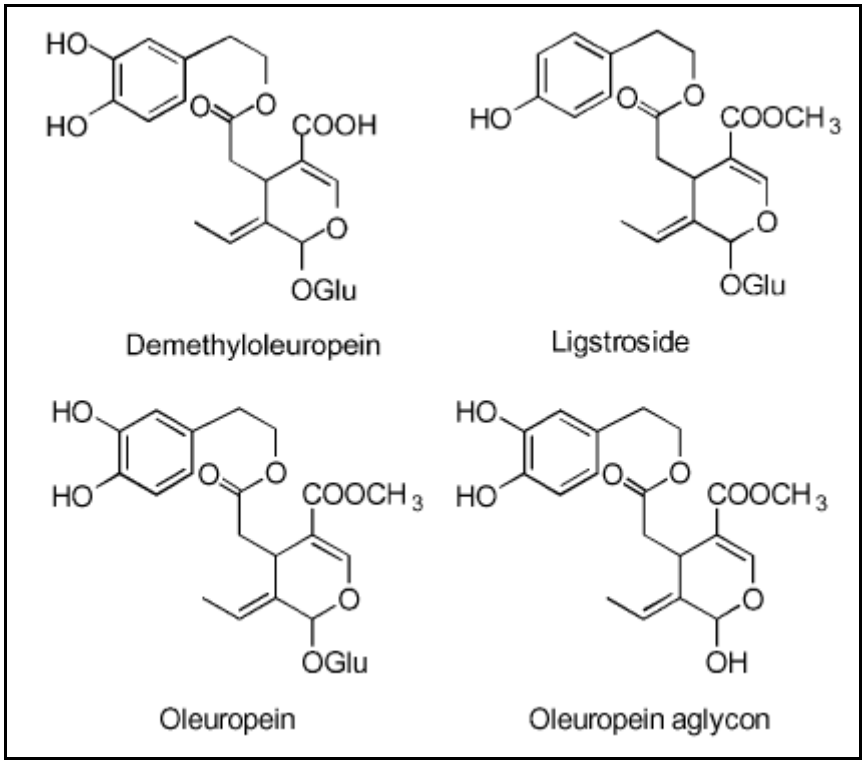


Figure 2-3 Structure of main olive secoiridoids (Ryan *et al.*, 2002).

We can group our simple substituted BP's as per the following (Ribereau-Gayon, 1972):

- C_6, phenols with no significant side chains.
- C_6-C_1, a phenol ring with a one carbon side chain.
- C_6-C_2, a phenol ring with a two carbon side chain.
- C_6-C_3, a phenol ring with a three carbon side chain.

These phenol species can also have more than one side chain giving combinations such as; C_3-C_6-C_3.

The majority of simple-substituted phenols present in *O. europaea* can be found below in Figure 2-4.

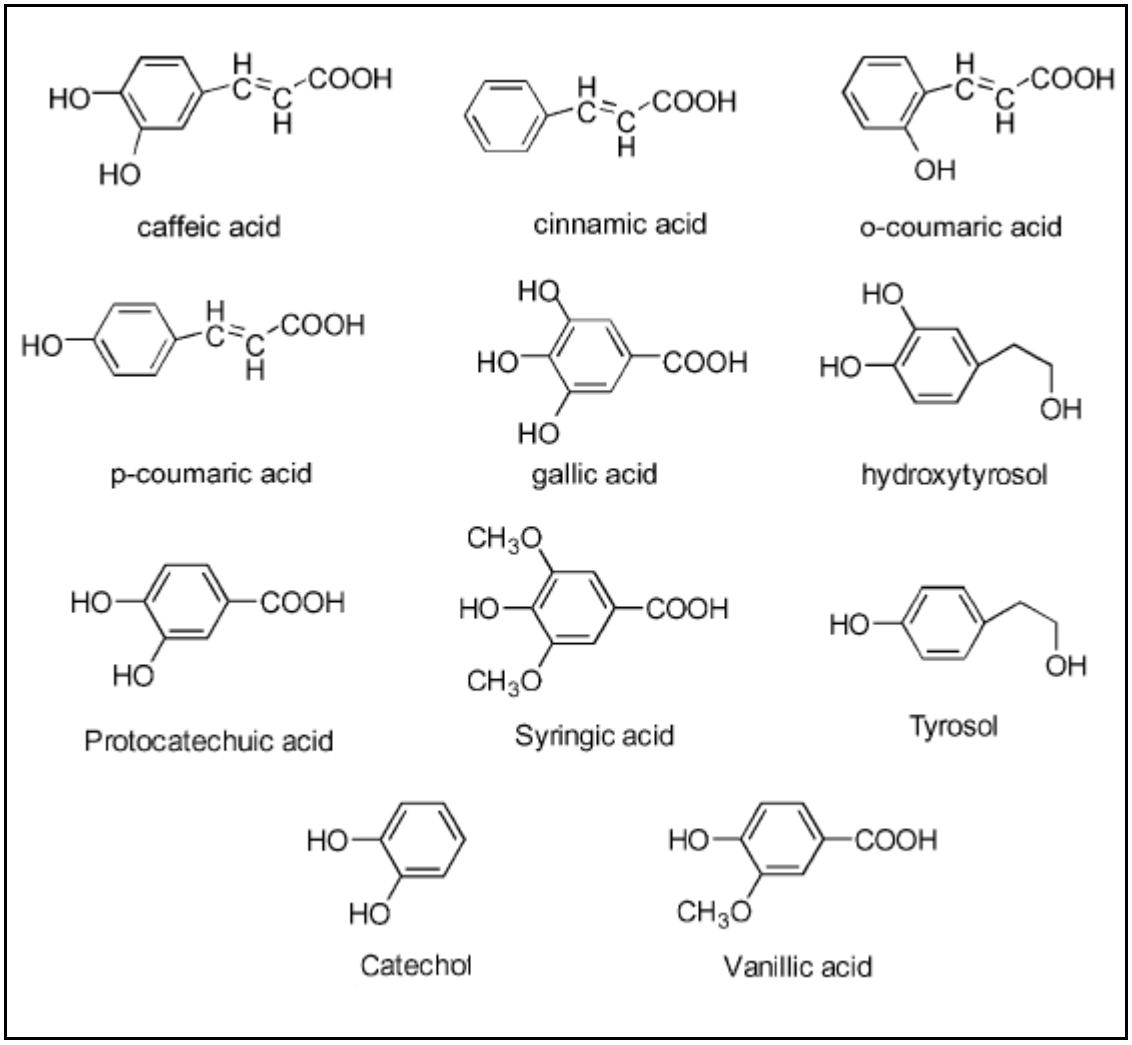


Figure 2-4 Simple substituted phenols present in olive plant material (Ryan *et al.*, 2002).

The flavanoid group of compounds is composed of flavones, flavanols, and flavan-3-ols. These are C_{15} structures with a three-ring conformation (two of which are of phenolic structure), where the variation occurs in the 3 carbon central unit (common short-hand is C_6-C_3-C_6). The flavones are of basic structure with no defining differences in the C_3 central unit. Flavanols have an extra -glycosyl functionality on the 4' carbon of the central unit, at which point it is common for glycosylation to occur. Flavan-3-ols are missing the 4' –glycosyl functionality replacing it with a 3' –glycosyl functionality. Flavan-3-ols are found predominantly in their aglycone forms while the former two are most often found as glycosidic compounds and rarely found as aglycones.

The main olive flavanoids can be found in Figure 2-5.

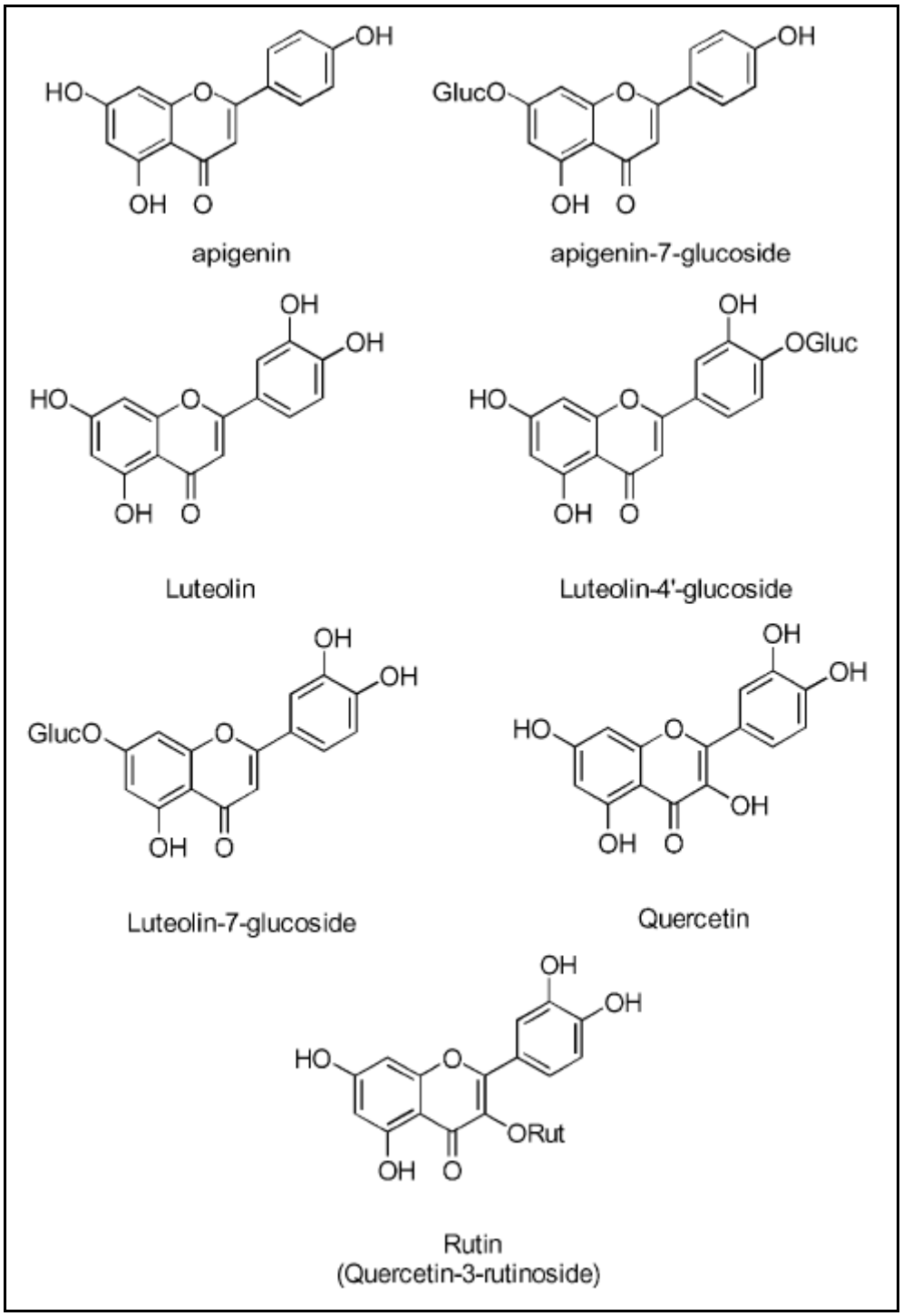


Figure 2-5 Main flavanoid compounds found in olive cultivars (Ryan *et al.*, 2002).

In addition, there are other BP's that cannot be characterised under these groups. Their presence may be significant in terms of activity and metabolic function, either as terminals or intermediates, so they are presented below in Figure 2-6.

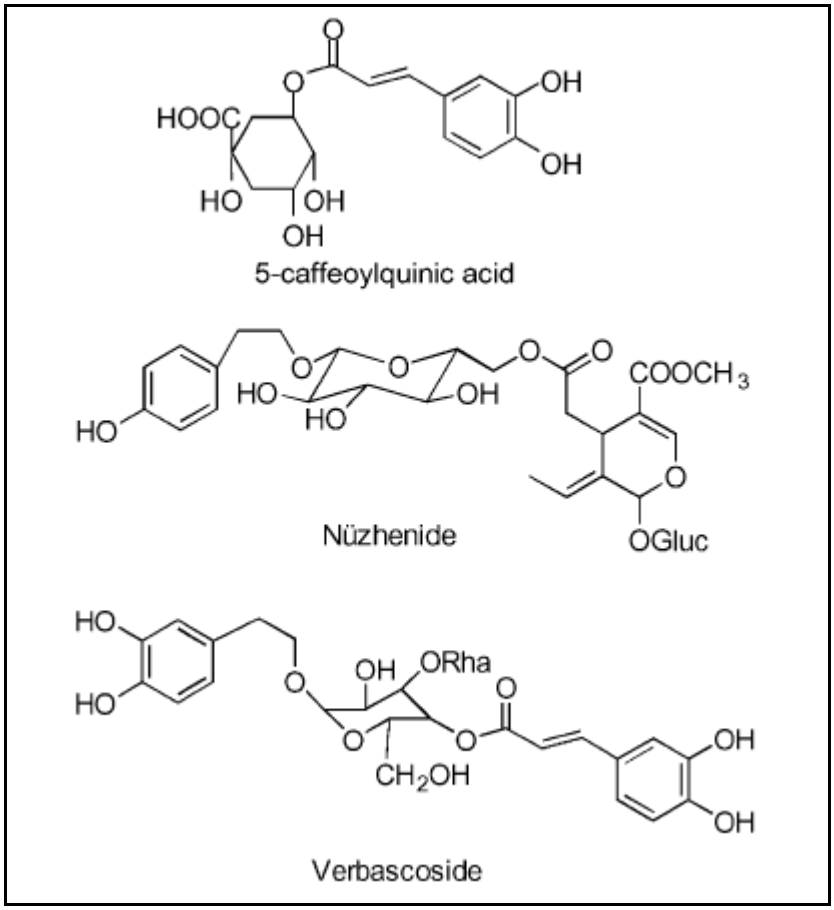


Figure 2-6 Other important BP's found in olives (Ryan *et al.*, 2002).
Note: 5-caffeoylquinic acid is chlorogenic acid (CGA).

It should be noted that there are significantly more phenolic compounds present in olives that will affect any biological activities studied. The phenols given above are consistently found in relatively high quantities in various olive constituents and make up the majority of phenolic species present.

2.3.2 **Phenolic Metabolism and Stability**

The phenolic profile of *O. europaea* is highly dependent on:

- The environmental conditions during maturation
- Phase of growth
- The plant tissue of interest
- The cultivar of interest

This is suggestive of significant differences in the metabolic and degradative processes of phenolic compounds with respect to the above factors. The metabolic

relationships between the various plant constituents and their metabolic profile is poorly understood with but a few mechanisms being elucidated, and then, only broadly at best. Due to the complexity of these biochemical relationships only the broad range effects will be discussed, the specific mechanisms are too complex to be outlined at this point.

It is simple to define these secondary metabolites from their origin through the shikimate pathway and phenylpropanoid metabolism (Ribereau-Gayon, 1972). These biosynthetic pathways are very complex with many interdependent reactions and alternate end products that are regulated differently with the aforementioned factors. This all combines to achieve a very wide range of possible BP structures (Ryan *et al.*, 2002).

The most widely documented, and arguably most significant, BP metabolic process that occurs in *O. europaea* is the conversion and degradation of oleuropein in the developing fruits during maturation. During the early stages of growth oleuropein content increases rapidly up to the point of green maturation. At this point we see a rapid decrease in oleuropein and chlorophyll with an increase in anthocyanins (pigment compounds), which are responsible for the black colour of ripe olives. The rate of reduction of oleuropein decreases during black maturation (if left on the tree for that length of time), where the quantity can often drop to zero for very ripe drupes (Macheix *et al.*, 1986; Macheix *et al.*, 1989; Bianco and Uccella, 2000). This reduction in oleuropein can be related to a proportionate increase in both hydroxytyrosol and elenoic acid glucoside (oleoside), which are the oleuropein constituent parts (Esti *et al.*, 1998), or demethyloeuropien, its methylated counterpart and degradation product.

An increase in the activity of hydrolytic enzymes, glycosidases in particular, could be the contributing factor to the increases in hydroxytyrosol and oleoside, although it has been suggested that an increase in this enzyme activity could be a result of the oil extraction process (Ryan *et al.*, 2002); thus analytical determinations may not be representative of the natural phenolic profile of the plant material. Yet other studies have recorded this BP when not subjected to malaxation conditions (Esti *et al.*, 1998), suggesting metabolic differences by cultivar. In cases where an increase in demethyloleuropein and other oleuropein derivatives occurs with oleurolein reduction,

it has been suggested that this could be the result of an increased esterase activity that was directly measured to coincide with its reduction (Macheix *et al.*, 1989; Esti *et al.*, 1998). Which mechanism of degradation is most significant in the reduction of oleuropein seems to be highly dependant on the cultivar, and can so be used as a species marker, although correlations with environmental factors cannot be ruled out.

The decline in oleuropein also coincides with the decrease of other less quantitatively important oleosides, such as ligustroside, shown to be an oleuropein precursor (Ryan *et al.*, 2002), and an increase in verbascoside and certain flavanoid compounds, anthocyanins in particular (Wichers *et al.*, 2000), which is understandable as these are the major pigmentation factors.

There is an interesting difference in the metabolic functions between the fruit and leaves with respect to oleuropein derivatives. While the fruit of *O. europaea* accumulates only glycosylated oleuropein derivatives, which may be less toxic than their respective aglycones, non-glycosylated secoiridoids are found in the leaf (Macheix *et al.*, 1989). This may have implications in the toxicological and plant protection activities between the respective extracts of fruit and leaves.

From analysing the various studies, it has been shown that the biological activities of these oleuropein derivatives are significantly different than that of the parent molecule.

2.3.3 **Phenolic Profile and Distribution**

BP's are present in all plant constituents, but the qualitative and quantitative differences are significant. As mentioned previously, oleuropein is by far the most predominant BP, with its quantity depending on degree of maturity, cultivar and extraction procedure, with literature indicating of up to 2.15 mg/g fresh pulp in a recent study (Bastoni *et al.*, 2001). Despite the great variations in absolute quantities of BP's in different studies, we do see that the relative proportions appear about the same with oleuropein often making up 25% of the total BP's in the leaves (Benavente-Garcia *et al.*, 2000) and the fruit (Bastoni *et al.*, 2001). Oleuropein is easily separated as a fraction of olive fruit, leaves and seeds, but it has not been recorded in EVOO, whereas various oleuropein derivatives have.

It is difficult to provide consistent data on the quantities of the various BP's and total BP's from the literature as the variations and contradictions are great. This is accentuated by the different isolation and purification schemes employed. Without careful attention to the reagents and possible *in-vitro* reactions, the characterisation of the chemical profile may provide results that are not representative to the raw material being assayed. Instead of conveying exact quantities of BP's found in various plant tissues, Table 2-1 summarises the rough trends in phenolic distribution found by a range of authors.

Table 2-1 BP content by respective plant constituent.

BP Group	BP Compound	Olive pulp (fruit)	Leaves	OMWW
C6	Catechol			(Tassou *et al.*, 1991; Capasso *et al.*, 1992; Capasso *et al.*, 1995)
C6-C1	4-Methylcatechol			(Tassou *et al.*, 1991; Capasso *et al.*, 1992; Capasso *et al.*, 1995)
	Vanillin	(Bianco and Uccella, 2000)	(Benavente-Garcia *et al.*, 2000)	
C6-C2	Hydroxytyrosol	(Esti *et al.*, 1998; Montedoro *et al.*, 1999; Bianco and Uccella, 2000; Bastoni *et al.*, 2001)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000)	(Tassou *et al.*, 1991; Capasso *et al.*, 1992; Lo Scalzo *et al.*, 1994; Capasso *et al.*, 1995; Limiroli *et al.*, 1996; Visioli *et al.*, 1999)
	Tyrosol	(Montedoro *et al.*, 1999; Bianco and Uccella, 2000; Bastoni *et al.*, 2001)	(Benavente-Garcia *et al.*, 2000)	(Tassou *et al.*, 1991; Capasso *et al.*, 1992; Capasso *et al.*, 1995; Limiroli *et al.*, 1996; Visioli *et al.*, 1999)
C6-C3	Caffeic acid	(Bianco and Uccella, 2000; Bastoni *et al.*, 2001)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	
	Courmic acid	(Bianco and Uccella, 2000; Bastoni *et al.*, 2001; Ryan *et al.*, 2002)		
Flavanoids	Apigenin		(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	

	Apigenin-glycosides		(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	
	Luteolin	(Montedoro *et al.*, 1999)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	
	Luteolin-glycosides	(Montedoro *et al.*, 1999)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	(Visioli *et al.*, 1999)
	Rutin (Quercetin-3-rutinoside)	(Montedoro *et al.*, 1999)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	
Secoiridoids	Demethyloleuropein	(Macheix *et al.*, 1989; Esti *et al.*, 1998; Montedoro *et al.*, 1999; Bianco and Uccella, 2000; Bastoni *et al.*, 2001; Ryan *et al.*, 2002)	(Le Tutour and Guedon, 1992; Ryan *et al.*, 2002)	
	Ligustroside	(Ryan *et al.*, 2002)	(Le Tutour and Guedon, 1992; Ryan *et al.*, 2002)	
	Oleuropein	(Kubo *et al.*, 1985; Macheix *et al.*, 1989; Esti *et al.*, 1998; Bianco *et al.*, 1999; Montedoro *et al.*, 1999; Bianco and Uccella, 2000; Bastoni *et al.*, 2001; Ryan *et al.*, 2002)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et al.*, 2000; Ryan *et al.*, 2002)	(Limiroli *et al.*, 1996)
	Oleuropein derivatives	(Bianco *et al.*, 1999; Bianco and Uccella, 2000; Bastoni *et al.*, 2001)	(Ryan *et al.*, 2002)	
	Oleoside	(Capasso *et al.*, 1995; Montedoro *et al.*, 1999; Benavente-Garcia *et al.*, 2000)		
Glycosides	Verbascoside	(Montedoro *et al.*, 1999)	(Le Tutour and Guedon, 1992; Benavente-Garcia *et*	

The profile of BP's of the fruit can be characterised as containing a wide range of phenols of different types and sub-types, from simple substituted phenols to more complex esterified glycosides. In contrast, the phenolic profile of the leaves is narrower, composed of the more complex flavanoids and secoiridoids glycosides and showing very small quantities of simpler phenols. The leaves have also been shown to accumulate secoiridoid aglycones, while the fruit accumulates mainly the glycosides, as mentioned earlier. This is suggestive of unique metabolic processes for each plant constituent.

Likewise, the phenolic profile of OMWW is significantly different to that of the fruit. We see far less complex secoiridoids and more of their simple degradation products, such as hydroxytyrosol, catechol and oleuropein aglycone and derivatives. The hydro- and lipo-solubility will also affect the profile of the BP's and the degree of separation between the aqueous phase and the oil phase of the must. The more hydrophilic BP's will be more prominent in the water phase than the oil phase. Some BP's are amphiphilic and can therefore be present in both phases, where the water distribution coefficient will determine relative proportions.

The variation in BP profile between various plant tissues is not the only source of variation that can be described. Each specific cultivar has a very unique BP profile, to the extent that rough species characterisation can be theoretically achieved by analysing their phenolic profile alone. Unfortunately, large environmental variations and the lack of any substantial databases can make this difficult. As a general observation, it appears that Italian cultivars (such as Taggiasca, Cassanese, Leccino and Pendolino) have the greatest phenol content followed by Greek (Thasos and Conservoila), Portuguese (Douro), and finally Spanish (Hojioblanca) cultivars (Bianco and Uccella, 2000; Bastoni *et al.*, 2001). The cultivars with the greatest BP content provide the greatest economic opportunity for BP recovery.

This last section has highlighted the problems associated with maintaining a consistent raw material for BP extraction and recovery for a novel product. The BP quantity and profile can vary with cultivar, plant constituent, and season. As a result, tests carried out on one plant source to determine the economic feasibility of BP extraction may not be comparable to tests on other plant sources, even if it's the same

plant tissue of a different harvest. The extent to which this will affect the final process must be analysed.

2.4 **Activity of Olive Biophenolics**

The biological activities of olive phenolics, as a crude extract or as pure compounds, have been well characterised in recent years. It is with these studies that the true diversity of phenolic functionality has been illustrated. A comprehensive summary of experimentally determined bioactivities of olive oil and its minor constituents is given by Visioli and Galli (2002):

- Inhibition of LDL oxidation, both in vitro and in vivo
- Scavenging of superoxide and other ROS (reactive oxygen species)
- Inhibition of apoprotein derivatization
- Inhibition of platelet aggregation
- Reduced Thromboxane-B2 and Leukotriene-B4 production by activated leukocytes
- Inhibition of peroxynitrate-induced DNA damage
- Inhibition of peroxynitrate-induced tyrosine nitration
- Scavenging of hypochlorous acid
- Increased nitric oxide production by lipopolysaccharide-challenged macrophages
- Inhibition of neutrophil respiratory burst
- Inhibition of microbial growth and activity
- Cytostasis
- Hypotensive action
- Decreased isoprostane excretion in humans and in sidestream smoke-exposed rats
- Increased rat plasma antioxidant capacity

In addition to this list, there is evidence that the BPs present in olives may also contribute a phytotoxic effect limiting the growth of selected plants (Capasso *et al.*, 1992), and a selective repellence to certain insects (Lo Scalzo *et al.*, 1994). In conjunction with research on the antimicrobial effects of olive extracts, some studies examined toxicity on human cells (Capasso *et al.*, 1995), claiming mixed results. It has been mentioned that phenolic compounds can bind to primary metabolites, such as proteins, which may in turn affect the function of both the protein and the BP and inhibit activity.

The activities that are of highest interest to the project are:

- Antioxidant activities
- Antimicrobial activities, encompassing antibacterial and antifungal effects

2.4.1 **Antioxidant Activity**

Defining antioxidant activity is made very difficult due to the large range of physio-chemical mechanisms, applications and testing systems, terminology, and with many possible sources and structures; a broad definition of what describes an antioxidant is required.

A biological antioxidant can be defined as “any substance that , when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substance” (Halliwell *et al.*, 1995). An oxidizable substrate can be almost any substance or compound found in food and living tissue, including fats and oils, proteins, carbohydrates and DNA. The term ‘activity’ also needs clarification as it can encompass multiple meanings. It could refer to mechanistic intervention, rate of scavenging, medium or substrate selectivity, concentration effectiveness, or synergistic effects (Antolovich *et al.*, 2002). The definition of ‘antioxidant activity’ is therefore dependent on the hypothesis being tested and the testing methodology used.

Given these broad underlying and flexible definitions, it is understandable that the majority of literature seems to lack consistency. This is due to the fact that antioxidant compounds can have notably variable results between tests; their ‘activity’ is dependent on which reactive oxygen species (ROS) is generated, how it is generated,

where it is generated, and what target of damage is measured (Halliwell *et al.*, 1995). Where a compound shows activity against one species and mechanism, it may not show the same level activity against certain others (Halliwell *et al.*, 1995; Frankel and Meyer, 2000). This can be seen in the following section outlining data from literature (Table 2-2 to Table 2-4), where the relative antioxidant activities of the olive BP's can be seen to vary between each set of results.

2.4.1.1 Data from Literature

The phenolic extract from olives has been shown to have a strong antioxidant action, as shown with free-radical scavenging and low density lipo-protein (LDL) oxidation testing (Papadopoulos and Boskou, 1991; Le Tutour and Guedon, 1992; Aeschbach *et al.*, 1994; Visioli *et al.*, 1998; Visioli *et al.*, 1999; Benavente-Garcia *et al.*, 2000; Keceli and Gordon, 2001; McDonald *et al.*, 2001; Andrikopoulos *et al.*, 2002; Briante *et al.*, 2002; Bouaziz *et al.*, 2005), with many studies employing a number of different methodologies, although the majority of data refers primarily to the quenching of ROS as opposed to the prevention of substrate oxidation.

The DPPH test is a common and simple test looking at an antioxidant's ability to scavenge a 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) compared to an experimental control. A study comparing common olive BP's to industry standard antioxidants is found in Table 2-2, with a lower EC_{50} (concentration required to quench the oxidative process by 50%) representing a stronger antioxidant activity. Hydroxytyrosol, a common simple substituted phenol found in olives, shows a remarkably strong antioxidant activity.

Table 2-2 DPPH radical scavenging of common olive BP's, (Visioli *et al.*, 1998).

Compounds	EC_{50} (M)
Oleuropein	3.63E-05
Vitamin C	1.31E-05
BHT	1.05E-05
Vitamin E	5.04E-06
Hydroxytyrosol	2.60E-07

Bouaziz *et al.* (2005) showed very similar results of the DPPH radical scavenging of olive phenols but covered a greater range of both simple-substituted phenols and the more complex flavanoids (Table 2-3).

Table 2-3 DPPH radical scavenging of common olive BP's, modified from (Bouaziz *et al.*, 2005).

Group	Compounds	IC_{50} (µg/ml)
Reference	*BHT*	0.91
Flavanoids	*Quercetin*	0.93
	Luteolin	2.05
	Luteolin-7-glucoside	3.35
	Rutin	3.4
	Apigenin	33.84
Simple	*Hydroxytyrosol*	0.57
	Caffeic acid	0.88
	Oleuropein	1.19
	p-coumaric acid	9.5
	Tyrosol	10.85

The results from another method, incorporating the scavenging of the $ABTS^{\bullet+}$ radical relative to a Trolox standard, can be seen in Table 2-4. The $ABTS^{\bullet+}$ test gives significantly different results relative to the DPPH results given above.

Table 2-4 $ABTS^{\bullet+}$ radical scavenging of olive phenolic compounds expressed in Trolox equivalent antioxidant capacity, modified from (Benavente-Garcia *et al.*, 2000).

Compounds	TEAC (mM)
Tyrosol	0.35 ± 0.05
Luteolin-7-glucoside	0.71 ± 0.04
Oleuropein	0.88 ± 0.06
Hydroxytyrosol	1.57 ± 0.12
Luteolin	2.25 ± 0.11
Rutin	2.75 ± 0.05

The presence of the o-diphenol moiety in hydroxytyrosol and caffeic acid appears to enhance the radical scavenging capacity of the compound significantly compared with its monophenol counterparts tyrosol and coumaric acid. This can also be seen when we compare quercetin and apigenin; quercetin has two more hydroxyl functionalities, one on the 3 carbon central unit and one on the terminal phenolic group, giving it a much greater reducing capacity. The addition of a glucoside side group can also negatively impact radical scavenging capacity as can be seen with luteolin-7-glucoside and its aglycone luteolin; the decrease in molar capacity is significantly greater than the expected decrease due to an increase in the molar mass.

2.4.1.2 Pro-oxidant Effects

There has been some concern cited that particular biophenolic compounds at particular concentrations actually exhibit a pro-oxidant effect (Briante *et al.*, 2001;

Briante *et al.*, 2004). This generally occurs when the antioxidant compound in question is present in a lower molar concentration than the oxidising species. The effect is evident during the initiation phase of oxidation and does not seem to influence the rate of ROS propagation (Briante *et al.*, 2004). However, when a crude leaf extract was analysed for antioxidant/pro-oxidant effects and compared to a pure sample of oleuropein and hydroxytyrosol, the pro-oxidant effect was apparent at a significantly lower concentration suggesting that synergistic effects work to increase the antioxidant activity (Briante *et al.*, 2004).

There have been some suggestions that the pro-oxidant effect can be attributed to the iron- and copper-reducing ability of some flavanoid compounds (Briante *et al.*, 2003), but a pro-oxidant action has been observed in other phenolics that do not exhibit such activities (Briante *et al.*, 2004). Consequently, more work needs to be carried out to elucidate the mechanisms that provide for this concentration-dependant pro-oxidant effect. Such a pro-oxidant effect could have a stark negative impact on the final product application.

2.4.2 **Antimicrobial Activities**

It has long been known that *Olea europaea* is resistant to microbial and insect attack through a multi-chemical defence mechanism. Two types of chemical protection have been identified in the plant; (1) through the bitter secoiridoids glycosides, as phytoalexin precursors (Kubo *et al.*, 1985; Uccella, 2001; Uccella and Saija, 2001); and (2) a physical barrier of crystalline oleanolic acid that coats the leaf (Kubo *et al.*, 1985). Oleanolic acid is not a BP and does not show any antimicrobial activity. It is suggested that the coat functions to limit microbial penetration into the leaf and helps in creating an environment that is not conducive to microbial growth by hindering moisture transport. The secoiridoids only show a minimal antimicrobial activity in their natural state. It is believed that highly hydrolytic enzymes, probably similar to β-glucosidase, are initially responsible for transforming the compounds into an active state, phytoalexins, as an induced or preformed reaction to microbial attack. This begs the question, what role do the other minor BP components have in the protection of the plant from microbial attack? Are they just present as secondary metabolites and intermediates with no direct function in plant defence? Is the antimicrobial activity of these compounds of no significance to overall plant protection? Of course the actual

presence of these compounds will affect the degree and likelihood of microbial infestation, but do they contribute to a formal plant protection system?

Examination of the *in-vitro* function of these minor components, show many of the isolated compounds with a wide range of antimicrobial activities, against many species of bacteria and, to a smaller extent, fungi. The antimicrobial activities of antioxidants has been known for some time and has been proven by studies that show common antioxidants used in the food industry, such as BHA and BHT, show a strong antimicrobial action (Tassou *et al.*, 1991; Tuncel and Negrez, 1993). This has sparked a lot of research into natural antioxidants for their use as microbial inhibitors for food systems.

Another interesting phenomenon when dealing with the activities of various BP's is their synergistic behavior. It is common for the total activity of an extract containing BP's to be greater than the activity of the sum of the individual parts. This is similar in nature to the synergistic behavior of vitamin C and E, with the addition of vitamin C increasing the activity of viamin E with respect to its antioxidant activity.

The range of bacteria and microfungi examined by various authors for susceptibility to olive BP's is large and includes human pathogens (Kubo *et al.*, 1985; Tuncel and Negrez, 1993; Tassou and Nychas, 1994; Bisignano *et al.*, 1999; Robles *et al.*, 2000), common plant pathogens (Northover and Schneider, 1993; Capasso *et al.*, 1995; Rodriguez-Kabana *et al.*, 1995; Fodale *et al.*, 1999), olive fermentation cultures (Fleming *et al.*, 1973; Jimenez-Diaz *et al.*, 1991), and bacterial spores (Tassou *et al.*, 1991). With BP's possibly having applications in the food industry, studies have also examined the toxicity of olive extracts on human cells (Capasso *et al.*, 1995).

2.4.2.1 Antifungal Activities of Olive Biophenols from Literature

In addition to studies on phenolic susceptible microbes, there has been interest in microbes that metabolically utilize olive BP's for biodegradation of olive-mill wastewater (Robles *et al.*, 2000; Robles *et al.*, 2000). The majority of these investigated microbes are fungi of various types. As a general observation, fungi appear to be more resistant to the antimicrobial activity of BP's with little data in literature showing olive BP-susceptible fungi. This is not entirely surprising considering that disease causing fungal species will have an evolutionary relationship

with the various olive BP's, or plant phenols in general, and would therefore be less sensitive to their deleterious effects (Appel, 1993).

The majority of studies relating fungi to olive BP's are with respect to the micro-organisms capability to degrade phenolic compounds, and as such, fungicidal properties are generally not considered. These studies are carried out to determine the biomass production and detoxification potential of various fungal strains isolated from olive mill wastewater (OMWW). A number of different fungal genera were isolated including *Penicillium* (Robles *et al.*, 2000; Robles *et al.*, 2000), *Acremonium, Alternaria, Aspergillus, Chalara, Fusarium, Lecytophora, Paecilomyces, Phoma, Phycomyces, Rhinocladiella* and *Scopulariopsis* (Robles *et al.*, 2000). Strains of *Chalara, Fusarium, Paecilomyces, Penicillium* and *Scopulariopsis* grew efficiently in undiluted OMWW and the latter four species showed the capacity for OMWW detoxification, depleting its antibacterial activity entirely. These strains show the ability to metabolise certain quantities of the BP compounds present in the wastewater; total initial phenol concentration was measured to be 1.6 ± 0.18 g/*l* (Robles *et al.*, 2000), which is good representation of most OMWW's.

As an example of antifungal activity, olive oil was applied to apple leaf tissue to determine the degree of control over diseases caused by *Podosphaera leucotricha, Ventria inaequalis,* and *Albugo occidentalis* (Northover and Schneider, 1993). Olive oil showed 99% inhibition of growth of *P. leucotricha*, only slight prophylactic activity against *V. inaequalis* and no inhibition of *A. occidentalis*. It should be noted that olive oil contains only small quantities of BP's and should only show a fraction of the activity of a specifically concentrated extract.

Verticillium dahliae Kleb. is a phyto-pathogenic fungus found in soil environments. A study was carried out to determine if watering soil with OMWW would increase the presence of this fungus to the detriment of the soil ecology (Fodale *et al.,* 1999). It was found that when OMWW is diluted in distilled water at the quantities of 400 ml/*l,* microsclerotia and conidia production was completely inhibited. This data suggests that OMWW could be used in combating this pathogenic fungus that is widespread in parts of western Sicily.

It should be realised that the antimicrobial activities of BP's are threshold phenomena (Appel, 1993), with ranges of sensitivity and tolerance. It is conceivable then, that with an increased dose of the active ingredients, we may see a considerable change in the susceptibility of the target fungal species. The concentrations of phenols in the OMWW detoxification studies above would be optimised, through dilution, for maximum growth and metabolic function, giving the study a unique focus. This project examines the levels of inhibitory concentrations in mediating the growth kinetics of these fungal species. More research is needed with this direct fungicidal approach to examine the possibility of using olive BP's in the control of problematic fungal species.

2.4.2.2 Antibacterial Activities of Olive Biophenols from Literature

An interesting discovery from these studies is that Gram-positive microbes are equally susceptible to attack from olive BP's as their Gram-negative counterparts, in some cases, even more so (Fleming *et al.*, 1973; Tuncel and Negrez, 1993; Markin *et al.*, 2003). Olive BP's appear to be capable of penetrating and disrupting structurally different cell membranes causing the leakage of cellular constituents, a common lethal mechanism for antibacterial agents (Tassou and Nychas, 1994).

Two commonly investigated olive BP's for antimicrobial activities are oleuropein and hydroxytyrosol. Although both compounds have an o-diphenol system common to highly active BP's, oleuropein was found to be significantly less toxic than hydroxytyrosol. It is speculated that the glycosidic functionality of oleuropein might render the compound unable to penetrate the cell membrane or to reach the target site, reducing the compounds efficacy (Bisignano *et al.*, 1999).

Various studies provide evidence for this where extracts have been treated with β̃-glucosidase, or other hydrolyzing compounds, show a higher potency or microbial inhibition (Fleming *et al.*, 1973; Kubo *et al.*, 1985; Rodrigeuz *et al.*, 1988). OMWW also tends to show a higher antimicrobial activity as a result of the hydrolysis of high molecular weight BP's during oil extraction procedures. Catechol, found primarily in the OMWW, is probably the most effective antimicrobial phenol investigated, and is a degradation product of higher molecular weight compounds.

The data that was obtained from various literature sources varies quite considerably with many contradictions. This emphasises the importance of a rigorous purification, characterisation and testing protocol. The data obtained from literature is summarised in Table 2-5. This data should be seen as only a tentative guide to the susceptibility of the various targets to BP attack.

2.4.3 **Phytotoxic Activities**

A phytotoxin can be loosely defined as any substance produced by higher plants that is capable of suppressing the growth of other plants. Many of these phytotoxic compounds isolated from plants are phenolic in nature. Whether or not the phytotoxic activity of olive BP's can be exploited as a herbicide or germination inhibitor depends on the potency of activity, either as pure compounds or as a raw extract, and the specificity of the compounds for various target weed species.

A substantial proportion of the literature data regarding the phytotoxic effects of olive BP's comes from studies on the ecological impacts of OMWW and olive pomace, either as raw waste products or as potential fertilizers. I have yet to find any literature dealing directly with the control of pest vegetation using olive waste products, although other data is suggestive of significant activity.

One study by Rodríguez-Kábana *et al* (1995) in which they were investigating control of root-knot nematodes on tomato using OMWW showed some phytotoxic effects on the plants. The addition of biuret or guanidine at 200-300 mg/kg soil, as a nitrogen source, reduced or eliminated the phytotoxic effects of the OMWW, controlled the root-knot nematodes, and showed an increase in esterase activity in the soil indicative of healthy microbial activity.

Capasso *et al* (1992) specifically investigated the phytotoxic effects of common OMWW BP's catechol, 4-methylcatechol, tyrosol and hydroxytyrosol as well as their respective acetates. Tests were carried out on tomato plants and vegetable marrow with phytotoxic effects analysed with respect to cellular collapse. Catechol, 4-methylcatechol and tyrosol all showed significant phytotoxicity against the tomato plants with hydroxytyrosol showing minimal effects at concentrations of 1-2 M.

Table 2-5 Standardised antimicrobial activities of various phenolic compounds. [a]

Bacterium		Analytically Pure Compounds						Olive Mill Waste Water		
		Caffeic	Catechin	Courmaric	Hydroxytyrosol	Oleuropein	Vanillin	Catechol	Hydroxytyrosol	Raw Extract
Gram-Positive	*Bacillus cereus*	++ [1]		++ [1]			++ [1]			
	Bacillus cereus (spores)					++ [4]				
	Bacillus megaterium									+++ [7], ++ [8]
	Bacillus subtilis									+++ [7]
	Corynebacterium michiganese							+ [6]	- [6]	
	Lactobacillus plantarium									
	Staphylococcus aureus	++ [1], +++ [2]	+++ [2]	- [1,2]	+++ [3]	++ [3,5]	+ [1]			+ [7]
Gram-negative	*Easchericia coli*	++ [1]		+ [1]			+ [1]			- [7]
	Haemophilus influenzae				+++ [3]	+ [3]				
	Pseudomonas aeruginosa	+++ [2]	+++ [2]	- [2]						
	Pseudomonas fluorescens									
	Pseudomonas savastonoi							+++ [6]	++ [6]	
	Pseudomonas salanacearum									
	Salmonella typherium	++ [1]		++ [1]	+++ [1]		++ [1]			- [7]
	Vibrio parahaemolyticus				+++ [3]	++ [3]				

Bacterium		Olive Fruit			Olive Leaf
		Oleuropein	Oleuropein (hyd)	Raw Extract	Raw Extract
Gram-Positive	*Bacillus cereus*				
	Bacillus cereus (spores)			++ [4]	
	Bacillus megaterium				
	Bacillus subtilis	++ [9]	+++ [9,11]	+++ [9]	- [12]
	Corynebacterium michiganese	+++ [9]	- [9]	+++ [9]	
	Lactobacillus plantarium	- [9]	++ [9]	++ [9]	
	Staphylococcus aureus	++ [9]	+++ [9]	+++ [5,9]	++ [12]
Gram-negative	*Easchericia coli*	- [9]	- [9,11]	++ [9]	++ [12]
	Haemophilus influenzae				
	Pseudomonas aeruginosa				+++ [12]
	Pseudomonas fluorescens	- [9]	- [9]	++ [9]	
	Pseudomonas savastonoi				
	Pseudomonas salanacearum	++ [9]	++ [9]	++ [9]	
	Salmonella typherium	- [9]	++ [9]	++ [9]	
	Vibrio parahaemolyticus				

[a] -, no activity (0-5%); +, minimal activity (5-25%); ++, moderate activity (25-75%); +++, strong activity (75-100%). (1) (Tuncel and Negrez, 1993), (2) (Paster *et al.*, 1988), (3) (Bisignano *et al.*, 1999), (4) (Tassou *et al.*, 1991), (5) (Tassou and Nychas, 1994), (6) (Capasso *et al.*, 1995), (7) (Robles *et al.*, 2000), (8) (Rodrigeuz *et al.*, 1988), (9) (Fleming *et al.*, 1973), (10) (Jimenez-Diaz *et al.*, 1991), (11) (Kubo *et al.*, 1985), (12) (Markin *et al.*, 2003)

Perez *et al* (1986) looked specifically at the effect of OMWW on seed germination and early plant growth. Three types of wastewater were examined; raw wastewater, wastewater with organic material removed and deionised wastewater. Results showed an inhibitory effect on seed germination by all treatments with raw wastewater having the greatest depressive effect, followed by deionised wastewater, and finally the wastewater with organic material removed. After application to soil, an initial inhibitory effect on seed germination is recorded. The activity of various micro-organisms in the soil degrades organic matter over time and tends to remove any phytotoxic effectors. Such processes require about 80-90 days for complete detoxification (Tomati and Galli, 1992).

Phytotoxic effects are most evident in herbage crops, particularly in potatoes, tomatoes and various weed species (Tomati and Galli, 1992). No harmful effects have been recorded on trees, particularly olive trees and vines; as such, the olive BP's may be able to be used to control various weed species or plant diseases without fear of affecting the tree.

2.4.4 **Human Toxicity Testing**

For any application of olive extracts where human contact is expected, possible toxic effects must be examined. In addition to experiments looking at toxicity to cultured human cells, we must also consider any pro-oxidant activities that might mediate DNA or protein damage.

Capasso *et al* (1995) examined the toxicity of OMWW BP's to cultured human Hep2 cells in relation to any possible antibacterial activity. His group found that raw wastewater, catechol, 4-methylcatechol, and various acidic BP's all showed significant toxicity to the examined cell line, whereas hydroxytyrosol and tyrosol showed no apparent activity. As hydroxytyrosol showed significant antibacterial activity with limited phytotoxic activity, the author suggests that this particular compound has the greatest potential for use in agricultural bacterial-pest management systems.

Aeschbach *et al* (1994) examined the antioxidant and pro-oxidant activity of hydroxytyrosol with respect to other commercially accepted and potentially

acceptable alternatives. They found that in addition to the strong antioxidant activity of hydroxytyrosol, the compound also shows a significant pro-oxidant activity, which can induce DNA, protein, or carbohydrate damage given the presence of certain reducible compounds. This effect appears to be an indirect result of the presence of complexes, particularly iron-complexes, which can be reduced by antioxidants and can therefore react with oxygen species to produce ROS that are the damage inducing species through oxidation mechanisms. A reduction in the pro-oxidant mediated damage can be observed with the addition of albumin. It is suggested that it is the protein itself that is damaged, in turn affording some protection to the DNA. Since only hydroxytyrosol was examined for pro-oxidant effects, it may be possible that other related BP species also exhibit this activity.

The BP species that can be extracted from olive waste products can show some negative, and potentially harmful, activities. Where human contact is expected or probable, assays should be carried out to determine the degree of toxicity and safe levels of human exposure. Very strong toxic effects may greatly reduce the list of possible applications of olive extracts or impose significant controls to their use.

2.4.5 **Factors Affecting Biophenolic Activity**

It is appropriate to examine various phenolic interactions that may affect the bioactivity of extracts obtained from olive waste products. Variation in the activity of phenolic compounds comes from three sources: (1) evolutionary differences in tolerance among organisms, (2) variation in phenolic structure and concentration, and (3) variations in conditions influencing the mode of phenolic action, i.e. oxidative activation and state (Appel, 1993). The idea that there is more to phenolic activity than just their respective concentrations and structures stems from recorded variations in phenolic activity where such aspects remain constant. Although it is stated that oxidative activation is the most important aspect affecting the variation in activity of phenolics, within the framework of this project, evolutionary relationships may prove to be a principle effecter.

It has been stated that organisms with an extended evolutionary relationship with phenolics would become, over time, less sensitive to any associated deleterious effects (Appel, 1993). An example used previously was that phyto-pathogenic fungal species

involved in plant disease would be less sensitive to phenolic defence systems. This could be a possible explanation for the resistance of some fungal species to phenolic attack. Another example would be the ability of certain soil microbes to survive in an environment with excessive phenolic content, e.g. after soil treatment with OMWW. After an initial period of decline, where the capacity of the microbe's ability to degrade the compounds is the limiting factor, the soil microflora adapts and recuperates and can exceed control conditions (Tomati and Galli, 1992). Such microbes have a historical role in the breakdown of plant material.

The concentration of the phenolic compounds will also play an important role in activity. Even for organisms that have a high tolerance for certain 'dietary' phenolics, there will be thresholds above which negative effects are observed. To this point, studies examining environmental phenomenon with respect to phenolic compounds must be carried out, and within ranges that will be of significant interest.

With the great diversity in phenolic structure, a great variation in their modes of action would be expected, and therefore, their ecological activities. Major structural classes of polymeric and monomeric phenols vary significantly in their bioactivities, as do differently substituted compounds and steroisomers. The two main groups of BP's of interest to this project are the glycosidic phenol species and the simple substituted phenol species. There is no doubt that these differently structured compounds play uniquely different roles within the olive plant. It has been suggested in previous sections that the glycosidically bound BP's are mainly responsible for the plants defence mechanisms and are bound to associated membrane molecules for bioavailability.

One of the most significant factors affecting ecological activity of phenolics is the variation in oxidation state (Figure 2-7). The oxidation state of phenolic compounds can be influenced by many biotic and abiotic factors, such as enzyme activity, pH and redox potential, producing phenolic, phenolate or quinone oxidation states.

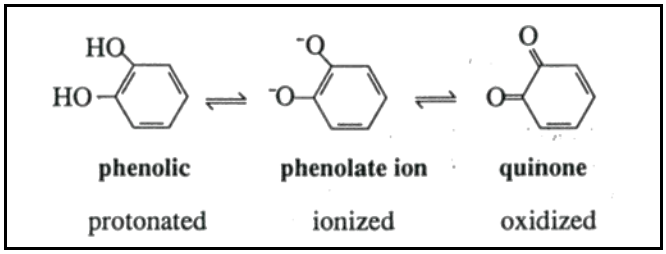


Figure 2-7 The oxidation states of phenolic compounds. (Appel, 1993)

The different oxidation states play vital roles in catalytic autoxidative chain reactions and in determining the types of bonds that the compound can participate in. The types of bonds in which phenols can participate in are given in Table 2-6 along with associated chemical conditions. As phenolic bioactivity is highly dependent on molecular binding and oxygen radical formation, these oxidation states will play a vital role in determination of antimicrobial and phytotoxic effects. In addition to this, the binding of BP's to macromolecular compounds will be determined by the oxidation state (Figure 2-8). The strength of these bonds, determined by oxidation state, will be a significant factor in the extraction and isolation of BP compounds from olive pomace and leaves. As such, the pH level during extraction may play a significant role in extraction. To prevent covalent bonding of the BP's, a pH of less than neutral should be used.

Table 2-6 The characteristics of bonds in which phenolic compounds can participate. (Appel, 1993)

	Type of bond			
Characteristic	Hydrophobic	Hydrogen	Ionic	Covalent
Strength (kJ/mol)	<4	10–40	100–1000	100–1000
Reversibility	reversible	reversible	reversible	irrevers.
Oxidation state	phenolic	phenolic	phenolate ion	phenolic or quinone
pH of formation	any	<8	>8	>8

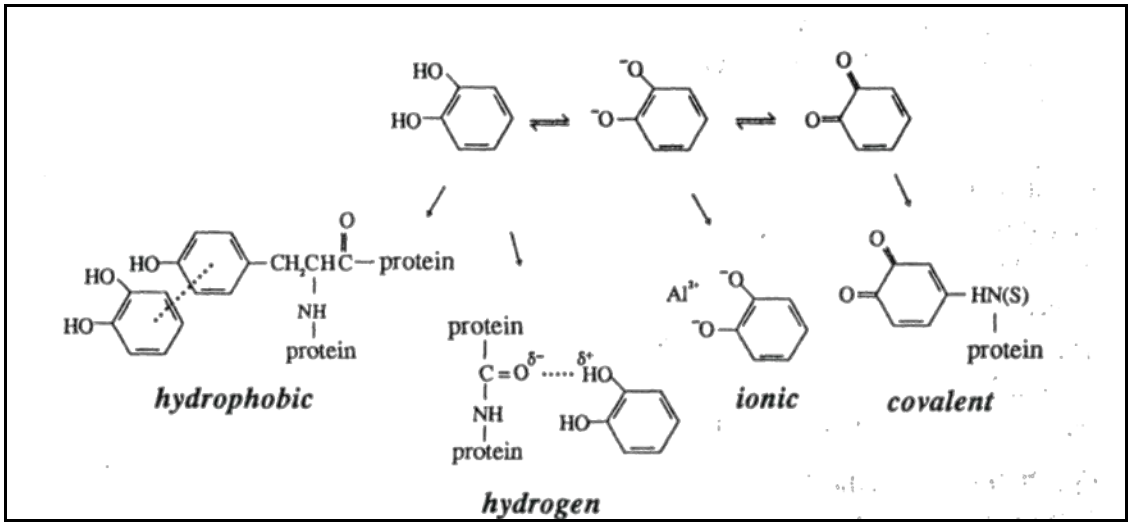


Figure 2-8 Potential modes of phenolic binding. (Appel, 1993)

2.4.5.1 Variations Due to Plant Constituent

As mentioned previously, the fruit of the olive accumulates mainly the glycoside variety of the more complex BP compounds, which have been found to be less active than their respective aglycones with respect to antimicrobial activity. On the other hand, leaves produce mainly the aglycones of the various secoiridoids, suggesting the possibility of a higher resistance to microbial attack and a greater opportunity for the isolation of antimicrobial species. The leaves are also known to accumulate mainly complex BP's with very few simple substituted phenols. As biological activity seems to increase with a decreasing molecular weight, the more complex BP profile in the leaves may offset the higher activity of secoiridoid aglycones with respect to extracts obtained from the fruit, which show a higher proportion of these simple compounds.

So the antimicrobial activity of a certain extract will be greatly determined by the source from which it was obtained. OMWW, leaves and the pomace remaining after must separation will all produce extracts of varying activities.

2.4.5.2 Variations Due to Extractive Procedures

The malaxation procedure in olive processing has been linked to an increase in the activity of certain hydrolytic enzymes. This directly results in the modification of the BP profile in all phases obtained, be it the oil, OMWW or the remaining husks (Uccella, 2001). This change in BP profile logically coincides with a change in the antimicrobial activity of the extract, usually increasing the activity due to a lower molecular weight and cleavage of glycosidic functionalities.

In addition to modifications of the chemical compounds affecting the antimicrobial activity of the extract, the BP profile, as a result of partition coefficients between water and oil, will also affect the extract activity. The more hydrophilic compounds will concentrate in the aqueous phase with the hydrophobic compounds concentrating in the oil phase. If the extraction procedures incorporate a higher water input, then it will be expected that more of the hydrophilic compounds will be extracted into the aqueous phase. This will reduce the total quantity of hydrophilic BP's left in the pomace for recovery, and will therefore affect the economic feasibility of any recovery process.

2.5 **Potential Applications for Olive Extract**

The various examples of olive BP bioactivity provide evidence that an extract from olive waste streams could present an economic opportunity for application in the horticultural or food industry. Such applications could make up part of a crop pest management system dealing with volunteer weeds, pathogenic diseases and/or insect pests. Post-harvest applications could help provide fruit or vegetable storage and distribution stability by reducing the possibility of microbial or insect contamination. Both applications in the primary cultivation, manufacture and storage and distribution of food products would be aimed at exploiting the current trends in organic food systems.

Each possible application has a number of positive and negative aspects that require examination and foresight; these will be covered in the following sections.

2.5.1 **Crop Management**

The use of fertilisers and pesticides is commonplace in horticultural enterprises and are important to ensuring a high crop yield by optimising nutrient supply and minimising pest emergence. As mentioned previously, the use of synthetic compounds in such systems has become a critical matter of retrospect for many consumers, thus the development of ecologically friendly or natural horticultural management tools has become a major area of interest for producers of such products.

In addition to the fertilising capacity of OMWW and pomace (Tomati and Galli, 1992; Rodriguez-Kabana *et al.*, 1995), extracts of olives show activity that may have applications in weed, microbial and insect pest management of horticultural, or even domestic, crop and plant systems.

With any horticultural crop management system we must consider how the sustained application of a product will affect the long-term stability of the ecosystem. It has been shown that certain soil microbiota breakdown olive phenolics through various pathways, thus neutralising the activity, over approximately 2-3 months (Walker, 1975; Tomati and Galli, 1992). The sustained application of any such fertiliser could result in a change in the microbiota population, with the emergence of more phenolic resistant and metabolising species. How this change in equilibrium would affect the crops is open to speculation, but any deviation from natural state would go against the premise of organic systems maintaining ecosystem equilibrium.

As with any horticultural food product, we must ensure that it is safe for human use and contact given the correct methods of application; this involves toxicology testing for acute, subacute, and chronic toxicity; mutagenicity (cancer); neurotoxicity (nerves); and metabolism studies with effects of ongoing worker exposure.

2.5.1.1 Weed Control

Weed control is a very important aspect of many crop systems, especially herbage crops where weed competition can be critical to crop yield. There are a number of strategies to minimise weed emergence in crops, which include various preventative, biological, cultural, mechanical and herbicidal options, each with their respective pros and cons and optimum points of use.

One of the best possible applications of an olive extract in weed management would be in pre-planting of herbage crops, where certain weed species require control prior to crop plantation. This would allow enough time for the soil microbiota to degrade the phytotoxic compounds to the point where the crop will not be adversely affected.

The aspects that require consideration for the application of olive BP's as a natural herbicide are the selectivity and efficacy of the product and the timing of application. We must also examine any affects on our crop of interest and the environmental fate

of the product including breakdown, groundwater seepage, and ecological effects on plants, animals, and soil microbiota.

2.5.1.2 Disease Control

The strong antimicrobial activity shown by olive BP's shows promise for the application of the extract to help manage various pathogenic, particularly bacterial, horticultural diseases.

The continuing activity of the extract applied to the plant will be a function of the environmental conditions, the stability of the active ingredients in the extract and the degree of contact between the extract and the target pest. Rain may play a large role in determining the contact time, and therefore, the absolute efficacy of the extract. The use of liposoluble active ingredients might decrease the rain-wash effect.

BP's are prone to oxidative chain reactions that may in turn alter the mode of action of the compound (Appel, 1993). As such, the activity of the product against certain pathogenic microbes may decrease with time in a photo-oxygen rich environment.

The degree of contact between the extract and the target will play a major role in determining the efficacy of the product. The site of disease will need to be targeted during application of the product; if the site of infection is not properly treated then the possibility of success is greatly reduced. Usually such fungal and bacterial infestation is site specific and as such requires attention at a plant-by-plant level as opposed to a crop level.

2.5.1.3 Pest Control

Insect pests can be responsible for significant fruit loss or variations in plant productivity. Such pests include certain pathogenic nematode species and various fruit fly species.

Horticultural cultivation encourages an increase in parasitic nematodes that feed on the crops being grown (Stirling, 1991). Nematodes parasitic to plants may cause yield losses by themselves or they may join with other soil-borne organisms such as viruses, fungi, and bacteria, to promote disease development in plants. Most often, nematode feeding reduces the flow of water and nutrients into the plant, increasing the

plant's susceptibility to other stress factors such as heat, water, and nutritional deficiencies.

Studies have shown that the proliferation of certain species of parasitic nematode, in particular the root-knot nematode *Meloidogyne ingognitaI,* is inhibited by the application of BP containing olive by-products (Perez *et al.*, 1986; D'Addabbo and Sasanelli, 1997). A decrease in nematode reproduction was evident at all concentrations and rates of application. One limiting factor in the application of olive extracts for its nemacidal activity is the inherent phytotoxicity of the active ingredients, pose a problem in sensitive herbage crops. It was found that mixing the extract with various concentrations of certain nitrogen sources, biuret and guanidine, significantly reduced the phytotoxic effects and increased soil esterase activity, indicative of microbial activity (Rodriguez-Kabana *et al.*, 1995; D'Addabbo and Sasanelli, 1997). It is suggested that the increase in N of such treatments increases the rate of phenol detoxification through microbial mechanisms, while maintaining control over the nematode population.

2.5.2 **Post-Harvest Stability of Fruit**

One of the more promising applications of an olive extract is in post harvest stability of fruit and vegetables. These products are prone to microbial attack, which can be responsible for greatly reducing the quality, and therefore market price, of the product. The antimicrobial activity of olive BP's suggests that it may be possible to greatly reduce the likelihood of microbial growth with the application of an olive extract. This will most likely come in the form of a fruit dip, whereby the fruit is immersed in the extract and left in storage, and then washed prior to distribution.

The bitter nature of the active BP compounds in the extract would mean that the sensory qualities of the product may be negatively impacted. The use of the extract on fruit where the skin is not consumed, as with avocados and citrus fruit, might provide a better opportunity. The permeability of the fruit skin might also pose difficulties in ensuring these bitter compounds are not absorbed into the mesocarp. The visual characteristics of the fruit may also deteriorate on treatment given the strength of the active ingredients and its nature to oxidise. If the quality of the fruit deteriorates with such treatment, then the feasibility of such an application would be greatly reduced.

Fungi are by far the most deleterious and commonplace microbial infections in post-harvest diseases of fruit and vegetables; where bacteria can cause disease in vegetables; they are generally rare in tree fruits and berries (Kader, 1992). As can be seen in the previous sections, olive BP's have a somewhat selective activity towards fungal species, some of which require high concentrations before any activity is observed. It should be realised that the fungicidal activity of olive BP's is a threshold phenomenon, whether or not the required concentrations for effective treatment are feasible requires experimental determination. The use of an olive extract with other commercial fungicides may provide a synergistic effect, whereby the olive extract can be used in conjunction with currently used fungicides to enhance antifungal activity for post-harvest applications, possibly allowing the reduction in inorganic or synthetic fungicide concentration.

It is a common understanding that as a fruit ripens and/or approaches senescence, its biochemical defence and fungal resistance decreases. The use of an olive extract might be most valuable during this time when climacteric fruit approaches its maximum rate of respiration and further shelf stability is required. Treatments with olive BP's will help create a synthetic chemical defence system when the natural fruit resistance decreases. Such a process will be most likely used during cold storage of the product, and as such, the activity of the olive extract should be tested against fungal species that are most likely to proliferate in these conditions, i.e. *Botrytis cinerea, Alternaria alternata* or *Penicillium expansum* (Kader, 1992).

Table 2-7 lists diseases and their respective trigger organisms that may provide targets for treatment with an olive extract.

Table 2-7 Post-harvest diseases caused by fungal species of certain fruit (Kader, 1992).

Fruit	Microbe	Disease Symptom
Kiwifruit	*Alternaria alternata*	Surface mould
	Botrytis cinerea	Stem-end rot
	Dothiorella gregaria	Soft-rot
	Phoma spp.	Phoma surface rot
	Penicillium expansum	Stem-end rot
	Typhula spp.	Buck-shot rot
Avocado	*Colletotrichum gloeosporioides*	Anthracnose of avocado
	Dothiorella gregaria	Stem-end rot
	Lasiodiplodia theobromae	Stem-end rot
Orange	*Alternaria citri*	Stem-end rot
	Botrytis cinerea	Stem-end rot
	Geotricum candidum	Sour-rot of Naval
	Lasiodiplodia theobromae	Stem-end rot
	Penicillium italicum	Blue rot
	Penicillium digitatum	Green rot
	Phomopsis citri	Stem-end rot
	Trichoderma viride	Yellow rot
Lemon	*Colletotrichum gloeosporioides*	Anthracnose of lemon
	Sclerotinia sclerotiorum	Sclerotinia rot of lemons
Banana	*Colletotrichum musae*	Anthracnose rot
	Fusarium roseum	Stylar-end rot
	Lasiodiplodia theobromae	Stem-end rot
	Thielaviopsis parqdoxa	Thielaviopsis rot
Grape	*Eutypa lata*	Grapevine dieback

These various fungal and fruit species have been isolated as the most viable targets for applications of olive extracts in post-harvest management systems. As noted, of these fruit species, avocado and the citrus fruit offer the best fruit targets. The testing of all of these fungal targets would be a very laborious task, so four targets were selected for testing; *Alternaria alternata, Botrytis cinerea, Penicillium digitatum* and *Eutypa lata*.

2.5.3 **Antioxidant Additive**

Food products, especially those with a high fat content, require protection from reactive oxygen species (ROS) due to the oxidative nature of their presence. This protection comes in the form of antioxidant compounds that readily reduce these ROS

to a more stable form, protecting the lipids and oils from attack. These compounds are usually synthetic in nature, but with the recent interest in food safety and consumer awareness, we have seen a resurgence in research and development of natural alternatives, of which, olive BP's pose a significant opportunity. Monomeric phenolic compounds are ubiquitous in nature and have been shown to have strong antioxidant activities.

Antioxidant compounds for food products have a number of requirements. In addition to a relatively strong antioxidant activity, the compound must firstly be devoid of human toxicity and disease. For organoleptic appreciation of the food product, the anti-oxidant compound must also be free of excessive colour and taste attributes that may negatively affect the food.

As olive BP's appear to quite strong in terms of bitter flavour, the possibility of using such compounds in consumed food systems seems unfeasible. Olive BP's may become acceptable for food use if the bitter flavour component can be masked in some way or used in a food product that is common to the source, i.e. reformulation of less pure olive oils.

It might also be possible to exploit these antioxidant activities in non-food systems, such as in therapeutics or controlled environment studies, where organoleptic qualities are of no consequence.

However, it is also unlikely that a raw mixture of BP's would be suitable for human consumption or for therapeutic applications. It has been shown that raw extracts of olive BP's may present a certain degree of human toxicity (Capasso *et al.*, 1995). Only a few of these BP compounds show no signs of toxicity, and would therefore require rigorous separation and purification procedures.

2.5.4 **Adjuvant of Pre-existing Product**

There is much evidence in the literature that suggests the possibility of synergistic behaviour between various BP's, and between various BP's and other compounds. It might be possible that an olive extract in combination with an active product might produce results that are greater than the sum of the respective parts.

An active olive extract could be combined with products such as Certified Organics Ltd. *Organic Interceptor*™ product in an attempt to increase the efficacy or selectivity of the product. In combination with the dehydrating capability of *Organic Interceptor*™, an olive extract might exhibit an increased phytotoxic activity as it initiates cellular collapse. The result may be a product that achieves plant death in a lesser time frame than with herbicide *Organic Interceptor*™ alone.

Fungicidal applications can also be examined with respect to synergistic activity. If the phenolic compounds in olives can be somehow bound to chitin targeting compounds of fungicides, then it may be possible to enhance the synergistic activity of these BP compounds.

2.6 Extraction/Purification Protocols for Recovery of Phenolic Compounds

The key to designing an extraction protocol for the economic recovery of a product of interest is in the rigorous analysis and characterisation of the starting material with a clear definition of the final product objective. This will help define possible separation techniques and identify any constraints regarding the product, operations and conditions to be used (Asenjo, 1990). The three following steps are used as a backbone from which to expand on separation protocol opportunities.

1. *Final Product Objective:*

The final product after processing will be a raw extract containing a given profile of olive phenolic compounds at a specified concentration showing a definable bioactivity. We wish to analyse the effect of different extraction techniques on the activity of the olive extract.

2. *Starting Material:*

The raw material for extraction of the phenol products can take two possible forms: a) a spent pomace from oil extraction, b) leaves remaining after pruning of the olive plant. Both of these are complex plant materials containing many possible contaminating species. At this point it is important to restate that the various BP

compounds in these plant matrices have a range of different polarities. This necessitates a compromise with either a focus on one group of compounds or two separate extraction protocols will need developing.

3. *Selection of Separation Sequence:*

It is difficult to define a complete separation sequence at such an early stage in the process, although a general sequence of processes can be described (Figure 2-9). There will be an initial raw material preparation process that will dry, grind, or process the raw material so that the following extraction step is as efficient as possible. After extraction from the plant material, the raw extract requires concentration, purification and finishing as required.

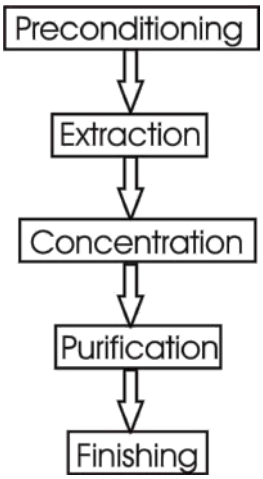


Figure 2-9 Basics steps in isolation of biological products from a plant source.

2.6.1 **Extraction of Bioactive Compounds from Plant Sources**

The use of plant material as a source for biologically and chemically important compounds has been exploited for millennia. Given this, it is surprising that the technology associated with extraction from plants has remained somewhat stagnant over recent years, with few avenues of new development. One reason for this lack of progress can be attributed to a preference for microorganisms as a preferred source for novel biological products given the relative ease of biomass production and genetic transformation. Separation technology, and research and development, have been more focussed on using microbiological sources for production of bioactive products than plants, leaving a gap in plant-based technology.

It is expected that the true value of plants for the production of proteins with complex tertiary structures will become realised in the near future and with it more efficient extraction procedures will be developed given that the associated mechanisms of product recovery are elucidated.

When analysing the process of extracting a product from plant material, at its simplest it can be defined as the removal of a solute from a solid matrix using a liquid solvent phase which shows affinity for the solute of interest. The two phases are in intimate contact allowing the solute to diffuse from the solid into the liquid phase. This is effectively a leaching process and is common in the extraction of oils from plant material as well as metal recovery from raw ores. The process of leaching from plant material will be discussed further in section 2.6.2.

In any extraction process we wish to minimise the application of organic solvents in our recovery methods to obtain a more concentrated product containing fewer additional toxins, and at the same time reducing the operational costs associated with solvent recovery, recycling, and replacement. As such, novel secondary metabolite extraction procedures have been developed to minimise the use of harmful reagents; ultrasound-assisted solvent extraction is one such operation. By modifying the extraction conditions, the quantity of organic solvents used in the process can be greatly reduced.

Another aspect of extraction that must be kept in mind is the possibility of modification of the product of interest during the isolation process. This is important with phenolic compounds as they can be sensitive to certain extraction conditions. The presence of reactive oxidising species, or even light and variations in pH, can modify structure of the phenol compounds, which can affect the bioactivity of the final product. We must also be sure that the extraction environments do not catalyse any unwanted chemical modifications of the product, including removal of sugar moieties and hydrolysis of ester linkages (although there is evidence suggesting an increase in activity given these modifications). In any case, we must be aware of the possibility that the extracted chemical profile might not be the true representation of the plant phenolic composition.

2.6.2 **Solvent Extraction**

By solvent extraction we are referring to the simple process of leaching of the plant material using a solvent as the carrier phase; this is briefly outlined below.

2.6.2.1 The Mechanics of Leaching

Leaching of a solute from a solid matrix can be generalised to include the following steps. The solvent must be transferred from the bulk solvent to the surface of the solid. The solvent then diffuses into the solid matrix and progressively dissolves the solute of interest, allowing the solute to move through the solid-solvent mixture to the surface of the solid matrix. Finally, the solute is transferred from the surface of the matrix to the bulk medium (Geankoplis, 1993). It should be noted that many different phenomenon encountered makes it almost impractical to apply any one theory to the leaching process.

Below are four different mechanisms known to play a role in leaching processes (Akgerman and Madras, 1994):

- When there are no interactions between solute and matrix, the process is simply dissolution of the solute into the solvent phase without an affect on the solid matrix.
- When interactions between solute and matrix are apparent, extraction is through desorption in the presence of the solvent where the adsorption isotherm determines the final equilibrium state. This mechanism is the most likely to be encountered in biological extractions.
- If the matrix undergoes destruction in the presence of the solvent, the solute is released into solution and extracted via the first two mechanisms.
- The final mechanism is known as reactive extraction where the solvent reacts with an insoluble solute to produce a soluble compound that is then extracted into the bulk fluid.

The rate of leaching will therefore be determined by the rate of solvent transfer from the bulk liquid to the solid, the rate of diffusion of the solvent into the matrix, and the dissolution and diffusion of the solute through the solid-solvent mixture. With biological materials, the presence of cellular material complicates the process and

greatly reduces the rate of diffusion into and out of the cell walls, especially when dealing with the thick cell walls of plants. Therefore the rate of diffusion of the solute through the solid-solvent mixture is generally the rate-limiting step. To overcome such problems, the raw material is often preconditioned by grinding and drying, which ruptures the cell walls bringing the solvent and solute into more intimate contact.

Equilibrium relationships between the solute and solvent, and the solute and solid matrix play a vital role in the recovery potential of the process. The driving force of the extraction system is based on the concentration differentials of the solute between the solid matrix and the bulk solvent, the greater the difference in concentrations, the greater the rate of extraction. The resistance to extraction was mentioned previously and is generally dominated by the rates of diffusion of the solute through the solid-solvent mixture. If the compounds are bound directly to the solid matrix, then solvent extraction is not possible unless the solvent itself can break the chemical bonds. Given this degree of binding, measures will be needed during the preconditioning stage to liberate these compounds for extraction.

As mentioned previously the leaching process is an equilibrium-based operation where the rate of extraction is determined by the driving force that is itself dependent on the degree of saturation of the solvent with the solute. As the extraction proceeds, the rate of leaching decreases so that fresh solvent must be added to maintain the desired rate of extraction. Optimisation of the process then becomes a matter of economic feasibility where the rate of extraction is counterbalanced by the cost of applying new solvent.

2.6.2.2 Solvent Selection

In solvent extraction systems, the selection of the solvent is the key decision. The following criteria should be used in selection of a solvent for leaching purposes (Humphrey and Keller II, 1997):

- *Distribution coefficient* – A high value for the distribution coefficient indicates high solvent affinity for solute, which will permit a lower solvent/feed ratio. A polar solvent will have a higher distribution coefficient for a polar solute than for a non-polar solute.

- *Recoverability of the solvent* – A clean and efficient separation of solute and solvent is desirable for solvent recovery, the greater the difference in volatility between the two species, the greater the achievable degree of separation.
- *Density and viscosity* – Viscosity plays an important part in determining the degree of contact and penetration of the solvent into the solid matrix, the lower the viscosity the greater penetration. The density of the solvent will determine the point of saturation of the solvent, a low-density solvent will reach saturation earlier, effectively reducing the rate of extraction and requiring the input of fresh solvent before leaching can proceed.
- *Safety and stability* – Toxicity and flammability are important health and safety considerations that should be tackled as early in the project as possible. The corrosive action of solvents may require special consideration to the materials of construction of the extraction or related vessels. Key questions include: Is the solvent stable and free from decomposition? Is the solvent prone to polymerization or interaction with components of the feed?
- *Compatibility* – Is the solvent compatible with the final application of the product? Some solvents might not be acceptable for use in the food or pharmaceutical industries or hold strict requirements for their removal.
- *Availability and cost* – One should make sure that the solvent of interest is commercially available and of an economically viable cost, as the solvent cost may represent a major initial expense as well as a heavy operational cost to replace lost solvent.

The importance of the above aspects of solvent selection is dependent on the application; in this case the distribution coefficient defining the degree of recovery is the most important aspect, which in turn will determine the feasibility of the any novel product developed based on an identified bioactivity.

2.6.3 **Ultrasound-Assisted Extraction**

In terms of product recovery, the use of ultrasound is an extension to the standard solvent extraction systems and is used to increase the efficiency of extraction processes by increasing rates of extraction and/or to reduce solvent input. The primary

benefit from the ultrasonic treatment of plant material prior to, or during, leaching processes, is the destruction of cellular material, particularly the cell wall, due to cavitation mechanisms. By destroying the cell wall, the resistance to diffusion of the solute through the solid matrix is greatly reduced; either allowing an increase in the rate of extraction, or acting to reduce the solvent input required to produce the same extent of product recovery.

2.6.3.1 Ultrasound – Glossary of Terms

Ultrasonic Intenstiy, $\boldsymbol{I_{diss}}$ *(W/cm*^{2}*).* This is a common term used to define the amount of energy input into a system relative to the area of the ultrasound probe submerged into the system. This is the intensity of the ultrasound at the surface of the ultrasonic probe.

Ultrasonic Power Dissipation, $\boldsymbol{P_{diss}}$ *(W).* This defines the total power flowing into the system released as heat. There are two main parameters that affect the power of dissipation: the number of cavitation events per volume, and the intensity of the collapse.

Transient Cavitation Threshold, $\boldsymbol{P_{tct}}$ *[W]*. The cavitation threshold can be defined as the value of the acoustic pressure amplitude that produces a detectable cavitation 'event' (Suslick, 1988). A cavitational event is one in which an unstable transient bubble is produced that proceeds to collapse, usually detected audibly as a 'click' or 'pop'.

2.6.3.2 Mechanics of Ultrasound

Ultrasound (US) can be defined as sound that is at a frequency beyond that of human hearing *i.e.* greater than 16 kHz. There are two distinct types of ultrasound: *diagnostic* (for physical measurement), and *power* (to influence chemical reactivity and cellular disruption), differentiated by the intensity of the power output, which is inversely proportional to frequency. It is the high intensity/low frequency power ultrasound for the disruption of a solid matrix that is of significance to this work, for which a frequency of 20 kHz is typical.

The mode of action for the destruction of a solid matrix using power ultrasound is through the process of cavitation. Sound is transmitted through a fluid as a wave

consisting of alternating compression and rarefaction cycles, analogous to the effect of a tuning fork in air, refer fig 6.2 below.

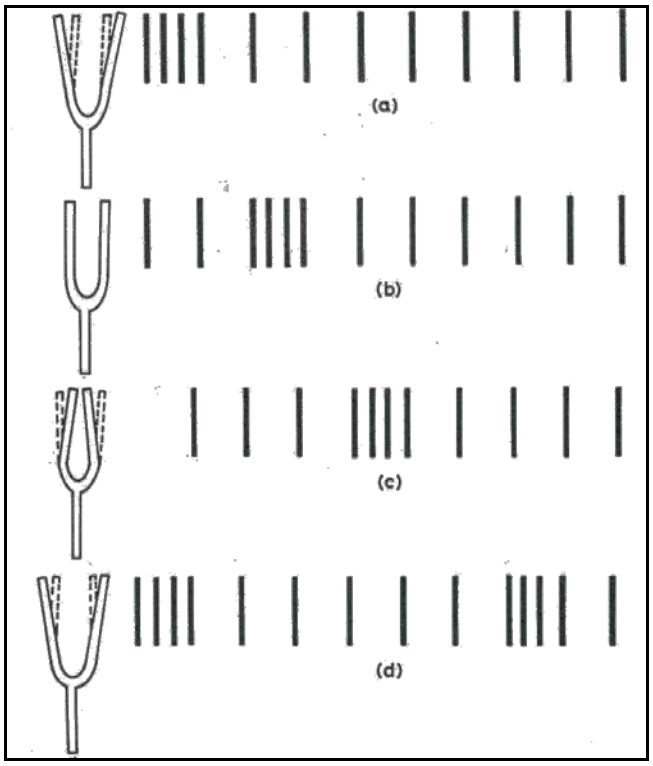


Figure 2-10 Pressure waves in air (Mason, 1990). Where (a) refraction, (b) standing, (c) compression, (d) refraction.

During rarefaction, the negative pressure developed by power ultrasound is strong enough to overcome the intermolecular bonds of the fluid, effectively tearing it apart at sites of nucleation (points where bubbles can form around existing gaseous areas, solid particles, and surface irregularities (Suslick, 1990; Thompson and Doraiswamy, 1999)). These voids can be either stable cavities that exist for many acoustic cycles, or transient cavities that exist only for a few acoustic cycles (Thompson and Doraiswamy, 1999). With high intensity ultrasound, these transient cavities can grow very rapidly as they do not have time to compress during the positive-pressure part of the acoustic cycle. The cavity then reaches a size where it can absorb energy from the acoustic field, with the size of the bubble being determined by the frequency of the waves. If in phase with the sound field, this cavity can expand very rapidly in the time of a single rarefaction cycle to a point where it can no longer absorb the energy from the sound field and can no longer sustain itself. At this point the surrounding liquid rushes into the cavity and the bubble implodes (Suslick, 1990).

This, almost instantaneous, implosion compresses the internal gas of the cavity generating temperatures of 5000 ºC with pressures exceeding 500 atmospheres (Suslick, 1990). In the presence of a solid-liquid interface, the collapse of these bubbles on or near the solid surface can cause the solid to break apart resulting in significant particle size reduction, as can be seen in Figure 2-11. The extent of particle size reduction is dependent on the physical properties of the solid and the ultrasonic conditions.

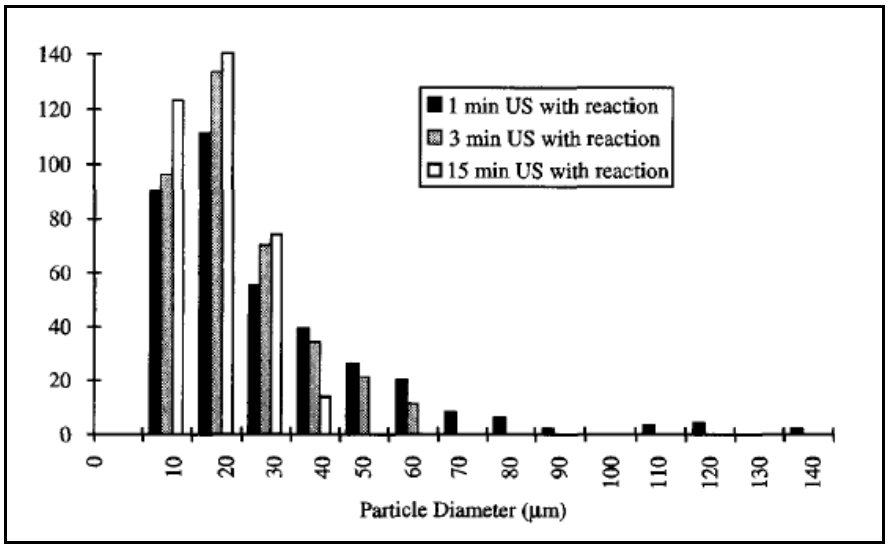


Figure 2-11 Size reduction of NaS under the influence of ultrasound (Hagenson and Doraiswamy, 1998).

It has been suggested by Suslick (1990) that there are two mechanisms contributing to this effect, although their relative contribution is, as yet, unknown. When in close proximity to an extended solid surface bubble collapse is non-spherical, driving high-speed jets of liquid into the surface (Figure 2-12). In the presence of particulate material, cavitation and the associated shockwave it creates can accelerate particles to great speeds inducing interparticle collisions capable of dramatic changes in the physical properties of the particles and a significant reduction in average particle size.

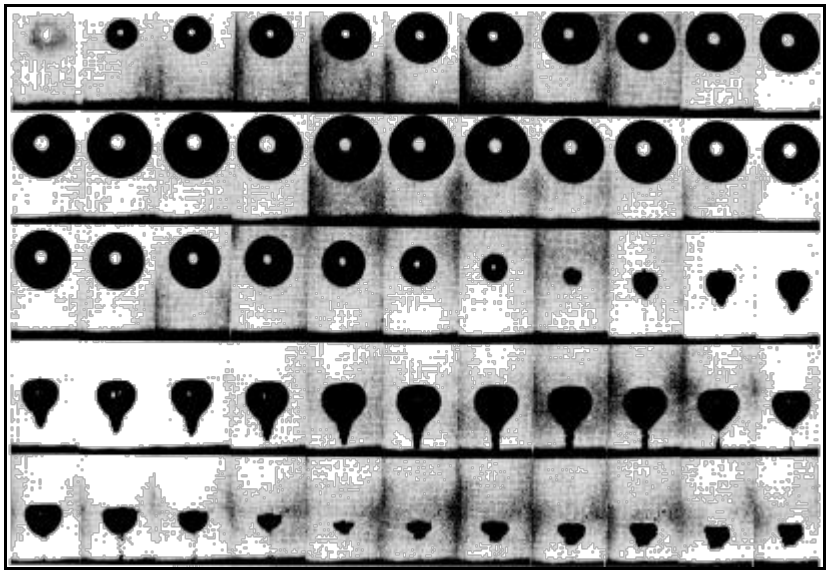


Figure 2-12 High-speed microcinemagraphic sequence of laser-induced cavitation near a solid surface showing microjet impact; 75,000 frames per second (Suslick, 1990).

The process of modelling cavitation can require quite an intense mathematical analysis, which is out of the scope of this report. There are, however, some important results from such analysis that requires mentioning.

Generally the formation of a bubble through cavitation in an ideal pure fluid can be calculated to require some very high-energy inputs, much higher than is found experimentally (Suslick, 1990; Thompson and Doraiswamy, 1999). The rate-limiting step associated with bubble formation is in the instant when the bubble is first created; this requires the greatest energy input. If a bubble is already present, such as those found attached to particulate material or lodged within a crevice, or any other entity that provides a suitable point of nucleation, then the process of cavitation during wave rarefaction requires a substantially smaller energy input.

2.6.3.3 Factors Affecting Cavitation

Cavitation from an acoustic field is a very complex process that is dependent on a wide range of process parameters and conditions. With such an extensive array of effectors and the wide range of US system set ups, it is difficult to make meaningful comparisons between data given in literature, where authors often do not outline the magnitudes of all the important processing variables. It is important in the study of ultrasonic systems to experimentally determine the magnitudes of important process parameters, as each US system is unique.

Below are the main factors that will directly affect the process of bubble formation and bubble fate during the process of cavitation (Mason, 1990).

Presence of gas – The presence of dissolved gas plays an important role in cavitation as a site for nucleation. The removal of gas from a liquid will reduce the available nuclei and it will become increasingly more difficult to induce cavitation within the system. Furthermore the passage of ultrasound through the liquid has an inherent degassing effect (Gondrexon *et al.*, 1997); therefore, the apparent power output from the horn, given the same electrical input into the transducer, will reduce. For this reason, some researchers apply a constant feed of gas into the system where constant power output is required over an extended period.

External pressure – A fluid will undergo cavitation given that the following relationship remains true:

$$P_a > P_h - P_v \qquad \textbf{Equation 2.1}$$

Where; P_a = the acoustic pressure,

P_h = the external pressure,

P_v = the liquids vapour pressure.

So, for an increasing external pressure, we will require a larger acoustic pressure to induce cavitation. But on the other hand, the energy released on implosion of the cavity increases significantly; up to a limit determined by pressure range and sample temperature (Raso *et al.*, 1999). After this point the increasing cohesive forces of the liquid counter balances the increase in implosive energy giving a downward trend.

Viscosity and surface tension – Since it is necessary for the rarefaction pressure to overcome the cohesive forces holding the fluid together, any increase in the surface tension or viscosity will require an increase in the amount of energy required to induce cavitation of the liquid, but the energy released during bubble collapse increases substantially, resulting in an overall increase in apparent power output for most systems (Raso *et al.*, 1999).

Choice of solvent – The point of issue with solvent selection is the vapour pressure associated with the solvent or solvent mixture. The lower the vapour pressure, P_v, for any given external pressure, P_h, results in an increase in the acoustic pressure, P_a,

required to satisfy Equation 2.1. Thus solvents with high vapour pressure cavitate with greater ease, but, the energy released during cavitation decreases. This is a very important observation and results from the presence of solvent vapour, within the cavity, cushioning the effect of cavitation (Raso *et al.*, 1999). The presence of vapour within the cavity also reduces the maximum pressure and temperature achieved during collapse as some of the energy produced goes into condensation of the vapour phase (Thompson and Doraiswamy, 1999).

Temperature – Raising the temperature of operation lowers the observed power output from cavitation due to a lowering of viscosity and surface tension, reducing energy of bubble collapse, and the increasing vapour pressure of the fluid, increasing the vapour-cushioning effects discussed above.

Frequency – To produce an observable effect of ultrasound, there must be sufficient time for the bubble to grow to an ample size prior to contraction. As the bubbles only grow during the rarefaction cycle of the sound wave, the length of this period will determine the size to which the bubble grows, in conjunction with the wave intensity. Increasing the frequency of the wave will decrease the rate of rarefaction/compression cycling and therefore the time the bubble has to grow before being compressed. Thus, as frequency increases, the production of cavitation becomes less likely as the threshold intensity increases; this can be counterbalanced to some degree by increasing the sound wave intensity, as expected. Most ultrasound processes use a frequency of 20 kHz.

Intensity – With an increasing intensity, the acoustic amplitude increases resulting in faster and more violent the collapse. Acoustic intensity also plays in role in determining the size that a cavity can achieve.

The variation of system variables can affect power output in two main ways; by changing the transient cavitation threshold or by changing the energy released at bubble collapse, as summarised in Table 2-8.

Table 2-8 Process parameters, their effects and magnitudes; modified from (Suslick, 1988; Raso *et al.*, 1999).

Extrinsic Variable	Physical Property	Effect	Proportionality[a]
Temperature	Liquid vapour pressure	Intensity of collapse	-
	Surface tension/viscosity	Transient cavtation threshold	+
	Dissolved gas tension	Transient cavtation threshold	-
Acoustic Intensity	Reaction zone size	Cavitation events per volume	+
Liquid Solvent	Vapour pressure	Intensity of collapse	-
	Surface tension	Transient cavtation threshold	-
	Dissolved gas content	Transient cavtation threshold	+
	Viscosity	Transient cavtation threshold	+
		Intensity of collapse	+

[a] As (physical property) increases, (effect) (+, increases, or -, decreases).

These effects can be represented graphically to visualise the effect they will have on the observed power output of the ultrasonic device. Figure 2-13 is a graphical representation of a system in which the transient cavitation threshold is increasing, i.e. due to the reduction in nucleation sites due to the degassing effect of ultrasound. With an increase in threshold required for cavitation, more energy goes into the generation of bubbles, giving less excess energy to maintain the same number of cavitation events per volume. Figure 2-14 shows a system where the observed power output decreases while the cavitation threshold remains constant, i.e. due to an increasing system temperature, which will act to increase the vapour pressure and therefore reduce the energy released at the point of bubble collapse.

Both of these scenarios define a system where the observed power output, or the power dissipated as heat, is reducing, but for very different reasons. This highlights the importance of understanding the effects of certain parameters on processing conditions and their interdependent nature.

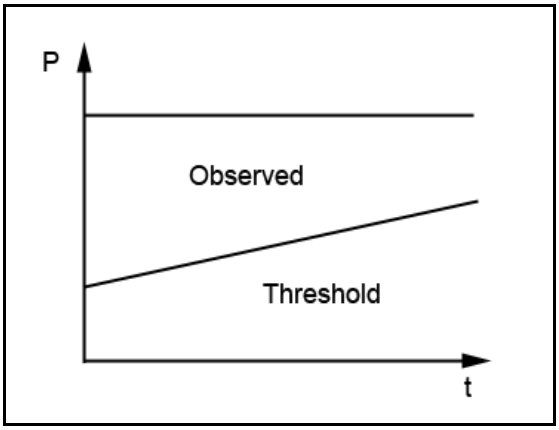


Figure 2-13 An increase in cavitation threshold.

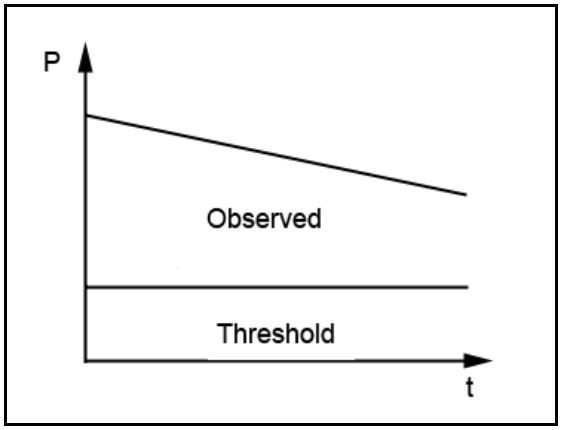


Figure 2-14 A decrease in cavitation intensity.

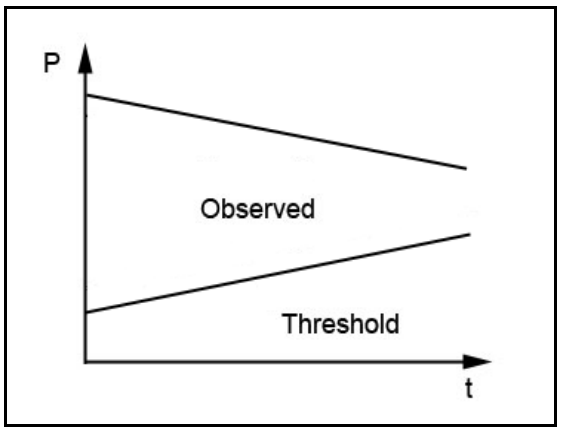


Figure 2-15 Combination of factors.

2.6.3.4 Degradation Effects of Ultrasound on Phenols

The process of ultrasound treatment produces extreme conditions of temperature and pressure at the point of bubble collapse; this can have deleterious effects on the nature of the olive extract both directly or indirectly. As the intention of US-treatment is to optimise recovery, the degradation mechanisms of phenolic species within these sound fields are a significant point of interest. Degradation of phenol can occur in the presence of ultrasonic irradiation through three mechanisms:

- Water undergoes direct thermal dissociation into atomic hydrogen and hydroxyl radicals under such extreme conditions (Suslick, 1990). These radicals, which are very strong and non-specific oxidising species, can act to oxidise the solutes in the liquid or vapour phases and inherently affect the overall activity of the BP compounds.
- The highly elevated pressures and temperatures associated with cavitation in liquid systems can also act to produce transient supercritical water, which can

act as another oxidising species (Thornton and Savage, 1992; Hua *et al.*, 1995).

- The direct thermal degradation of phenol compounds due to the highly elevated temperatures also acts as another mechanism for phenolic degradation (Wu *et al.*, 2001).

It is generally believed that these degradation effects occur in three distinct zones of an ultrasound irradiated sample: in the cavity itself, at the interface between the cavity and the bulk solution, and in the bulk solution (Thompson and Doraiswamy, 1999); this is shown schematically in Figure 2-16.

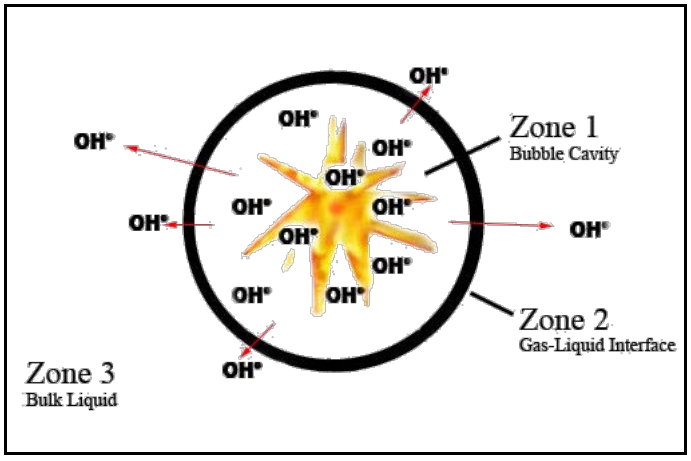


Figure 2-16 Zones of reactivity under sonochemical irradiation. →, diffusion from bubble cavity.

At each zone, the prominent mechanism for phenolic degradation will be different; depending on factors affecting cavitation dynamics and on the characteristics of the compound in question, specifically the volatility of the compound. A highly volatile or hydrophobic organic compound can easily pass the gas-liquid boundary and enter the vapour region – the cavity itself (Thompson and Doraiswamy, 1999; Vassilakis *et al.*, 2004). If not, the main reaction zones will be the gas-liquid interface and the bulk liquid. If the reactant does not exhibit volatility to any great extent, and cannot enter the interfacial boundary, remaining predominantly in the bulk solution, then US will not chemically enhance the reaction (Thompson and Doraiswamy, 1999).

In terms of phenolic degradation mechanisms mentioned previously; direct thermal decomposition occurs within the bubble itself and to a lesser extent at the boundary layer. Oxidation resulting from contact with supercritical water occurs at the

interfacial boundary (Hua *et al.*, 1995), while radical-induced oxidation occurs predominantly at the interfacial boundary and in the bulk solution (Thompson and Doraiswamy, 1999).

As phenol species are not particularly volatile (that is, they have a low vapour pressure), they do not easily cross the interfacial boundary layer and enter the cavity (although it has been suggested that phenolic compounds do undergo direct thermal decomposition to a small extent due to the discovery of residual acetylene in the bulk solution, a product of phenolic thermal degradation (Currell *et al.*, 1963)). In any case, it has be shown that the mechanism of direct thermal degradation does not play a major role in US treatment (Vassilakis *et al.*, 2004). The degradation process of *p*-coumaric acid has been shown to proceed predominantly via radical-induced reactions at the interfacial boundary and in the bulk solution (Vassilakis *et al.*, 2004). The presence of the phenolic species at the interfacial layer suggests that supercritical water would also play a role in the oxidative degradation of the compounds under the influence of an ultrasound field, but the magnitude of this has yet to be determined.

The rate of phenol degradation has been found to be a function of pH, frequency, ultrasonic intensity and operational atmosphere. Under an inert atmosphere, the rate of phenol degradation can be significantly reduced, by as much as 33% when compared to a standard natural atmosphere (Wu *et al.*, 2001). It was also found that the more acidic the conditions of reaction, the higher the rate of phenolic degradation (Wu *et al.*, 2001). This is due to the proliferation of the atomic phenol species as opposed to the phenolate, or ionic, species. The phenolate compounds are concentrated around the gas-water interfaces of bubbles, where the hydrophobicity is strong, and cannot vaporise into the cavitation bubble. At the gas-liquid interface they can only interact with available hydroxyl species. The molecular state phenols can more easily enter the gas-liquid interface and become vaporised in the bubble where the compound can undergo thermal dissociation as well as hydroxyl oxidisation.

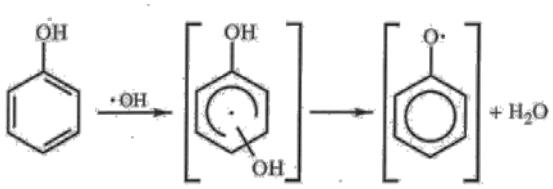
Such phenomena may only be apparent in the presence of water; by removing the moisture in the sample the rate of phenolic degradation may greatly decrease. We must therefore look for solvents that do not undergo the sonochemical dissociation that water can. It has been shown that in alcohol-water mixtures of between 96 and 50% by volume showed no chemical modification by GC-MS, although a small

change in electrical conductivity was observed during sonication (Vinatoru *et al.*, 1997). In addition, sonochemical degradation is known to have a synergistic effect on photolysis of phenolic species (Wu *et al.*, 2001). As such, it is important to keep the sample free from UV irradiation during the extraction process.

2.6.3.4.1 Phenol Degradation Pathways

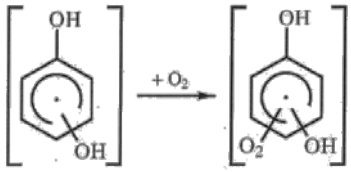
As mentioned previously, the main degradation pathways through which phenols are decomposed in ultrasound irradiated systems are oxidative reactions involving hydroxyl radicals produced from thermal degradation of water within the bubble cavity. A radical oxidative degradation pathway for a simple phenol is given in Wu *et al.* (2001), and can be found below composing several related equations.

Radical oxidation of phenol from hydroxyl radicals leads to the formation of dihydroxyl cyclohexadienyl radicals shown below.



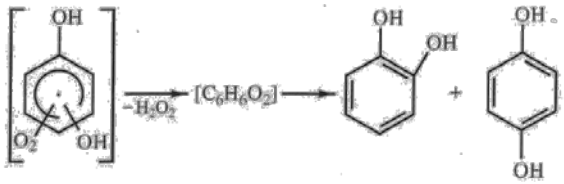
Equation 2.2

With the addition of molecular oxygen, the dihydroxyl cyclohexadienyl radicals form peroxyl radicals, refer to Equation 2.3.



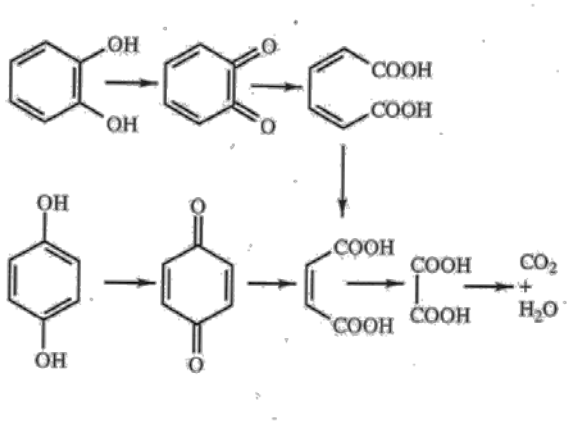
Equation 2.3

These peroxyl radicals are known to form both hydroquinone and catechol after the elimination of superoxide radicals and rearrangement of the aromatic ring; this is given in Equation 2.4.



Equation 2.4

Now the aromatic compounds are in a conformation that allows for the cleavage of the aromatic ring, it can be broken down into carboxylic acids; refer to Equation 2.5.



Equation 2.5

So, even though the degradation pathway contains a number of intermediate antioxidant compounds, if allowed to proceed to completion, then the activity would be lost due to the cleavage of the aromatic ring and oxidation of the hydroxyl groups.

The above mechanism is sufficient for a simple phenol system, but an olive extract has a very broad range of different phenol species, composing a number of different conformations and incorporating a wide range of different side-chains. As such, one pathway describing a ubiquitous degradation pathway would not suffice. Figure 2-17 shows a proposed degradation process of *p*-coumaric acid under the influence of ultrasound irradiation as defined by Vassilakis *et al.* (2004). The point of interest here is the process for breakdown of the side chain into simple carboxylic acids.

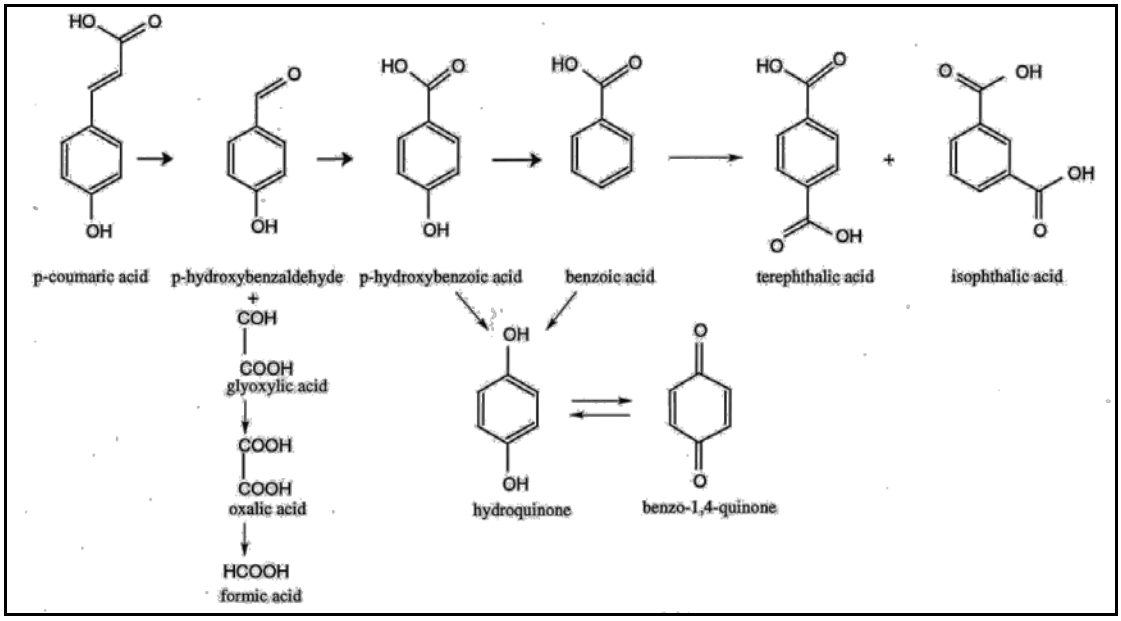


Figure 2-17 Proposed oxidative degradation for *p*-coumaric acid. Modified from Vassilakis C. *et al.* (2004).

Once the side chain as been removed, the degradation process proceeds through a similar process to hydroquinone allowing ring cleavage and giving the intermediates shown in Figure 2-18. These biodegradable products can then be easily mineralised.

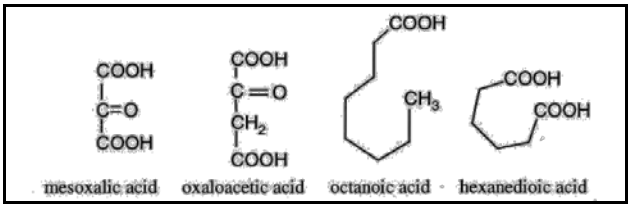


Figure 2-18 Proposed ring cleavage intermediates from the degradation of *p*-coumaric acid. Modified from Vassilakis C. *et al.* (2004).

2.6.3.4.2 Rates of Sonochemical Degradation of Phenol

The mechanisms of ultrasonic degradation of phenol in an aqueous solution have been given, but of greater interest to this project is the rate at which such degradation occurs. There are not a significant number of studies that deal directly with the sonochemical degradation of olive phenolic constituents; rather the majority of studies look at the rate of degradation of pure phenol solutions.

The literature examined all agree that the rate constants of phenolic degradation in US systems are relatively low when compared to optimised US-photochemical systems incorporating UV irradiation. Petrier *et al.* (1994) showed that US at 20 kHz with a power output of 70 W achieved a rate constant of 0.3×10^{-4} s^{-1}. Entezari *et al.* (2003)

showed that under similar conditions, but with a lower output of 50 W, a rate constant of only 0.05 x 10^{-4} s^{-1} was achieved. This equates to approximately a 60 % reduction in observable phenol content over 100 minutes of operation; at optimised conditions, phenol is completely removed, including any mechanistic intermediates and phenol derivatives, after 80 minutes of operation. Drijvers *et al.* (1999) showed only an approximate 20 % reduction in phenol over 100 minutes of operation at 20 kHz and 12.0 W. So we can see a definite relationship between power output and the rate of phenolic degradation with a higher power output resulting in a greater rate of degradation.

Vassilakis *et al.* (2004) also showed that the rate of degradation is dependent on concentration of the phenol species in question. His study of *p*-coumaric acid showed a substantial decrease in rate constant with increasing phenol concentration; a possible explanation being an increased compound stability due to H-bonding dimer formation. An increase from 10 mg/L to 100 mg/L reduced the extent of phenolic degradation by over 50% under identical conditions.

Wu *et al.* (2001) showed that decreasing the pH towards more acidic conditions (and less than the pKa of the compound in question) results in significant increase, up to 50 %, in the extent of phenolic degradation. This is due to the fact that at a pH of less than the pKa of the compound we see the predomination of the molecular state. As this state exhibits a slightly higher hydrophobic tendency, it can more easily enter the gas-water interface of the cavity allowing it to undergo both interaction with hydroxyl radicals and vaporisation into the cavity instigating direct thermal cleavage.

2.6.3.5 Effects on Adsorption Characteristics

It has been suggested that the application of ultrasound waves can greatly influence the adsorption characteristics of phenols on a polymeric resin (Li *et al.*, 2002). As the possibility exists that our BP compounds of interest may be bound to various macromolecule species, such a phenomenon may be of significance. As the bubbles collapse, microjets of solvent are formed perpendicular to the solid matrix. When these jets of solvent continually impinge on the surface of the solid, it results in the breaking of some chemical bonds between the adsorbate and adsorbent, releasing the

adsorbate into solution. This will result in a new adsorption isotherm that will eventually reach equilibrium as long as the ultrasound treatment continues.

Whether such reactions will be of significance in our extraction processes will depend on the strength of the BP-protein/carbohydrate complexation. There is evidence that weak van der Waals forces and/or hydrophobic effects maintain these complexes, but the accuracy of this model system has yet to be realised (Romeo *et al.*, 1997). If the compounds are simply adsorbed onto the surface of the molecule, then this sonochemical effect will be significant. If stronger covalent or ionic interactions are prevalent, then such phenomenon will not be significant.

Chapter 3

Analytical Methodologies

3.1 Introduction

This chapter deals with the standard analytical techniques used to characterise the olive extracts. It is the combination of these analytical techniques that will be used to define the product quality. The subsequent sections describe the application of these analytical techniques throughout the project. These discuss any problems associated with the standard protocols and the actions taken to remedy such issues.

For the assessment of extract quality there were three key indicators used in the context of this project. They were total biophenol (TBP) yield determined spectrophotometrically using the Folin-Ciocalteu reagent, the comparative phenolic profile given by HPLC, and bioactivity determined through bioassays. In this chapter the protocols for the estimation of TBPs and analysis of the BP profile will be explained.

3.2 Analysis of Total Biophenol Content

A common tool for the quantification of total phenols, the Folin-Ciocalteu reagent (FCR) method provides a reproducible and relatively robust technique giving results comparable to other methods with fewer of the specific problems (Singleton *et al.*, 1999). Its mode of action is well understood and the assay and measurement of total phenols includes mono-phenols, as well as the more readily oxidised poly-phenols, which other techniques do not always fully quantify. As the olive phenolic profile generally has a significant proportion of mono-phenol compounds, this was an important factor in choosing an analytical technique for total phenol determination.

FCR is a phosphotungstate-molybdate complex with the addition of lithium sulphate. The chemistry of the reaction is complex, but deals with the reduction of these complex metal compounds by the oxidation of phenols and other reactive species. The reduction products have an intense blue colour allowing the degree of reduction to be determined through spectrophotometric techniques relative to a standard. The absorption peaks are generally quite broad for these blue products with 760nm generally used for analysis with the FCR. Due to the breadth of the absorbance peaks and the fact that other components of biological samples do not absorb in this region, measurements can be taken over a range of wavelengths (Singleton *et al.*, 1999). For the analysis of phenols from olives, a wavelength of 725nm was used. Due to the fact that FCR can be reduced by any oxidising species present in the sample, the final measurement is of all oxidising species, not just phenolic compounds (refer to next section).

Although it is possible for some simple-substituted phenols to form complexes with the components of the FCR, it appears that the phenolic compound being oxidised has no effect other than to supply electrons to the oxidant (Singleton *et al.*, 1999). This is due to several observed factors:

- The absorbance spectrum of the FCR induced colour product is essentially the same for different phenolic substrates (Singleton *et al.*, 1999).
- The increase in absorbance produced by the addition of gallic acid to wine can be quantitatively separated (Singleton and Rossi, 1965).
- The total absorbance produced by a phenolic mixture is equivalent to the sum of its individual parts (Singleton, 1974).

3.2.1 **Sources of Error**

There are a number of possible interfering effects that can influence the accuracy of phenol determination using the FCR. These can be classified as additive, inhibitory, and augmenting or enhancing (Singleton *et al.*, 1999). It appears that additive effects are most significant in terms of the effect upon accurate measurement of phenol content, depending on the state of the sample, which means that the total phenol content is often overestimated.

3.2.1.1 Sugars

Sugars are known to have an additive effect on phenol determination but only if the sugar content is relatively high, over 25 mg/ml (Slinkard and Singleton, 1977). Generally the molar absorptivity is very low for sugars, meaning that on a molar basis its ability to reduce the FCR is limited. In alkaline solutions, it is also known that sugars slowly convert to enediols that can have an appreciable additive effect on the measured absorbance, but such a mechanism is very slow at room temperatures (Slinkard and Singleton, 1977). The effect of fructose is higher than that of glucose, which is suggestive of the enediol reductone mechanism of additive interference as this reaction proceeds at a higher rate with fructose (Singleton *et al.*, 1999).

Considering the high content of phenols in the plant extracts, the low molar absorptivity and the slow pace which such sugar-based reductone reactions take place, and the fact that the extract has an acidic character (dependant on concentration; usually between pH of 3.5 and 6), the interferences resulting from sugar content should be minimal. But it should be noted that aqueous ethanol extracts of olive wastes do contain an appreciable quantity of reducing sugars; 5.0 and 6.5 %DW in olive fruit mesocarp (Proietti and Antognozzi, 1996) or between 8 and 20 %DW in olive leaf (Proietti and Famiani, 2002).

3.2.1.2 Ascorbic Acid

Ascorbic acid (AA), an enediol, is a very common additive interference with the FRC method of phenol determination, as is has a high molar absorptivity; comparable to that of many mono-phenolic compounds (Singleton *et al.*, 1999). As such, AA content should be measured to determine if it is present at levels that will significantly interfere with TBP measurement, and if so, to correct for this.

It has been shown in literature that the ascorbic acid content of an extract can be accurately determined through HPLC analysis (George *et al.*, 2005; Lopez *et al.*, 2005). It has also been shown that by taking advantage of the thermolabile nature of AA we can indirectly measure its content using the FCR and calorimetric methodology (George *et al.*, 2005), reducing the cost of analysis greatly in both time and money. The results obtained from this method slightly underestimate the true

value obtained via HPLC (George *et al.*, 2005), but if only an estimate of magnitude is required, then it provides a simple and robust technique.

The protocol for the calorimetric determination of AA is as follows (George *et al.*, 2005):

I. The TBP content is measured using the standard protocol incorporating the Folin-Ciocalteu reagent.

II. 3 ml of a 20% dilution is added to a test tube, in triplicate.

III. The test tubes are covered and placed into a test tube holder in a water bath set at 85 ºC.

IV. The samples are left for 2 hours, allowing enough time for the ascorbic acid to be thermally degraded.

V. The test tubes are removed and TBP content measured again.

The difference in absorbance between the initial and the heat treated sample is due to the thermal degradation of AA. Given the molar absorptivity of AA, 17.5×10^3 abs units/mol (Singleton *et al.*, 1999), an estimate of content in can be found given the following relationship:

$$AA = \left(\frac{\Delta Abs}{\varepsilon} . Mr . \frac{Vt}{D} \right) . \frac{1}{m}$$

Equation 3.1

Where; AA = ascorbic acid content, g/g leaf DW

ε = molar absorptivity, abs units/mol

Mr = molar mass of ascorbic acid, g/mol

ΔAbs= the difference in Abs of initial and heat treated sample, abs units

Vt = the total volume of the extract, mls

D = dilution factor between the extract and the sample

m = dry mass of material, g

If an appreciable quantity of AA was present a significant reduction in absorbance should have been observed. It can be seen from Table 3-1 that this was not the case, the observed difference being negative and not within the 5 % experimental error for TBP determination (section 3.2.2.1), so it must be assumed that the content of AA

present in the sample did not produce an appreciable additive interference in the determination of TBP.

Table 3-1 Heat treatment of olive leaf extract for ascorbic acid determination.

	Absorbance at 100% Dilution	TBP (mg GAE/ml)
Initial	3.8	2.99
Heat Treated	3.867	3.04
Change	-0.067	-0.05
[a]*Ascorbic acid concentration (mg/ml)*	N/A	

[a] Determined from Equation 3.1.

Examination of literature shows a small AA content in fresh olives, 9 mg AA/100 g fresh weight (Lopez *et al.*, 2005). After 2 weeks storage at 6ºC, the AA content dropped by approximately 10% (Lopez *et al.*, 2005), but compounded for extended periods of storage, it can be expected that the AA content would drop markedly, or even degrade completely.

3.2.1.3 Organic Compound Inhibition

Organic matter has a pronounced inhibitory effect on the development of colour in the determination of TBP content using the FCR. This effect can be highlighted by Figure 3-1: the more concentrated an extract, and hence the higher concentration of organic material, the more pronounced the deviation from linearity. Each extract underwent identical extraction conditions, except one is subject to rotary evaporation for a longer period of time. A pure standard curve of gallic acid was shown to retain linearity up to approximately 0.500mg/ml. This is the limit of linearity for a pure sample with no interfering effects.

For the two extracts, of two different concentration states, there is a pronounced depression in the rate at which the curve deviates from linearity. In the more concentrated of the samples this depression is more pronounced suggesting that the higher concentration of organic compounds blocks colour development to a greater extent than with the more dilute sample.

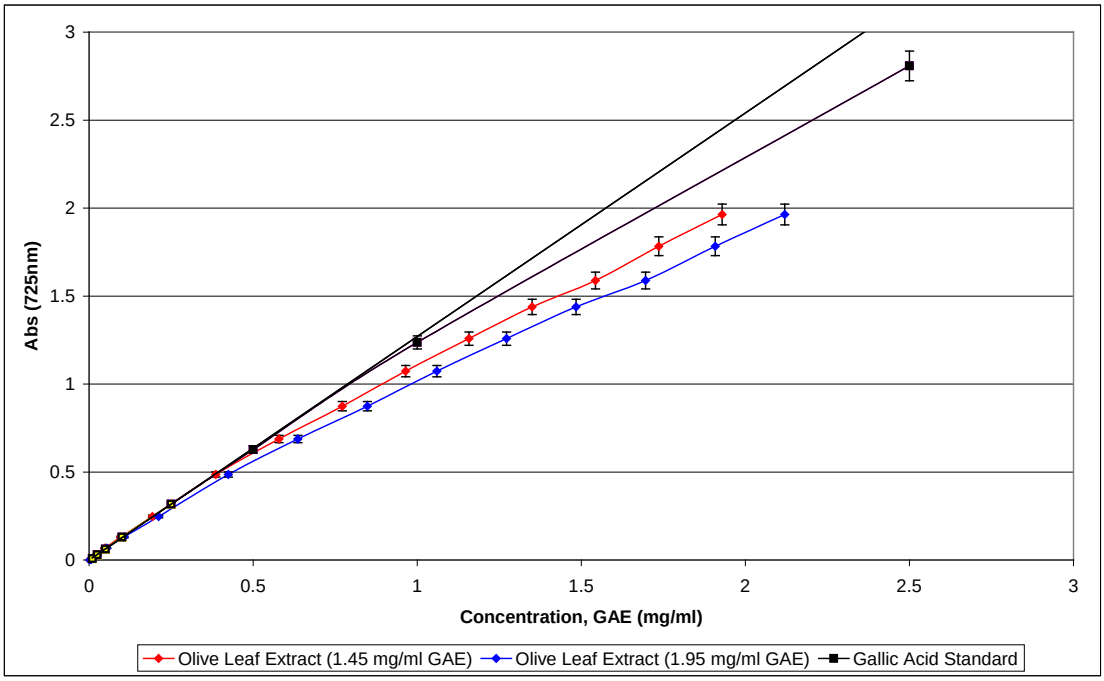


Figure 3-1 Depression of colorimetric TBP determination in two olive leaf extracts, of different concentrations states, due to organic effects.

To determine the degree of colour suppression and assess the extent of linearity for the determination of TBP's using the FCR, spike tests were carried out. A standard, concentrated, olive leaf extract (6.75 mg/ml GAE) was spiked with a known quantity of gallic acid of a known concentration (5.0 mg/ml). TBP content was then determined via the FCR method and compared to the expected concentration. It can be seen from Figure 3-2 that any dilutions greater than 3% deviate significantly from the expected concentration. From these results, we can be confident that dilutions of less than 3% of a concentrated olive extract will produce results that will allow accurate extrapolated determination of the TBP of the sample.

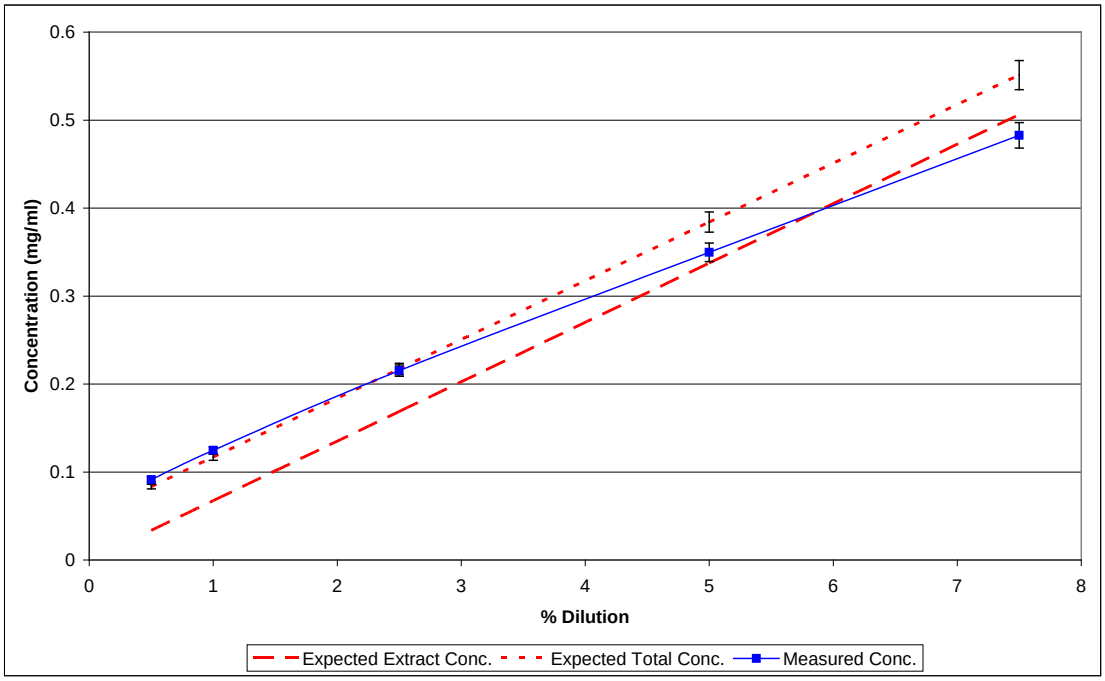


Figure 3-2 Spike test of olive leaf extracts (6.75 mg/ml GAE) with Gallic acid (5 mg/ml).

In Table 3-2 we see the percentage of the gallic acid that shows up in the analytical determination of TBP. We see at low concentrations that the gallic acid is overrepresented suggesting the dilution of the olive leaf extract with gallic acid and water may have increased the observed TBP content that the crude extract provided to the total phenolic content of the solution. There is no synergistic effect as it is known that gallic acid can be quantitatively separated when added to a phenolic extract (Singleton and Rossi, 1965). If we increased the extract concentration to above 2 %, the gallic acid that was added to the solution is lost due to organic inhibition.

Table 3-2 % recovery of gallic acid added to different concentrations of olive leaf extract, exhibiting degree of organic suppression.

Extract Dilution %	% Recovery of Gallic acid
0.5	116.0
1	115.6
2.5	96.5
5	31.3
7.5	-36.7

The measurements were replicated, two sets of three measurements for each dilution, and the variations were found to be very small. All measurements made were within 3% of the mean at 95% confidence, which shows an acceptable reproducibility.

3.2.2 **Method Development**

To accurately determine the concentration of TBPs in a complex extract, it is important to have an idea of how concentrated the extract is. This gives an idea of what dilutions are needed in the sample set so that extrapolation of the results gives an accurate estimation of TBPs of the extract; the more concentrated an extract, the larger dilution factor is required to ensure the measurements are made in the linear range.

The extracts generally went through two concentration states; an initial dilute state where the TBP content was in the range of 1.5-2.0 mg/ml, and a concentrated state where the concentration was 8-10 mg/ml or higher. For the crude state the linear range was 0-10% dilution of the extract. For the concentrated state the linear range was 0-3% dilution of the extract. A number of dilutions are made within this linear range, and then triplicate measurements of TBP are made for each dilution. As long as the results provided a linear relationship, then an extrapolation to the pure sample concentration was made, and the total biophenols determined.

For the determination of TBPs in a selected sample, the following methodology was used:

I. Depending on the concentration state of the extract, crude or concentrated, dilutions were made up covering the expected linear range of the Folin-method absorbance output, up to 3% and 10% respectively.

II. 0.1 ml of each dilution was taken in triplicate, and pipetted into a test tube.

III. 0.5 ml of Folin-Ciocalteu reagent (FCR) was pipetted into each test tube and vortexed. The tubes are then left for 5 min at room temperature, but no longer than 8 min, to allow the formation of colour.

IV. Once the colour has formed, 3.0 ml of 20% sodium carbonate was added to quench the reaction and colour of the FCR.

V. The solution was then made up to 10 ml with distilled water and centrifuged at 1500 rpm for 10 min.

VI. After 50 min, the absorbance of the solution at 725 nm was determined relative to a blank solution with no sample.

VII. The average of the absorbance of each triplicate set was determined and concentration of TBPs expressed in gallic acid equivalents (GAE) mg/ml. (Regression of the gallic acid standard curve between 0 and 0.25 mg/ml gave a linear gradient of 1.2703 abs units/mg.ml with an R2 of 0.9994.)

VIII. The results were then plotted against the dilution, and an analysis of the linearity of the results made.

IX. Regression analysis of the linear data points then allows the determination of the concentration of TBPs in the pure sample.

3.2.2.1 Repeatability and Reproducibility

Due to the requirement for extrapolation beyond the range of linearity, reproducibility for the analysis of TBPs using the Folin-method was of high importance. It must be shown that variation of experimental conditions was not magnified so much as to bring into question the validity of the analytical methodology. There are two areas of variability that needed to be addressed; the variation within a test, and the variation between tests.

Variation within the test, i.e. between test replicates, defines the variation of the triplicate samples and stems mainly from the inherent experimental uncertainty of the methodology. The variation between two identical tests analyses the repeatability of the tests and makes sure that the results achieved are consistent enough to be reliable indications of TBP content.

Table 3-3 shows the 95% confidence intervals, as a % variation from the mean, of the test triplicates, where 5 dilutions are analysed in triplicate for each run.

Table 3-3 95% confidence intervals (C.I.) for triplicates in TBP determination. % variation from mean.

	20-30 minutes			**60 minutes**		
Replicate	*Run 1*	*Run 2*	*Run 3*	*Run 4*	*Run 5*	*Run 6*
C. I. (95%)	4.52	6.24	9.58	1.87	2.93	2.64

From these results it was quite clear that the variation of measurements was dependent on experimental methodology. The time dependence of the absorbance measurements indicates that it was very important, in terms of consistency of results, to maintain a defined experimental method to obtain experimental reproducibility. We can see that if the sample was not left for the full 60 min after centrifugation the variation between

the triplicates was significant. The longer the solution was left, the smaller the standard deviation, which was in agreement with Singleton (1999). If the TBP determination methodology was adhered to, the variation between replicates was smaller and the results substantially more reliable (runs 4-6).

With respect to the variation between two identical runs, the TBP content was determined through the normal methods, and the differences between the results analysed.

Table 3-4 95% confidence intervals (C.I.) for replicate determinations of TBP content.

	20-30 minutes			**60 minutes**		
Replicate	*Run 1*	*Run 2*	*Run 3*	*Run 4*	*Run 5*	*Run 6*
Concentration (mg/ml)	9.53	9.81	10.83	9.85	10.16	10.49
C.I. (95%)		0.77 mg/ml			0.36 mg/ml	
		7.61%			3.57%	

Analysis of the variability of the above replicates found that runs 1 through 3 had a mean value of 10.06 mg/ml ± 0.77 mg/ml, while runs 4 through 6 has a mean value of 10.17 mg/ml ± 0.36 mg/ml. Both sets of data gave acceptably similar results, but the first set (runs 1-3) showed an unacceptable degree of variability. It is desirable to have this technique give results within 5% of mean value at 95% confidence; the second set of results clearly show that this was possible. It also shows, once again, the importance of a consistent test methodology to this analytical technique.

3.3 **HPLC Analysis of Extracts**

In the framework of this project high-performance liquid chromatography (HPLC) was used primarily as a qualitative analysis tool allowing examination of the phenolic profile of olive extracts to indicate the effect of processing on the integrity of the phenolic composition of the sample. A quantitative analysis of the samples requires both identification of all peaks and pure samples that these can be referenced against. This is an intensive and costly exercise and as this was outside the scope of this project, HPLC was used purely as a qualitative tool.

HPLC analysis was undertaken at Crop and Food Research, Palmerston North, under the supervision of Dr Julian Heyes and Tatayana Pinkney.

3.3.3 **Protocol**

For the identification of phenolic compounds using HPLC, the method of (McDonald *et al.*, 2001) was used. This achieved clear separation of peaks and consistent retention times. The method incorporated the following parameters:

- Separation of phenolics was achieved using a Waters 7/7 HPLC fitted with an autosampler with detection of peaks made using a Waters 996 photodiode array detector.
- Luna C18(2) column (150 x 4.6 mm; 5µm, particle size).
- As phenolic compounds exhibit polar behaviour to different extents, a combination of water:acetic acid (100:1 v/v) as Solvent A and methanol:acetonitrile:acetic acid (95:5:1 v/v) as Solvent B was selected.
- The elution gradient comprised of an initial isocratic period of 2 min at 5% B followed by a linear increase to 25% B at 10 min, with further linear increases to 40% B at 20 min, 50% at 30 min and 100% at 40 min, hold for 5 min and return to initial conditions over 10 min.
- Flowrate was set at 1.5 ml/min.

Due to the sensitive nature of HPLC analytical techniques, every effort must be used to ensure that the methodology remains consistent and that the system was in perfect working order. To ensure that the column was in the same preconditioned state prior to sample injection, a methanol blank is always run through the column immediately prior to the sample. A methanol blank was also injected at the end of each run to ensure the complete removal of all species applied to the column during the sample run.

3.3.4 **Analysis of Results**

The absorbance of the peaks was measured at three wavelengths; 280, 313 and 350 nm. The peak height and area of each peak within the detection tolerances was given at each wavelength. The 3-D Waters 996 photodiode array detector also allows the examination of the entire wavelength spectrum at a given time, which is very useful when attempting to fingerprint and compare a particular peak between samples.

3.3.4.1 Standards for Extract Characterisation

Due to the fact that HPLC is being used primarily as a qualitative tool, standard selection only needs to cover the main phenol species and a range of different phenolic structures. This will allow identification of compound groups by reference to the standard compound absorbance spectrum and retention time, and therefore identify processing effects on the phenolic profile of the extract as a function of structural modification.

In an attempt to cover the main areas of phenol structure known to be present in olive extracts, the following standards were selected:

- Simple substituted phenol:
    - C_6-C_1; Gallic acid, Sigma-Aldrich
    - C_6-C_3; Caffeic acid, Sigma-Aldrich
- Secoiridoid phenol; Oleuropein, Extrasynthase
- Flavanoids; Quercetin, Luteolin and Apigenin, care of Crop and Food Research
- Flavanoid glycoside; Rutin, Sigma-Aldrich
- 5-caffeoylquinic acid (chlorogenic acid)
- An oleuropein hydrolayse giving a number of products, representative of oleuropein degradation products, synthesised by protocol outlined below.

Each standard is applied at a concentration of 1 mg/ml and analysed first individually, and then in two groups, shown in Figure 3-3 and Figure 3-4.

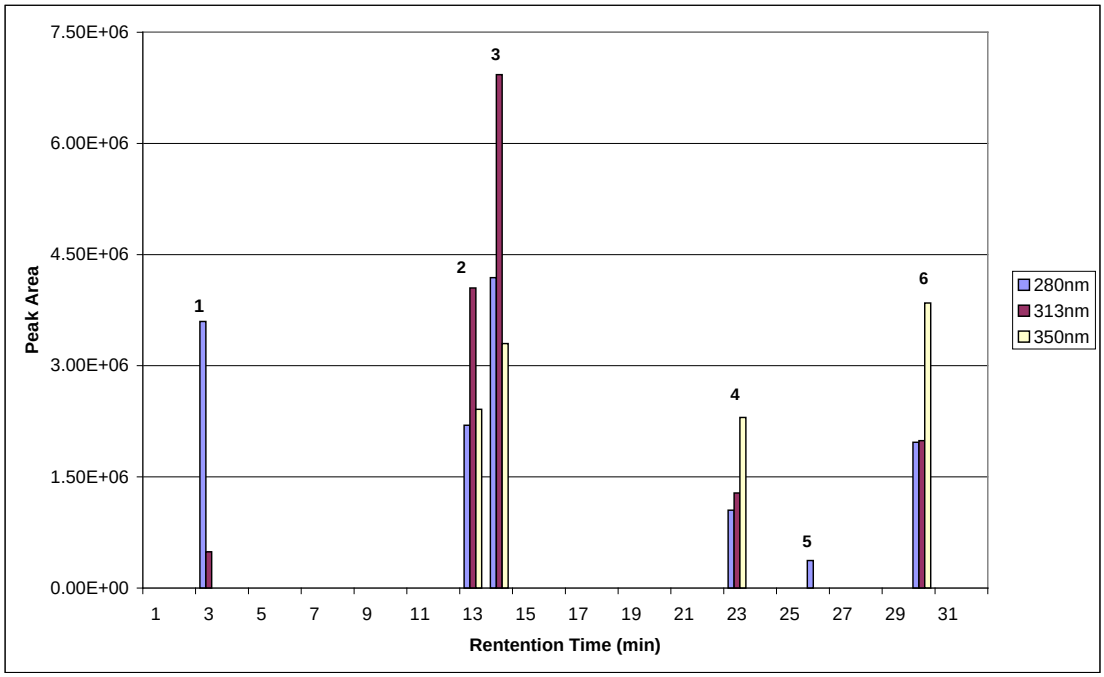


Figure 3-3 Retention and absorbance spectra of HPLC standards: peak (1) Gallic acid; (2) 5-caffeoylquinic acid; (3) Caffeic acid; (4) Rutin; (5) Oleuropein; and (6) Quercetin.

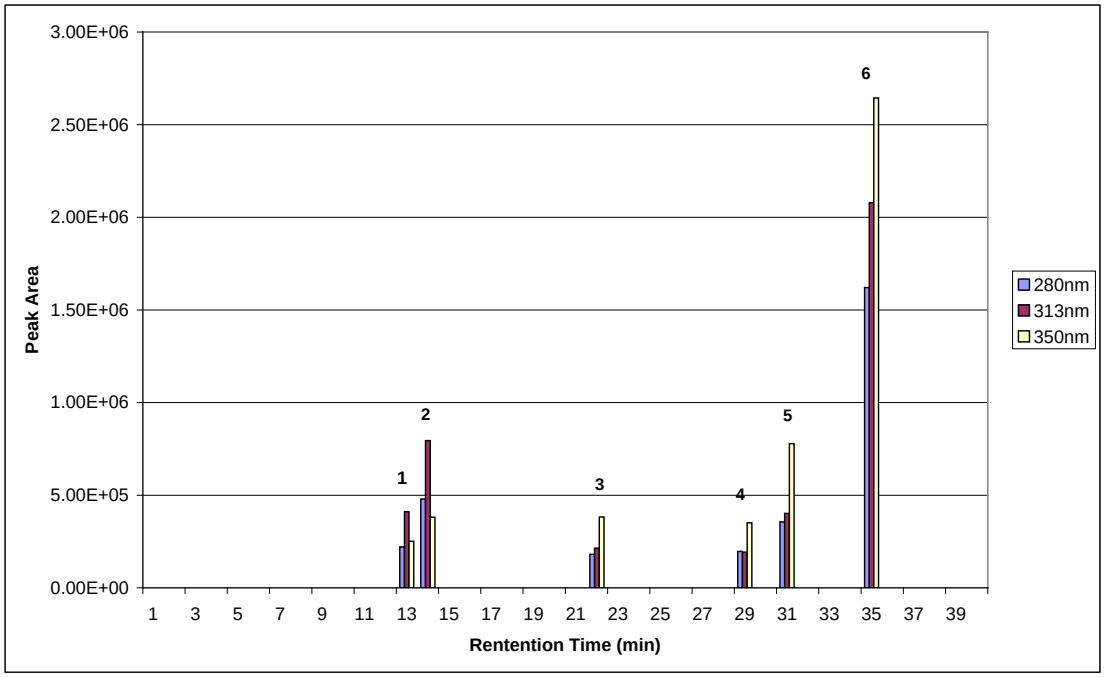


Figure 3-4 Retention and absorbance spectra of HPLC standards: peak (1) 5-caffeoylquinic acid; (2) Caffeic acid; (3) Rutin; (4) Quercetin; (5) Luteolin; and (6) Apigenin.

In addition to this spectrum of standards, an Oleuropein hydrolayse was analysed through HPLC to determine the retention time of some oleuropein degradation products. The procedure used to obtain the acid hydrosylate was as follows:

I. 10 mg of oleuropein was added to 2 ml of 1 M H_2SO_4 in a test tube and placed in a 100 ºC water bath for 1 hour.

II. The solution was then allowed to cool and adjusted to pH 2.0 with KOH.

III. 3 ml of ethyl acetate was added and vortexed at low speed to mix thoroughly.

IV. The ethyl acetate phase was then collected and most of the liquid removed under nitrogen, reducing the volume to less than 0.2 ml.

V. The hydrosylate was then made up to 1 ml with 85% methanol/15% acetic acid solution, filtered and applied to the column.

The resulting profile is given in Figure 3-5. The main degradation products of the acid hydrolysis of oleuropein are hydroxytyrosol, elenolic acid and the aglycone of oleuropein (Briante *et al.*, 2001). There is only one distinct peak to speak of in the oleuropein hydrosylate, RT 9.27 minutes. With hydroxytyrosol being the main degradation product of oleuropein hydrolysis, we can tentatively state that this might be hydroxytyrosol.

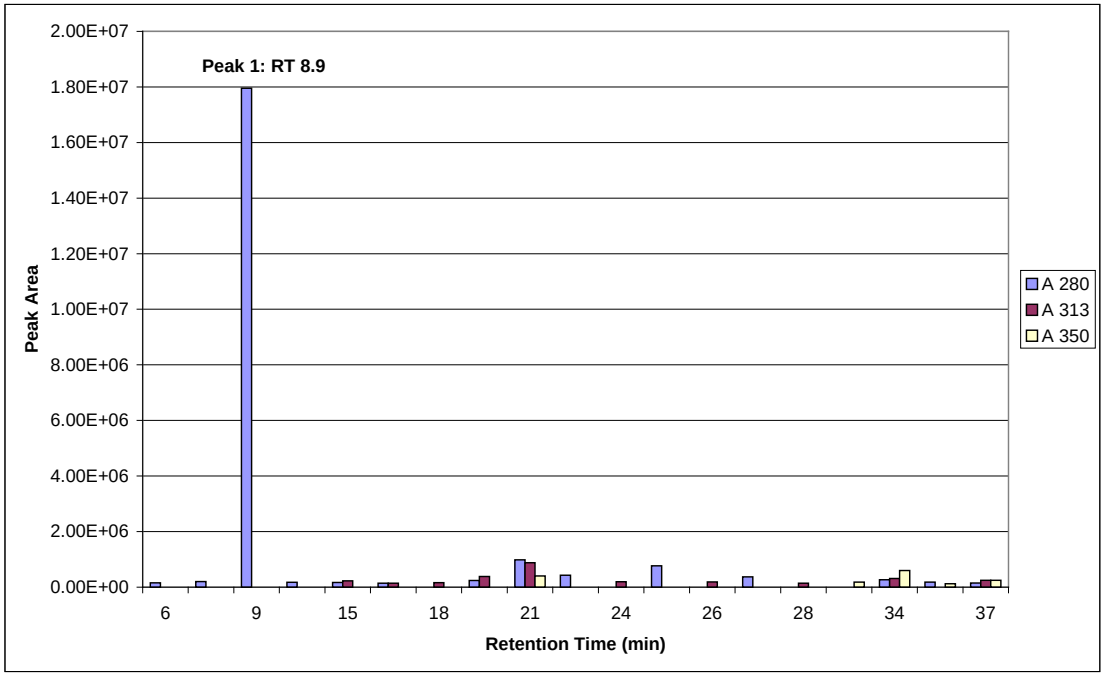


Figure 3-5 HPLC profile of Oleuropein hydrolayse.

3.3.4.2 Olive Extract

The protocol selected for phenol determination provided very good initial results, with clear, symmetrical and consistent absorbance peaks. A typical output from the analysis of olive phenols of olive leaf can be seen in Figure 3-6.

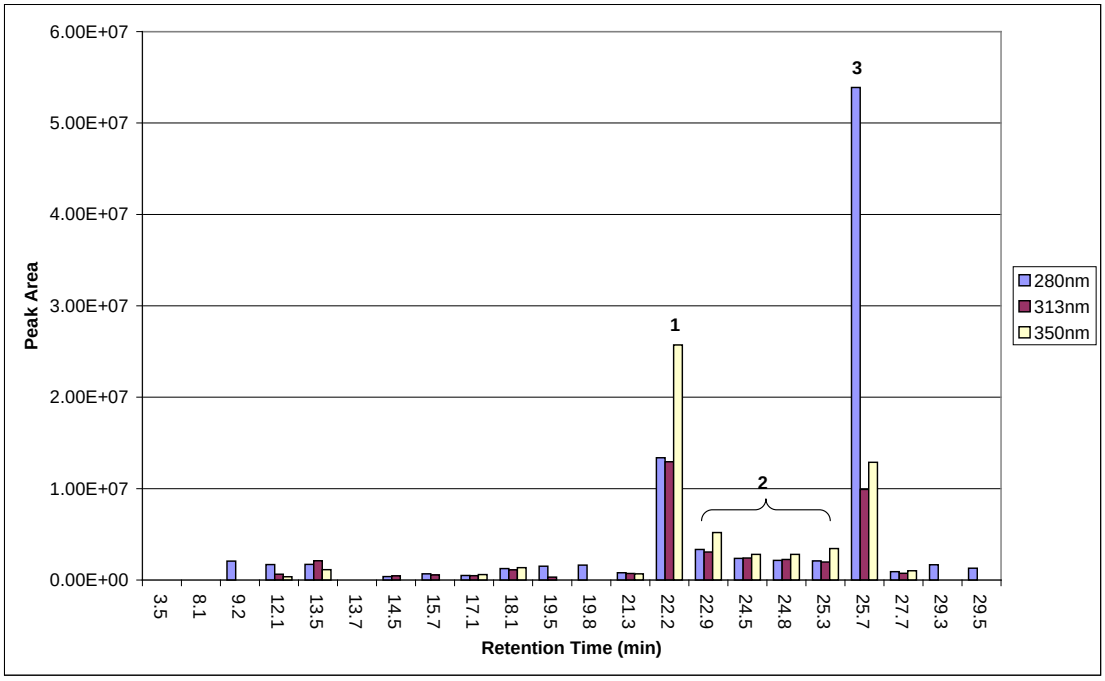


Figure 3-6 HPLC profile of crude extract of olive leaf.

This figure usefully presents of data from multiple wavelengths in a clear and simple fashion. It is then possible to distinguish between specific substances and/or groups of compounds by the shape of the output at a given retention time. For example; peak 1 and peak group 2 share a very similar absorbance spectrum, it is therefore likely that these compounds share similar structural moieties. It must be kept in mind that the presence of other eluting compounds may modify the apparent shape of the absorbance spectrum, giving a sum-total of absorbance, not just the absorbance of the compound of interest.

Peak 1 and 3 are the most pronounced peaks in the spectrum. They show very different absorbance spectrums, so it is unlikely that they share significant structural similarities. Peak 1 is proposed to be the dialdehydic form of the oleuropein aglycone, following McDonald *et al.* (2001).

Peak 3 is made up of two distinct peaks that have overlapped into one. There is a distinct difference between the RT at the 280 nm wavelength, and 313 and 350 nm

wavelengths. The RT of oleuropein at 280nm is typically between 25.6 and 25.8 minutes, the corresponding peaks at 313 and 350nm precede the peak at 280nm by 0.1 to 0.2 minutes. This is not a large difference, and is not significant enough to be deemed separate peaks given experimental tolerances, but is much greater than any differences in RT observed with any other peaks. Hence it is very likely that the large absorbance at 280nm of oleuropein was shielding another significant peak at a very similar retention time.

3.4 **Miscellaneous Methods**

3.4.5 **Moisture Content**

Moisture content was determined using the standard method for plant material. Approximately 5 g of the sample was weighed into an aluminium moisture dish and lid with a known weight. The sample was placed in a vacuum oven set at 110 ºC and left for 24 hours. The samples are then left to cool in a desicator before the difference in weight was determined; the difference being weight lost due to moisture loss.

Each measurement of moisture content was made with 5 replicates with the final value determined by the mean of the proportional difference in weight loss of the replicates.

3.4.6 **Measurement of pH**

The measurement of pH was carried out in the usual manner incorporating an Orion SA520 digital pH meter. It should be noted that the pH electrode was sensitive to high concentrations of alcohol. As such, samples must be in a pure water matrix before any readings can be taken. This put limits on when an analysis of pH could be undertaken.

3.4.7 **Ionic Composition**

For the determination of salt levels in the extract, elemental determination using plasma emission spectrometry at Grasslands Research Centre, Palmerston North, was used. This gave an elemental breakdown of the extract and allowed a rough estimate of the ionic composition, and therefore, salt in the extract.

Chapter 4

Extract Preparation and Solvent Extraction

4.1 Introduction

One of the specific aims of this project was to examine novel extraction methodologies to determine which method provides the greatest opportunity for the recovery of an active phenolic fraction. This will be determined by examination of the fractions in terms of composition and yield of the phenols, as well as biological activity of the active components. The extraction methods examined are:

- Solvent extraction
- Ultrasound-assisted solvent extraction

This chapter will begin by discussing the initial raw material sourcing and the necessary pre-treatment and storage to ensure that the raw material does not undergo excessive deterioration prior to testing.

During the course of this project, it was necessary to develop a strict extraction/purification protocol for the phenols to ensure that the samples were as consistent as possible with minimal variation due to the extraction and purification conditions.

The yields and typical phenolic profile from the solvent extraction of olive solid wastes is examined. Allowing comparisons to be made against extracts obtained from ultrasound-assisted extraction detailed in Chapter 5.

4.2 **Materials and Preparation**

In this project, waste streams from the olive oil processing industry are analysed for their potential bioactivity for use as a raw material for a novel product. To ensure that the raw materials do not undergo excessive deterioration during storage and prior to extraction, processing and testing, the leaf material was pre-treated. This will help ensure that results of temporally separated tests can be compared.

4.2.1 **Sources and Harvesting**

Leaf material was supplied by Bridgegegrove, a small olive grove situated in Otaki in the lower half of the North Island of New Zealand. They cultivate a number of different varieties of olives, specifically *Barnea*, but also *Leccino* and *Pendolino*. Harvesting took place early May of 2004 and May of 2005.

Leaves were picked at random from all peripheral parts of the tree in an attempt to simulate pruning. Leaves were not shown any selection bias in terms of size, colour or health selection; all leaves were kept for pre-treatment and processing with no sorting of the leaves taking place.

During the May 2004 harvesting and processing, *Barnea* leaves were picked, treated and stored separately while the *Leccino* and *Pendolino* varieties were picked but mixed together in unknown proportions prior to treatment and storage.

During the May 2005 harvesting, only *Barnea* leaves were harvested.

4.2.2 **Pre-treatment and Storage**

4.2.2.1 Leaf Material

Leaf material was dried on perforated metal trays at 37^{o}C in the absence of light until they were brittle and could be easily crushed by hand. Leaves were checked every 24 hours; after 48 hours they were sufficiently brittle. Weight loss during drying was determined to be 43.3% w/w. The initial total moisture content, determined through the methodology outlined in section 3.4.5, was found to be 57.5% w/w. This shows that leaf material was sufficiently dry with 75% of the water being removed. Removal of a large proportion of moisture from the raw material will prevent localized

shielding of the solid matrix and allow extraction of the compounds of interest to proceed relatively unhindered.

To facilitate the extraction of phenol compounds and to prevent any variation due to particle size, the leaves were reduced to a fine particle consistency. This was achieved with a 15 cm diameter circular plate grinder. The leaf material was applied to the grinder a number of times until reduced to an acceptably fine and consistent level. After the final grinding the particle size distribution appeared to be relatively narrow with a bias towards the finer particles, but was not measured accurately. Ground leaf material was stored in a plastic container at $4^{o}C$ in the complete absence of light.

4.2.2.2 Pomace Material

The pomace material had very high water content with large quantities of free water. As such, water removal was most efficiently achieved via freeze drying on a Cuddon Ltd FD 57 plate-bed freeze dryer. Initial moisture content was determined to be 73.2% w/w and the pomace was processed to complete dryness.

After drying, the pomace material had a highly matted and fibrous texture, with the crushed seeds easily visible as small white particles. To reduce particle size the pomace was also ground in the plate grinder. After two or three applications the pomace was reduced to a semi-fine powder.

The pomace material was stored at $4^{o}C$ in the complete absence of light.

4.3 Extract Preparation Protocol

In the early stages of the development of analytical and biological testing protocols, it was imperative to maintain a consistent sample quality so that variations due to experimental methodology could be isolated. This led to the development of a standard protocol for the isolation and concentration of olive plant extracts. At this point it was important to define the final product objective as well as the starting raw materials in terms of acceptable limits of impurities and contaminants. Once this was done, the process of defining a product recovery protocol was undertaken.

The final product objective in this project is a crude olive leaf extract that is rich in biophenolic compounds that can be applied to bioassays without the complication of interfering substances.

4.3.3 **Contaminants and Impurities**

The main contaminants and impurities in the olive extracts are the aforementioned organic compounds that affect TBP (total biophenol, mg/ml GAE [gallic acid equivalents]) determination, lipids and oils, and ethanol and salts that can interfere with bioactive testing on microbial cultures.

4.3.3.1 Lipids and Oils

Lipids and oils can have a number of deleterious effects on both the analytical methods for extract analysis and the stability of the extract itself. The presence of lipids in extracts analysed through HPLC can have a marked affect on the performance of the column, and as such they need to be removed prior to injection.

Evidence in literature exists that both complex polyphenolic flavanoids and simple substituted phenolic compounds can bind to protein (Pripp *et al.*, 2005) and lipoprotein macromolecules (Vinson *et al.*, 1995). If a crude olive extract is stored at low temperatures for an extended period of time, then a heavy oily residue layer forms at the bottom, this goes in turn with a greater than expected reduction in the TBP content of the sample, determined by the Folin-method (data not shown). This reduction in TBP content could be the result of such phenol-lipid/protein complexation and coalescing, removing the phenols from solution.

There are a number of ways to remove lipid material from plant extracts, usually incorporating organic solvents. Two methods were tested in the purification of olive extracts. One used pure hexane as a medium for lipid and colour removal, the other used an ethanol:water:chloroform mixture in the ratios of 1⅓:1:1 respectively, this is a modified-Folch method (Washburn, 1989).

The hexane system achieved a good recovery of the phenol species but required a significant input of organic solvent. The modified-Folch method allowed for a very clear separation of phases with much lower input of organic solvents, requiring a single wash phase compared to the three required for hexane extraction, but some

phenol compounds were extracted along with the contaminant material, as can be seen in Table 4-1.

Table 4-1 Phenolic fractionation during lipid removal.

Solvent Phase	Initial Yeild (mg GAE)	Extraction Yeild (mg GAE)	
		Aqueous Phase	Organic Phase
E.W.C (Folch)	398	140	258
Hexane	936	0	936

The objective of this step was to ensure that the extract integrity is retained as much as possible while removing all the lipid, oil and colour contaminant material. Given these objectives, it is clear that the hexane system is the best option as the modified-Folch method fractionates the compounds of interest.

4.3.3.2 Salt and Inorganic Compounds

Certain salts can have a significant antimicrobial activity, and as such they should be removed prior to testing to eliminate the potential for any false positive results. The inorganic mineral content is removed from the extract by contacting it with a resin, Sepabeads SP207, which selectively binds phenols of interest while allowing salts to pass through.

4.3.3.3 Ethanol

Ethanol is a highly active antimicrobial substance. As such it must be removed prior to any biological testing. This is achieved through rotary evaporation under vacuum. Ethanol has a higher vapour pressure than water; and as such it can be removed from water through simple evaporation. Evaporation under a vacuum has the added advantage of decreased partial pressure allowing the use of lower temperatures, which can be highly beneficial when dealing with biological samples prone to pyro-degradation.

4.3.4 Recovery and Purification Method Development

The final recovery and purification methodology is represented in Figure 4-1; descriptions of each step are outlined below.

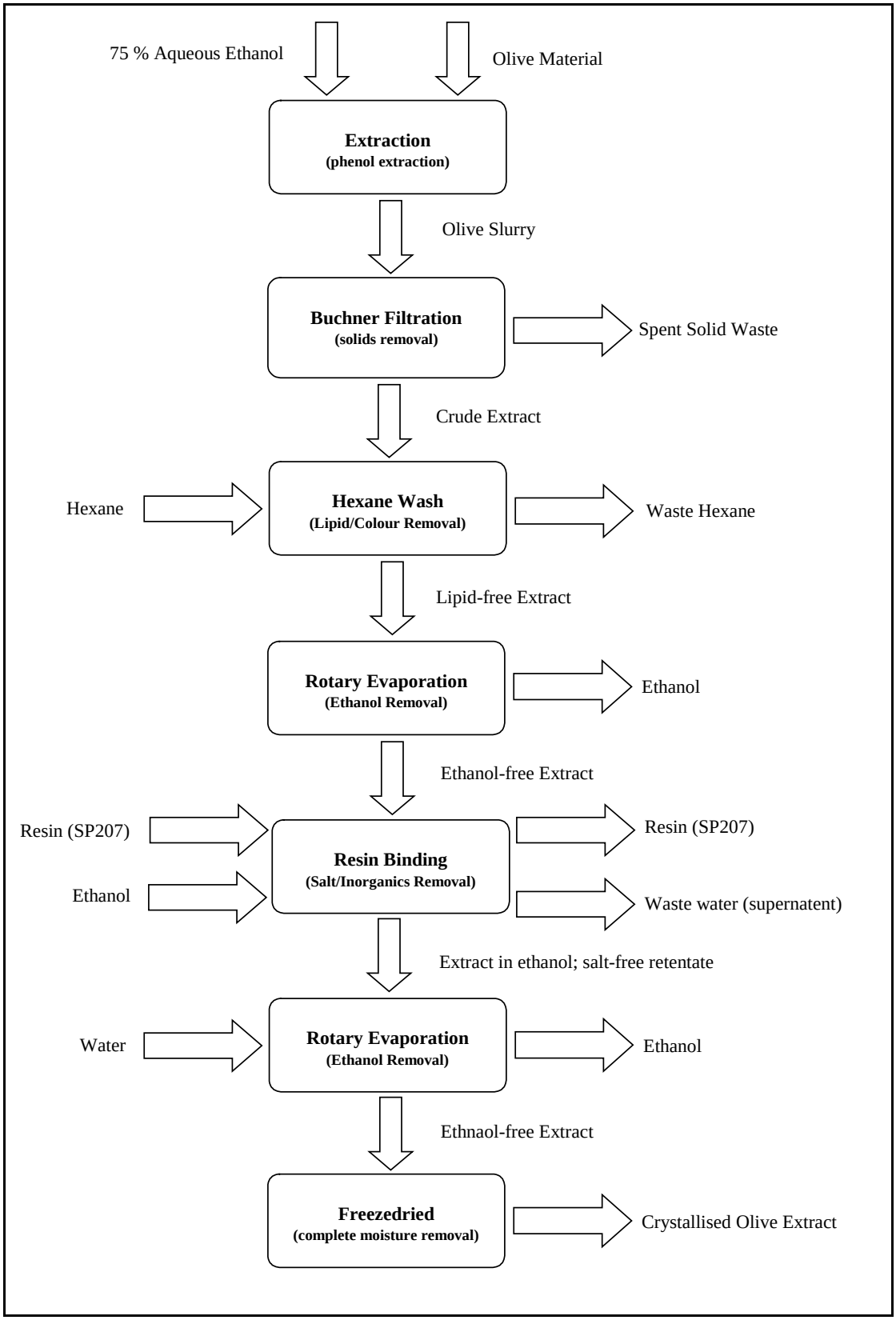


Figure 4-1 Schematic of the phenol purification protocol.

4.3.4.1 Preconditioning

The two main aspects to be considered in raw material preconditioning are the presence of water to create localised shielding of the solid matrix solvent contact, and a wide particle size distribution creating qualitative differences in extract quality due to variations in extraction dynamics. As mentioned previously, localised shielding was prevented by removing the water from the raw material. This was achieved by either freeze drying or air drying the raw material. To ensure that particle size variation had as little effect as possible on extraction dynamics, the raw material was ground down to a fine powder. This provided equal opportunity for solute removal irrespective of size and localisation in various plant tissues.

4.3.4.2 Extraction

For the extraction of phenol compounds from olive plant tissues, the following experimental parameters were set:

- Batch extractions were undertaken with powdered olive leaf material and 75% aqueous ethanol at a leaf to solvent ratio of 5 g to 100 ml.
- Temperature set at room temperature.
- Well-mixed in a conical flask of the appropriate volume.
- Covered in aluminium foil to prevent light exposure.

4.3.4.3 Purification and Concentration

Lipid and Colour Removal: Hexane was found to be the best solvent for the removal of lipid and colour impurities. For complete removal, more than one washing of the extract is necessary; 3 aliquots of hexane equal to the extract volume were used to wash the extract. Hexane was contacted with the extract for 30 min prior to being

separated in a separation funnel. The heavy aqueous phase was kept while the light organic phase is discarded[1]. The process is repeated with each hexane aliquot.

Ethanol Removal: Ethanol removal was achieved in a Büchi Rotovapor R110 rotary evaporator where solvent removal can be achieved in a vacuum allowing the use of lower temperatures. Temperatures of between 60 and 70 ºC are used depending on the decreasing content of ethanol in the sample.

Salt Removal: Salt removal was achieved through the binding of the phenol fraction of the extract, allowing the flow through of the salts in the supernatant. Binding and recovery parameters were optimised for the recovery of the phenol fraction whilst removing as much of the salts as possible. A number of different resins were tested; refer Table 4-2. Diaion SP207 achieved the greatest absolute binding of gallic acid given preliminary conditions. This resin was selected for optimisation studies.

Table 4-2 Recovery of gallic acid from solution using various polymeric adsorbent resins.

Resin	% Gallic acid bound
XAD-4	25.48
XAD-7	45.99
Diaion SP207	65.25
Dowex 88	27.66

Optimisation studies were undertaken with gallic acid as the target compound. The main experimental variables were defined as temperature, phenol concentration, ratio of resin to solution, pH of solution, and the proportion of ethanol in solution and in the recovery elution phase. The results of the studies can be seen in Figure 4-2 through Figure 4-7. With each of the tests all experimental variables were kept constant with modification of the variable of interest only.

[1] After the final separation stage, the recovered extract often has a turbid appearance; this is residual hexane suspended in the solution. Centrifugation at 3000 rpm for 5 min and removal with a pipette allows complete removal of this residual hexane and any associated lipid and colour impurities.

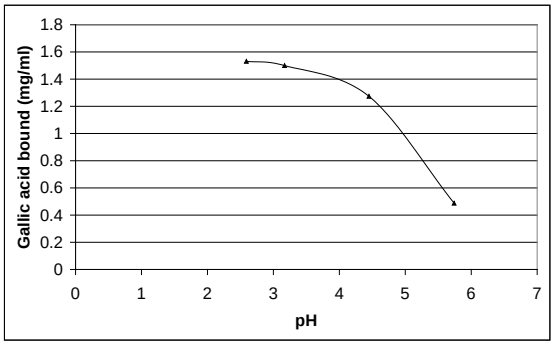


Figure 4-2 Effect of pH on gallic acid binding.

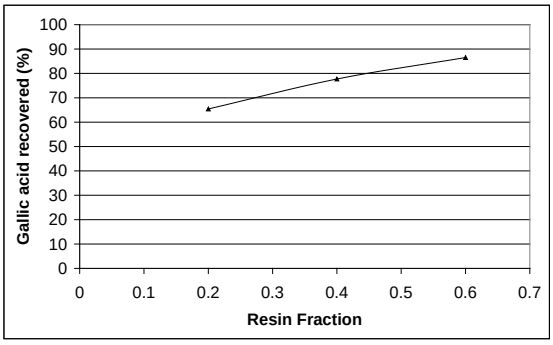


Figure 4-5 Effect of resin ratio on recovery of gallic acid.

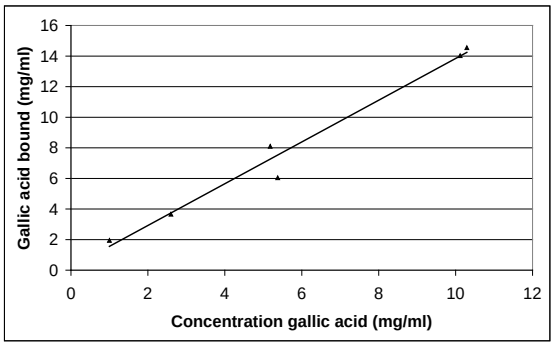


Figure 4-3 Effect of concentration on gallic acid binding.

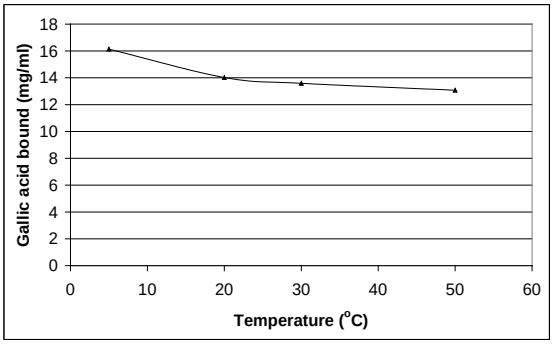


Figure 4-6 Effect of temperature on binding of gallic acid.

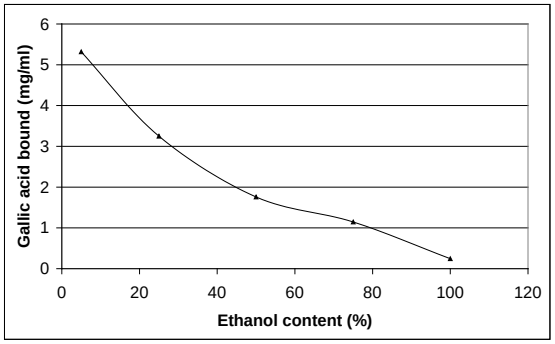


Figure 4-4 Effect of EtOH content on gallic acid binding.

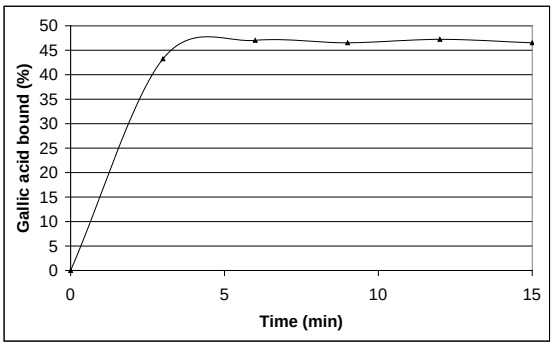


Figure 4-7 Kinetics of the binding of gallic acid.

From these results, defining the optimum conditions for phenol binding to SP207 was quite straight forward. These optimum conditions were defined as follows:

- pH adjusted to 3.0 using 1.0 M hydrochloric acid.
- As high a concentration as possible. This allows minimisation of resin input and increases the relative efficiency of binding.
- The complete absence of ethanol greatly enhances phenol binding to SP207.

- The higher the resin ratio, the greater the recovery of phenol. A resin to solution ratio of 1:2 v/v provides complete binding of phenol compounds in a two stage process.
- Low temperatures facilitate the binding of phenolics, i.e. temperatures in the range of 0-5 ºC.
- Binding is effectively complete within 5 min at these conditions. So leaving the resin and sample contacted for 10 min will give a good safety margin for recovery.

To determine if the phenolic profile of the extract remains unchanged, an HPLC analysis of the extract prior to application, after recovery, and the discarded overflow was undertaken. This is to ensure that certain phenol species are not selectively bound and not fully recovered during the phase of washing the resin. HPLC profiles can be seen on Figure 4-8 to Figure 4-10.

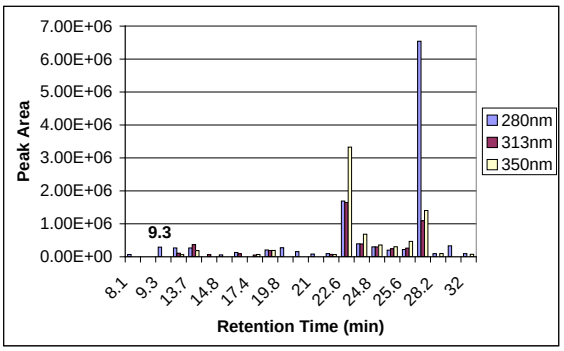


Figure 4-8 HPLC profile prior to resin application.

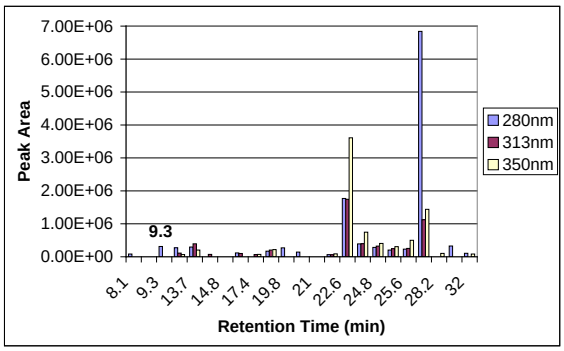


Figure 4-9 HPLC profile **a**fter resin application.

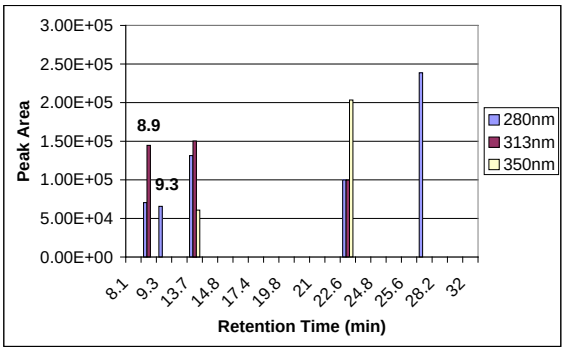


Figure 4-10 Resin overflow.

The application of the extract to resin SP207 did not modify the phenol profile to any great extent, the small variations in peak size and absence of smaller peaks can be attributed to experimental error and low HPLC detection tolerances. The resin

overflow, or unrecovered supernatant, does contain some specific phenol species, notably oleuropein and its hydrolysis product (most likely to be hydroxytyrosol), but also chlorogenic acid and the large peak that may be the dialdehydic form of the oleuropein aglycone, refer to section 3.3.4. The relative sizes of these peaks are very small though, representing a fraction of the input; but it should be noted that the first three peaks are present in quantities not proportional to the input suggesting selective recovery, albeit a minor effect.

In addition to carrying out experimental runs using an ideal solution of gallic acid, an experiment was designed to analyse the effects of some factors on the binding and recovery of olive plant phenols from a crude lipid-free extract. The factors examined were concentration, pH, and proportion of ethanol in the eluting stream. The experiment was designed as in Table 4-3. Note that the levels of C are compounded with the interaction effects of AB, therefore these secondary interaction effects can not be isolated.

Table 4-3 Treatment levels of leaf extract binding and recovery experiment.

	A[a]	**B**[a]	**C**[a]
Run 1	-	-	+
Run 2	+	-	-
Run 3	-	+	-
Run 4	+	+	+

[a] Where; - and + refer to the low and high value of the following respectively. A, gallic acid concentration (5.425,7.1264 mg/ml); B, pH (3.00,4.54-4.79); C, eluting aqueous ethanol % (75 %,100 %).

A Yates analysis of these results, found in Table 4-4, suggests that factor C, the % of ethanol in the eluting phase, has the strongest positive effect of the examined factors at 5.83. Factor A, the concentration of phenols in the extract, had a small positive effect of 0.35. And factor B, the pH of the applied extract, had a significant negative value of -1.27. Hence we can come to the conclusion that for the elution of phenols from Diaion SP207 pure lab grade ethanol should be used to ensure phenol recovery.

The degree of salt removal was only moderate, with generally only 43 % of the ionic content of the sample being removed during treatment, from an initial salt concentration of 2.04 to 1.13 mg/ml, measured as ionic composition, section 3.4.7.

This figure could be greatly increased if the resin was immobilised on a column, and elution steps are used to recover the salt and then the phenol.

Table 4-4 Yates analysis of phenol binding to and recovery from SP207.

Design Table

	A	B	C
Run 1	-	-	+
Run 2	+	-	-
Run 3	-	+	-
Run 4	+	+	+

Where, A = Concentration (5.425,7.1264) V (extract) = 25 ml
B = pH (3.00,4.54-4.79) V (resin) = 12.5 ml
C = Recovery EtOH % (75,100) T (^{o}C) = 0-5 ^{o}C

Treatment Table

	TBP (mg/ml)	pH	EtOH %	TBP (mg)
Run 1	5.425	3	100	135.625
Run 2	7.1264	3	75	178.16
Run 3	5.425	4.79	75	135.625
Run 4	7.1264	4.54	100	178.16

Binding Table

	Supernatent				
	Dilution	Average Abs	TBP (mg/ml)	Bound (mg/g)	% Bound
Run 1	0.05	0.0603	0.950	8.950	82.490
Run 2	0.05	0.0947	1.490	11.272	79.085
Run 3	0.05	0.0647	1.018	8.814	81.233
Run 4	0.05	0.0920	1.448	11.356	79.674

Recovery Table

	Eluent					
	Dilution	Average Abs	TBP (mg/ml)	V (ml)	TBP (mg)	% Recovery
Run 1	0.05	0.113	1.784	57	101.708	**74.992**
Run 2	0.05	0.138	2.173	57	123.845	**69.513**
Run 3	0.05	0.106	1.674	55	92.078	**67.892**
Run 4	0.05	0.150	2.356	56	131.958	**74.067**

Yates Analysis (of TBP recovery)

	A	B	C
	0.697	-2.547	11.655
Effect	**0.348**	**-1.273**	**5.827**

[a] Effect is the sum of squares of the positive effects minus the negative effects divided by the half the number of treatment rows. 0 is no effect, -ve number is a negative effect, +ve number is a positive effect with the number representing magnitude of effect.

Ethanol Removal: As the recovery of phenols from SP207 requires pure ethanol as an eluent, this must be removed prior to bioactive testing. RO water at 25% of the initial volume was added to the solution so that the phenols can be recovered in a pure water matrix. The process parameters for ethanol removal are the same for all operations, as outlined above.

4.3.4.4 Product Finishing

Once the ethanol has been removed, the sample required preparation for bioactive testing. It was ideal to have a concentrated sample allowing dilution to a concentration

suitable for testing. This was achieved by freeze-drying the sample in a Telstar Cryodos manifold freeze dryer. The sample was then diluted to the desired concentration when required using distilled water.

To prevent any spoilage from fungal species and to avoid any sample degradation at the high temperatures of an autoclave, the sample was filter sterilised through a 0.45 micron filter into a pre-sterilised 10 ml glass container. Samples were stored at 4 °C in the absence of light until required[2].

4.4 Solvent Extraction

By definition, solvent extraction is simply the extraction of solutes from one phase to another using a solvent mix as the transferring medium. In this particular case the aim was to extract phenol species from a solid olive matrix using a solvent or solvent mix.

The first phase in developing a method for the extraction of phenolic compounds from olive tissues is to compare different solvent systems to determine which gives the highest recovery of phenols.

4.4.5 Solvent Selection

The analysis of phenol recovery, as a function of solvent system, was undertaken using a Soxhlet extractor schematically represented in Figure 4-11. This apparatus allows the periodical contacting of the dried plant material with fresh solvent. The plant material is loaded into a porous cellulose thimble filter. Fresh solvent is loaded into the lower round bottomed flask with a heat supply of a heating mantle. A condenser is placed above the extraction chamber. The solvent is heated into a vapour that rises through the vapour tube into the condenser unit where it condenses and drips down into the extractor and contacts with the plant material. The solvent builds up in

[2] During cool storage of a liquid extract, some oily residue often formed on the bottom of the flask. When samples are taken for testing, this oil layer was not resuspended to prevent any inconsistencies in the test.

the extractor unit until the level reaches that of the return tube, at which point the extract overflows back into the round bottomed flask.

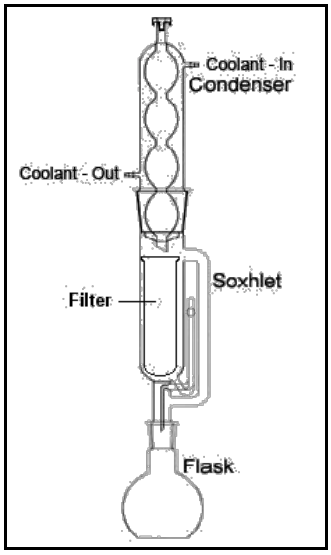


Figure 4-11 Schematic of a Solxhlet extractor.

One difficulty when attempting to make comparisons with extraction rates in a Soxhlet extraction apparatus is that each solvent has a unique rate of evaporation at a given temperature. To ensure that the extraction rates are independent of contact time and solvent residence time, defined as times between solvent overflows, a variable temperature heating mantle was used. This allowed control of the degree of solvent heating and therefore allowed the solvent flowrates to be standardised to 0.15 ml/s, giving approximately 330 s for solvent overflow to occur, refer to Figure 4-12.

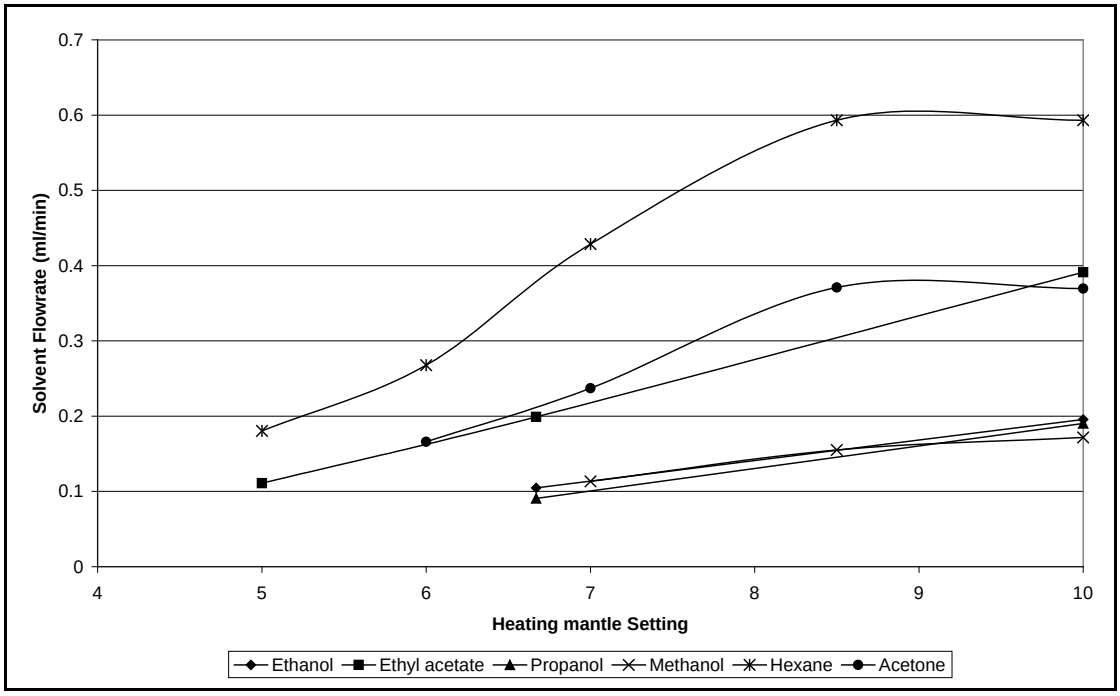


Figure 4-12 Solvent flowrates through a Soxhlet extraction vessel.

Figure 4-13 and Figure 4-14 show the TBP yields from olive pomace and olive leaves respectively; presented as polynomial models of best fit. In both cases extraction is not complete at the conclusion of the experiment.

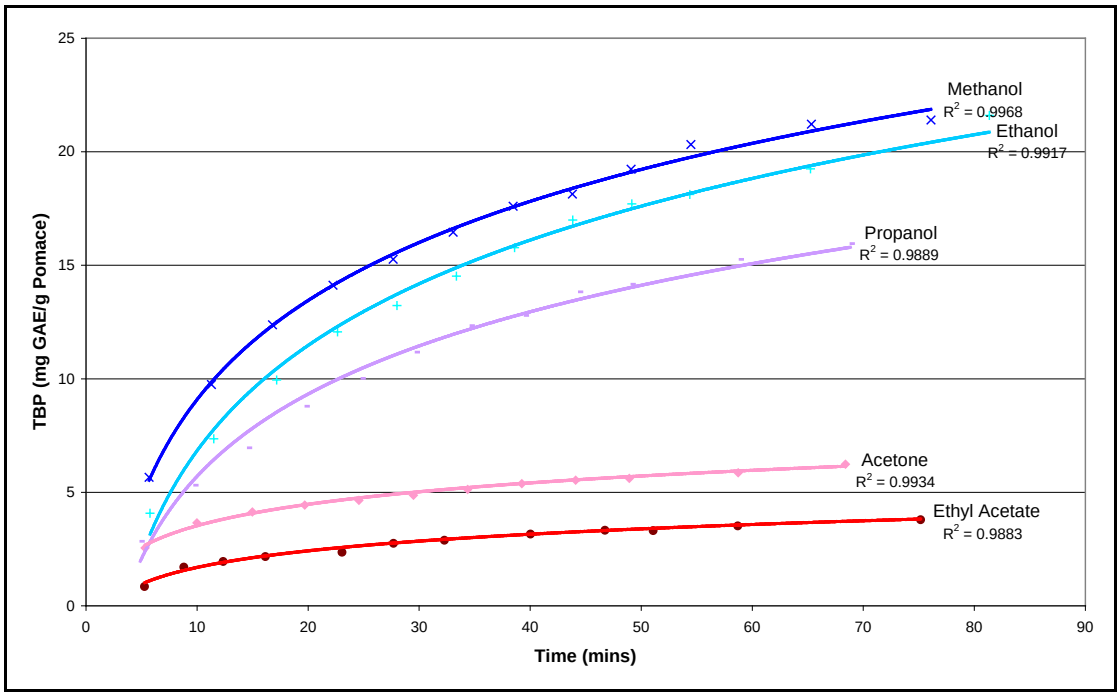


Figure 4-13 Extraction yields of phenol compounds from olive pomace in different pure solvent systems: 10.0g pomace, 300 ml solvents, solvent flowrate 0.15 ml/s.

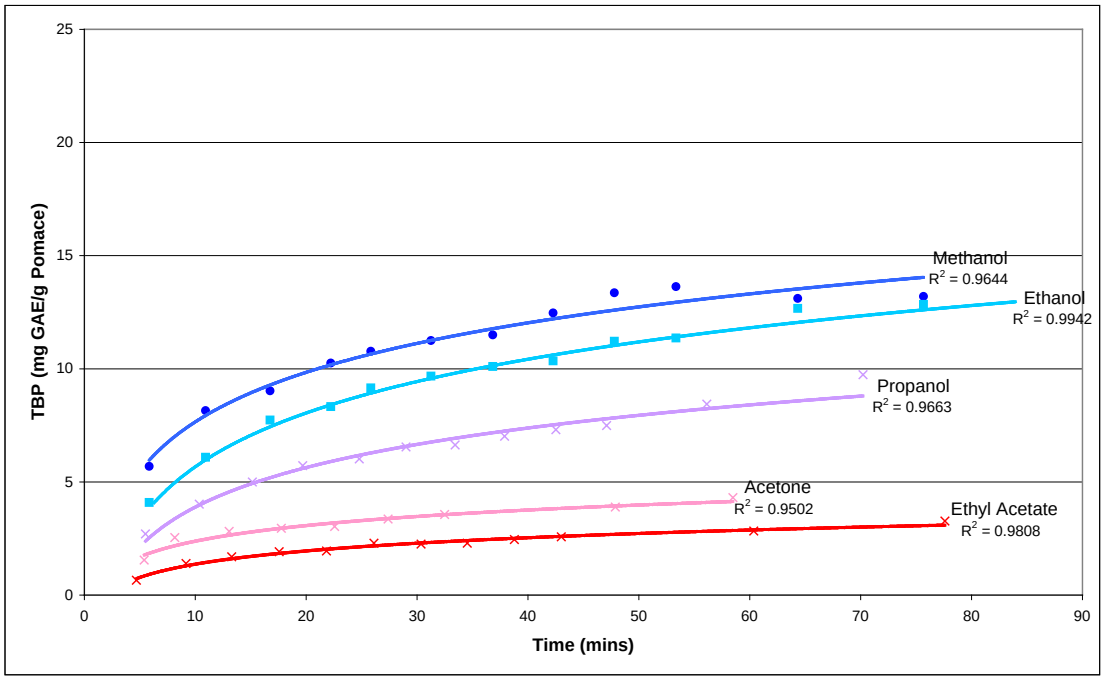


Figure 4-14 Extraction yields of phenol compounds from olive leaf different pure solvent systems: 10.0 g leaf, 300 ml of solvent, solvent flowrate 0.15 ml/s.

It can be seen that ethanol and methanol solvent systems achieve the greatest yield of phenols from both the olive pomace and olive leaf material. The degree of phenolic recovery from the leaf material is significantly greater than that from pomace. This result suggests a smaller quantity of recoverable phenols from the 'spent' fruit material, which is understandable given that it has already undergone an extraction process for the recovery of the oils.

The extracts from the olive pomace extracts were then concentrated and a comparison of the degree of phenol recovery with the associated solvent polarity, defined by the Hildebrand solubility parameter, was made, Figure 4-15. It shows a trend of increasing phenolic recovery with increasing solvent polarity, which is expected given the hydrophilic nature of phenols.

Whilst phenol recovery was greater with methanol, it was decided that ethanol will be used due to its lower toxicity and established safety in food systems.

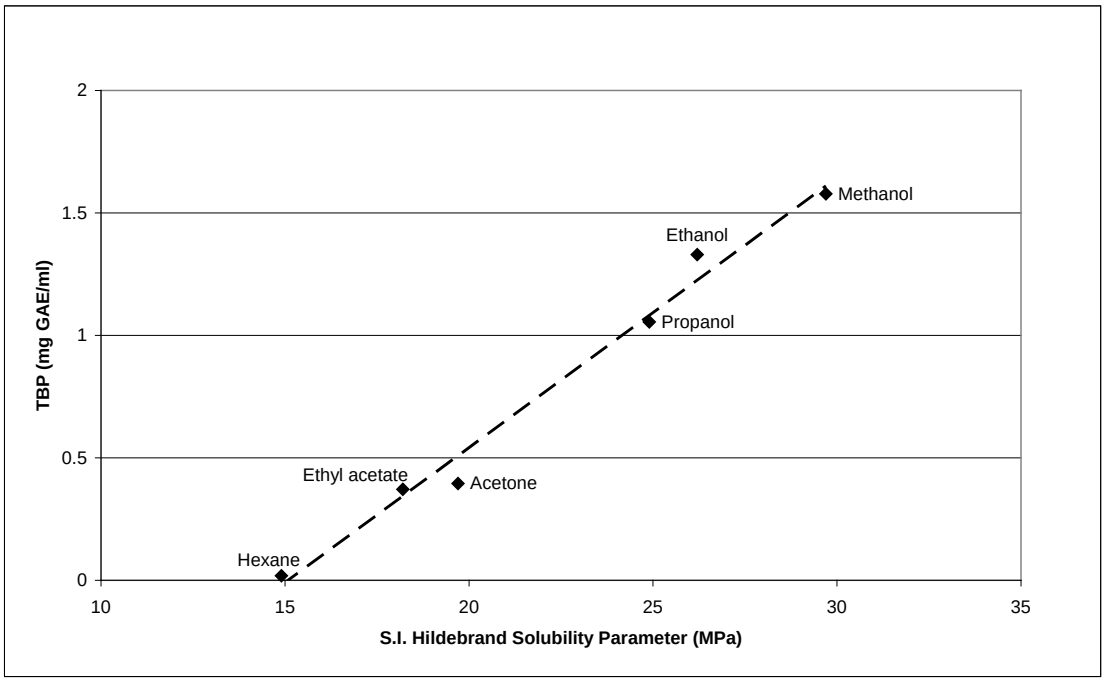


Figure 4-15 Effect of solvent polarity, defined by Hildebrand Solubility Parameter, on the final of TBP content of concentrated extracts of olive pomace.

Aqueous ethanol solvent systems achieved a greater extraction of phenol species than pure ethanol, with a maximum recovery at 70% aqueous ethanol, as shown in Figure 4-16. The experiment was conducted in a completely stirred 50 ml beaker at room temperature, as opposed to a Soxhlet apparatus, resulting in different rates of extraction and total phenolic recovery due to the higher degree of contact between the plant material and the solvent mixture.

The ethanol concentration achieving the highest recovery of phenols was very similar for both olive pomace and leaf raw materials, which was surprising considering that the different olive tissues have decidedly different phenolic profiles as outlined in section 2.3.3. Nevertheless, it was convenient that a standard optimised solvent matrix could be used for both plant tissues.

For the extraction of biophenolic compounds from preconditioned olive wastes, 75% aqueous ethanol was used.

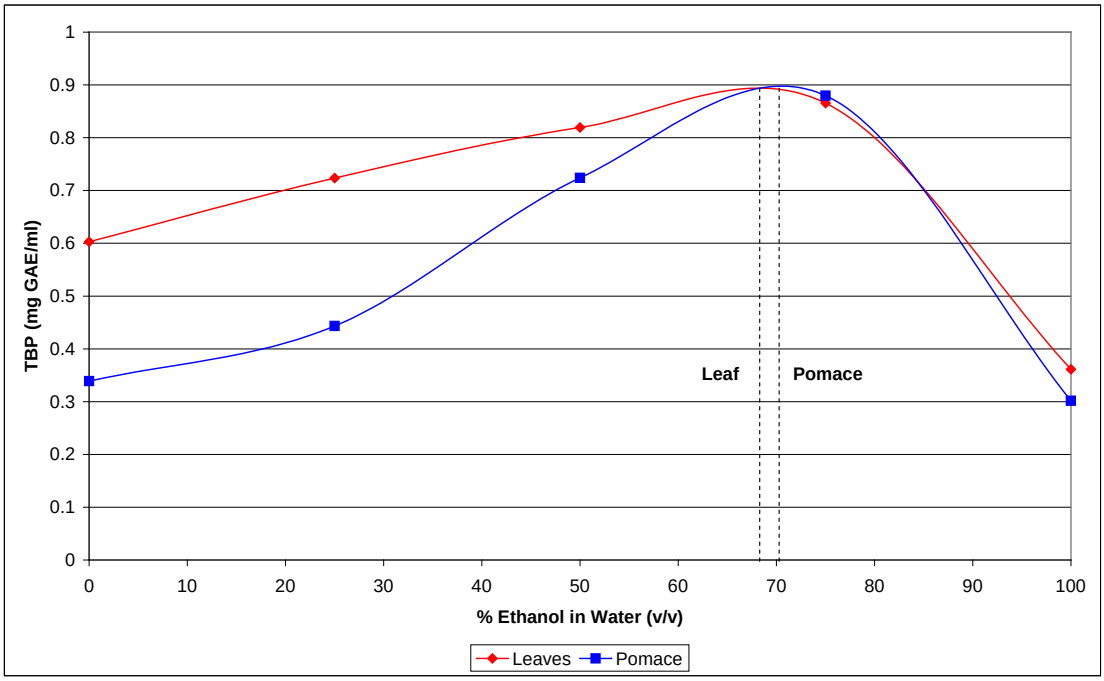


Figure 4-16 Effect of ethanol concentration on the recovery of phenols from plant tissues: 5 g plant material, 100 ml solvent mixture, contact time of 30 mins.

4.4.6 **Yield and Phenol Profile**

The yield and phenolic profile was determined as indicated in section 3.2.2 and 3.3.3 respectively. The yield of phenols is given as gallic acid equivalents (GAE) and is typically expressed as a concentration, mg GAE/ml, or as a yield, my GAE/g leaf material.

The yields and phenol profile for the standard solvent extracts will serve as a control to which the US-assisted extract will be compared.

4.4.6.1 Recovery of Phenols

A kinetic study of the phenol yield indicates the efficiency of the extraction system. A yield relative to the input of raw material will allow a comparison of extraction effectiveness. Figure 4-17 shows phenolic yield of a simple solvent extraction system in a completely stirred reactor incorporating 50 ml of 75% aqueous ethanol and 2.5 g of pre-conditioned leaf material.

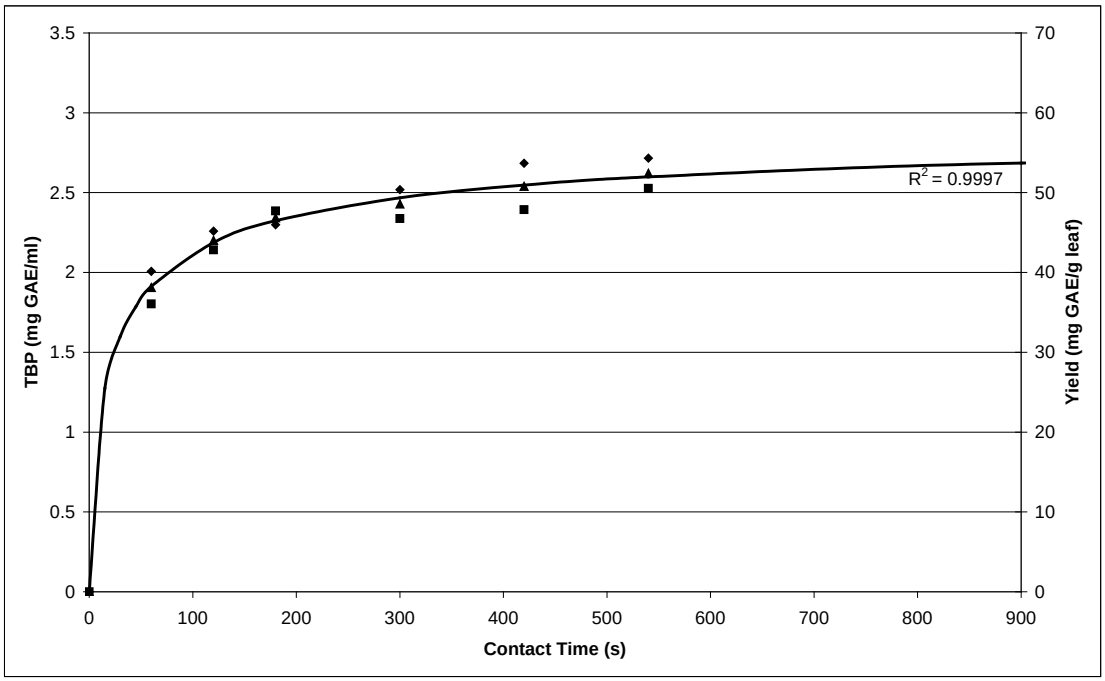


Figure 4-17 Phenol content vs. time over the first 900 seconds of extraction: 2.5 g leaf material, 50 ml 75% aqueous ethanol.

The data in Figure 4-17 is fitted using non-linear regression with a Morgan-Mercer-Flodin model, Equation 4.1, in Curve Expert 3.1. This model is part of the sigmoidal family of growth curves that are common in biology, engineering, and economics. This model increases monotonically to an asymptotic final value.

$$y = \frac{\left(ab + cx^{d}\right)}{\left(b + x^{d}\right)} \qquad \textbf{Equation 4.1}$$

It can be seen that the majority of phenol recovery is achieved within the first 10 minutes of solvent contact. A slight gradient on the extraction curve from this point onwards suggests that the raw material is not exhausted but the concentration gradient between the olive leaf and the solvent, which is the driving force for phenolic extraction, is insufficient to maintain the high rate of extraction.

It can be seen that after 15 minutes of contact, a yield of 53 mg GAE/g leaf material was achieved.

McDonald *et al.* (2001) achieved a TBP content of 150 mg GAE/g of dried extract, which itself was 32% of the initial dry weight of the olive material used. This would culminate in a phenolic recovery of 48mg GAE/g of freeze-dried olive tissue.

Briante *et al.* (2004) measured the TBP content of a concentrated *O. europaea* cv. *Moraiolo* extract to be 280mg tyrosol equivalents/g extract, without any information concerning the yield of extract from the leaf material.

Le Tutor and Guedon (1992) identified high oleuropein content in *O. europaea* leaves of 60-90 mg/g DW (although this data was never published formally). Briante *et al.* (2002) measured oleuropein content of the leaves of a number of different cultivars showing contents of 1-14.35 mg/g (with no indication of results relative to dry or fresh weight).

There is a lack of data for comparison for the extent and rate of phenolic extraction in literature, due primarily to interest in activity of the compounds of interest opposed to quantifiable recovery. This is compounded by the wide range of extraction methods, the large differences in phenolic profiles of different cultivars, and standards used to quantify the TBP content. Common standards used include oleuropein (Briante *et al.*, 2002), gallic acid (McDonald *et al.*, 2001) and tyrosol (Briante *et al.*, 2004). Given the different molar absorbance of these standards with the Folin-Ciocalteu reagent, the measured TBP content would vary considerably depending on the standard used.

From the information in literature it can be assumed that the yields obtained in Figure 4-17 are in the right degree of magnitude.

4.4.6.2 Profile of Phenolic Compounds

The HPLC profiles of phenol compounds from the standard solvent extraction are very consistent and effectively identical to that of given in 3.3.4.2. The plot used as the reference profile to compare extraction methodologies is given in Figure 4-18.

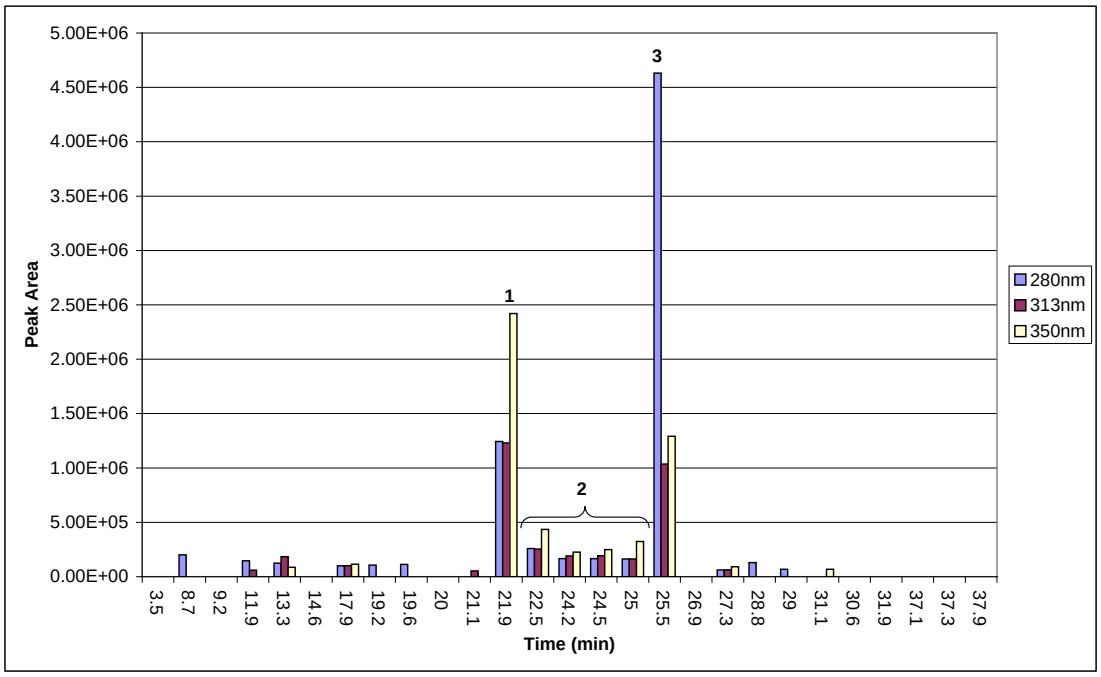


Figure 4-18 HPLC phenolic profile of a standard solvent olive leaf extract.

4.5 **Conclusions**

An extraction protocol was developed that allowed the consistent and optimised recovery of biophenols from solid wastes of the olive oil industry. This ensured that there were minimal variations between extracts and significant removal of contaminants such as chlorophyll, lipids and salts.

75% aqueous ethanol was found to achieve the greatest recovery of olive biophenols with yields of 53 mg GAE/g DW of olive leaf.

A standard HPLC profile was developed showing the phenolic profile of a standard olive leaf extract.

Chapter 5

Ultrasound-Assisted Solvent Extraction

In this section, the yield and phenolic profile obtained from the ultrasound-assisted solvent extraction of olive leaf will be examined. The conditions for extraction are examined first, identifying the effects of the main process variables, and then the properties of the extracts are examined.

What distinguishes ultrasound-assisted (US) solvent extraction from standard solvent extraction is the implementation of acoustic cavitation to reduce the resistance of solvent penetration into the solid plant matrix by cellular destruction; thereby enhancing solute dissolution and recovery. This work examined the effectiveness and efficiency of US-assisted extraction of biophenols from olive wastes, relative to standard solvent extraction, in terms of phenolic yield and composition of the extract.

5.1 Defining Conditions for Ultrasound-Assisted Extraction

Cavitation is quite a complex phenomenon that is dependent on fundamental process parameters, including temperature and pressure, as well as the apparatus setup, such as the positioning of the disruptor horn. Given the large number of significant factors in ultrasound operation, it is very important to maintain a consistent operation protocol to ensure system variation does not become a factor in the comparison of results.

5.1.1 **Apparatus**

A Virsonic Digital 475 system was used incorporating a 475 W generator that allows control of power output using a 0-10 power setting dial. The nominal power output was displayed as a % of max power output, although it will be shown that this reading significantly overestimates the actual acoustic energy output. The electrical energy is converted into acoustic energy through an electrical-mechanical energy transducer known as the converter. This mechanical energy is focussed through either a 1/2" titanium disruptor horn or a 1/8" microprobe that is placed within the extraction chamber: a perfectly tuned unit should resonate at 20 kHz. This is all mounted within a sound enclosure to prevent any discomfort associated with cavitation noise via harmonics eliminating through the sample vessel walls and fluid surface. Experimental runs were carried out to compare the performance of the standard 1/2" horn and a 1/8" micro probe in 50 ml of water (Figure 5-1). The method for calculating power output from the probe is discussed in section 5.1.2.

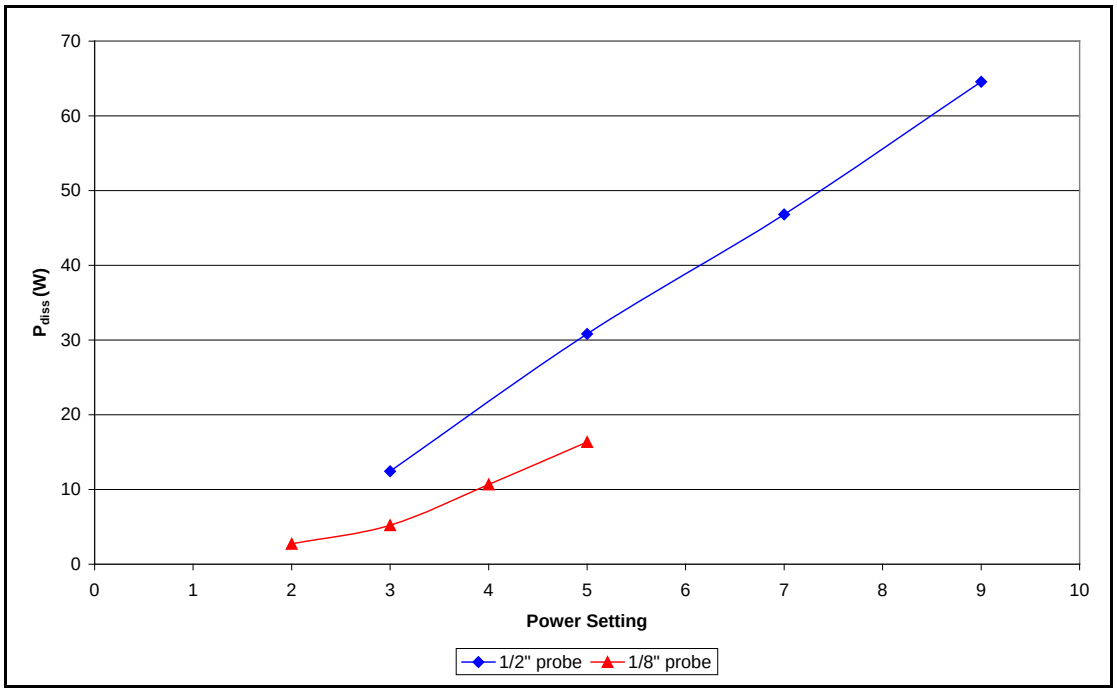


Figure 5-1 Power output of different probes[3].

It can be seen that the acoustic power output profiles of the two probes are quite different. It should be noted that the 1/8" probe can only be operated up to a power

[3] Note: these power outputs have not been adjusted to include heat loss and absorption into apparatus.

setting of 5 as given in the standard operation manual. Given this and the higher power outputs of the larger probe, the 1/2" horn was selected for further work.

The extraction chamber was a simple beaker of suitable volume, generally 100 mls, and temperature was monitored using a Grant 1250 series Remote Squirrel meter/logger incorporating a metal cased smooth-ended 321 stainless steel thermistor temperature probe (3.2 mm diameter, 50 mm length). To ensure that the temperature profile and particulate suspension was homogenous, the extraction vessel was placed on a magnetic stirrer.

Prior to operation, the ultrasonic device must be tuned following the standard procedure in the operating guide. Tuning ensures the highest efficiency of operation where that the greatest tip amplitude is achieved at the lowest possible power input. Once the unit is properly tuned, operation can commence.

It must be noted that when utilizing the ½" disruptor horn, the tip must be submerged 1-1.5 tip diameters below the surface of the medium. The horn positioning can have an effect on the degree of cavitation and acoustic power output as the surface area of the probe submerged in the extraction vessel determines the power dissipation (W) relative to the acoustic intensity (W/cm^2), as well as the effects of acoustic echoing and formation of standing waves. As such, the horn should always be placed in the centre of the extraction chamber as shown in the schematic Figure 5-2 below.

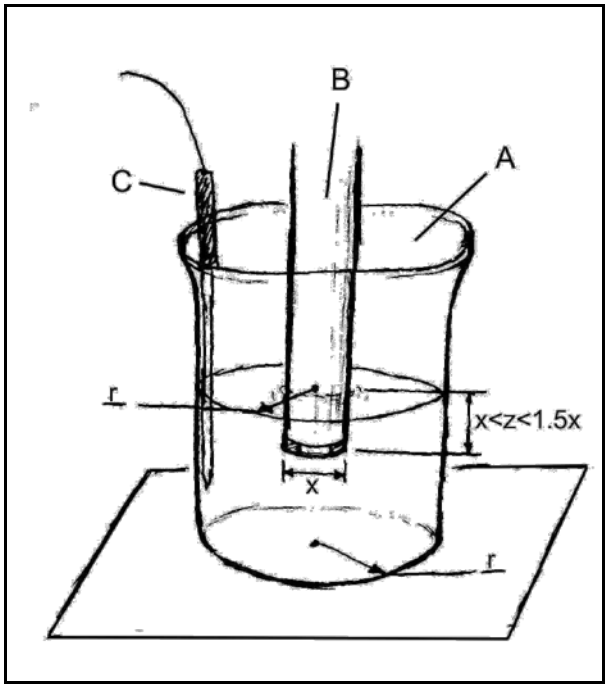


Figure 5-2 Schematic representation of horn placement. Where A is the extraction vessel, B is the ½" disruptor horn and C is the temperature probe.

5.1.2 **Measuring Ultrasonic Power Output**

An ultrasonic generator transforms electrical energy into acoustic energy, which is a form of mechanical energy. The efficiency of the transformation of this energy is dependent on a number of conditions, including the equipment itself, but also the conditions in which sonication is implemented (Raso *et al.*, 1999). It is therefore erroneous to assume that the electrical output from the power supply into the transducer equates to the acoustic energy delivered to the liquid medium.

If we assume that all the acoustic energy delivered to the system is eventually transformed into heat, we can measure the actual energy input into the system using calorimetric techniques: i.e. the change in temperature over time of the liquid medium can be transformed to power in Watts, Equation 5.1.

$$P_{diss} = \left(\frac{dT_s}{dt}\right)_{t=0} m_s Cp_s$$

Equation 5.1

Where; P_{diss} = the power dissipated in the solution as heat, W

T_s = the temperature of the solvent, ºC

Cp_s = the weighted sum of all component specific heat capacities, J/gºC

m_s = the mass of the sample, g

Equation 5.1 assumes that heat loss to the environment and absorbed into the extraction vessel is negligible. If these factors are to be included into the model, the equation must be modified. Including heat absorbed into the extraction vessel we get Equation 5.2 (Hagenson and Doraiswamy, 1998).

$$P_{diss} = \left(\frac{dT_s}{dt}\right)_{t=0} m_s Cp_s + \left(\frac{dT_v}{dt}\right)_{t=0} (A_{ws} x_w)\rho_v Cp_v$$

Equation 5.2

Where; T_v = the temperature of the reaction vessel, ºC

A_{ws} = the surface area of the wetted surface of the vessel, m^2

x_w = the thickness of the vessel, m

P_v = the density of the reaction vessel, kg/m^3

Cp_v = the heat capacity of the reaction vessel, J/gºC

This equation can also be modified to include the heat lost from the reaction vessel to the atmosphere if it is not completely insulated, yielding Equation 5.3.

$$P_{diss} = \left(\frac{dT_s}{dt}\right)_{t=0} m_s Cp_s + \left(\frac{dT_v}{dt}\right)_{t=0} (A_{ws} x_w)\rho_v Cp_v + UA_{ws}(T_w - T_a)$$

Equation 5.3

Where; U = the heat transfer coefficient from the wall of the vessel to the atmosphere, $W/m^{2o}K$

T_w = the vessel wall temperature, ºC

T_a = the ambient air temperature, ºC

The power dissipated as heat is determined as follows. The temperature profile is recorded during ultrasound treatment, Figure 5-3, and a 3rd order polynomial is fitted using Curve Expert 3.1.

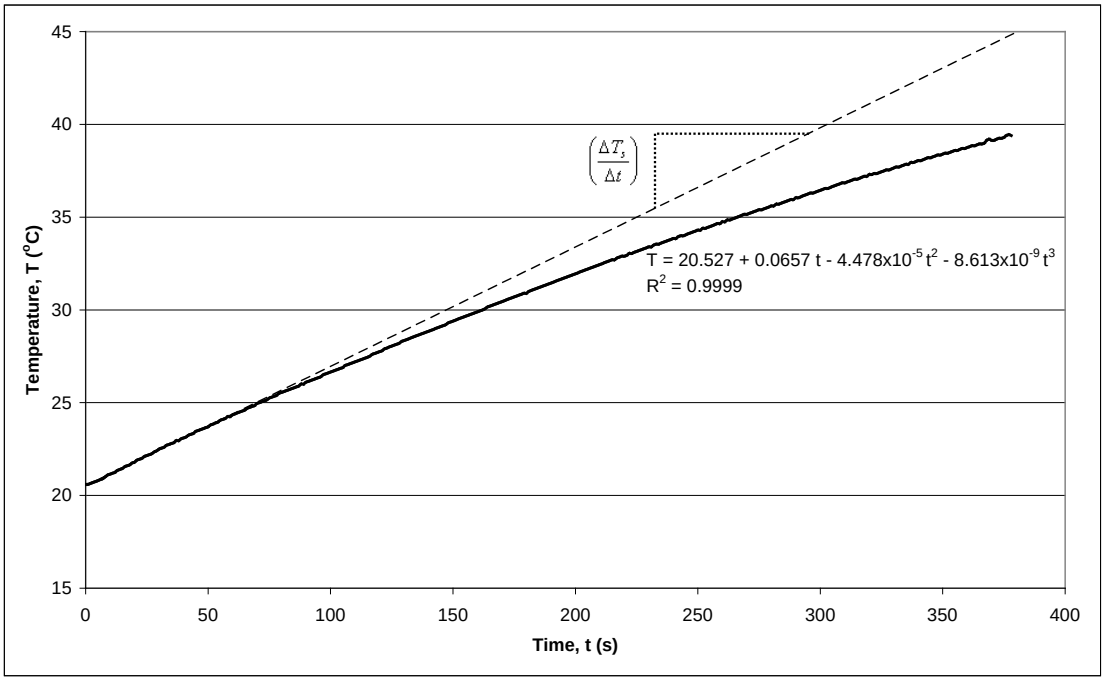


Figure 5-3 Temperature profile during ultrasound treatment of 50ml of water at power setting of 3.

From the temperature profile equation given in Figure 5-3, the rate of temperature change can be derived for every given time, Figure 5-4.

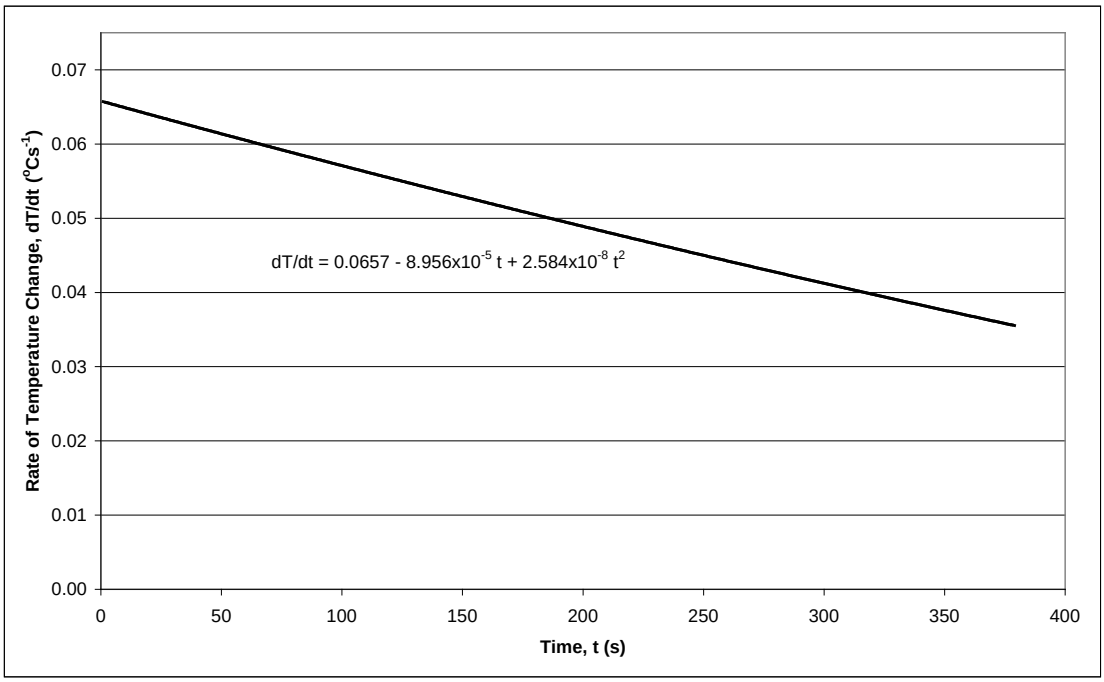


Figure 5-4 Rate of temperature change during ultrasound treatment of 50ml of water at power setting of 3.

With information on the physical properties of the system, Equation 5.3 can be used to describe the power input into the system at any particular time, Figure 5-5.

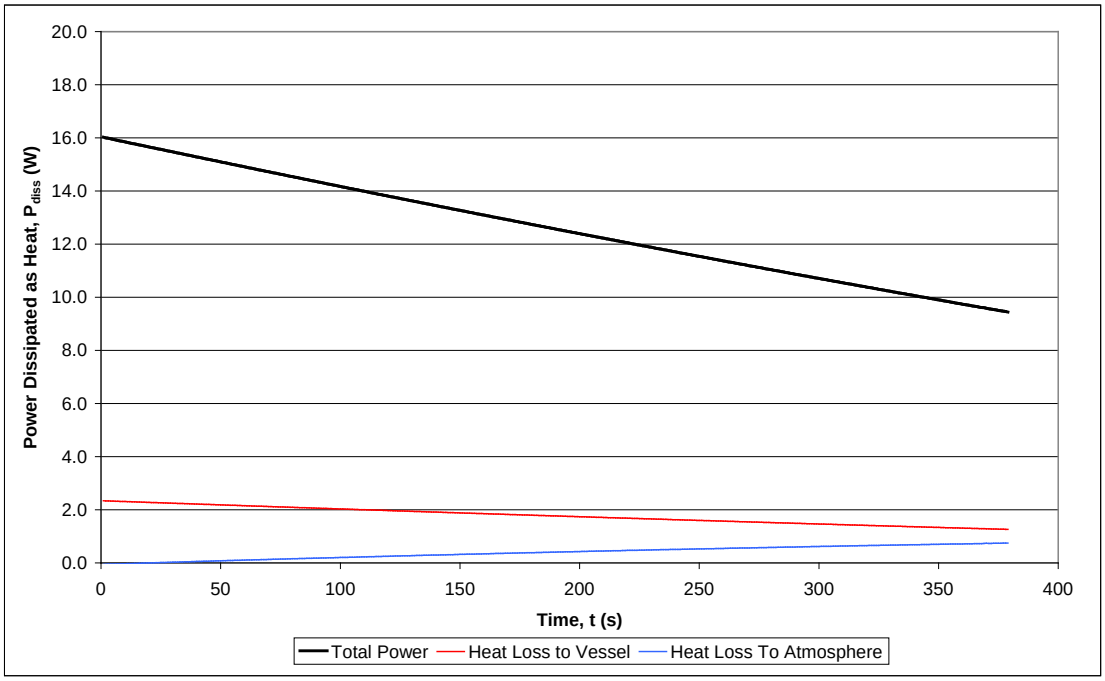


Figure 5-5 Power dissipated into system as heat during ultrasound treatment of 50ml of water at power setting 3: shows contribution of heat losses to total power dissipation.

The quantity of heat absorbed by the extraction vessel is significant, comprising 15% of the thermal capacity of the system, and although at low temperatures the loss of heat from the vessel to the atmosphere is negligible, at higher temperatures the total heat lost to the surroundings could prove significant as the driving force for heat loss increases. Heat losses to the environment and absorbed by the extraction vessel are significant so should be incorporated into the power model.

It has been stated in literature that this method of power output determination provides satisfactorily repeatable results with variation coefficients always being less than 5% (Raso *et al.*, 1999).

During ultrasound extraction, the temperature of the system will be maintained between 10ºC and 25ºC due to thermo-depressive effects on power output, so tracking power dissipation into the extraction chamber using the calorimetric technique will be impossible.

So to accurately determine the power output of the horn for a particular medium composition, the power was calculated before the system parameters change enough to influence the acoustic energy delivered (i.e. from the initial linear temperature change as shown in Figure 5-3, using Equation 5.4).

$$P_{diss} = \left(\frac{\Delta T_s}{\Delta t}\right)_{t=0} m_s Cp_s + \left(\frac{\Delta T_s}{\Delta t}\right)_{t=0} (A_{WS} x_{ws}) \rho_V Cp_V + UA_{WS}(T_V - T_a)$$

Equation 5.4

Where, $\left(\frac{\Delta T_s}{\Delta t}\right)_{t=0}$ = linear change in temperature with time over the first 60 seconds of ultrasound treatment, $^oCs^{-1}$

5.1.2.1 Power Setting

As the power setting on the generator gives no absolute indication of the actual power transferred to the medium, a series of trial runs were carried out to define the relationship between power setting and power output of the ½" disruptor horn calorimetrically, Figure 5-6. Power dissipation was determined by Equation 5.4.

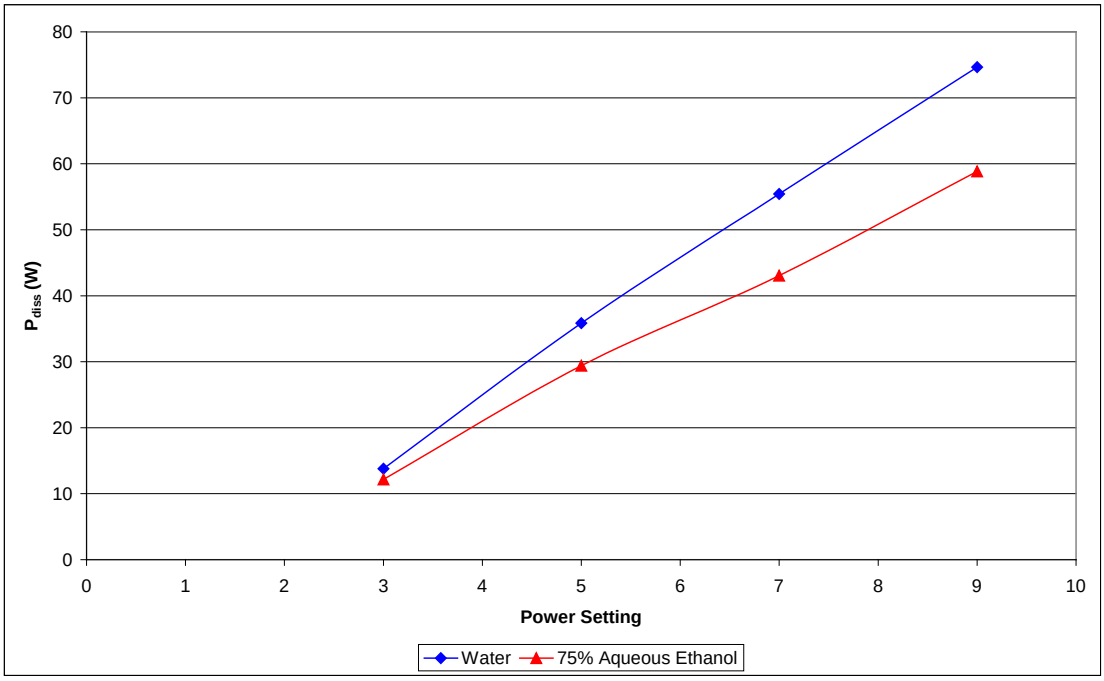


Figure 5-6 Initial power output of ½'ultrasound horn at different power settings dependant on solvent composition.

5.1.2.2 Solvent Composition

The solvent and medium composition has a significant impact on the power output of the disruptor horn. To induce cavitation, the acoustic pressure must be greater than the difference between external pressure and solvent vapour pressure, fulfilling Equation 2.1. As ethanol and water have significantly different pure solvent vapour pressures, approximately 57 and 23.7 mmHg respectively at 25ºC (Perry and Green, 1984), it is

expected that the dynamics of cavitation between the various solvent compositions are going to be considerably different. The acoustic power required to induce cavitation in an aqueous ethanol system is smaller than that of water, given Equation 2.1. But, due to the presence of higher quantities of vapour in the system, cushioning effects from the vapour contained within the bubble during cavitation significantly hinders the power output of the disruptor horn (Raso *et al.*, 1999). So the overall effect is a reduction in observed power, shown clearly in Figure 5-6, with the ethanol-water mixture showing a smaller power output.

The presence of particulate material, as 5 g of dried and powdered olive leaf, did not significantly influence power output of the system, Figure 5-7. It was expected that at least a slight increase in power output would be observed with the addition of leaf material to the system, with an increase in nucleation sites for cavitation.

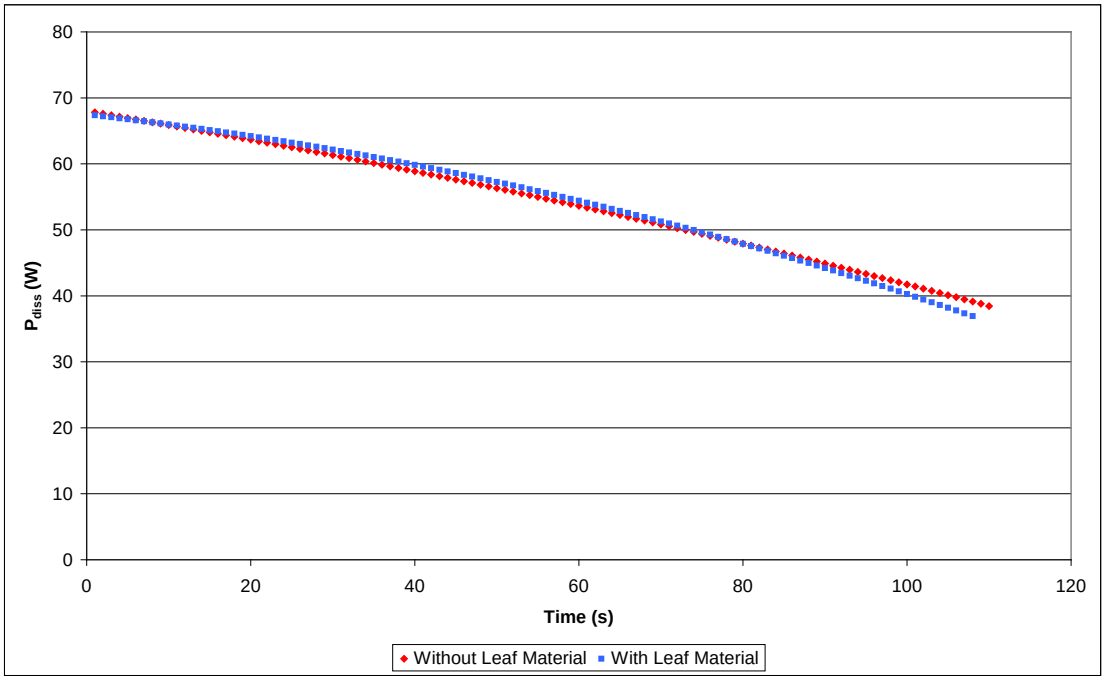


Figure 5-7 Power dissipation with and without 5g leaf material in ethanol/water (75:25) at power setting 9.

5.1.2.3 Temperature Effects

Increasing solvent vapour pressure, resulting from increasing system temperature, also contributes to observed reduction in power output. As system temperature increases, so does the vapour pressure of the solvents present, which in turn reduces power output due to vapour cushioning effects. This can be seen in Figure 5-8.

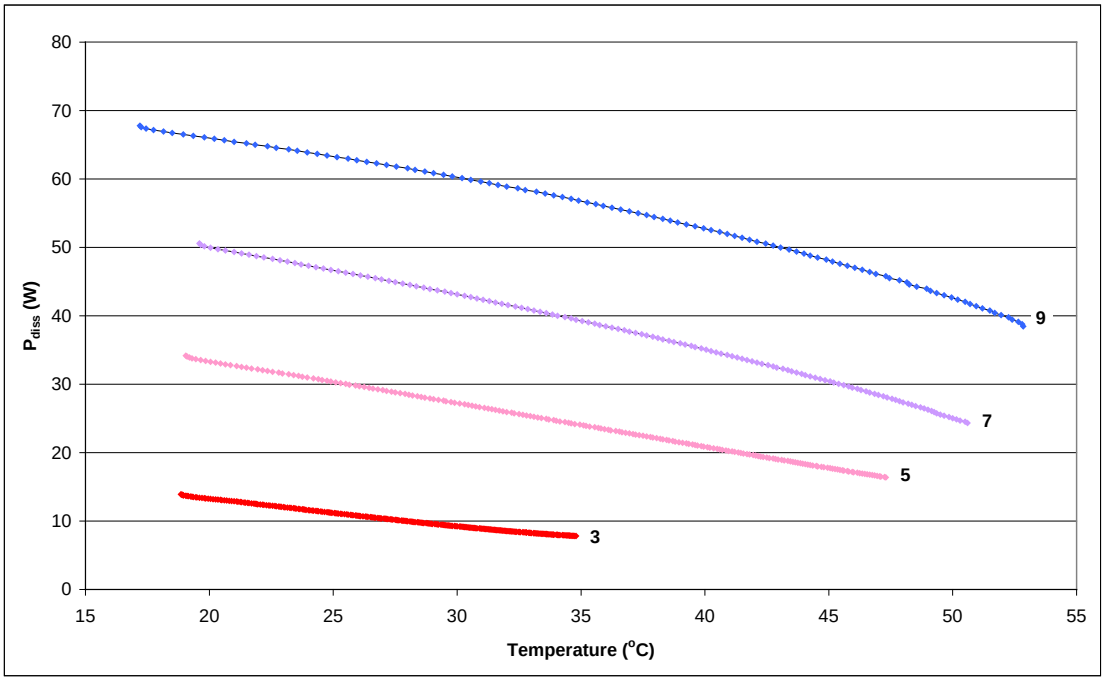


Figure 5-8 The effect of temperature on the power dissipated in ethanol/water (75:25). Where 3, 5, 7, and 9 are the ultrasonic power settings.

Reductions in fluid viscosity and surface tension can also have a negative impact on the power output of the system with increasing temperature by reducing the energy released on cavity implosion during a cavitation event. This may also have the indirect action of decreasing the transient cavitation threshold, inducing bubble shrouding of the horn-tip (Mason, 1990).

5.1.2.4 Degassing Effects

It has been shown in literature that there is an inherent degassing effect associated with cavitating liquids under an ultrasonic field, and that this effect is observed most dramatically during the first 5 minutes of operation (Gondrexon *et al.*, 1997). This degassing effect is independent of acoustic intensity, with the time required to reach a gaseous equilibrium being unaffected by ultrasonic power for a given volume and composition, although the position of the gaseous equilibrium can be influenced by power dissipation.

Two samples were analysed and replicated; one being deionised water, the other being deionised water degassed under a vacuum for approximately 3 hours until no further traces of gas could be observed to be forming at sites of nucleation. With the removal of dissolved gas, and all other factors remaining constant, the reduction in power

output with time can be assumed to be entirely from the increase in temperature and the chain of effects that such an increase in temperature brings about. The resulting difference in power dissipation between the gassed and degassed sample can therefore be entirely attributed to the reduction in dissolved gas in the control sample. Hence it is possible to quantify the inherent degassing effect on an ultrasonic system and the resulting increase in cavitation threshold as outlined in section 2.6.3.3. Figure 5-9 shows the effect of reducing dissolved gas composition on the effective power output of the probe in a low-power water system.

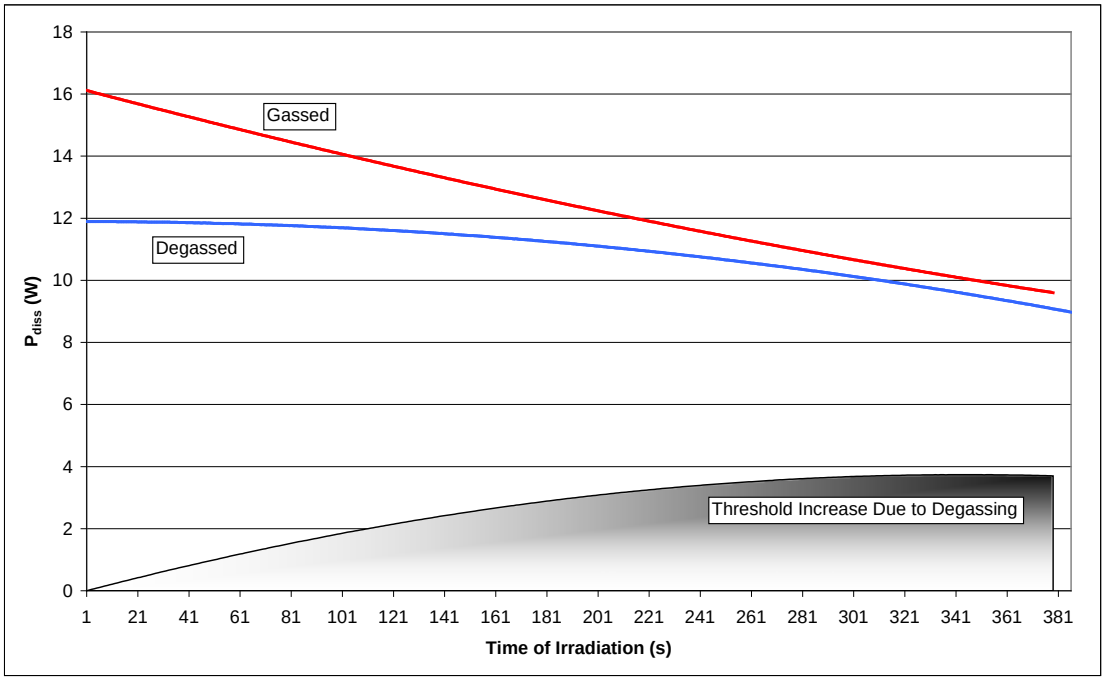


Figure 5-9 Power decrease due to inherent degassing effects of sonication. Power setting of 3 and treatment volume of 50ml of water.

It can be seen from Figure 5-9 that the degassing effect levels out after 300s, in agreement with Gondrexon *et al.* (1997).

If the temperature remains constant, it can be assumed that the only factor effecting power dissipation into the system is the degassing effect, resulting in an increase in cavitation threshold, which in turn reduces the amount of power that can be used for developing cavitation events. For an isothermal application of ultrasound treatment, a power dissipation profile shown in Figure 5-10 can be expected. This will give an average input power of 12 W over the first 900 seconds of extraction.

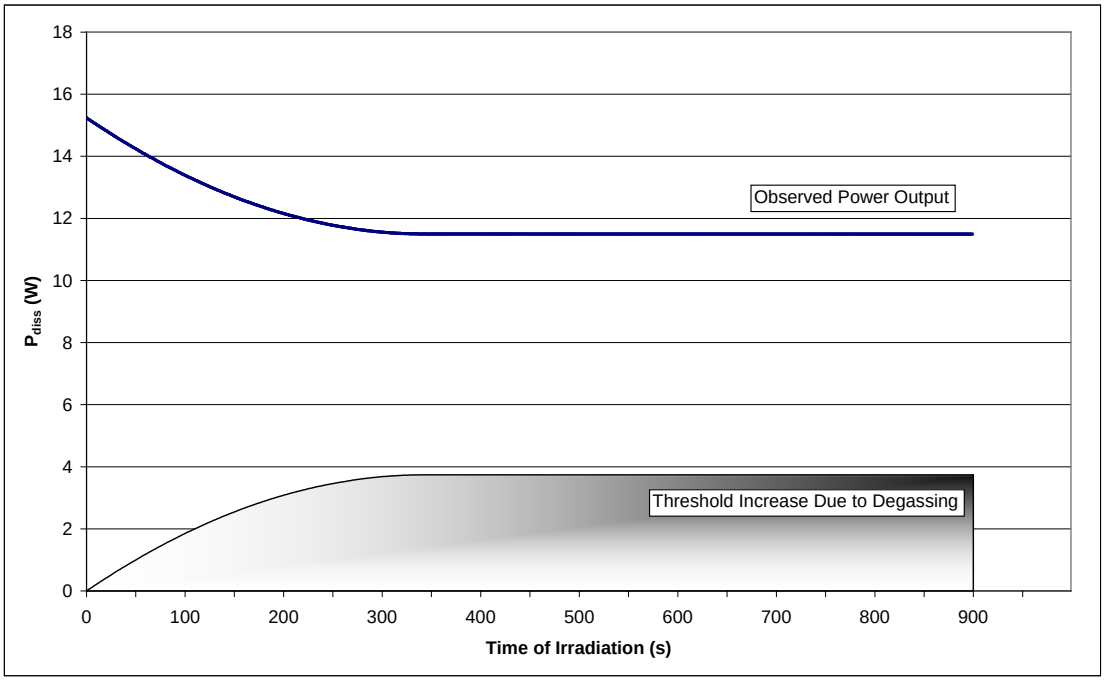


Figure 5-10 Expected power dissipation profile of an isothermal water system at power setting 3.

During the extraction of biophenols from olive leaf using ultrasonic irradiation, the power dissipated into the system will not remain constant, but will slowly decrease during the first 3 minutes to an asymptotic point. It is assumed that this power dissipation into the sample will follow this profile given the same extraction conditions; power setting of 3, 50ml of 75% aqueous ethanol.

5.1.3 **Conditions for Extraction**

When examining the rate and yields of extraction, we wish to minimise the effects of temperature and promote sufficient mixing to ensure that only the ultrasonic irradiation effects are measured.

- The extraction vessel must remain at a constant temperature between 10 and 25 ºC to prevent any effects on power output as outlined previously. To maintain a constant temperature during the course of the extraction, the extraction vessel was submerged in an ice/water bath. This was a crude form of temperature control but it helped maintain extraction temperature at approximately room temperature; the variation was never more than 2 ºC at low power outputs (PS 3).

- To maintain a constant degree of mixing, a magnetic stirrer and magnetic flea were used. This was due to the fact that at lower ultrasonic power outputs the velocity profiles created by ultrasonic turbulence were insufficient to suspend the leaf material in solution, and therefore, create conditions that were comparable to the control.

5.2 **Yield and Phenolic Profile**

5.2.4 **Recovery of Phenols**

Examination of differences between the concentrations of phenols, as a function of time, from standard solvent extraction and US-assisted solvent extraction allowed estimates to be made about the extent to which a US system enhanced the recovery phenols from olive leaf. This could happen in two ways; by increasing the rate at which phenols are transferred into the bulk solution, and by increasing the yield of phenols by making them available for extraction due to cellular disruption.

Firstly, the difference in the concentration of phenols with time was examined. Figure 5-11 shows the experimental data from extraction of phenols from 2.5 g of olive leaves using both standard solvent extraction and US-assisted solvent extraction at a power setting of 3 (providing an initial and average power of 15 W and 12 W respectively, refer Figure 5-10), incorporating an aqueous ethanol (75% v/v) solvent mixture.

It can be seen that the phenolic concentration is higher with US-assisted extraction than with standard solvent extraction at all measured times. It is also apparent that the sample sets extracted under the influence of ultrasound achieves a higher asymptotic equilibrium point. This is strong evidence that there are some phenols that are not available for recovery using standard solvent extraction, and in some way ultrasound allows these phenols to be recovered.

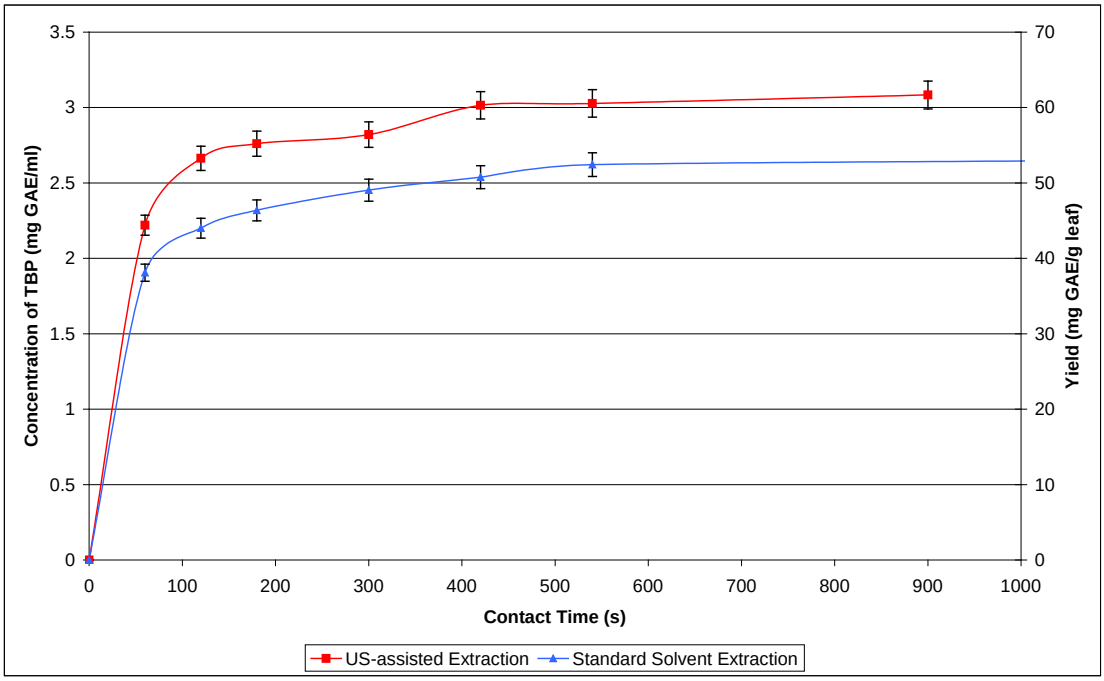


Figure 5-11 Yield of phenols during standard solvent extraction and US-assisted extraction, average of triplicate runs: 50ml 75% aqueous ethanol, 2.5 g olive leaf, power setting 3 (for US).

The relative yield achieved under the influence of ultrasound was found to be 61.5 mg TBP/g leaf material after 15 minutes of contact, compared to 53 mg TBP/g leaf material for the standard extract. The action of ultrasound on the solid olive matrix increases the absolute degree of phenolic extraction by 16% compared to standard solvent extraction.

This effect is most likely due to mechanism of particle destruction under the influence of ultrasound. The disruption of the plant cell walls and organelles allows the recovery of the intracellular constituents, in this case intracellular phenolics; thus giving a higher yield of phenolics per gram of leaf input.

In order to examine the premise that the action of US increases the rate of phenolic dissolution and diffusion into solution, a leaching model was constructed on a solids-free basis to examine the changing mass fraction by time. The leaching model was constructed under the following premises:

- phenols are infinitely soluble in solution, with no preferential binding to leaf matrix
- no solid dissolution in solution, leaf material is insoluble

- phenolic composition of the solution in the leaf and bulk solution is identical at equilibrium
- complete leaf removal from solution during phase separation
- 35% (w/w) solution retained in leaf material, average from analysis (data not shown)
- sufficient contact time to allow equilibrium

Given these assumptions, it was possible to express the regression data as a mass fraction by time, allowing the construction of Figure 5-12. The mass fractions are split into two streams, or phases, and compared between US-assisted extraction and standard solvent extraction; the two phases being the bulk solution, and the leaf matrix with any associated solution that cannot be effectively separated from the leaf matrix.

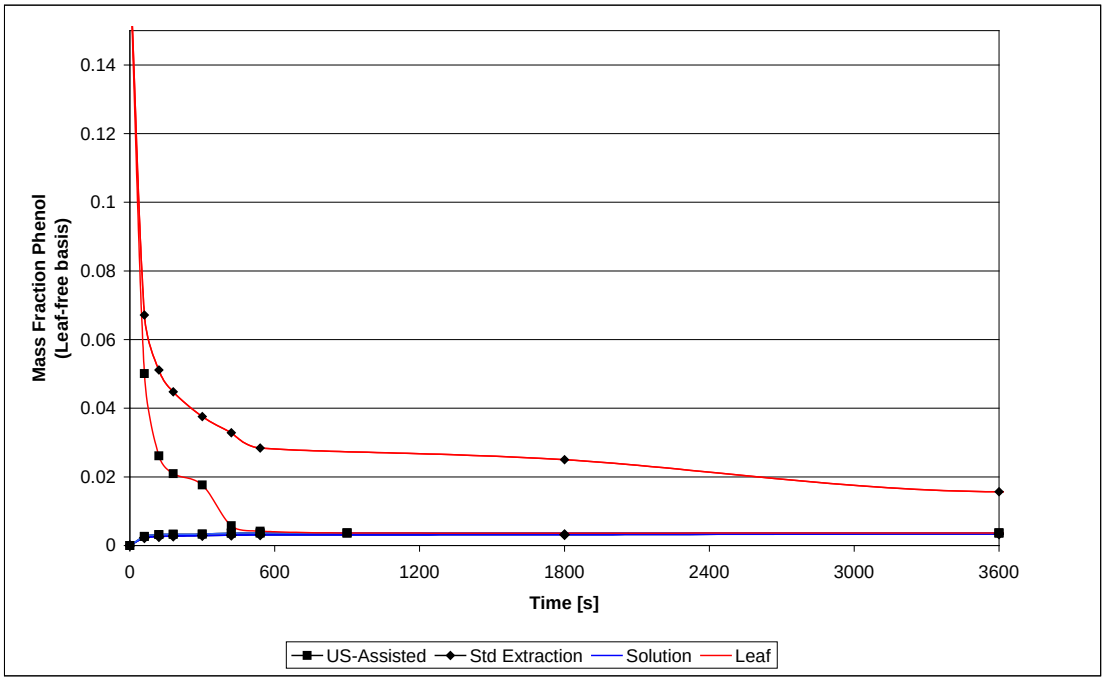


Figure 5-12 Mass fraction of phenols between solid matrix and solution, comparison between control and US extractions.

It can be seen quite clearly that there is a difference between the rates of reduction of the mass fraction of phenols in the olive leaf; under the influence of US, the mass fraction of phenols approaches the asymptotic equilibrium point at a greater rate. Given the similar hydrodynamic behaviour of the systems, it is feasible that the degree of contact between the bulk solution and the leaf matrix is comparable. If we

also agree with the reasonable assumption that phenols are effectively infinitely soluble in the solution, then the resulting increase in extraction rate would be primarily due to the reduction in *diffusive resistance* through the solid matrix to the bulk solution: please refer to 2.6.2.1.

The extent to which this affect may influence systems of different particle size distributions is unclear. It is clear that the relative influences of the rate effecting factors, particularly the rate of diffusion through the matrix and the degree of solid-liquid contact, could vary significantly. In these experiments, the leaf material was effectively powdered prior to extraction, reducing the diffusion path for the solute into the bulk solution. It could be envisioned that with larger particle sizes, the effect of US could be substantially greater.

In Figure 5-13, we can see that to achieve a 95% recovery of leaf phenol content using US-assisted extraction, it would require a contact of only 350 seconds, a fraction of the time required for standard solvent extraction, often left to 'mature' for hours to achieve a comparable phenolic recovery (Hagenson and Doraiswamy, 1998).

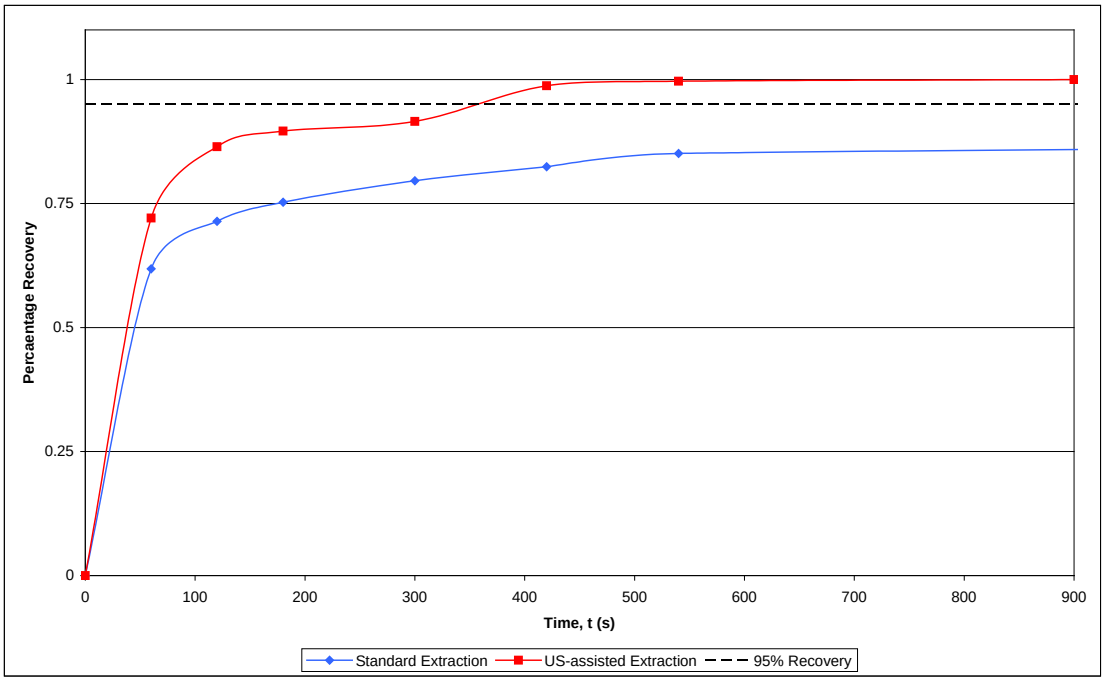


Figure 5-13 Recovery of phenol as % remaining in leaf

Under the conditions used in these experiments, phenol recovery was increased by just over 15% at 15 minutes of extraction.

5.2.5 **Profile of Phenolic Compounds**

In the previous section we saw that an ultrasound-assisted extraction system can effectively increase the yield and availability of BP's from olive leaf. But are the phenolic compounds extracted free from chemical modification? Here we wish to ensure that the phenols we extract during US-assisted extraction are the same as those extracted during standard solvent extraction. We do this by comparing the phenolic profile established with HPLC. Figure 5-14 shows the comparison of two HPLC profiles.

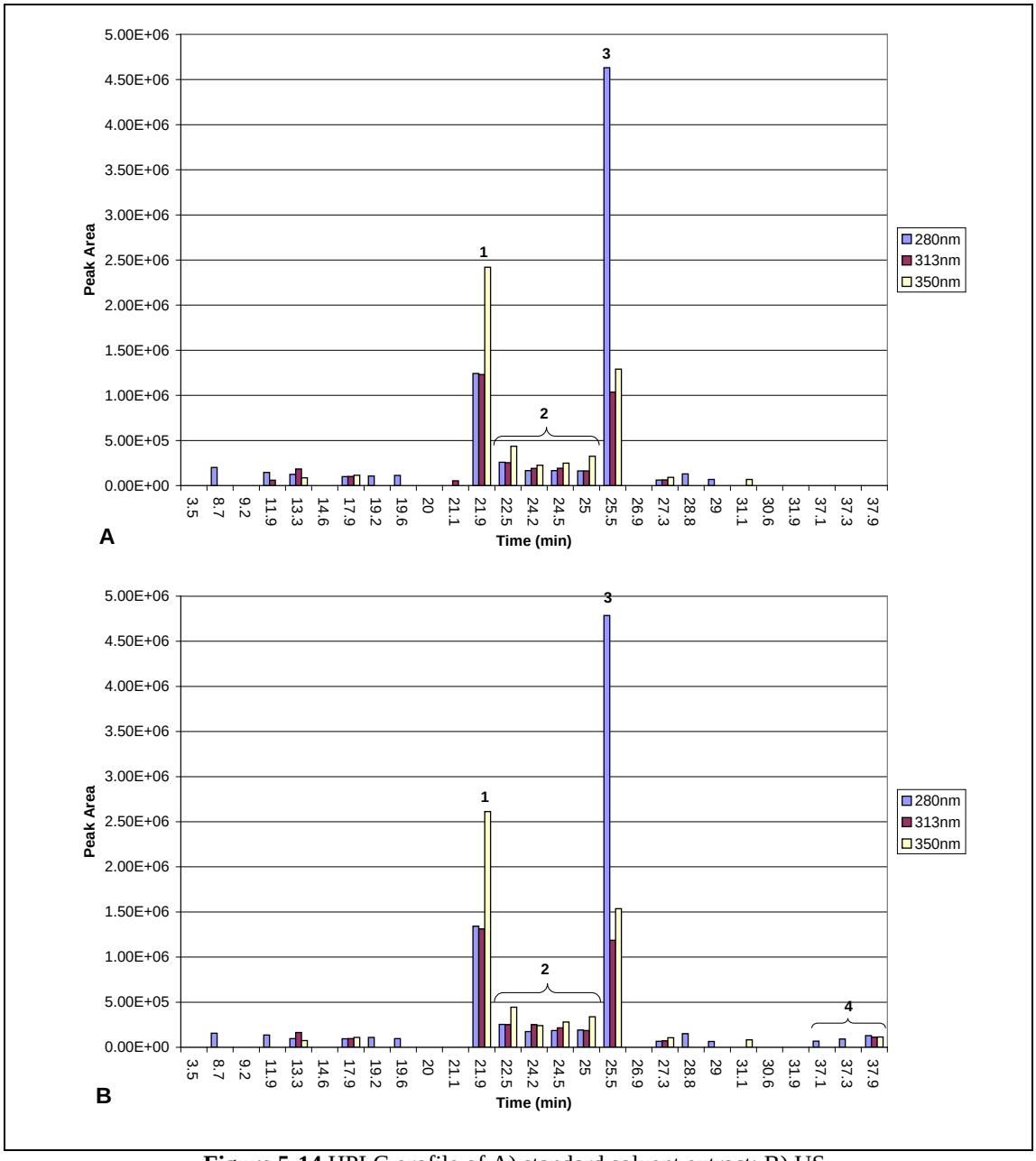


Figure 5-14 HPLC profile of A) standard solvent extract; B) US-assisted extract.

It can be seen that the two HPLC profiles a very similar with all the main peaks of interest present in relative proportions, but there are a couple of inconsistencies that are not present in the previous figure.

Most of the small differences between these two plots can be justified with the high peak tolerances for height and area. It is quite conceivable that if the peak is on the boundary to being or not being significant, relative to set tolerances, then it could appear on one schematic and not the other. So we will not focus on the small fluctuation with the minor peaks; for example the 313 nm wavelength absorbance at RT 11.9 minutes and the peak at RT 21.2 minutes. Peak set 4 is one group of peaks that are of definite interest; they are only present in US-sample profiles and they are of a significant size and location (no previous peaks were found at retention times greater than 30-31 minutes).

So the action of US does modify, to some extent, the natural physio-chemical make-up of the phenols in solution. Are these unidentified compounds degradation products and is their presence important to the bioactivity of the extract sample?

5.3 **Stability of Phenol Species during US Extraction**

It has been shown in literature that under the influence of ultrasound phenol species can be subject mechanisms of degradation; refer to 2.6.3.4. If these deleterious mechanisms are significant, then they must be taken into account when analysing the recovery of phenols. Experiments on two sets of samples were carried out; one on 50 ml of pure oleuropein solution (1 mg/ml) and the other on 50 ml of a complex leaf extract.

The aim of this experiment was to identify any changes in the phenolic yield and profile of a standard olive leaf extract and oleuropein before and after the application of the ultrasound field. The TBP content and the phenolic profile by HPLC were determined for each subject prior to sonication. The sample was then subjected to US for 15 minutes, and then analysed again for discrepancies.

We can see by Figure 5-15 that the TBP content of the leaf sample was effectively unchanged during US processing; after 15 minutes of contact, the sample has not changed in any appreciable way.

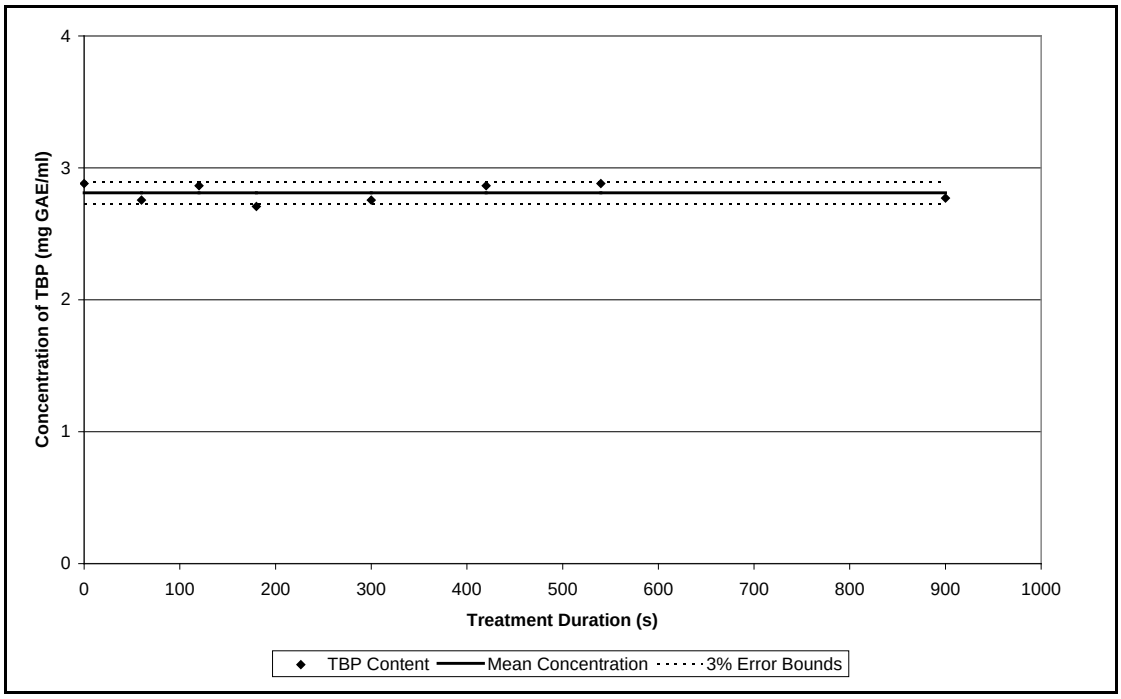


Figure 5-15 Degradation of phenols in a leaf extract during ultrasound treatment.

Likewise, the content of oleuropein before and after US treatment, determined through TBP analysis, remains unchanged within experimental error, as shown in Table 5-1.

Table 5-1 TBP content of 1 mg/ml pure oleuropein, before and after 900s US treatment.

	TBP Content (GAE mg/ml)		
	Before	After	% Loss
Oleuropein	6.077	6.062	0.25

As mentioned previously, the profile of phenolic species is a different story. The conditions allow for what appears to be a small modification to one, or a group, of phenol compounds, bringing into being a new set of compounds represented on Figure 5-14 as peak set 4. The compounds were not identified and are not of a significant quantity, so the effects on bioactivity testing would be limited at best; with any changes covered in Chapter 5: *Bioactivity Testing.*

Oleuropein showed no variation in the HPLC profile before and after US treatment. There were no extra peaks present, suggesting no degradation products, with the

oleuropein peak heights and areas being comparable with an identical RT. This brings me to the conclusion that oleuropein, the most abundant of olive leaf phenols, is not modified during the first 15 minutes of the US-assisted extraction process.

Given this information, we can tentatively conclude that the degradation of phenols in this US system is insignificant under these conditions and that the yields and recoveries presented in the previous section are a true representation of the action of US in the recovery of phenolic compounds.

5.4 **Conclusions**

It is apparent from the results presented above that the application of ultrasound can enhance the recovery of phenol from the solvent extraction of olive leaf. This may be due to two physical effects resulting from the destruction of cellular material:

- The reduction of diffusive resistance to extraction; increasing the rate of extraction.
- Increasing the quantity of phenols made available to extraction; increasing the absolute yield.

The increase in the recovery of TBP (total biophenolic) compounds under the influence of ultrasound was over 15% compared to standard solvent extraction under identical conditions, refer to .

Table 5-2 Summary of yields from extraction protocols after 900s of extraction.

	Yield (mg GAE/g DW)
Standard Solvent Extraction	53
US-Assisted Extraction	62

The biophenolic profile captured by HPLC was very similar in both the ultrasound and standard extracts, with only a few minor variations. There does appear to be a physio-chemical reaction taking place modifying the extract, although in the time frames tested, it does not appear greatly significant.

Phenolic degradation studies show that over the course of extraction under the influence of ultrasound, the phenolic component of the extract remains viable with no reduction in TBP as measured spectrophotometrically. After 15 minutes of operation,

no reduction in TBP content was observable with no observable modifications to the HPLC phenolic profile.

Chapter 6

Biological Assays

6.1 Introduction

The analysis of extract bioactivity is one of the most important indicators of extract quality within the framework of this project. It is within these tests that a potential application for olive waste extracts will be found. Bioactive testing was targeted towards both fungal and bacterial species with a focus on postharvest fruit quality and horticultural diseases. Given this focus, a number of number of target cultures were identified. Examination of literature uncovered a number of different testing methods that were investigated for their applicability.

This chapter is presented as two main sections, the first pertaining to antibacterial testing, and the second pertaining to antifungal testing, with discussions on method development given in each section.

6.1.1 Target Cultures

There are a large number of potential targets for bioactive testing; with only a few fungal species given in Table 2-7, and a plethora of potential bacterial pathogens and spoilage organisms. With such a large number of options available, only a selection of targets could be tested. An initial figure of four fungal cultures and four bacterial cultures is an appropriate starting point for bioactive testing.

The fungal species selected were:

- *Alternaria alternata*, a postharvest rot of the horticulurally important food crop of kiwifruit. Sourced from Landcare Research Ltd, culture #13947.

- *Botrytis cinerea*, a common postharvest rot of citrus species. Sourced from the culture collection of the Institute of Technology and Engineering, Massey University.
- *Eutypa lata*, a fungal disease of the genus *Vitis*, or grape vine. Care of Dr. Peter Long of the Plant Protection Department, Institute of Natural Resources, Massey University.
- *Penicillium digatatum*, another common postharvest rot of citrus species. Sourced from Landcare Research Ltd, culture #10737.

The target bacterial species are as follows:

- *Escherichia coli* NCTC 8196, a Gram-negative and indicator organism. Sourced from the culture collection of the Institute of Technology and Engineering, Massey University.
- *Staphylococcus aureus*, a Gram-positive and indicator organism. Sourced from Mike Sahayam, Institute of Technology and Engineering, Massey University.
- *Bacillus megatarium*, soil-borne Gram-positive bacteria. Sourced from Mike Sahayam, Institute of Technology and Engineering, Massey University.
- *Erwinia amylovora*, Gram-positive bacteria responsible for fire-blight in apples, pears, raspberries and blackberries. Sourced from Landcare Research Ltd, culture #9755.

These cultures gave a good range of bacteria and fungi, phyto-pathogenic and post-harvest organisms, Gram-positive and negative bacteria and food-borne pathogenic indicator organisms.

6.1.2 **Types of Bioassays**

There are a vast number of different protocols for the testing of bioactive substances against fungal and bacterial species. As the two groups of micro-organisms vary substantially in their growth requirements and dynamics, the procedure for bioactive testing will vary accordingly between fungi and bacteria, with the final test protocol given in the respective sections.

The objective of the bioassay should be well defined with the form of response that is required to fulfil the objective also well defined. The question of what form the results must be in has to be asked, and a protocol selected that can achieve this form. The main groups of biological assays can be defined as quantitative, quantal, and direct (Roberts and Boyce, 1972).

Direct assays are probably the simplest testing method with the tested target receiving an increasing dose until some measurable response can be determined. This is also, typically, the least practical method as it is difficult to modify the dosing during testing, especially when using solid media and extended time frames.

Quantal assays deal with an all-or-nothing response as in mortality testing; either the target responds or no response is measured. In this case, the degree of response cannot be deduced from individual subjects, but subjects must be pooled into identical treatments and an observed percentage response determined for each dose step.

Quantitative assays on the other hand have a graded response where each individual subject gives a direct measure of the degree of response. A difficulty with quantitative assays is that one of the extreme values is not well defined, such as maximum growth rate, and therefore requires control groups with a zero dose to get an average maximum growth.

Quantitative assays seem to be most suited to antimicrobial testing as growth measurements as a response can be easily measured, allowing a graded response curve for each test subject or test group to be drawn up with ease.

6.2 **Antibacterial Testing**

6.2.3 **Method Development**

The antibacterial tests can be separated into two different groups of analysis, growth inhibition studies and bactericidal studies. The degree of growth inhibition is defined as the dosing required to completely retard the growth of the bacteria, while bactericidal activity is the dosing required to render the microbe unviable.

6.2.3.1 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of a substance is the minimum concentration required to completely retard the growth of the organism in question. Assays are carried out using micro-titration plates allowing the analysis of multiple extracts or cultures using very little of the extract sample. Growth is determined by visual observation with a cell pellet aggregating at the bottom of the well indicating cell growth; the MIC is defined as the minimum concentration of the sample where no growth occurs. This method does not allow the analysis of kinetic growth effects but offers a quick and efficient determination of the MIC.

The protocol for the determination of MIC in bacterial assays was as follow:

I. The extract or sample to be tested was standardised to a known concentration determined by FRC-method.

II. A single colony from the master cell culture plate was transferred into 4.5ml of Mueller Hinton broth (MHB) and incubated overnight at 30^{o}C. This culture was known as the overnight culture.

III. 100µl of the overnight culture was added to 4.5ml of MHB and incubated for 3 to 4 hours at 30^{o}C.

IV. The absorbance of this culture was measured at 600nm. From this result, the culture was then diluted to an absorbance of 0.1 at 600nm and further diluted 1000 fold. This final culture was the working culture to be used in the assay.

V. 50µl o f buffer solution composed of 2mg/ml of bovine serum albumin and 0.01% acetic acid was placed in each of the micro-titration plate wells through which the sample is diluted, excepting the first.

VI. 100µl of buffer solution is added to the first well of the blank row, while 100µl of the sample to be tested was place in the first well of the sample rows. The rows were then serially diluted through the buffer giving a final volume of 50µl in each well.

VII. 50µl of the working culture was then placed into each of the wells.

VIII. The plate was incubated at 30^{o}C in the complete absence of light for 24hrs.

IX. A visual inspection of plates comparing the presence of growth to the blank; bacterial growth appeared as a pellet of cells at the bottom of the well.

A typical micro-titration plate can be seen in Figure 6-1. After the 24 hour incubation period, the plates are placed over a UV light making the cell pellet much easier to recognise.

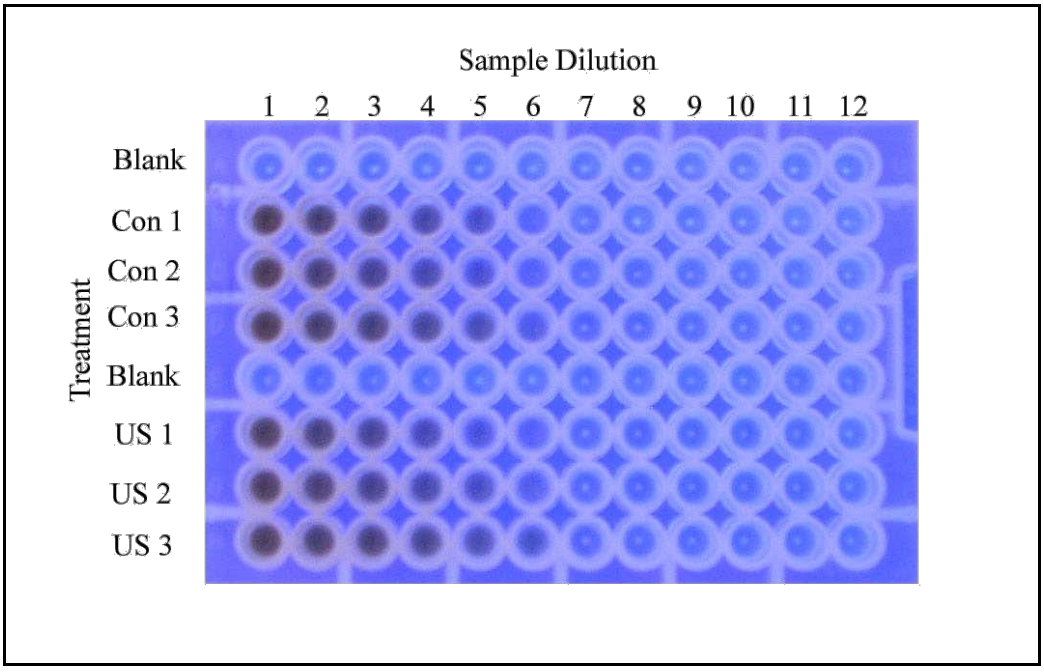


Figure 6-1 Microtitration plate used in MIC testing of bacterial cultures. *Staphylococcus aureus* shown as example.

It is quite obvious, that the lowest dilution in which an observable pellet is present is the 7th column of wells; in this particular case, all wells of the 7th column. This means that the 6th column has a level of TBP's at which the bacterium can not grow, hence this is the MIC.

Due to the fact that the samples are serially diluted through the row of wells by a factor of 2, the final concentration of phenols in each well is given by the relationship:

$$[TBP] = \frac{[TBP]_i}{2^{DF}}$$

Equation 6.1

Where; [TBP] = the concentration of total biophenols in the well, mg/ml (GAE)

$[TBP]_i$ = the initial sample concentration of total biophenols, mg/ml (GAE)

DF = Dilution Factor, the column number of the well with the lowest [TBP] with no growth, referred to as the dilution factor

6.2.3.2 Bactericidal Activity

Bactericidal activity is the measure of the efficacy of an olive extract to reduce the viability of the cells to grow and multiply. This differs from the determination of MIC, because although the results suggest that cell growth may be inhibited, they may still be viable and grow normally if cultured in fresh medium. The measurements of optical density at 600nm (OD_{600}) in this experiment will give an indication of cell viability as cellular destruction will result in a reduction of OD_{600}.

I. The extract or sample to be tested was standardised to a known concentration determined by FRC-method.

II. A single colony of the organism was transferred into 4.5 ml of MHB and incubated overnight at 30ºC to achieve a high cell count.

III. The OD_{600} of the cultures were measured. The cultures are then diluted to an optical density of 0.1 absorbance units. This was the working culture.

IV. 1 ml of a 2 mg/ml BSA, 0.01 % acetic acid buffer was added to four sterilised 10 ml bottles. Into the fifth bottle, 2 ml of the sample extract to be tested was added. In the sixth bottle, 2 ml of buffer was added; this was the control giving the maximum limit of growth.

V. 1 ml of the extract was then taken from the fifth bottle and serially diluted through the buffer in the four bottles containing 1 ml of buffer. These were the treatment bottles.

VI. 0.9 ml of the working culture was added to each of the treatment bottles and the control bottle.

VII. 3 ml of each treatment was measured into 3 separate 3 ml plastic cuvettes, giving three replicates of each treatment. One 3 ml aliquot of the control treatment I placed into an additional cuvette.

VIII. The OD_{600} of the treatments is then measured against a blank containing 1 ml of buffer and 0.9 ml of MHB.

IX. The treatments are incubated at 37 ºC and OD measurements taken every hour.

Due to the high colour content of the olive leaf extracts, control treatments were required to distinguish between the absorbance of the cells in suspension and the

sample extract itself. For the sake of simplicity, a single control using 1 ml of a 1:1 mixture of the two leaf extract sets, the US-assisted extract and the standard extract, diluted with 9 ml of pure Mueller Hinton broth was used. It was assumed that the difference in absorbance and kinetic effects between the two extracts in the control treatments would be minimal. Results can be seen in Figure 6-2.

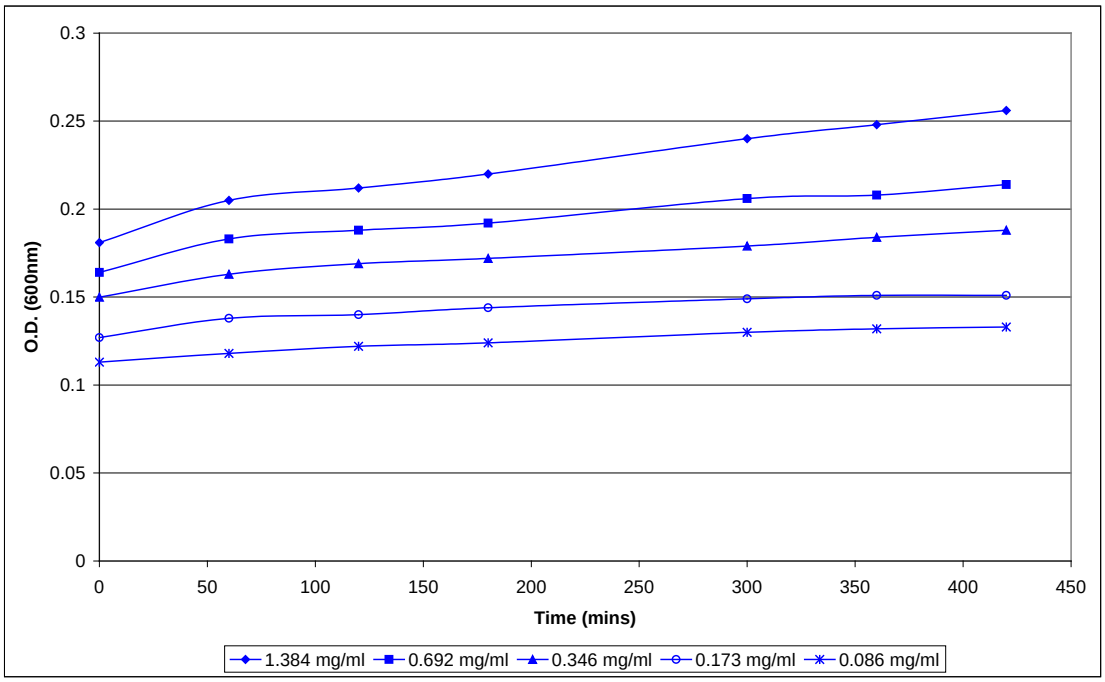


Figure 6-2 Optical density of TBP control samples.

Surprisingly, the control treatments did not maintain a consistent optical density during the trials; after an initial increase over the first 60 minutes, a decrease in the rate of OD_{600} levels to a constant value that is greater at higher concentrations of the TBP content. This is indicative of a reaction taking place in which the phenols are interacting with other compounds in solution, each other, or undergoing chemical modification, resulting in an increase in the OD_{600} of the solution. This increase in OD_{600} from the concentration controls was subtracted from the OD_{600} of the inoculated treatments to obtain an expression of OD_{600} dependent on the variation of cellular density only. This is given as a corrected OD_{600} in the pertinent bactericidal figures.

Using this method, it is expected that the results achieved will give us an understanding of kinetic effects of the leaf extracts on the growth characteristics of the target organisms, with any bactericidal activities being self-evident.

6.2.4 **Analysis of Antibacterial Activity**

The following section outlines the results of the antibacterial testing. The first section outlines results from the minimum inhibitory testing, followed by the results from the bactericidal, or kinetic, testing.

6.2.4.1 Minimum Inhibitory Testing

In the previous chapter it was shown that there are minor modifications to the biophenolic profile of phenols in a sample extracted under an ultrasonic field. Minimum inhibitory testing was undertaken to examine if these slight modifications produce any discernable variations in the bioactivity of the sample. The standard solvent extractions will effectively act as controls to which the US treated samples will be compared.

The minimum inhibitory concentration of the various extracts against the target bacterial cultures is given below in Table 6-1. Each trail was undertaken in triplicate. In these trails, a TBP concentration of 14.33 and 13.34 mg/ml GAE was used for the US and standard extract respectively.

Table 6-1 Minimum inhibitory concentrations of US treated extract and standard solvent extract, average of two different leaf extracts tested in triplicate

	MIC (mg GAE/ml)	
	US Extract	Standard Extract
Erwinia amylovora	0.15	0.15
Bacillus megatarium	2.78	2.99
E. coli	1.67	1.79
S. aureus	0.35	0.35

B. megatarium showed the highest degree of resistance to the olive leaf extract of the bacteria tested, having an MIC of close to 3 mg GAE/ml. A higher resistance compared to other Gram positive bacteria has also been shown in literature for bacteria of the *Bacillus* genus (Markin *et al.*, 2003). Markin *et al.* (2003) showed that an olive leaf extract had a minimal bactericidal activity against *B. subtilis*, while Tassou *et al.* (1991) showed that certain phenolic compounds can act to inhibit the germination of *B. cereus* spores. Rodriguez *et al.* (1988) showed that uncharacterised extracts of olive mill waster waters can have a minimal bactericidal activity against *B. megatarium*, although no specific phenol concentrations are given.

Its higher resistance to the inhibitory effect of olive phenolics is presumed to be attributed to its strong evolutionary relationship with plant compounds, due to its soil environment, and its ability to form spores.

Both *S. aureus* and *E. amylovora* have a relatively low tolerance to the olive BP's, registering MIC's well below 0.5 mg/ml GAE.

It was expected that *E. coli*, being a Gram negative bacteria, would have a higher MIC than the Gram positive bacteria, as this is generally stated in literature when comparing the two structural types (Fleming *et al.*, 1973; Tuncel and Negrez, 1993; Markin *et al.*, 2003). The results showed a higher resistance to BP's than the *S. aureus* and *E. amylovora*, but a lower MIC than *B. megatarium*.

From the results given in Table 6-1, the difference between the US-assisted extracts and the standard extract is minimal. By comparison of the dilution factors (refer to Table 6-2), we can see that the dilution required to breach the MIC is very similar between both sets of samples. This indicates that any difference in MIC between a sample extracted under the influence of ultrasound and one that has undergone standard solvent extraction is smaller than the resolution of this test, i.e. the difference in. MIC between the two sample sets is less than a 2-fold dilution. This stems from the discrete nature of the test results.

Table 6-2 Comparison between dilution factors of US-treated and standard extract required to breach the MIC, average of 2 sets tested in triplicate.

	Dilution Factors	
	US Extract	Standard Extract
Erwinia amylovora	6.56	6.783
Bacillus megatarium	2.33	2.33
E. coli	3.33	3.33
S. aureus	5.33	5.44

6.2.4.2 Bactericidal Testing

Bactericidal tests were undertaken to examine if the olive leaf extract would act to render the cells unviable,= and to get a description of the kinetic behaviour of the cultures in the presence of olive leaf extracts. In this set of experiments OD_{600}, referenced to pure MHB broth, was used as a rough estimate of viable cell count opposed to the labour intensive plate count method.

Two cultures were selected for testing, *E. amylovora* and *S. aureus*, as they showed the lowest measured MIC's and were examples of known plant and human pathogens respectively. Concentration ranges of the US-assisted extract and the standard extract covered the MIC of *E. amylovora* and *S. aureus*, as given in Table 6-3.

Table 6-3 Concentrations used in bactericidal testing.

Treatment Ref.	US (mg/ml)	Con (mg/ml)	Nisin (IU/ml)	Concentration Blank (mg/ml)
A	1.334	1.433	100.00	1.384
B	0.667	0.717	50.00	0.692
C (S. aureus MIC)	0.334	0.358	25.00	0.346
D (E. amylovora MIC)	0.167	0.179	12.50	0.173
E	0.083	0.090	6.25	0.086

In Figure 6-3 and Figure 6-4 we see the results from the positive control where the cultures were contacted with the given concentrations of Nisin. Concentrations of nisin are given in international units per ml.

Figure 6-3 shows the effect of nisin on the kinetic growth rate of *Erwinia amylovora*. From this figure we can conclude that the effect of nisin on the growth kinetics of *E. amylovora* is not strongly dependant on concentration, only giving a small overall reduction in cellular material.

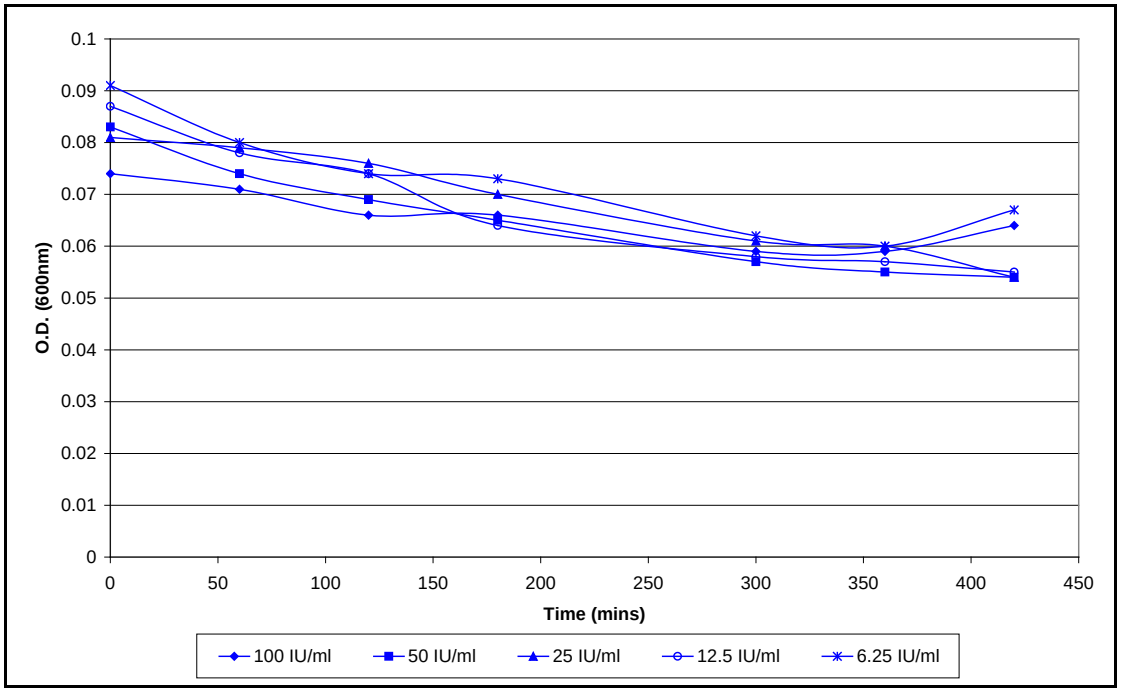


Figure 6-3 Effect of nisin on growth of *E. amylovora*.

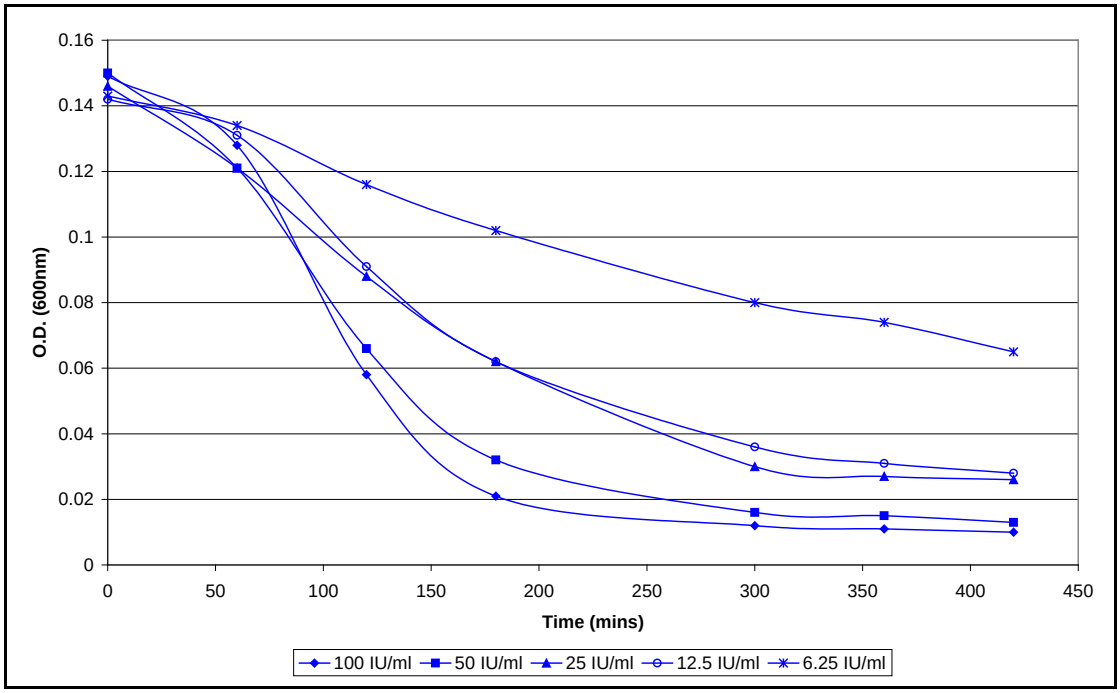


Figure 6-4 Effect of nisin on growth of *S aureus*.

The results from the *Erwinia amylovora* trials, with the standard extract and the US-assisted extract, can be seen in Figure 6-5 and Figure 6-6 respectively.

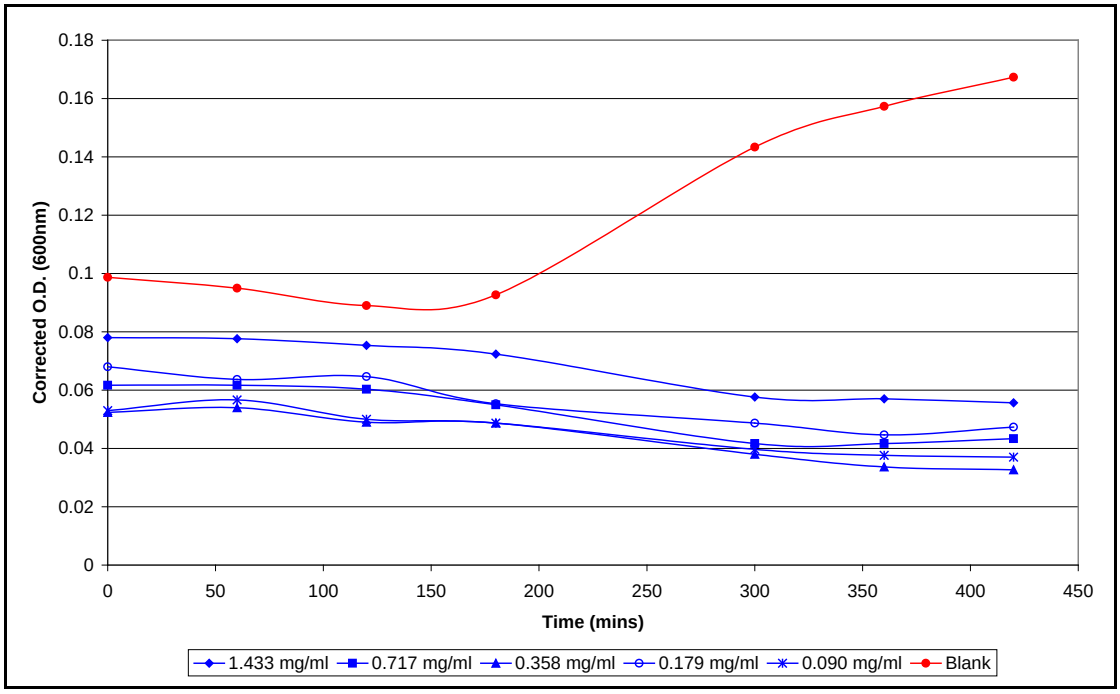


Figure 6-5 Optical density of standard extract with *E. amylovora.*

From the blank in Figure 6-5, we can make out the distinct pattern of lag and then exponential growth typical of inoculation with an overnight culture opposed to a log culture. This confirms that the culture is healthy and the inoculum viable.

It was expected that the high concentration of TBP in the treatments would reduce the optical density of the samples, which is indicative of cellular collapse and destruction. It was also expected that at relatively high concentrations of TBP the reduction in OD_{600} would be considerable compared to the samples with lower concentrations.

We can definitely see that there is a reduction in optical density, but the increase in concentration does not seem to increase cellular destruction to any great extent. The rate of reduction in OD_{600} of the highest TBP concentration is not that much higher than the lowest concentration over the range of time measured.

Figure 6-6 shows the results from the trial incorporating the US-assisted extract. The pattern is very similar to that of the standard extraction, with an initial lag period prior to a slow reduction, and then levelling off after a 60-75% reduction in OD_{600}.

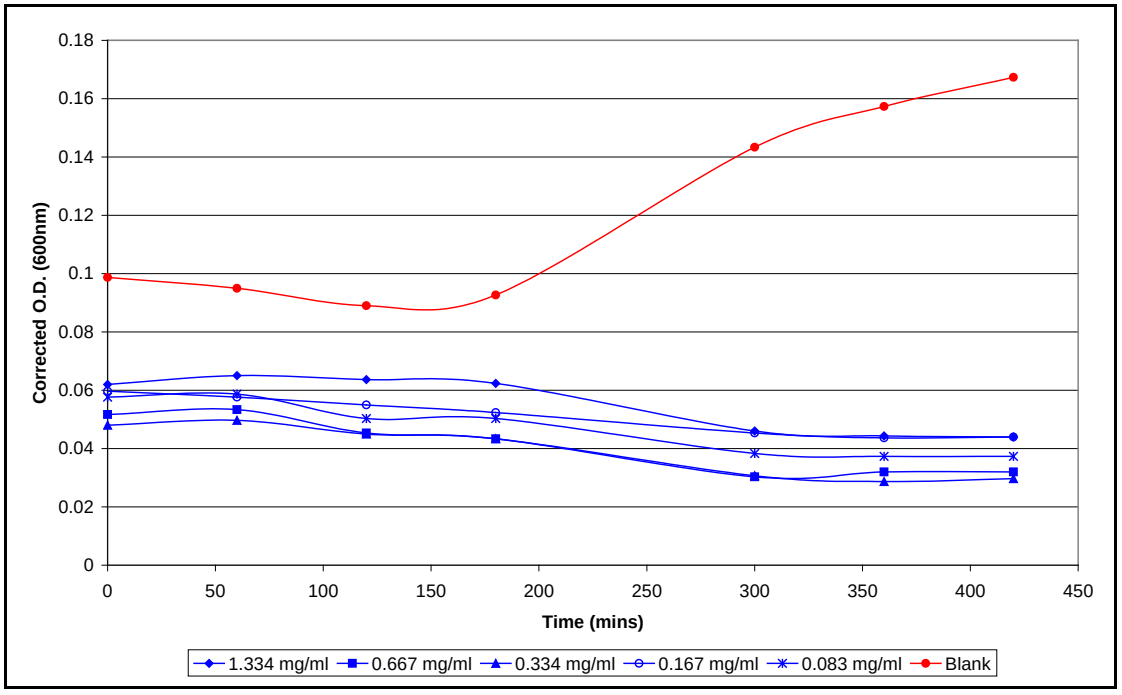


Figure 6-6 Optical density of US-assisted extract with *E. amylovora*.

In terms of the absolute values of OD_{600} with time, the US-assisted extract appears to give a slightly greater reduction in OD_{600}. But relative to the initial starting value of OD_{600}, the difference in the rate of reduction is not significant.

In Figure 6-7 below, we see the fractional reduction in OD_{600} with respect to TBP concentration at four points in time during the trials.

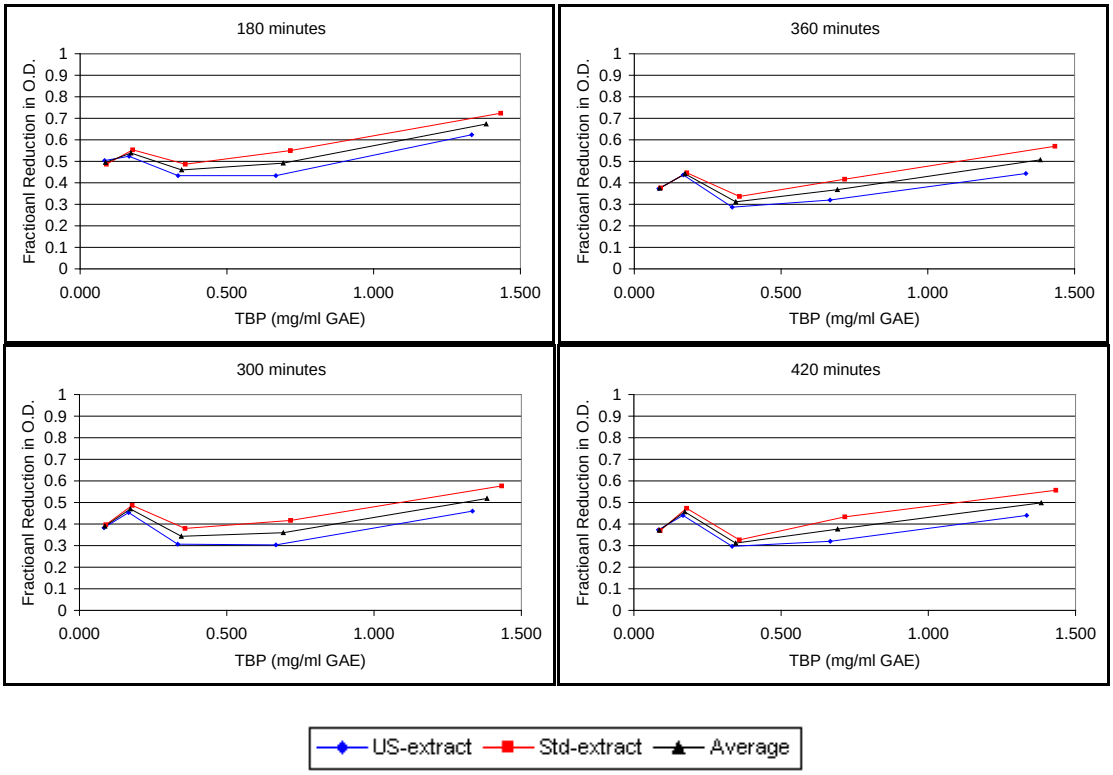


Figure 6-7 Fractional reduction in O.D. by TBP concentration at 180, 300, 360 and 420 minutes for *E. amylovora*.

Again we can see that the US-assisted extract appears to give a slightly greater reduction in OD than the standard extract. Another point of interest from this figure is that at higher concentrations of TBP we see a corresponding increase in OD_{600}. This maybe due to the high nutritional load of the extracts as mentioned previously. We can see that at a concentration of about 0.400 mg/ml, the greatest degree of OD reduction is observed. It is at this point that the bactericidal activities of the BP's and the nutritional components of the crude extract are balanced.

Nisin appeared to have a slight effect on the reduction of OD of an *E. amylovora* culture, but not as great as that seen from the application of olive leaf extract Figure 6-8. It appears that the olive leaf extract has a higher relative bactericidal activity compared to nisin against *E. amylovora*.

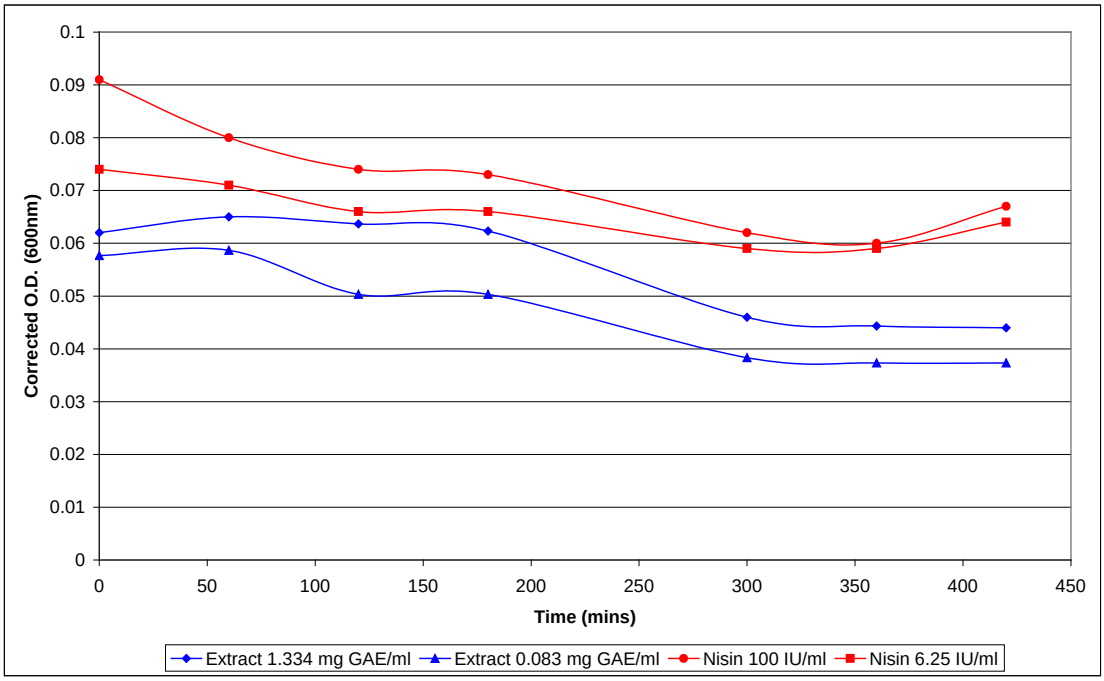


Figure 6-8 Comparison of nisin and leaf extract in rate of cellular collapse of *E. amylovora* measured as OD_{600}.

The results from the *Staphylococcus aureus* trials, with the standard extract and the US-assisted extract, can be seen in Figure 6-9 and Figure 6-10 respectively, with the blank culture shown in red.

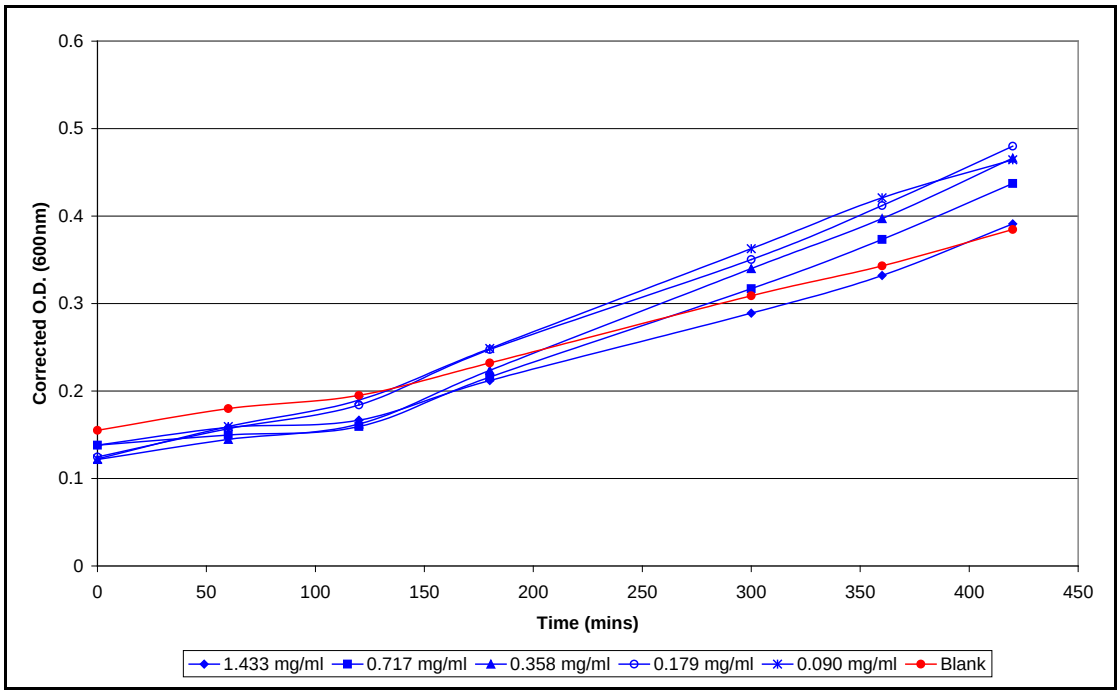


Figure 6-9 Optical density of standard extract with *S aureus.*

The bactericidal effect of the standard olive leaf extracts on the growth of *S. aureus* is non-existent, and the inhibitory effect minimal at best, from the results given in Figure

6-9; the rate of cellular growth actually increases with the addition of the extract, compared to the blank. This is most likely due to the high nutrient load of the crude sample. There are concentration effects that are apparent in the *S. aureus* tests though. It is evident that at higher concentrations, the rate of cellular growth is lower than at lower concentrations. This provides evidence that the biophenol components of the extract are acting to inhibit the growth of *S. aureus*, just not to an extent that is significant in the tested concentration range.

In the MIC testing of *S. aureus*, TBP concentrations in the range of 0.2 to 0.35 mg/ml completely inhibited growth, where in the bactericidal tests *S. aureus* propagated in TBP concentrations exceeding 1 mg/ml, although evidence exists that increasing the TBP concentration reduces the rate of cellular growth. This rather large discrepancy might come from the differences in the initial bacterial inoculum, which plays a very important part in the culture behaviour during testing.

For the MIC tests, the inoculum was in log-phase growth and in 100 times more dilute than the inoculum for the bactericidal tests, which was not in log-phase growth. This much greater cellular count in the bactericidal tests may have resulted in a higher proliferation than cellular disruption ratio, resulting in an overall increase in cell count. At lower initial cell counts, the rate of cell growth may be lower than the rate of cellular collapse, resulting in a decreasing viable cell count, and an observable bactericidal activity.

The US-assisted extract was tested for bactericidal activity on *S. aureus* with the results shown in Figure 6-10. The results are very similar to the results of the standard extract shown above.

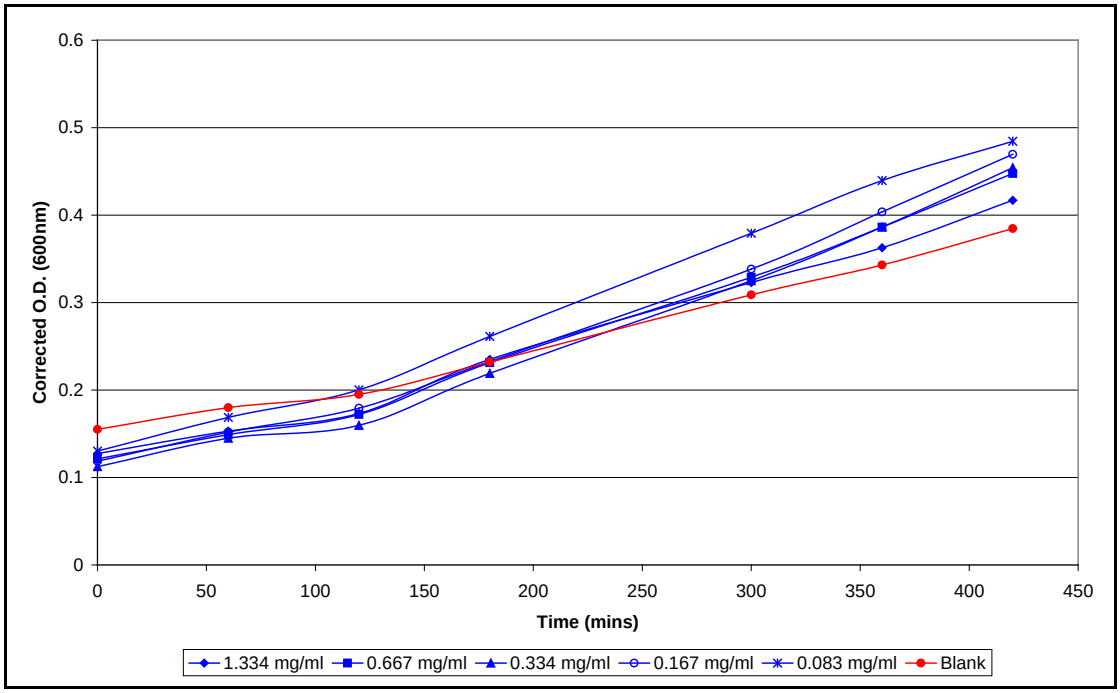


Figure 6-10 Optical density of US-assisted extract with *S aureus.*

Relative to the nisin control, the crude olive leaf extracts tested had no effect on the growth kinetics of *S. aureus*, but to enhance the rate of cellular propagation; which is counter to the aims of this chapter.

The experimental factors that are likely to affect the rate of cellular propagation in these tests, and force an observable increase in cell count in the presence of BP's are as follow:

- High initial cell count in the inoculum resulting in a much greater rate of cell growth.
- High nutrient content of crude extract supplementing growth requirements.

6.3 **Antifungal Testing**

6.3.5 **Method Development**

With the considerable differences in physiology between fungal and bacterial species, it is expected that methods of bioassay would be likewise different. It is not easy to undertake and achieve reproducible results for antifungal activity in liquid medium; so a solid medium is generally incorporated. There are at least two specific ways of conducting a quantitative bioassay on solid media, firstly by mixing the sample

extract with the agar solution and then measuring degree of growth from a specific point, or by placing a sample on an inoculated bed of agar and measuring the zone of inhibition.

With the first method, the degree of response is measured as growth by time from the point of inoculation. A problem with this method stems from the tendency of fungi to have a very narrow range of graded responses, from maximum growth to no growth with increasing dose. This will mean that the range of responses will be very narrow, and possibly out of the range of precision for the test.

The second method has a degree of inhibition as a response, measured from either the middle of the paper disc or well. Problems can arise with this method when attempting to get an even lawn of fungi forming on the surface of the agar, around the points of sample loading. With fungi it may be necessary to produce a spore solution to be added to the agar prior to setting, this maybe difficult depending on the fungi in question. Another issue surrounding this method involves the diffusion of the sample into the surrounding agar giving a graded concentration from the point of loading. Therefore, the inhibition measurement is not a direct measure of the response to a given dose, but a measurement of the response to the graded concentration. One benefit of this method is that narrow dosing range for extreme responses as given in the first method can be overcome.

It is of interest of this work to produce non-arbitrary results that are transparent; the second method given above does not fulfil this requirement as the response is not directly related to dose given the diffusion effects, therefore the first option is considered most suitable.

The testing of fungal strains was achieved using a method allowing the direct measurement of fungal growth inhibition. Growth of the fungal culture from the point of inoculation was measured and compared to a blank allowing the determination of a degree of inhibition relative to concentration of the sample. The method was as follows:

I. The extract or sample to be tested was standardised to a known concentration determined by FRC-method.

II. 2 ml of the sample to be tested was measured into a sterile 10 lm bottle and 1 ml of buffer solution (0.1 % acetic acid, 2 mg/ml BSA) measured into further sterilised bottles. 1 ml of the sample was then serially diluted through all bottles depending on concentration ranges tested.

III. The diluted samples were then made up to 10 ml with Potato Dextrose Agar (PDA) and subsequently poured into petri dishes and allowed to set.

IV. Corks of fungal culture were taken from the edge of a fungal working culture grown on PDA using the top end of a 1 ml pipette tip and carefully placed 15mm from the edge of the petri dish in triplicate and equi-spaced (refer to Figure 6-11) with every caution used to maintain sterility.

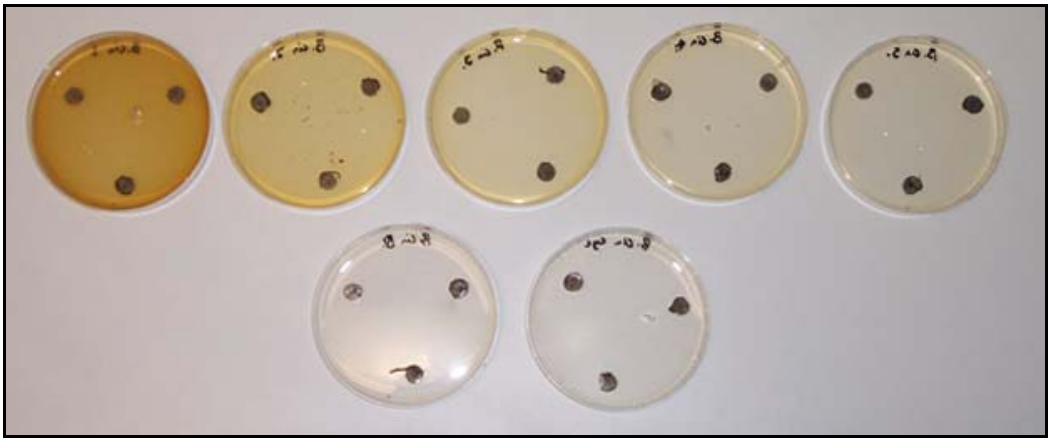


Figure 6-11 Layout of plates for minimum inhibitory concentration antifungal testing.

V. The plates were then incubated at 20-25 ºC in the complete absence of light.

VI. The degree of growth of the culture from its inoculation point was measured every 2 days, using a Mitutoyo digital vernier calipar, and compared to a blank with no sample present.

VII. At the point where fungal growth can no longer grow, due to the growth from the other replicate corks on the plate, the test was concluded and photos taken of the plates.

VIII. The minimum concentration of extract that gives no growth can be defined as the minimum inhibitory concentration (MIC) for that particular fungal strain.

6.3.6 **Analysis of Antifungal Activity**

In this section the results of the antifungal testing of minimum inhibitory concentration will be outlined.

6.3.6.1 *Alternaria alternata*

A. alternata is a relatively slow growing fungal species, with Figure 6-12 showing the extent of growth after 7 days.

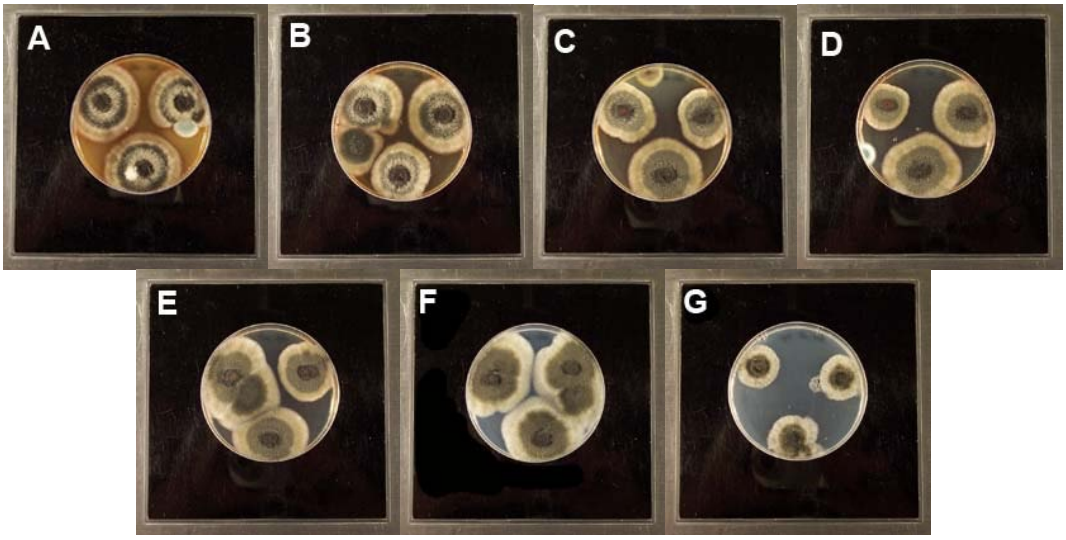


Figure 6-12 The extent of growth of *Alternaria. alternata* in; A 2.500mg/ml; B 1.250 mg/ml; C 0.625 mg/ml; D 0.313 mg/ml; E 0.156 mg/ml; and F 0.0 mg/ml of extract GAE; with G 1.0 mg/ml cycloheximide after 7 days.

The extent of growth of the colonies began with a high degree of consistency, but as the experiment progressed, contaminating spores and irregular colony shapes made it difficult to take consistent measurements over the three plate triplicates.

Given this, it could still be observed that there was a slight reduction in the growth of the fungal colonies in the presence of the olive leaf extract. This observation was more pronounced in the samples with the lower TBP concentrations, as is shown in Table 6-4 below; on days 2, 3 and 5 a distinct pattern on increasing inhibition with decreasing TBP concentration was observed. By the cessation of the experiment on the 7^{th} day, the extent of this inhibitory effect had been reduced significantly to the point where only the lowest TBP concentration shows a significant inhibitory effect.

Table 6-4 Growth and relative growth of *A. alternata* in the presence of different concentrations of olive leaf extract

Growth mm				
Extract TBP mg GAE/ml	Day 2	Day 3	Day 5	Day 7
2.500	2.80	5.60	11.63	18.27
1.250	2.47	4.87	11.80	18.25
0.625	2.03	4.33	12.40	18.30
0.313	1.43	3.40	10.70	18.40
0.156	1.97	4.53	9.15	14.35
Blank	3.33	7.73	15.27	21.60
Cycloheximide (1 mg/ml)	0.00	0.00	5.17	8.57

Growth relative to blank mm/mm				
Extract TBP mg GAE/ml	Day 2	Day 3	Day 5	Day 7
2.500	0.84	0.72	0.76	0.85
1.250	0.74	0.63	0.77	0.84
0.625	0.61	0.56	0.81	0.85
0.313	0.43	0.44	0.70	0.85
0.156	0.59	0.59	0.60	0.66
Blank	1.00	1.00	1.00	1.00
Cycloheximide (1 mg/ml)	0.00	0.00	0.34	0.40

The cause of the suppression of fungal growth at lower concentrations of the leaf extract compared to the higher concentrations is unknown. This maybe attributed to the reducing concentration of extract nutrients present in the treatment, as samples are serially diluted for each treatment, allowing the inhibitory action of the biophenols to become observable.

6.3.6.2 *Botrytis cinerea*

B. cinerea is a very fast growing fungal species with Figure 6-13 showing the extent of fungal growth after only the 3rd day.

The effect of the olive leaf extract on *B. cinerea* was minimal. In most cases it appeared to actually increase the degree of colony growth compared to the blank as can be clearly seen in Table 6-5 below. It could be once again, that the increase in fungal growth can be attributed to the high nutrient content of the olive leaf extract. Whatever the cause, we can be sure that at the concentrations tested the olive leaf biophenols do not adversely affect the growth of *B. cinerea*.

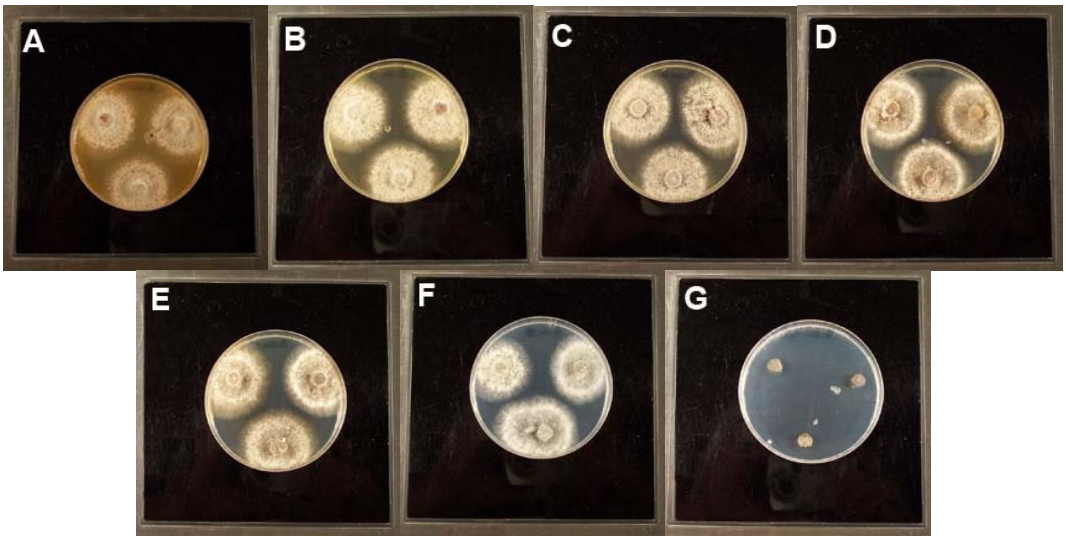


Figure 6-13 The extent of growth of *Botrytis cinerea* in; A 2.500mg/ml; B 1.250 mg/ml; C 0.625 mg/ml; D 0.313 mg/ml; E 0.156 mg/ml; and F 0.0 mg/ml of extract GAE; with G 1.0 mg/ml cycloheximide after 3 days.

Table 6-5 Growth and relative growth of *B. cinerea* in the presence of different concentrations of olive leaf extract

Growth mm		
Extract TBP mg GAE/ml	Day 2	Day 3
2.500	10.87	19.13
1.250	12.13	20.43
0.625	11.37	18.67
0.313	11.83	20.13
0.156	10.50	20.10
Blank	7.97	17.40
Cycloheximide (1 mg/ml)	0.00	0.00

Growth relative to blank mm/mm		
Extract TBP mg GAE/ml	Day 2	Day 3
2.500	1.36	1.10
1.250	1.52	1.17
0.625	1.43	1.07
0.313	1.49	1.16
0.156	1.32	1.16
Blank	1.00	1.00
Cycloheximide (1 mg/ml)	0.00	0.00

6.3.6.3 *Eutypa lata*

A slow growing fungus, it took up to 5 days for the growth of *Eutypa lata* to be accurately gauged across the plate. Figure 6-14 shows the extent of growth on the 7th and final day of the experiment.

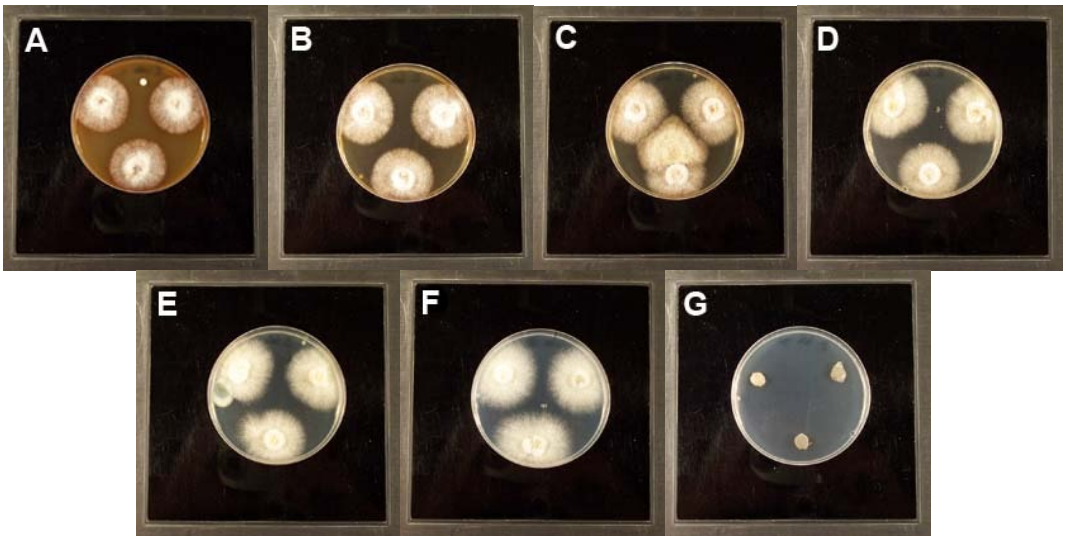


Figure 6-14 The extent of growth of *Eutypa lata* in; A 2.500mg/ml; B 1.250 mg/ml; C 0.625 mg/ml; D 0.313 mg/ml; E 0.156 mg/ml; and F 0.0 mg/ml of extract GAE; with G 1.0 mg/ml cycloheximide after 7 days.

It is apparent from Figure 6-14, comparing frames A and B to frame F that the olive leaf extracts exhibit a slight inhibitory effect on the growth of *Eutypa lata* without completely suppressing it at the TBP concentrations tested. This is highlighted by Table 6-6 below where it can be seen that at Day 7 the extent of growth of *Eutypa lata* has been depressed in the presence of the olive leaf extract.

Table 6-6 Growth and relative growth of *E. lata* in the presence of different concentrations of olive leaf extract

Growth mm			
Extract TBP mg GAE/ml	Day 3	Day 5	Day 7
2.500	0[a]	11.03	14.73
1.250	0[a]	10.47	15.20
0.625	0[a]	10.25	15.40
0.313	0[a]	10.43	15.03
0.156	0[a]	9.57	15.07
Blank	0[a]	10.00	16.80
Cycloheximide (1 mg/ml)	0.00	0.00	0.00

Growth relative to blank mm/mm			
Extract TBP mg GAE/ml	Day 3	Day 5	Day 7
2.500	0.00	1.10	0.88
1.250	0.00	1.05	0.90
0.625	0.00	1.03	0.92
0.313	0.00	1.04	0.89
0.156	0.00	0.96	0.90
Blank	1.00	1.00	1.00
Cycloheximide (1 mg/ml)	0.00	0.00	0.00

[a] Significant growth around the inoculum cork is apparent, but no expansion across the plate.

6.3.6.4 *Penicillium digitatum*

Figure 6-15 below shows the extent of growth of *Penicillium digitatum* after 5 days of growth. Table 6-7 shows the extent of growth at varying concentrations of TBP over the course of the experiment.

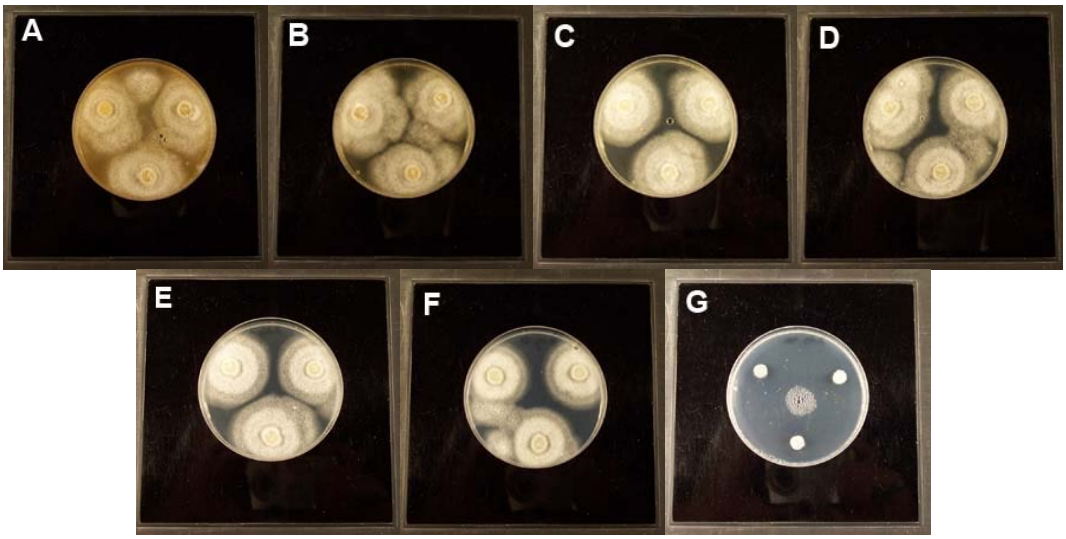


Figure 6-15 The extent of growth of *Penicillium digitatum* in; A 2.500mg/ml; B 1.250 mg/ml; C 0.625 mg/ml; D 0.313 mg/ml; E 0.156 mg/ml; and F 0.0 mg/ml of extract GAE; with G 1.0 mg/ml cycloheximide after 5 days.

Table 6-7 Growth and relative growth of *P. digitatum* in the presence of different concentrations of olive leaf extract

Growth mm			
Extract TBP mg GAE/ml	Day 2	Day 3	Day 5
2.500	2.83	8.73	17.63
1.250	3.40	9.97	18.53
0.625	3.97	10.80	19.47
0.313	4.60	11.10	18.53
0.156	6.17	12.37	19.20
Blank	4.97	10.53	17.27
Cycloheximide (1 mg/ml)	0.00	0.00	0.00

Growth relative to blank mm/mm			
Extract TBP mg GAE/ml	Day 2	Day 3	Day 5
2.500	0.57	0.83	1.02
1.250	0.68	0.95	1.07
0.625	0.80	1.03	1.13
0.313	0.93	1.05	1.07
0.156	1.24	1.17	1.11
Blank	1.00	1.00	1.00
Cycloheximide (1 mg/ml)	0.00	0.00	0.00

The comparison of frames A-E with frame F shows that the growth of *P. digitatum* is not inhibited at all by the olive leaf extract with a greater extent of growth seen in the treatments containing the olive leaf extract. This was not the case over the whole experiment, as can be seen in Table 6-7.

Day 2 shows what appears to be strong inhibitory effects dependant on TBP concentration. It was not until after the 2nd day did the fungal growth appear to 'overcome' inhibitory mechanisms to achieve a growth rate greater than the blank.

6.4 **Conclusions**

Examination of the antibacterial bioassay results allows the following conclusions to be made.

- Olive leaf extracts have the greatest inhibitory affect against *Erwinia amylovora* and *Staphylococcus aureus* with MIC's of 0.15 and 0.20-0.35 mg GAE/ml respectively.
- The difference between samples extracted under an US field and those extracted using standard extraction techniques showed the same MIC's giving the same level of activity.
- *Bacillus megatarium* showed a high resistance to olive leaf extracts compared to the other bacteria assayed.
- Olive leaf extract showed a strong ability to reduce cellular viability of *E. amylovora* at a TBP concentration as low as 0.09 mg/ml GAE.
- *S. aureus* showed the ability to maintain cellular viability up to TBP concentrations of 1.43 mg/ml GAE.

Examination of the antifungal assay results elucidated the following conclusions.

- The behaviour of the tested fungi all exhibited unique growth characteristics in the presence of olive leaf extracts.
- *Alternaria alternata* and *Eutypa lata* both showed a minimal inhibitory effect in presence of olive biophenols after prolonged contact.

- *Penicillium digitatum* showed a strong inhibitory effect in the early stages of testing, but recovered to show minimal inhibition.
- *Botrytis cinerea* showed no evidence of biophenol inhibition.

From these results we can comfortably state that the crude leaf extract tested showed a higher activity against bacteria than fungi, and that the antibacterial affect is stronger against Gram positive than Gram negative bacteria with the exception of *B. megatarium*.

From the whole series of bioassays, it can also be stated that the crude form of extract used in these tests beguiles the true activity that the biophenols present could have on these target organisms. A more concentrated and purified sample may produce significantly more promising results.

Chapter 7

Implementation

7.1 Introduction

In this section, the information gathered in the preceding chapters will be put into an industrial light and the feasibility of a final recovery process for olive leaf biophenols defined.

The ultrasound-assisted solvent extraction of olive leaf is modelled against experimental data and comparisons drawn, allowing examination of the mode of action of US in the enhancement of the extraction process.

7.2 Models for Extraction of Phenols from Olive Leaf

Development of a mathematical model for the extraction of biophenolic compounds from olive leaf will simplify process design for the large scale industrial recovery of the compounds. Of specific interest in the design process is the rate of recovery of the product of interest, change in concentration, which is in turn dependent on the mass transfer diffusion coefficient related by Equation 7.1 (Tzia and Liakdakis, 2003).

$$\frac{\partial C_{BP}}{\partial t} = K_c \Delta C_{BP} \qquad \textbf{Equation 7.1}$$

Where, $\frac{\partial C_{BP}}{\partial t}$ = Rate of extraction of biophenols from olive leaf, mg/g s^{-1}

K_c = global mass transfer coefficient, s^{-1}

ΔC_{BP} = Concentration gradient, or driving force, mg/g

Examination of literature shows very little examination of the extraction kinetics of ultrasound-assisted extraction. Only one study investigating the US-assisted extraction from plant material could be found; the kinetic modelling of substances extracted from sage (Velickovic *et al.*, 2006). The mechanism of extraction was confirmed to occur in two phases; and initial washing phase, and a slow diffusion phase. It was found that this extractive mechanism could be described mathematically by the concepts of unsteady state diffusion, film theory and Ponomaryov empirical equation. In this work, all models were found to effectively describe the system.

What differentiates the modelling of the extraction data in this project opposed to other data sets presented in literature is the characteristic two-stage extraction process where the rate of extraction increases at point A as shown in Figure 7-1 below, after reaching an apparent equilibrium.

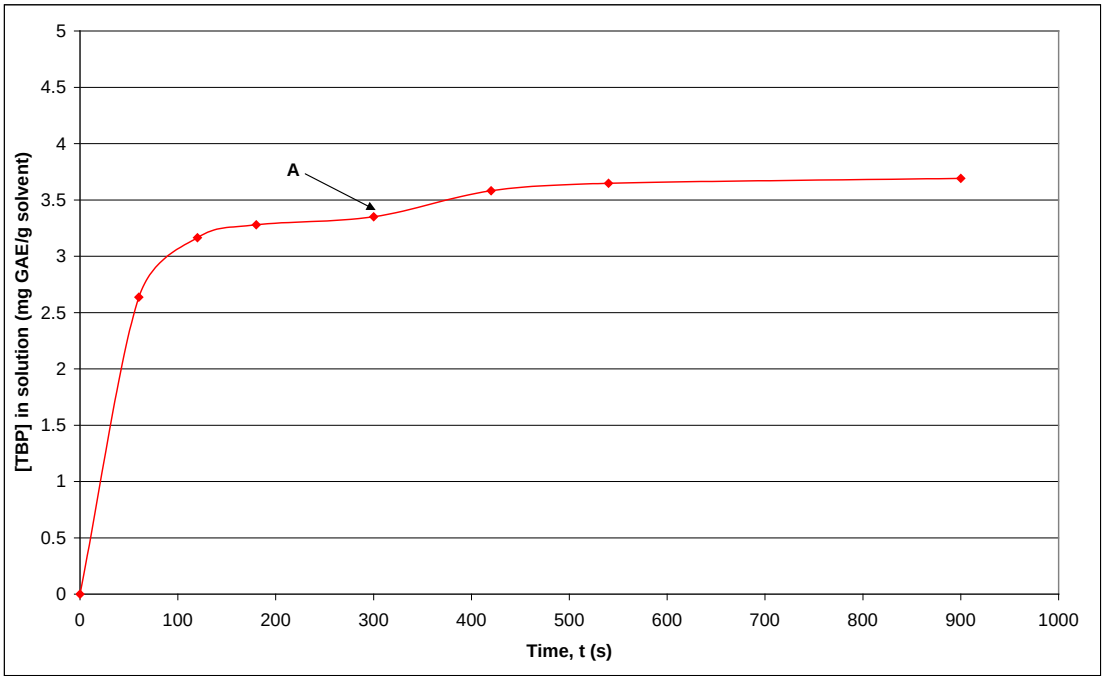


Figure 7-1 Ultrasound-assisted extraction yields of olive leaf.
Characteristics of extraction yields.

The US-assisted solvent extraction of phenols from olive leaf moves towards an initial equilibrium point of 3.1-3.3 mg GAE/g solvent at 200 seconds before a significant increase in phenolic extraction towards a final equilibrium concentration of just over 3.6 mg GAE/g solvent. This has also been shown in literature where the extraction of hyperoside (quercetin-3-o-galactoside) and rutin (both olive flavanoid biophenolics) show the same sharp increase in equilibrium concentration, as shown in Figure 7-2.

One article also shows this characteristic increase in flavanoid glycosides after 3-5 minutes of micro-wave irradiation (Japon-Lujan *et al.*, 2006).

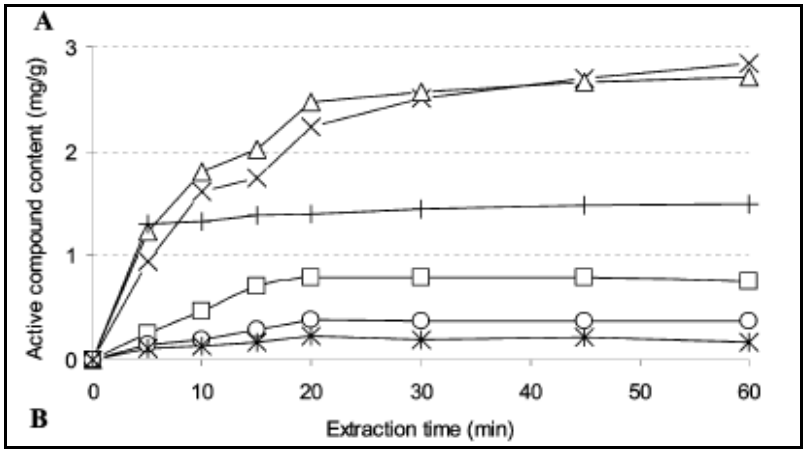


Figure 7-2 Characteristic two-stage extraction of (x) rutin and (Δ) hyperoside (Smelcerovic *et al.*, 2006).

Attempts to model the data set using standard models of 1st and 2nd order rate constants do not fit the data satisfactorily. Attempts to fit the entire data set to the integrated rate functions of unsteady state diffusion, film theory and the Ponomaryov empirical equation, as in Velickovic *et al* (2006), do not achieve straight lines allowing the identification of rate constants.

It is obvious that a number of effects are working in unison to create this distinctive shape of extraction yield versus time.

A model was constructed from mass balances over solvent and olive leaf and from Equation 7.1, giving Equation 7.2. The assumption that all biophenolic compounds present in the leaf material are free to form an equilibrium concentration with the solvent was made.

For the 1st order rate of extraction of phenols from olive leaf:

$$M_S \frac{\partial C_S}{\partial t} = k\left(M_L C_{Seq} - M_S C_S\right) \qquad \textbf{Equation 7.2}$$

Where, $\frac{\partial C_S}{\partial t}$ = Rate of change of concentration of biophenols in the bulk solvent, mg/g s^{-1}

k = Mass transfer coefficient, s^{-1}

M_L = Mass of leaf material, g

M_S = Mass of solvent, g

C_{Seq} = Concentration of biophenols in the bulk solvent at equilibrium, mg/g solvent

C_S = Concentration of biophenols in the bulk solvent at time t, mg/g solvent

Simplifying to:

$$\frac{\partial C_S}{\left(C_{Seq} - C_S\right)} = k\left(\frac{1}{M_S} + \frac{1}{M_L}\right)\partial t \qquad \textbf{Equation 7.3}$$

Integrating:

$$\int_{C_{Si}}^{C_S} \frac{\partial C_S}{C_{Seq} - C_S} = k\left(\frac{1}{M_S} + \frac{1}{M_L}\right)\int_0^t \partial t \qquad \textbf{Equation 7.4}$$

$$-\ln\left(\frac{C_{Seq} - C_S}{C_{Seq} - C_{Si}}\right) = -k\left(\frac{1}{M_S} + \frac{1}{M_L}\right)t \qquad \textbf{Equation 7.5}$$

Where, C_{Si} = Concentration of biophenols in the bulk solvent at time 0, mg/g solvent

Plotting $-\ln\left(\frac{C_{Seq} - C_S}{C_{Seq} - C_{Si}}\right)$ versus time achieves a straight line with the 1st order rate constant of $-k\left(\frac{1}{M_S} + \frac{1}{M_L}\right)$ being the slope, K.

For a second order equation, Equation 7.4 becomes:

$$\int_{C_{Si}}^{C_S} \frac{\partial C_S}{\left(C_{Seq} - C_S\right)^2} = k\left(\frac{1}{M_S} + \frac{1}{M_L}\right)^2 \int_0^t \partial t \qquad \textbf{Equation 7.6}$$

Integrating:

$$\left(\frac{C_{Seq} - C_{Si}}{C_{Seq} - C_S}\right) = -k\left(\frac{1}{M_S} + \frac{1}{M_L}\right)^2 t \qquad \textbf{Equation 7.7}$$

Plotting $\left(\frac{C_{Seq}-C_{Si}}{C_{Seq}-C_S}\right)$ versus time achieves a straight line with the 2nd order rate constant of $-k\left(\frac{1}{M_S}+\frac{1}{M_L}\right)^2$ being the slope, K.

Manipulation of the data to attempt to extract a rate constant was carried out, with examination of the data using 1st and 2nd order rate kinetics over different time intervals of the extraction.

Figure 7-3 shows that linear relationships could be extracted from the US-assisted solvent extraction data using both 1st and 2nd order kinetics over different times.

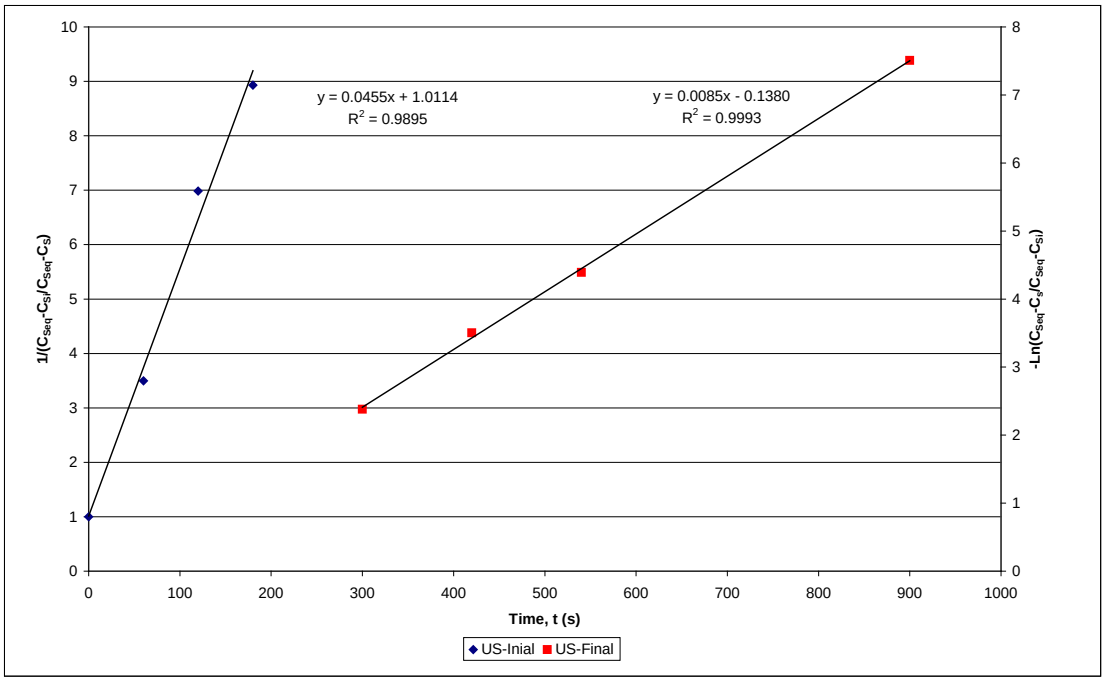


Figure 7-3 Linearised plot for identification of extraction rate constants for US-assisted solvent extraction.

Figure 7-4 shows that linear relationships could be extracted from the control extraction data using different order kinetics over different times.

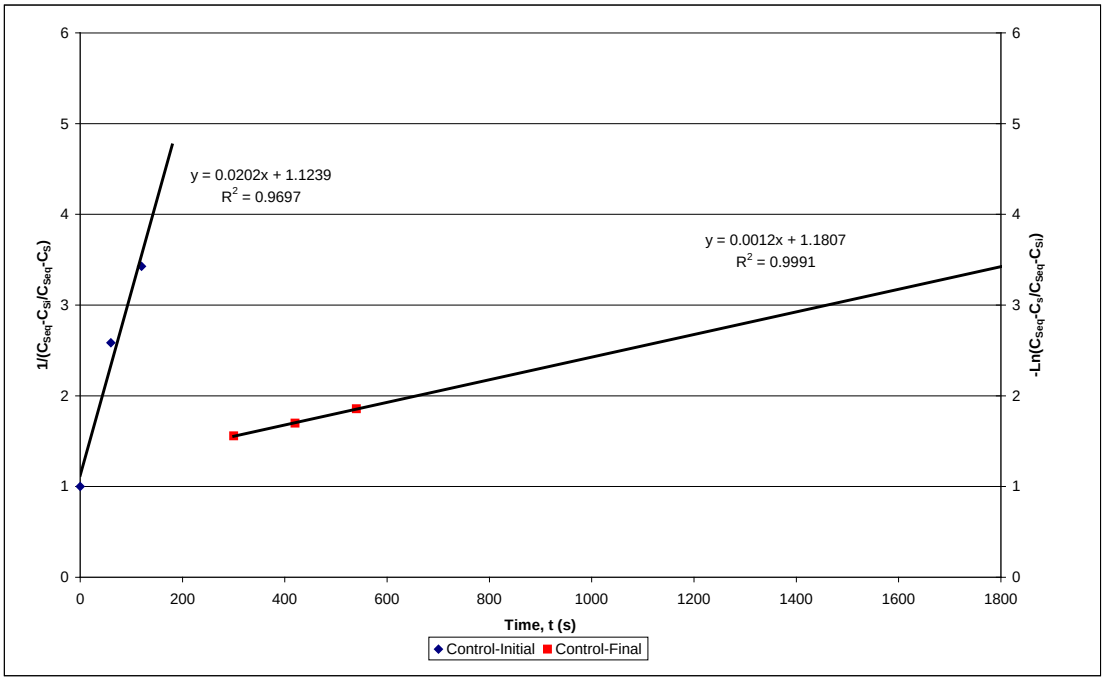


Figure 7-4 Linearised plot for identification of extraction rate constants for control solvent extraction.

From Figure 7-3 we can see that for the first 300 seconds of extraction the rate of extraction follows 2nd order kinetics and from 300 seconds onwards the rate of phenolic extraction follows 1st order kinetics. It can then be suggested that the first phase of extraction the mechanisms are a combination of two 1st order rate equations (defined as K_{total}), one relating to the washing of phenolics from the proportion freely available to the bulk solvent (defined as K_W) and the other relating to the slow diffusion of phenolics from the plant matrix (defined as K_{SD}), with the second phase of extraction relating solely to 1st order slow diffusion.

In other words, during the first phase of extraction, the washing of TBP's from the plant matrix/solvent interface is the rate limiting factor, in the second phase of extraction the slow diffusion of TBP's from the plant matrix to the plant matrix/solvent interface is the rate limiting factor; where the initial free total biophenol content determines the contribution of the washing phase to the overall rate of phenolic extraction.

Given this, K_W and K_{SD} can be related by:

$$K_{Total} = K_W + K_{SD} \qquad \textbf{Equation 7.8}$$

From Figure 7-3, under the influence of ultrasound, K_{Total} over the first 300 seconds of extraction is 0.0455 s^{-1}. K_{SD} is the rate from 300 seconds onwards, assuming K_W is too large to be significant, and is shown to be 0.0085 s^{-1}. From Equation 7.8, K_W is calculated to be 0.0370 s^{-1}. Table 7-1 shows the final values for the rate constants for both standard solvent extraction and US-assisted solvent extraction.

Table 7-1 Rate constants for US-assisted extraction and standard solvent extraction.

	Rate Constants		
	K_{Total}	K_W	K_{SD}
US-assisted	0.0455	0.0370	0.0085
Standard Solvent	0.0202	0.0190	0.0012

The rate constants under the influence of ultrasound are far greater than the rate constants under standard solvent extraction. The high rate constants for slow diffusion were expected for the US-assisted system as the destruction of plant cell material would greatly reduce the resistances to mass transfer.

It was expected that given the high degree of mixing in both US-assisted extraction and standard solvent extraction, that the rate constant of washing, K_W, would be quite similar. This is not the case; it may be presumed that under the influence of US, the degree of contact between the solid plant matrix and the bulk solvent is greater than in a well-stirred reactor. This is not difficult to imagine given the high amount of energy dissipated in the system in addition to the mixing found in the standard solvent extraction. It is also proposed in literature that sonication may also increase the rate of dissolution of solutes, which could also produce the observed effect (Thompson and Doraiswamy, 1999).

We have now defined the extraction process to occur in two 1^{st} order phases, the washing phase reaches a limit defined by the initial free content of biophenols in the system and the slow diffusion phase reaches a limit based on the total phenolic fraction available for extraction. As the process of slow diffusion progresses, the free content of biophenols limiting the washing phase increases.

Given these assumptions, the TBP concentration in solution can then be modelled from Equation 7.9.

$$C_S = \left(1 - e^{(-K_W t)}\right)\left(C_{FA} + \left(1 - e^{(-K_{SD} t)}\right)C_{NA}\right)$$ **Equation 7.9**

Where, K_W = rate constant of washing of free solutes, s^{-1}

K_{SD} = rate constant of slow diffusion, s^{-1}

C_{FA} = concentration of phenols freely available to be washed from the surface of the plant matrix, mg/g

C_{NA} = concentration of phenols only available for slow diffusion from plant material, mg/g

Where C_{NA} and C_{FA} are related by the following:

$$C_{Seq} = C_{NA} + C_{FA}$$ **Equation 7.10**

Where, C_{Seq} = final equilibrium concentration of phenols in the system under the influence of ultrasound, mg/g

The incorporation of Equation 7.9 requires the estimation of an initial quantity of freely available phenols (C_{FA}). This can be estimated from the second phase plot of Figure 7-4, where the intercept of the slow diffusion line gives the initial phenol content.

$$-\ln\left(\frac{C_{Seq} - C_S}{C_{Seq} - C_{Si}}\right) = 1.1807$$ **Equation 7.11**

Solving Equation 7.11 for C_{Si}, an initial free phenolic content, C_{FA}, of 2.56 mg/g was found. The model given in Equation 7.9 was then compared to experimental data achieving results shown in Figure 7-5.

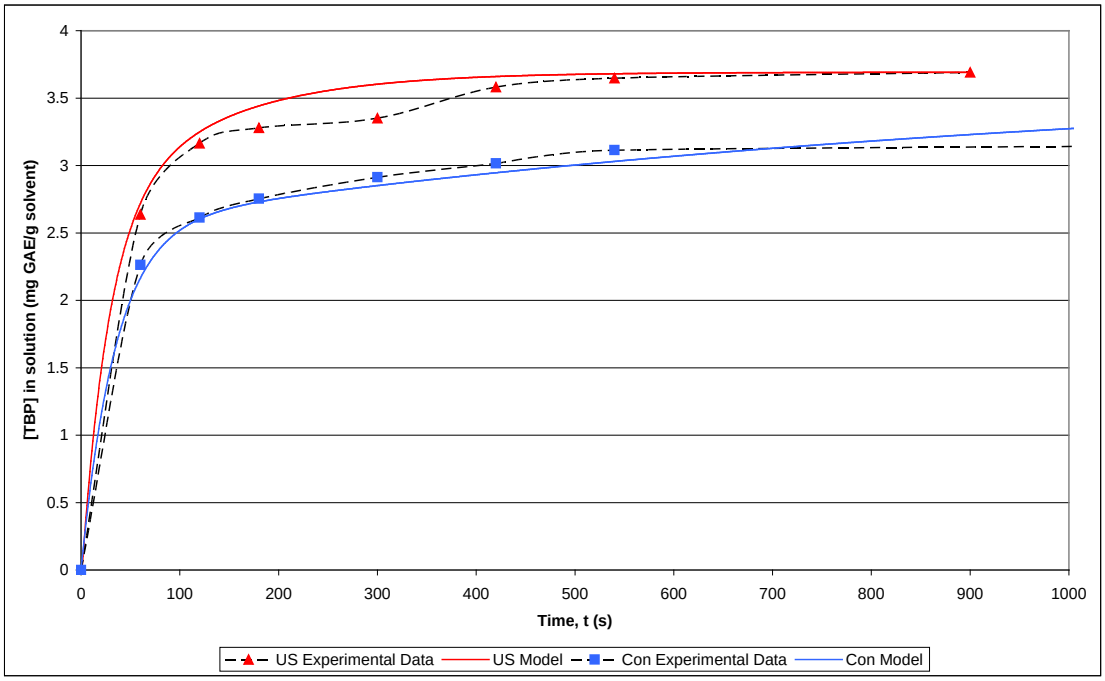


Figure 7-5 Comparison of extraction models with extraction data.

The model fits the initial increase and final equilibrium concentrations of the experimental US-assisted extraction data, but fails to explain the characteristic 'bump'. The model approximately fits the control extraction data initially, but greatly overestimates the increase of TBP concentration during the slow diffusion phase of phenolic extraction.

Attempts to model the unique characteristics of the US-assisted extraction of biophenols form olive leaf were not entirely successful. The increase in equilibrium conditions at 300 seconds may be the result of a lag period in which the biophenols are made available when they were previously unavailable, as opposed to a slow increase in equilibrium as modelled here. This could be explained by the release of bound biophenols that can only be achieved given a certain degree of cellular destruction, after a certain period of ultrasound treatment. This observation may also be a result of the setup of the apparatus. Without further experimentation, the specific cause can not be identified.

What is certain is that there are unique characteristics in the US-assisted solvent extraction of biophenolic compounds from olive leaf when compared to standard solvent extraction, with much promise for further study.

7.3 **Process Design**

The process design for the lab-scale recovery of a product of interest is different to the process design for large-scale recovery of the same product in terms of both objectives of the design process and the feasibility of certain design elements.

In lab-scale design, the main objectives are generally focussed towards identifying product activities and efficacy, ensuring the product is not modified during the recovery process, and testing limits of its physio-chemical stability. In the large-scale design process, the objective is taking those elements learnt in the lab-scale recovery, ensuring a high yield of the product of interest, while simultaneously increasing the process output.

In this project the main objectives were to compare US-assisted solvent extraction with standard solvent extraction in terms of yield and to examine potential antimicrobial activities of said extracts. It was found that the action of ultrasound during the extraction of olive leaf enhances the recovery of biophenols whilst not adversely effecting bioactivity. An increase in yield of 15% was achieved over the first 15 minutes of extraction compared to the control extraction, with the system coming to a chemical equilibrium at a much faster rate.

On a laboratory scale, US-assisted extraction shows promise for enhancing the recovery of olive biophenols, but can this technology be used at an industrial scale? Thmpson and Doraiswamy (1999) discuss a number of scale-up considerations and the importance clearly defining the role of ultrasound in the enhancement of the process. If the enhancement is entirely physical and dependant solely on the destruction of cellular material, then the solids may be treated prior to extraction, allowing a substantial reduction in treated volume and therefore energy inputs.

The experiments undertaken in this work did not provide conclusive evidence that the enhancement in extraction is entirely physical; the hydrolysis of bound phenolic entities may also play a crucial role in the process.

There is also evidence that the action of ultrasound increases the rate of mass transfer in the system, as can be seen in Table 7-1 where the rate constants for the initial washing phase of extraction is substantially greater for US-assisted solvent extraction

than for standard solvent extraction. Given this, sonication would be required over the entire course of extraction process to maintain the enhancement effects.

There are a number of different types of batch and continuous-flow reactors to examine. Two of which are reactors with imbedded transducers, as seen in Berger's sonochemical reactor (Figure 7-6), or reactors with an external flow loop, as in the Harwell sonochemical reactor (Figure 7-7). For investigation of this application, only these two reactors were considered.

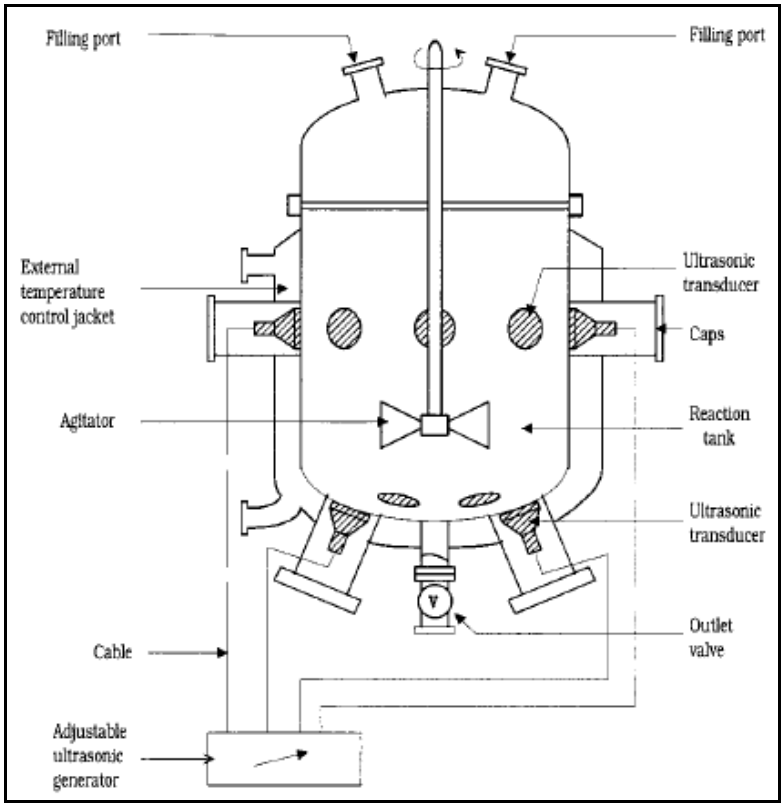


Figure 7-6 Sonochemical stirred tank reactor (Berger *et al.*, 1996).

The Berger reactor was designed to overcome problems of efficiency and reproducibility associated with ultrasonic baths and the propensity for probe-type systems to deliver ultrasonic energy to only a small band of liquid. It has a mechanical agitator, external jacket, and ports allowing for batch, semi-batch and continuous operation. It has 6-8 ultrasonic transducers built into the wall and 3-5 transducers built into the bottom of the vessel.

The Harwell-type external loop reactor vessel has a number of advantages. Including well characterised sound fields and operation mechanisms given the small volume of treated material in the external loop and clear control of residence time within the

sonochemical area. Its modular design also allows for easy maintenance. One rather large deficiency in the system is the enhanced action resulting from the sonochemical treatment may be short-lived, especially if the enhancement mechanism is chemical in nature, such as radical species formation.

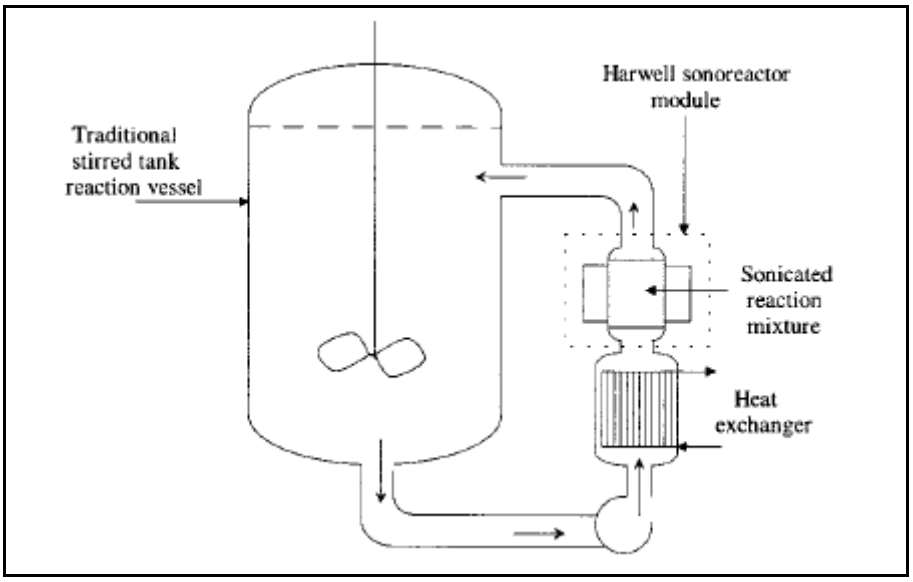


Figure 7-7 The Harwell sonochemical reactor (Thompson and Doraiswamy, 1999)

In respect to this project, we cannot be sure of the contributions of purely physical and physio-chemical mechanisms to this extraction enhancement. If we were certain that the resulting increase in phenolic extraction from olive leaf was entirely physical in nature, with the destruction of cellular material providing access for the solvent to dissolve previously unavailable phenolic species, then an external loop system might be an option. However, given that the mechanics of ultrasonic enhancement of extraction cannot be well defined, constant application of ultrasonic energy to the system is recommended. Coupled with the flexibility of the Berger reactor, a final design akin to this system would be ideal.

No information was found in literature outlining a specific process for the scale up of ultrasonic reactors. There were, however, a number of articles explaining the complexity of the problem (Thompson and Doraiswamy, 1999; Gogate *et al.*, 2001; Gogate and Pandit, 2004). Gogate and Padnit *et al.* (2004) outline a number of deficiencies in theoretical models for examination of cavitional events based on operating and geometric properties and a lack of design strategies linking theoretical models to experimental data. This is exasperated by the reliance on lab-scale data and

information resulting in very large scale up ratios, and therefore, very high uncertainties.

Given the difficult nature of sonochemical reactor scaling, an examination of the pilot plant scale recovery of biophenolic compounds from olive leaf using a Berger reactor was undertaken. A rough extraction/purification protocol is given in Figure 7-8.

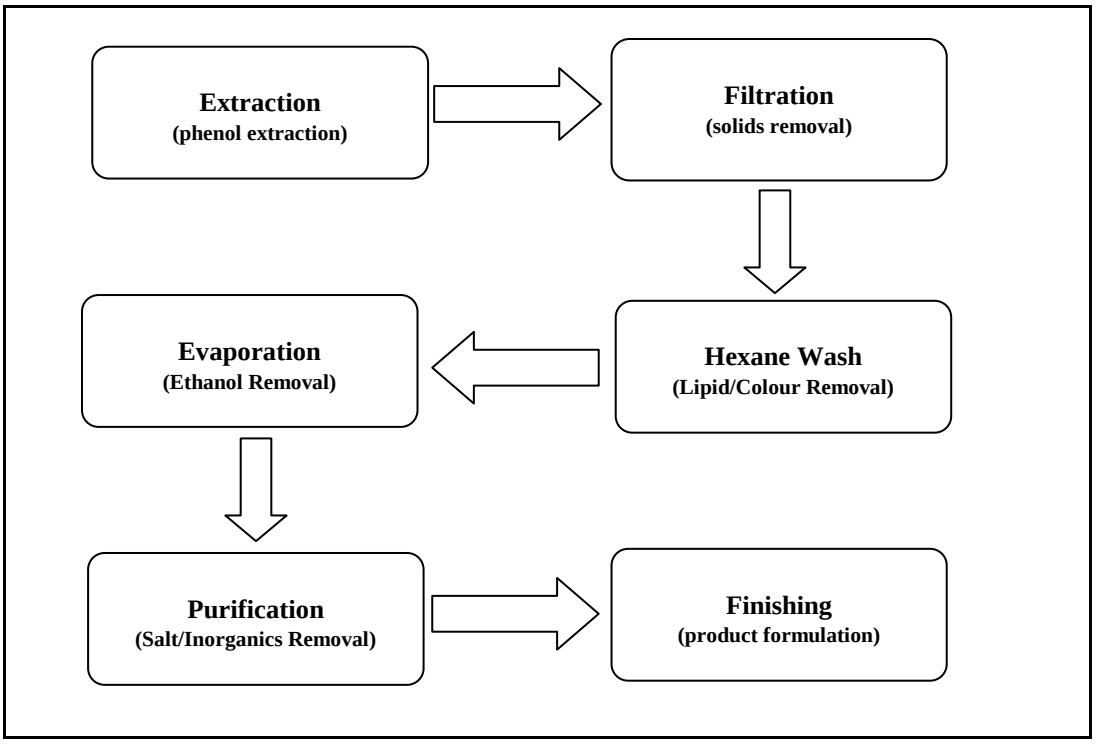


Figure 7-8 Extraction/purification protocol for the recovery of olive leaf biophenolics.

The following assumptions are made:

- The reactor will operate in batch mode.
- Heat loss to the extraction vessel and atmosphere are negligible.
- Each transducer on the Berger reactor contributes equally to the total power input into the system; 10 in total.
- The efficiency of the Berger reactor shall be comparable to that of the lab-scale apparatus with equal distribution of energy dissipation within the reactor and no wave cancellation and standing wave effects.
- A final product concentration of 1 mg GAE/ml will provide a viable product.

- The yield of biophenolic compounds after 900 s of US-assisted extraction is 63 mg GAE/g DW of leaf material.

- Scale-up effects in the ultrasound system are negligible.

The initial inputs into the extraction vessel are:

- Solvent mixture; 75 l ethyl alcohol and 25 l deionised water.
- 5 kg pre-treated olive leaf.

Given the same required power input on a mass basis as the experimental runs at 16 W and 45 g total mass (42.5 g solvent and 2.5 g of leaf material), we can estimate the power input required in the large-scale operation using Equation 7.12.

$$\left(\frac{P_{diss}}{m}\right)_{LabScale} = \left(\frac{P_{diss}}{m}\right)_{IndustryScale} \qquad \textbf{Equation 7.12}$$

If Equation 7.12 remains true, then we can expect a total P_{diss} requirement of 35.5 kW. With the above assumptions, each transducer can be expected to provide 3-4 kW of power. This is not out of the realms of possibility, but the sourcing of US-probes in with a power in the range of kW is very difficult. As of 1997, the largest commercially available transducer was 2.5 kW that functioned at 60% efficiency. When dealing with such high power outputs of transducers, power efficiency becomes a real issue. The huge power output at the tip of the probe will likely create excessive bubble shrouding of the tip which in turn reduces the effectiveness of the probe.

Given an initial input of dry leaf material of 5 kg and a final biophenolic yield of 62 mg/g DW, we can expect the final yield from the extraction process to be 310 x 10^3 mg.

For the chlorophyll/lipid extraction set, an equal volume of hexane is required to wash the extract solution. The Berger reactor may also be used in this phase of recovery, with the degree of contact between the hexane and extract solution greatly enhanced, possibly allowing for a reduction in input volumes. Even a possible reduction in solvent volumes, the total volume of hexane and the extract solution will be greater than the volume of the Berger reactor. Hence a secondary holding tank will be

required, with the solution being recycled between the two. 100 % recovery of the biophenols is expected from this step.

The hexane can then be removed by firstly mechanical means, and then by evaporation to ensure complete removal. The hexane can then be recycled for use in following batches.

The ethanol component of the extract solution is likely to need removal as well. This can be achieved using a number of different evaporation techniques. Large-scale rotary evaporators are available on the market and finding one to handle volumes of 100 l should not be difficult. This will ensure that excessive heat is not put into the product which may cause degradation. The ethanol can then be recovered for future use.

It is the belief of the author that a single chromatographic step can be used to purify the extract solution by a) further concentrating the product, and b) removing any contaminants that are present in solution i.e., salts and organic contaminants. This would most likely involve the binding of the biophenolic component to a chromatographic resin, and washing away the remaining solution, allowing the elution of the biophenolic fraction with great ease.

Given a relatively pure biophenolic solution, the final finishing step would simply require dilution of the product to the desired concentration, and the addition of any adjuvant or preservative compounds.

A final required biophenolic concentration of 1 mg GAE/ml has been stated. Given a 100% recovery during the finishing processes, we can expect a final product yield of 310 litres from 5 kg of leaf material, or 62 litres per kg of leaf material.

With the complete internal recycle of hexane and ethanol, and minimal chemical inputs for the chromatographic purification of the extract solution, and the fact that the initial raw material is a waste product, the greatest operating cost in the recovery of biophenolic compounds from olive leaf would be the energy requirements for evaporation and the ultrasound probes.

7.4 **Feasibility of Application**

With a rough breakdown of a potential recovery process, it can be seen that the recovery of a biophenolic fraction from the extraction of olive leaf can be a relatively cost effective exercise.

The viability of the recovery process is then dependant on the identification of an appropriate application for the product. The biophenolic fraction of olive leaf does show an antimicrobial activity, whether this can be exploited as a potential target for the application of this product requires expansive screening of wide varieties of microbial pests and pathogens.

The biophenolic fraction of olive leaf does not show any antifungal activity towards the fungi tested in this project. As a result, the application in the post harvest protection of fruits and vegetables looks limited; although the examination of specific phytopathngeic bacterial disease may elucidate a potential and specific role for the extract.

As it stands, with the current knowledge of the bioactivity of this olive leaf extract, the large scale recovery of the product does not look feasible. More work on identifying potential applications is required. On the other hand, the recovery of the biophenolic fraction from olive leaf looks like a potentially cost effective process.

7.5 **Conclusions**

A model was developed on the basis of 1^{st} and 2^{nd} order rate kinetics based on simple mass transfer. This model showed a good agreement with the experimental data in parts, but does not explain some interesting characteristics of the ultrasound-assisted extraction of biophenols from olive leaf. Further experimental investigation will help define the exact role that US plays in the enhancement of the extraction of biophenols from olive leaf, whether it be a purely mechanical process of cellular disruption, or if US plays a part in actively freeing biophenols from bound positions where they are unable to be extracted into the bulk solution.

It appears, from rough process design, that the recovery of biophenols from olive leaf could be a very cost effective process. Given that the initial raw material is a waste product and that the majority of the processing aids can be recycled and reused from batch to batch, the cost of operation would be minimal allowing the quick recovery of capital investment.

Further work is required to identify a single step purification process that will allow the complete removal of any contaminants.

Assuming a final, effective, product concentration of 1 mg GAE/ml, and complete phenolic recovery during processing, 62 litres of product can be recovered from 1 kg of olive leaf.

The one aspect for which the economic recovery of a product from olive leaf extracts is clearly dependant on is the identification of an appropriate application. This project has failed to make a clear identification of a target, although there is evidence that a solution of less than 1 mg GAE/ml will show an antimicrobial activity towards certain bacterial species.

Further work is required to identify a clear broad or highly specific activity to which this olive leaf extract could be applied.

Chapter 8

Conclusions and Recommendations

The extraction of olive waste products for the use as a novel product has seen mixed results.

A rigorous extraction and purification and testing protocol was developed. This allowed the recovery of extracts of a consistent quality determined through reproducible and accurate analytical techniques. Accuracy for the determination of TBP using the Folin-Ciocalteu reagent was reduced to below ± 3% at 95% confidence while the HPLC analysis of the phenolic profile allowed consistent qualitative characterisation of the extracts.

Results from the extraction of phenolic compounds from olive wastes show that the use of ultrasound-assisted extraction enhances both the rate of extraction and the absolute yield of phenols. Under the experimental conditions used, the recovery of total biophenolic compounds, expressed as gallic acid equivalents (mg GAE/ml), was 15% greater under the influence of ultrasound. Under the influence of an ultrasonic field, 95% of the total biophenolic fraction can be extracted in a fraction of the time of standard solvent extraction. Both of these phenomena can be explained by the destruction of cellular material; releasing biophenolic components not available for standard extraction, and increasing the rate of mass transfer to the bulk solution.

The quality of the biophenolic fraction of the extract does not appear to be significantly affected by the action of ultrasonic irradiation under the conditions tested. After a 15 minute exposure time, no reduction in total biophenol content was observed, with only a very minor change in the biophenolic profile.

Bioactivity testing on various bacteria and fungal species showed that this slight modification to the biophenolic profile of the fraction had no impact on the antimicrobial activity of the samples.

Targets for antimicrobial testing were chosen based on their action as a post-harvest or horticultural disease. These cultures gave a good range of bacteria and fungi, phyto-pathogenic and post-harvest organisms, as well as structurally different Gram-positive and negative bacteria.

Antibacterial testing showed the olive leaf extract had an adequate inhibitory activity against the tested bacteria, with *Erwinia amylovora* and *Staphylococcus aureus* being the most susceptible. Minimum inhibitory concentrations were in the range of 0.15 and 3.50 mg GAE/ml. Discounting the *Bacillus* sp., which showed a high resistance to the inhibitory activities of the extract, as shown in literature, Gram-positive bacteria appear to be more susceptible to the action of the biophenolic fraction of the extract compared to Gram-negative bacteria.

Bactericidal testing showed results somewhat contradictory to the minimum inhibitory tests. Significantly high concentrations of biophenolic compounds did not inhibit the growth, let alone reduce viable cell count, of *Staphylococcus aureus*. This inconsistency is believed to stem from the much stronger inoculum used in the antibacterial tests. In most cases the olive leaf extract enhanced the growth rate of *S. aureus* suggesting that nutrients in the sample were creating an additive interference to the test. *Erwinia amylovora* showed a reduction in cellular material in the presence of the leaf extract at a level similar to the nisin control. It can be concluded that *Erwinia amylovora*, responsible for the horticultural disease known as fire-blight, shows the greatest opportunity as a potential bacterial target for a novel product form olive wastes.

Against the fungal targets, the olive leaf extract showed minimal activity. In most cases the leaf extract showed not only a lack of activity, but in some instances actually created an increase in the growth of the fungal culture. This may be due to the high nutrient content of the crude olive leaf extract as previously mentioned. *Eutypa lata* and *Alternata alternaria* showed a degree of growth less than that of the blank, over the range of concentrations tested and after 7 days of incubation, suggesting some

inhibitory action, but at 90% growth of the blank, it was a minimal effect. Up to concentrations of 2.50 mg GAE/ml, the extract shows no activity against fungal species although there is some evidence that an inhibitory effect might be apparent at higher concentrations against *E. lata* and *A. alternaria*.

It was decided that a crude extract should be used to simplify the purification procedures in an attempt to retain all active fractions. Results indicate that some fraction of the crude extract increase microbial growth, creating an interference from the biophenolic fraction of interest. The use of a further purified olive leaf extract will reduce the effect of such interfering effects and allow the accurate determination of the bioactivity of the olive phenolic fractions.

This project aimed at examining the potential for using olive wastes as a raw material for a novel product, and to examine novel methods for the recovery of active agents and any differences in product quality resulting from the use of these methods compared to standard techniques. The use of ultrasound to enhance the recovery of biophenolic components has been shown to be significant. The mechanism of increased recovery is thought to be based on the destruction of cellular material, enhancing rates of diffusion and increasing phenolic exposure to the bulk solution, but certain intrinsic properties of the time/yield curve are unexplained and require further examination.

From this work, it can be concluded;

- The recovery of biophenolic compounds from olive wastes is enhanced by the action of ultrasound with little negative impact on product quality.
- The olive biophenols tested in a crude extract showed a varied activity against bacterial species with minimal activity against fungal species.
- Given the low potential cost for recovery and a waste product raw material, there exists and economic opportunity for the recovery of olive biophenols, given a suitable application can be identified.

It is recommended that;

- A new purification protocol needs to be developed so that active compounds can be tested free of interferences.

- A large-scale screening process for the identification of potential target uses needs to be undertaken.
- More work needs to be done examining the mode of action of ultrasound on the recovery of biophenolic compounds from olive wastes.

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