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PhD dissertation

Virgin olive oil and biophenols in oil-in-water food emulsions: stability and interactions in relation to the release of aroma compounds

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List of abbreviations:

ANOVA	Analysis of variance
CAR	Carboxen
DVB	Divinylbenzene
EVOO	Extra virgin olive oil
GC/MS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
HP-LP	High/Low whey protein (WPI) concentrations
HS-SPME	Headspace solid-phase microextraction
HX-LX	High/Low Xanthan gum
MUFAS	Monounsaturated fatty acids
MWP	Milk whey proteins
O/W	Oil-in-water
OHTy	Hydroxytyrosol
OHTy-EA	Aldehydic form of elenoic acid linked to hydroxytyrosol
OHTy-EDA	Dialdehydic form of elenoic acid linked to hydroxytyrosol
OMW	Olive mill wastewater
PDMS	Polydimethylsiloxane
P-OMW	Powder extracts from OMW
PUFAs	Polyunsaturated fatty acids
PV	Peroxide Value
RAS	Retronasal aroma simulator
ROO	Refined olive oil
ROOP	Refined olive oil with added polyphenols
RSM	Response surface methodology
SIM	Selected Ion Monitoring
TBARS	Thiobarbituric Acid Reactive Substances
Ty	Tyrosol
Ty-EA	Aldehydic form of elenoic acid linked to tyrosol
Ty-EDA	Dialdehydic form of elenoic acid linked to tyrosol
VOO	Virgin olive oil
W/O	Water-in-oil
W/O/W	Water-in-oil-in-water
WPI	Whey protein isolate

Preface

In past few years, the consumers' interest for "healthy" fats lead food manufacturers to add these ingredients to their products, for example higher amounts of oleic acids, as well as plant secondary metabolites with biological functions such as natural phenolic compounds. However, the addition of these ingredients to foods is not straightforward as some of them can easily undergo lipid oxidation and also the effects on the physical stability of the food products over storage must be assessed. Lipid oxidation is a major problem and economic concern in the food industry because it changes taste, texture and appearance of products, develops undesirable flavours and decreases nutritional value and shelf-life of products. Hence, food manufacturers have been seeking for reliable and feasible methods to incorporate vegetable oils into food products and specifically emulsion-based foods.

With specific regard to olive oil, moreover, the aim is to preserve its characteristics and its appreciated flavour, both in terms of aroma and taste. In fact, virgin olive oil aroma and phenolic compounds are the most appreciated quality characteristics of this products, both with its fatty acid composition (high oleic acid content). Usually, emulsions used as sauce or dressing creams are not formulated with olive oil, mainly due to its price. However, during the past few years its use has been increasing particularly for some niche markets like in top restaurants. However, little is known about the possible interaction of constituent phenolic compounds of virgin olive oil on the emulsions properties, both in terms of physical stability and oxidation rate, but also for its impact on aroma release. Moreover, there is a huge demand for the recovery and valorisation of waste products from the food industry, and particularly the olive oil industry for responsible of the production of high amounts of wastewater. The presence of valuable phenolic compounds also in the by-products of olive oil extraction, i.e. olive mill wastewater, is of great importance in terms of environmental sustainability for its high pollutant risk, but it is also interesting for the recovery and application of these biophenols to be further used in food products to design functional foods.

In this context, the use of olive phenolic compounds is of great interest for their *in vivo* biological properties responsible for their positive effects on human health and their importance in terms of overall flavour to virgin olive oils. The use of biophenols from natural sources and in particular from olive mill by-products is one of the main challenges in olive industry. This issue has been linked to the use of "green" hydrocolloids and in particular of whey protein isolate in emulsions, and the interaction of these hydrocolloids also with salivary proteins has been further investigated to understand the in-mouth aroma release, for the maximisation of the aroma impact in food products based on olive oil or virgin olive oil. Moreover, olive phenolic compounds have been shown to protect some key aroma compounds in virgin olive oils, with consequent impact on the headspace aroma release, recently demonstrated by our research group.

Oil-in-water (O/W) emulsions have a wide application in the food industry, as many products are emulsions, e.g. sauces, mayonnaise, milk, vinaigrette, etc., but also crema in the espresso coffee. The addition of ingredients such as phenolic extracts in O/W emulsions might be useful for the development of functional food products with improved quality characteristics. However, the addition of these extracts might cause changes with respect to the effect of other ingredients commonly used in O/W emulsions as stabilisers such as proteins or polysaccharides.

The interaction of food ingredients is a very interesting topic, which has been increasing in the past few years also in parallel with the development of a new scientific discipline called "molecular gastronomy". The study of the interaction between ingredients, or the use of new and uncommon ones, is within the scopes of molecular gastronomy. In particular, the effect of these interactions on the release of aroma compounds and therefore on the final aroma and flavour of the product is particularly relevant.

The present thesis deals with olive oil and emulsions made from olive oil, when other ingredients are added in these foods systems, particularly whey protein isolate and phenolic compounds (also called biophenols) derived from olive.

The first chapter presents an overview of the most up-to-date researches on olive oil chemistry and flavour, on food emulsions characterisation and on the emulsion destabilisation phenomena. Moreover, the interaction of emulsion ingredients, particularly phenolic compounds and proteins (especially milk proteins) have been detailed, with emphasis on their effect on emulsion stability and on aroma compounds. In this context, the study of headspace aroma release from emulsions and dispersions is relevant in food science as this strongly influence the final impact of a food product. In fact, in a vision of the so-called "molecular gastronomy", the use of new ingredients but also the application of an uncommon combination of ingredient in a food product must be studied to assess the final flavour effect, based on the food pairing theory, which has been detailed in the introduction chapter. Being the study of aroma release particularly important, the latest research papers about food aroma release and model systems simulating mouth conditions, also regarding the interaction of phenolics and proteins, have been reviewed.

The third chapter presents an experimental study where a model olive oil-in-water emulsion has been created by using refined olive oil and the following ingredients: olive mill wastewater phenolic extracts, whey protein isolate, xanthan gum. The phenolic compounds extracts were added to create a functional food product with possibly effect on the lipid oxidation status and potential human health benefits, but the effects on physical stability should be also studied. For this reason, the creaming rate, mean particle size, particle size distribution and other physical properties were assessed and reported in order to verify the effects of the hydrocolloid, proteins and phenolic added.

A mathematical model has been then created and presented in chapter four to study the emulsion behaviour by means the response surface methodology to better describe the effect and interaction of these ingredients. Not discrete concentrations but ranges were used for each of the compound in the same emulsions system, by using the statistical design known as central composite design. The model performance and the full description of the emulsions was reported and discussed.

Finally, in chapter five the headspace aroma release from emulsions/dispersions was the object of the study. Dispersed systems were created by using olive oil with added olive phenolic compounds and whey protein isolate. Their effect on the retronasal aroma release was studied *in vitro* by a retronasal aroma simulator device. This system used human saliva and therefore also the effects of salivary proteins and other components on the mixture was studied, in relation to the release of olive oil aroma compounds.

These studies will help in the understanding of emulsion physical and chemical stability, but also in the optimisation of olive oil aroma release and in food pairing of olive oil with other food products. The addition of olive phenolic compounds might be also a useful "green" mean to preserve the aroma compounds in emulsions, and the uses of phenols from olive mill wastewater is expected to help in the future for the reduction of the pollution and consequent environmental issues.

Keywords: olive oil, food pairing, oil-in-water emulsions, emulsion stability, aroma release, protein-polyphenols interaction.

Prefazione

Negli ultimi anni, l'interesse dei consumatori per i “grassi sani" ha portato le aziende alimentari verso l’utilizzo di grassi piú salutari nei loro prodotti, ad esempio con un maggior contenuto di acido oleico, nonchè l’uso di ingredienti con funzioni benefiche sulla salute umana ottenuti da fonti naturali come i composti fenolici dalle acque di vegetazione dei frantoi oleari. Tuttavia, l'aggiunta di questi ingredienti negli alimenti non è semplice, dal momento che i prodotti alimentari con alto contenuto lipidico sono facilmente suscettibili all’ossidazione. Inoltre l’effetto dell’aggiunta di composti biofenolici sulla stabilità fisica dei prodotti alimentari necessita di studio, in quanto questo aspetto, insieme all’ossidazione dei lipidi, è di fondamentale importanza per l'industria alimentare dal momento che può avere influenza sulle caratteristiche sensoriali e nutrizionali del prodotto e sulla sua accettabilità. La ricerca negli ultimi anni si è focalizzata sulle proprietà dell’olio extravergine di oliva e sulle interazioni che può avere con gli altri alimenti e nei prodotti alimentari in particolare sotto forma di emulsione.

Relativemante agli oli di oliva, lo scopo dei produttori e in genere dell’industria alimentare è di preservarne le caratteristiche, sia in termini di aroma che di gusto. Infatti, l’aroma e i composti fenolici dell’olio vergine di oliva sono caratteristiche di qualità del prodotto molto apprezzate dai consumatori. Normalmente, emulsioni usate come salse o *dressing* non sono formulate con olio di oliva, dovuto al suo prezzo elevato. Tuttavia, nel corso degli ultimi anni il suo uso è in costante aumento, soprattutto per alcuni mercati di nicchia e di ristoranti di alta gamma. Ancora limitati sono gli studi circa la possibile interazione dei composti fenolici dell’olio extravergine di oliva sulle proprietà di emulsioni olio-in-acqua, sia in termini di stabilità fisica e grado di ossidazione, che di rilascio di composti aromatici nello spazio di testa o durante il consumo. Inoltre, vi è una grande richiesta per il recupero e la valorizzazione dei sottoprodotti dell'industria alimentare, e in particolare dell’industria olearia, la quale è responsabile della produzione di elevate quantità di acque reflue. La presenza di composti fenolici nei sottoprodotti di estrazione dell'olio di oliva, i.e. acque reflue, è di grande importanza in termini di sostenibilità ambientale a causa del loro alto potere inquinante, ma risulta interessante anche il loro recupero e la loro applicazione in prodotti alimentari allo scopo di formulare alimenti funzionali.

In questo contesto l'uso dei composti fenolici, da olio di oliva o suoi sottoprodotti, è di grande interesse sia per le loro proprietà biologiche positive in vivo sulla salute umana sia per la loro importanza sensoriale negli oli vergini di oliva. L'uso dei polifenoli estratti da fonti naturali e in particolare dalle acque reflue dei frantoi è una sfida nell'industria olearia. Il loro utilizzo è stato legato all'uso di idrocolloidi ed ingredienti "verdi" in emulsioni, come quelli usati nel presente lavoro di ricerca, ovvero isolati di proteine del siero del latte in emulsioni. L'interazione degli idrocolloidi con le proteine della saliva è stata poi studiata *in vitro* per comprendere il rilascio dei composti aromatici nella bocca durante il consumo e la conseguente percezione dell’aroma, allo scopo di massimizzare l'impatto aromatico di prodotti alimentari a base di olio di oliva, o di oli vergini di oliva. Inoltre è stata dimostrata la capacità dei composti fenolici dell’olio di oliva di proteggere alcuni composti aromatici “chiave” negli oli vergini di oliva, con conseguente impatto sul rilascio dei composti aromatici nello spazio di testa, recentemente dimostrato dal nostro gruppo di ricerca.

Le emulsioni olio-in-acqua (O/W) hanno una vasta applicazione nell'industria alimentare, dal momento che una vasta gamma di prodotti sono sotto forma di emulsione, come salse, maionese, latte, vinaigrette, caffè espresso, etc. L'aggiunta di ingredienti quali emulsioni O/W con estratti fenolici potrebbe essere utile per lo sviluppo di prodotti alimentari funzionali, con migliori caratteristiche qualitative. Tuttavia, l'aggiunta di questi estratti potrebbe avere impatto significativo sulle caratteristiche fisico-chimiche delle emulsioni a causa dell'interazione con gli altri ingredienti presenti in emulsioni o usati come stabilizzanti, ad esempio proteine o polisaccaridi.

L'interazione tra i vari ingredienti alimentari è un argomento molto interessante nell’ambito delle scienze degli alimenti, e la ricerca in questo ambito è incrementata negli ultimi anni anche in parallelo allo sviluppo di una nuova disciplina scientifica denominata "gastronomia molecolare". Lo studio dell'interazione tra ingredienti, o l'uso di nuovi e insoliti ingredienti, è diffusa ed è fra gli scopi della gastronomia molecolare. Particolarmente rilevante è l'effetto di queste interazioni sul rilascio di composti aromatici e quindi sull’aroma ed il gusto del prodotto finale.

La presente tesi affronta il problema dell'uso dell'olio d'oliva e delle emulsioni a base di olio d'oliva, in sistemi alimentari in cui sono aggiunti altri ingredienti, quali isolati proteici del siero di latte e composti fenolici (chiamati anche biofenoli) derivati dalla lavorazione delle olive.

Il primo capitolo presenta una panoramica dello stato dell'arte sulla tecnologia e chimica dell'olio vergine di oliva e sulle sue pecularità e caratteristiche sensoriali, sulle emulsioni alimentari e la loro caratterizzazione e sui fenomeni di destabilizzazione dell'emulsione. Inoltre sono riportati in dettaglio le interazione degli ingredienti in emulsione, quali proteine (proteine del latte e composti fenolici, con particolare attenzione al loro effetto sulla stabilità dell'emulsione e influenza sui composti aromatici. In tale contesto, lo studio dell'aroma nello spazio di testa di emulsioni e dispersioni alimentari è importante nella scienza degli alimenti, dal momento che i composti aromatici hanno forte impatto sull'accettabilià dei prodotti alimentare. Infatti, nella visione della cosiddetta "gastronomia molecolare", l'uso di nuovi ingredienti ma anche la combinazione di ingredienti non comuni in un alimento, deve essere studiata anche in relazione alla teoria denominata "food pairing theory", che è stata riportata nel capitolo introduttivo. Essendo lo studio del rilascio dell'aroma particolarmente importante, ulteriori dettagli sono presentati per riportare i più recenti lavori di ricerca sul rilascio dei composti volatili dagli alimenti e in sistemi modello che simulano le condizioni della bocca, anche in relazione all'interazione di composti fenolici e proteine.

Il terzo capitolo presenta uno studio sperimentale in cui si è sono studiate emulsioni modello O/W formulate utilizzando olio d'oliva raffinato ed i seguenti ingredienti: estratti fenolici ottenuti dalle acque di vegetazione dei frantoi oleari, isolati delle proteine del siero di latte e gomma xantano. Gli estratti di composti fenolici sono stati aggiunti per creare un prodotto alimentare funzionale per valutare l'effetto sullo stato di ossidazione dei lipidi e l'effetto sulla stabilità fisica. Per questo motivo, l'indice di cremaggio, la dimensione media delle particelle e la loro distribuzione, la viscosità ed altre proprietà fisiche sono state misurate per verificare gli effetti degli idrocolloidi aggiunti, delle proteine e dei composti fenolici.

Un modello matematico è stato poi creato e presentato nel quarto capitolo per studiare il comportamento delle emulsioni attraverso la metodologia statistica denominata "response surface methodology" con il disegno statistico composito centrale, allo scopo di descrivere meglio l'effetto e l'interazione di questi ingredienti durante la conservazione in condizioni di ossidazioni accelerate. Sono state usate concentrazioni di ciascun composto in intervalli di concentrazione scelti in base a lavori precedenti e per simulare una vasta gamma di emulsioni alimentari. La validazione del modello statistico e la descrizione completa delle emulsioni sono state riportate e discusse alla fine del capitolo.

Infine, nel quinto capitolo, l'oggetto di studio è stato il rilascio di composti aromatici da emulsioni/dispersioni. I sistemi dispersi sono stati creati utilizzando olio di oliva con aggiunta di composti fenolici estratti dall'olio vergine di oliva e proteine del siero di latte. Il loro effetto sul rilascio dell'aroma è stato studiato mediante un sistema *in vitro* di simulazione dell'aroma retronasale è stato applicato per valutare il possibile rilascio retronasale in vitro. In questo sistema si è impiegata saliva umana e quindi gli effetti delle proteine salivari e degli altri componenti della miscela sono stati valutati in relazione al rilascio dei composti aromatici dell'olio d'oliva.

Questi studi aiuteranno a comprendere meglio la stabilità fisica e chimica di emulsioni olio-in-acqua, nonché l'ottimizzazione del rilascio dell'aroma dell'olio vergine di oliva, simulato mediante aggiunta di composti aromatici puri, in abbinamento con altri prodotti alimentari. L'aggiunta di composti fenolici estratti dai residui della lavorazione delle olive potrebbe essere anche un utile metodo, ecosostenibile, per preservare i composti aromatici nelle emulsioni. Questo utilizzo degli estratti fenolici da acque di vegetazione potrebbe anche aiutare, in futuro, la riduzione dell'inquinamento e i conseguenti problemi ambientali legati a questi sottoprodotti.

Parole-chiave: olio di oliva, emulsioni olio-in-acqua, stabilità di emulsioni, rilascio di aroma, interazione proteine-polifenoli.

State of the art

Chapter 1

Virgin olive oil and its phenolic compounds

Olive oil is the product obtained from olive fruit and it is mainly composed by triacylglycerols, which accounts to 98% of the total composition. The remaining fraction comprises free fatty acids, phenols, tocopherols, sterols, phospholipids, waxes, squalene and other hydrocarbons. For "olive oil", many commercial categories exist: olive oil itself has been defined as a mixture of oil obtained from olives and that undergo refining process, with virgin olive oil (VOO). This latter is a product that is extracted from olives without any chemical process. Among VOOs, extra virgin olive oil has particularly high standards both in terms of composition, namely acidity, peroxide values and oxidation indices, as well as sensory characteristics assessed by recognised panels (EC Reg. 2568/91).

Several studies have tried to understand the quality of extra VOOs from the retail market, as this is relevant for the consumers in terms of the oil composition, health benefits and organoleptic properties (Caporaso et al., 2015a). However, it is important to understand the compositional changes due to other factors that influence VOO composition, including the agronomical practices and extraction technology. The olive variety, or "cultivar", the climatic conditions and geographical location of the olive orchard, as well as the agronomic practices and olive ripening degrees influence the final composition of the oil and therefore its flavour (Angerosa et al., 1999; Benincasa et al., 2003). All these factors will be described in the present review paper.

Virgin olive oil (VOO) contains an unsaponifiable fraction including waxes, phospholipids, phenolics, pigments and carotenoids. The most abundant ones are represented by phenolic compounds (oleuropein aglycon, hydroxytyrosol, lignans, etc.), tocopherols, squalene and β-sitosterol (Boskou, 2008). The known antioxidant activity of olive oils is mainly due to the presence of phenolic and ortho-diphenolic compounds, and secondarily to the fatty acid composition (Visioli, Poli and Gall, 2002). Olive fruit - contrary to other vegetable fats which usually derive from seeds - is properly a fruit. The major olive components are water (40-70%) and fat (6-25%), mainly present in the mesocarp. The fruit also contains simple sugars, organic acids, nitrogen compounds and biophenols.

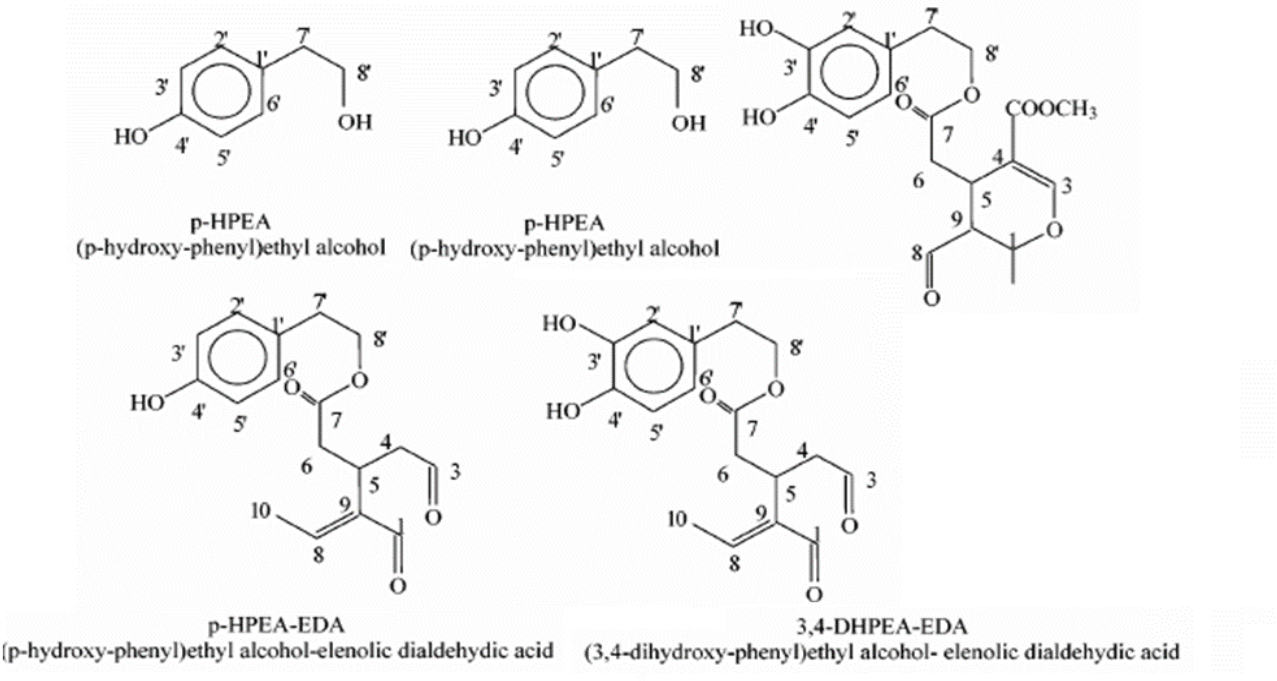

Figure 1.1. Secoiridoid compounds occurring in Virgin Olive Oil (from Servili et al., 2003).

Particularly important for olive composition is the phenolic fraction, mainly constituted by oleuropein, demethyl oleuropein and verbascoside. These compounds are present in concentration comprised between 0.5 and 2.5% of the fresh weight. The oil fraction is present as oil droplet in the pulp (16.5-23.5% fresh weight), while little amounts are also found in the seed (1-1.5%). An eco-physiological role of the phenolic compounds in many fruits and vegetables seems to be related to their protection against insects or other plants "enemies". Indeed, the reaction of polyphenols after their esterification by esterases during fruit drupe maturation exerts a plant defence mechanism against biotic attacks (Piperno et al., 2004). After oleuropein and ligstroside accumulation, the enzymatic hydrolysis produces hydroxytyrosol, tyrosol, demethyloleuropein and secoiridoide glycoside, arising from the degradation of complex polyphenols (**Fig. 1.1**).

Recovery of phenolics from olive mill wastewater

The olive oil extraction system can be divided into two main systems, i.e. the traditional pressing, and the centrifugation systems. These latter group is classified according to the by-products obtained, into three- and two-phases systems. The three-phase system generates three fractions at the end of the process: a solid (olive husk or olive pomace) and two liquids (oil and wastewater). This technology considerably decreases the volume of plant effluents and the disposal problems (Moreno et al., 2000). In addition, this extraction system releases a liquid/solid by-product rich in hydroxytyrosol, with concentration 10–100-fold higher than in olive oil (Visioli, Vincieri, & Galli, 1995). The majority of VOO in the Mediterranean area is currently extracted by centrifugation system, which are different from the traditional discontinuous systems based on pressure. In Italy this technique represents more than 80% of total production (Roig, Cayuela, & Sanchez-Monedero, 2006). The three-phase centrifugation system provides a dilution of the olive pastes producing 0.50–0.90 liters of OMW per kg of olive paste. A general picture of the products and by-products obtained from the two centrifuge systems is shown in **Fig. 1.2**.

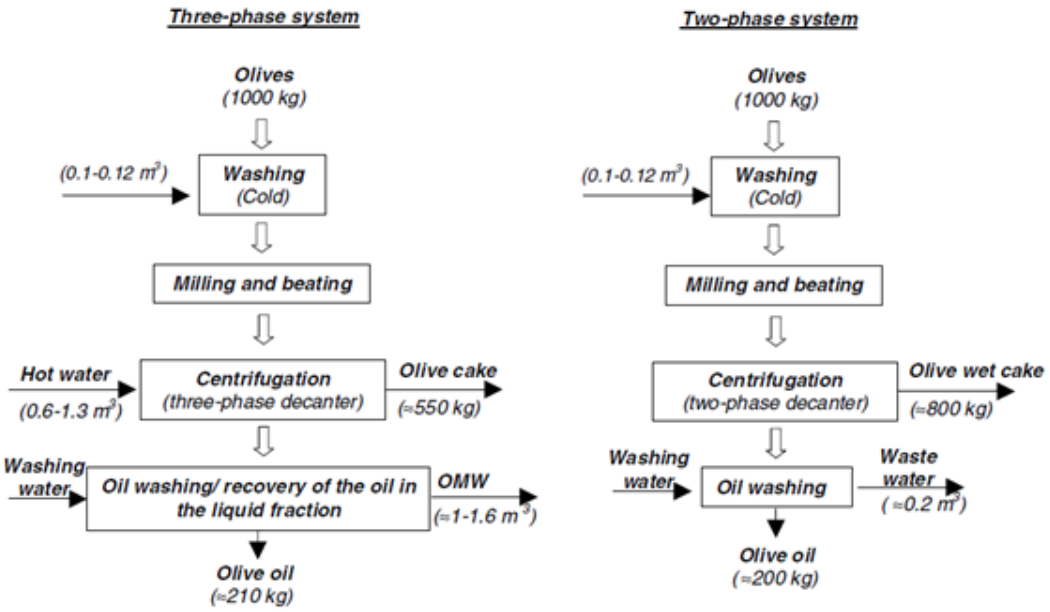

Figure 1.2. Comparison of the production of olive mill wastewater (OMW) and other products and by-products from the olive mill industry, in the case of three- and two-phases extraction systems. The traditional press system is not indicate (from Alburquerque et al., 2004).

The extraction of the olive oil usually yields olive oil (20%), a semi-solid waste (30%), and an aqueous liquor (50%) called olive mill wastewater (OMW). The OMW comes from the vegetation water and the soft tissues of the olive fruits in addition to the water used in different stages of oil production. The olive fruit is very rich in phenolic compounds, in addition to containing other components, but only 2% of the total phenolic content of the olive fruit passes into the oil phase, while the remaining amount is lost in the OMW (approximately 53%) and in the pomace (approximately 45%) (Rodis et al., 2002; El Abbassi, 2012).

Olive mill wastewaters (OMW) are the main pollutant from three-phase extraction systems and traditional mills (Roig et al., 2006). They are constituted by vegetable water of the fruit and the water used in different stages of oil extraction. They hold olive pulp, mucilage, pectin, oil, etc., suspended in a relatively stable emulsion (Paredes et al., 1999). Their chemical composition is variable depending on olive varieties, growing techniques, harvesting period and especially the technology used for oil extraction (Roig et al., 2006).

The main characteristic of OMW is the presence of organic compounds such as organic acids, lipids, alcohols and polyphenols that turn OMW into phytotoxic materials, representing a great environmental hazard when it is not managed properly (Roig et al., 2006).Olive processing wastes are produced in huge amounts (6–7 million tons/year) and are characterized by negative environmental effects, with a highly diverse organic pollutant load (Ginos, Manios, & Mantzavinos, 2006; Capasso et al., 1992).

Olive oil extraction process involves the production of high amounts of olive mill wastewater, which implicates serious environmental problems especially in the Mediterranean area. Mediterranean countries produce about 11 million of tons of olive are per year from which about 1.7 million tons of olive oil is extracted (Aktas et al., 2001). As several millions of cubic meters of wastewater produced in three months by OMW in the Mediterranean countries as Spain, Italy and Greece, Morocco, etc., this represent one of the greatest environmental problems of olive agro industry in the Mediterranean basin. The pollution charge of OMW is well represented by its COD value that ranges between 80 e 160 g/L of O_2.The chemical composition of OMW, and consequently the value of COD depends on different parameters, first of all the process of olive crushing, the so-called “three-phases” or “two-phases”: the first one produces olive oil, olive mill wastewater and olive paste (semi-solid) as products; while the second one produces olive oil and a semi-solid by-product named “*alperujo*”, being mostly applied in Spain. The OMW shows a brown-red colour, weakly acid pH, about 83.4 % of water, inorganic salts (1.8%), organic compounds (14.8%) and traces of olive oil (fats) (Cimato et al., 2001; Vitagliano, 2013). The pH of OMW is slightly acidic, with about 83.4 % of water, inorganic salts (1.8%), organic compounds (14.8%) and traces of lipids are present (Cimato et al., 2001).

The management of agroindustrial wastes is a serious economic and environmental problem. On the other hand, the valorisation of such by-products through the recovery and/or the biotransformation of their organic matter is a relevant opportunity, since it can combine the waste treatment to the production of added-value chemicals and biofuels (Scoma et al., 2011). The COD charge is due to the high organic content related to molecules as sugars, organic acids, oils, proteins and polyphenolic compounds that are present in concentrations ranging between 3 to 11 g/L. These chemicals represents the main obstacle to OMW disposal because they show antibacterial and phytotoxic properties, they can inhibit both aerobic and anaerobic fermentation processes (Ranalli et al., 2003; Rozzi et al., 1996), and consequently they require development of a specific treatment processes. Where allowed by law, OMW can be spread in the agricultural fields (ferti-irrigation) and in many countries this is most common way of OMW disposal. Conventional treatment methods as: biological and phisico-chemical depuration are efficient for OMW. Some negative consequences on the soil properties have been reported in research papers (Fiorentino et al., 2003; Sierra et al., 2001).

Most of processes focus on the polyphenols degradation through oxidation, performed by air-oxygen or ozone insufflation, and very few of them are based on the application of microorganisms as selected yeasts able to metabolize polyphenols. More specifically, the major studies carried out on the recovery of polyphenols from OMW include: i) adsorption on vegetable matrices; ii) solid phase extraction; iii) membrane processes. The energetic exploitation of this by-product (co-generation from wood and plant material) has been also proposed as a valid alternative. However since olive leaves contains high levels of polyphenols (mainly oleuropein, luteolin-7-glucoside and verbascoside) (Silva et al., 2006) other approaches have been investigating during recent years, for example the recovery of valuable compounds from this matrix through membrane separation.

Membrane technology is a term that refers to a number of different filtration processes that are used to separate substances. With this technology, membranes are used as filters in separation processes, with a wide variety of applications, both industrial and scientific. They provide effective alternatives to related technologies such as adsorption, ion exchangers, and sand filters. The membranes used in membrane technology may be regarded as barriers separating two fluids and allowing certain substances to be transported across the membrane. The technology is based on permeable membranous filters which allow water to flow through, but traps suspended solids. Membrane are manufactured using inorganic materials as

alumina, zirconia, or metals, or organic materials as carbon, cellulose, or complex polymeric molecules such as polysulphone (PS) or polytetrafluoroethilene (PTFE), polyamide (PA), etc.

There are several ways to let the water phase/mixture penetrate through the membrane, e.g. gravity, pressure, electrical current, or maintaining a concentration gradient across the membrane. One of the major uses of membrane filtration is for water purification, including desalination, or creation of drinking water from salt water, and purification of ground waters or wastewaters. Other areas of industry that utilize membrane technologies include biotechnology, food and drink manufacturing, and medical uses such as dialysis.

Membrane filtration system are classified into micro-, ultra- and nano-filtration, with a further technique called reverse osmosis. Microfiltration and ultrafiltration are applied to remove larger particles, while nano-filtration and reverse osmosis is applied to remove salts. The first two techniques need lower working pressure than the other ones. Microfiltration and ultrafiltration are able to remove suspended or colloidal particles via sieving mechanisms based on the membrane pore size. The range of the first one is comprised between 0.1 and 0.2 μm, while ultrafiltration pores ranges from 0.01 to 0.05 μm. Details on membrane process technology has been widely reported and reviewed by others (Paraskeva and Diamadopoulos, 2006; Galanakis, 2012), however, in the following paragraph the characteristics of microfiltration and ultrafiltration are briefly reported.

Microfiltration (MF) and Ultrafiltration (UF) are the two processes that are most often associated with the term "membrane filtration". MF and UF are characterized by their ability to remove suspended or colloidal particles via a sieving mechanism based on the size of the membrane pores relative to that of the particulate matter. However, all membranes have a distribution of pore sizes, and this distribution will vary according to the membrane material and manufacturing process. MF membranes are generally considered to have a pore size range of 0.1 – 0.2 μm (nominally 0.1 μm). For UF, pore sizes generally range from 0.01 – 0.05 μm (nominally 0.01 μm) or less. Because some UF membranes have the ability to retain larger organic macromolecules, they have been historically characterized by a molecular weight cutoff rather than by a particular pore size. The concept of the molecular weight cutoff (expressed in Daltons, a unit of mass) is a measure of the removal characteristic of a membrane in terms of atomic weight (or mass) rather than size. Typically the cutoff levels for UF membranes range from 10'000 to 500'000 Daltons, with most membranes used for water treatment at approximately 100'000 Daltons. UF membranes remove particulate contaminants via a size exclusion mechanism and not on the basis of weight or mass. UF membranes used for drinking water treatment are also characterized according to pore size with respect to microbial and particulate removal capabilities (Vitagliano, 2013).

The use of OMW extract in food or pharmaceuticals is a new trend in the food science sector to formulate new foods with positive effect on consumers' health. Consequently, the production of functional foods from OMW extracts constitutes a viable alternative for transforming this agro-industrial waste stream into a useful and relevant ingredient (Obied, Bedgood, Prenzler, & Robard, 2008).

Identification of phenolic compounds in OMW

At present, more than 50 different phenolic compounds have been identified in OMW, being oleuropein, hydroxytyrosol and tyrosol the major phenolic compounds found in this matrix. Hydroxytyrosol has been described to possess the strongest antioxidant phenolic compound in olive oil (D'Antuono et al., 2014).

However, the phenolic composition of OMW varies strongly between studies, as it is characterized by a significant complexity (Bianco et al., 2003; Obied, Bedgood, Prenzler, & Robards, 2007) and several new compounds have been recently identified and reported (Cardoso, Falcão, Peres, & Domingues, 2011). Hydroxytyrosol acyclodihydroelenolate and p-coumaroyl-6′-secologanoside (comselogoside) were recently identified in OMW and their antioxidant and antiproliferative activities was studied (D'Antuono et al., 2014). Other phenolics identified in OMW are verbascoside, tyrosol, catechol, 4-methylcatechol, p-hydroxybenzoic acid, vanillic acid, syringic acid, and gallic acid (Capasso et al., 1992; Visioli et al., 1999).

De Marco et al. (2007) reported the characterization of the OMW phenolic compounds, obtained by liquid–liquid extraction, after the optimization of the extraction parameters and conditions, for the analysis by HPLC. Hydroxytyrosol is the most abundant biophenol in ethyl acetate extracts from an acidified OMW.

Many other biophenols (tyrosol, caffeic acid, vanillic acid, verbascoside, luteolin-7-glucoside, dialdehydic form of decarboxymethyl oleuropein aglycon, ligstroside, luteolin) were identified. Fractionation of OMW phenolic extracts by reversed phase solid phase extraction (RP-SPE) allowed the separation of eight different phenolic fractions, whose radical scavenging activity was measured by the 2,20-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid) (ABTS) assay. Hydroxytyrosol, in particular, was purified from all the other biophenols, giving a fraction with a very high antioxidant activity (De Marco et al., 2007).

The authors obtained about 1.2 g of hydroxytyrosol and 0.4 g of flavonoids from one litre of OMW. The extraction and purification of hydroxytyrosol from OMWW may represent, then, a rapid, cheap and simple alternative to chemical or enzymatic synthesis. The authors also commented that this procedure may be transferred to industrial scale and OMW may become a valuable source of natural antioxidants, object of great interest for pharmaceutical, food and cosmetic industries (De Marco et al., 2007).

Encapsulation of food bioactive constituents

Encapsulation is a process by which small particles are entrapped in a wall material in a homogeneous or heterogeneous matrix to form microcapsules. The core may be composed of just one or several ingredients and the can be single or double-layered. Several method for encapsulation of food ingredients have been proposed and/or applied also at food industry level. A common one is to produce encapsulated products by using spray-drying which involves the conversion of liquid oils in the form of an emulsion into dry powders. This technique is widely applied the cosmetics and drugs industry in order to provide protection against oxidation, increased solubility, and increased activity when consumed orally. In the food industry, encapsulation is used for several purposes, e.g. as flavours carrier and to impart of protection against evaporation of volatile compounds, reaction, or migration in a food, and it has been used successfully for the creation of functional foods. During the recent years nutraceuticals are considered as health-promoting ingredients of food and therefore encapsulation can provide a certain protection against oxidation.

It has been reported that encapsulation may lead to dissolution rate enhancement, increased membrane permeability and bioavailability of low-solubility nutraceuticals. For example, cyclodextrins were reported to also act as flavour carriers and provide protection against lipid oxidation and heat-induced changes, so they can prolong the shelf life of food products and mask or reduce undesired taste and odour. Calvo et al, (2010) studied the influences of emulsion conditions and the effect of different wall components such as proteins (sodium caseinate and gelatin), hydrocolloids (Arabic gum), and hydrolysed starches (starch, lactose, and maltodextrin). Hydrolyzed starches (glucose, lactose, corn syrup solids, and maltodextrin) are generally added as a secondary wall material to improve drying properties of sprayed droplets (Re, 1998).

The molecular encapsulation of olive leaf extract into β-cyclodextrin was reported by Mourtzinos et al. (2007), and it result in increased aqueous solubility or increased partitioning in the oil/water system, improved protection against oxidation during storage and, possibly, better bioavailability. β-Cyclodextrins in emulsion can provide a higher solubility of the polyphenolic residue from olive leaf, up to 150% their initial value. Therefore, encapsulated olive leaf extract was proposed as a food additive with the advantage of higher aqueous solubility. The solid complex of encapsulated olive leaf extract were also proposed to be used in the future to fortify foods or as a food supplement as the leaf extract alone with the advantage of increased stability (Mourtzinos et al., 2007). However, it is noted by the author of this thesis that olive leaf phenolics are mainly represented by oleuropein, which is a much complex compound, and by other glycosylate derivatives which might have further functional properties; this system is strongly different from olive mill wastewater phenolic, which have simpler molecular structure and undergo dramatic changes during and after olive processing.

The choice of wall material is of crucial importance for the quality of the final product, as the efficiency of the protection or controlled release mainly depends on the composition and structure of the established wall. This wall could act as a barrier and it may protect against oxygen, water, light or could avoid contact with other ingredients or control diffusion (Calvo et al., 2010). In fact, Calvo et al. (2010) reported that a better microcapsule yield, efficiency, and internal and external fat ratio were achieved when a combination of proteins and polysaccharides was used as the wall component.

Molecular Gastronomy: definition, aims and applications

The food industry is one of the most important industries in the European Union, which spans a wide range of economic activities, with a high relevance for employment and economic output (Wijnands et al., 2007). Food industry is always trying to create and develop alternative food processing methods, but also combination of existing methods (Rastogi, 2010). This is also one of the objectives of Molecular Gastronomy.

Gastronomy (Greek gastèr = stomach, and nomìa = law), *i.e.* the set of techniques and culinary arts to prepare good food, is, in a broad sense, the study of the relationship between culture and food. The new scientific discipline named "Molecular Gastronomy" (MG) has been defined as "the scientific activity consisting in looking for the mechanisms of phenomena occurring during dish preparation and consumption" (Vega and Ubbink, 2008). The application involves transferring empirical kitchen practices to a science-based approach when preparing a dish. This research area is regarded as molecular gastronomy. Molecular cooking or science-based cooking is the application of MG to prepare a dish (Vega and Ubbink, 2008).It is a branch of food science that studies the physical and chemical transformations of food during cooking, and sensory phenomena associated with consumption. MG is considered as a part of food science and technology, and aims to apply gained knowledge to improve dishes, focusing mainly on home cooking and restaurants (Barham, Skibsted, Bredie, Bom Frost, Moller, Risbo, et al., 2010; Myhrvold and Smith, 2011).

According to This and Rutledge (2009),the official appearance of this discipline was generally recognized in 1992, when the first international conference of Science and Gastronomy, entitled "Molecular and Physical Gastronomy" was held in Erice, Sicily (Italy). Other sources based on official documents report that the meeting was firstly called "Science and gastronomy" and this name remained until early 1992, when it was converted into something that sounded less frivolous for the academic world. The adjective "molecular" was chosen because it was similar to "molecular biology". The two people who mostly contributed to the organization of the workshop in Erice were Elizabeth Cawdry Thomas and Nicholas Kurti (Myhrvold and Smith, 2010).

"Molecular cooking", which is the application of MG principles to cooking, can be differentiated from traditional cooking methods by its use of tools adapted from scientific laboratories, such as rotary evapourators, sintered glass filters, ultrasound probes, etc. Ingredients not commonly used in the kitchen but are frequently applied in the food industry, *e.g.* sodium alginate, calcium lactate, phenols extracted from grape juice, flavours, ascorbic acid, etc. are also used in molecular cooking (This and Rutledge, 2009).

The recent developments of MG in some countries have been reviewed by a number of papers published over the last few years. The outcomes of the collaboration between scientists and chefs, some examples of MG studies and the potentially exciting topic of food pairing theory, which have been recently reported by some scientists, are reported in this section. The aspects of the hedonistic impact and consumer's behaviour has also been presented, and finally, the possible future trends of MG have been discussed.

The purpose of the chefs and restaurateurs for applying MG is to create high quality foods having high sensory properties, consumer acceptability and satisfaction (van der Linden, McClements and Ubbink, 2008). Scientific researchers and chefs working in the field of MG have the following objectives: to research and study the sayings, proverbs and popular culinary knowledge; expand the classic recipes and invent new ones; and to introduce new ingredients, methods, equipment and processes in the kitchen (This and Rutledge, 2009). These specific aims can be studied from the perspective of scientists or chefs. The former group is more interested in the science behind the culinary phenomena, while chefs are more focused on its applications (e.g. molecular cooking) to innovate the way they create their dishes. The principles of MG have led to the invention and testing of new methods of preparation, cooking, presentation and combination of foods such as flash freezing using liquid nitrogen, the use of tobacco as a food, "frying" in sugars, the use of vacuums for the preparation of mousse and meringues, etc. (This and Rutledge, 2009).

The goal of MG is also to the construct culinary tests in scientific laboratories to assess the so-called "culinary myths". Some of these myths have been found to be false statements even though they can be found in traditional cookbooks. The researchers scientifically verify the traditional cooking practices to check their truthfulness (de Solier, 2010). Another aim of this discipline, according to This and Rutledge, (2009), is to create a universal culinary scientific language, which implicates the translation of recipes from

everyday language into the language of science. Dishes are neither solid nor liquid but "complex disperse systems" (CDS), therefore some authors have tried to develop formalisms to describe them in a scientific language called 'CDS formalism'. A second formalism was proposed to describe dishes overall structure, called "nonperiodical organization of space" (NPOS). Nevertheless, no information has been found in literature so far about the application of these formalisms outside the works published by the cited author.

de Solier (2010) argued that MG has different aims: for the scientist it is the culinary enlightenment, for chef the culinary creativity, and for the "foodie" the goal is the gastronomic education. For example, in the laboratory MG is used by scientists to discover chemical and physical reactions or the effects of different culinary practices, while for chefs it is related to the purpose of 'culinary creativity'. Lastly, in home practice, MG has the objective of entertaining food passionates and educating consumers about food composition and innovation. According to This and Rutledge (2009), culinary activities have three components, *i.e.* the social, artistic and technical parts. Indeed, the MG scientific program is composed of three parts: exploring the technical component of cooking, exploring the artistic component of culinary activities and exploring its social component.

The theory and principle of food pairing in MG studies

Pairing two or more ingredients to create a dish has always been the "problem" of every chef, cook or food lover. It has particular relevance in MG, as new combinations or new ingredients are tested, and therefore their final effects in terms of aroma and sensory properties have to be deepened. Some authors tried to approach the problem from a scientific point of view, and a theory was proposed for food pairing. This concept has been attributed to the empirical research on flavour improvement by chef Heston Blumenthal and chemist François Benzi. The first scientific reports on this topic were published by Ahn et al (2011) and Kort et al., (2010).

Kort et al (2010) applied the principle of food pairing to verify its validity from experimental tests, using a combination of ingredients and sensory analysis. According to the authors, there are foods where flavour pairing is particular important, for example the combination of wines with food products. The results of the experiment, however, showed that the food pairing theory was not valid, at least for those foods tested. Therefore, the authors concluded that the following assumption could be debated: "a successful combination of two ingredients is obtained when the score for the combination is higher than the average of the scores of the separate ingredients". In fact, the way cooks use food pairing theory is almost the opposite of the basis of sensory test (Kort, Nijssen, van Ingen-Visscher and Donders, 2010).

The principle of food pairing, an area of MG used to describe scientifically the fundamental "laws" of pairing flavours of a dish, was also addressed from another perspective, *i.e*. using the concept of networks and comparing the ingredients and recipes in many parts of the world. Ahn et al. (2011) created a flavour network (**Fig. 1.3**), where the ingredient sharing the highest number of aroma compounds were linked together to verify whether stronger links correspond to a higher number of recipes. A considerable number of ingredients, *i.e.* 381, were studied together with 2,021 flavour compounds considered as food odorants. The authors stated that the scientific analysis of any art, including the art of cooking, is unlikely to be capable of explaining every aspect of the artistic creativity involved. Indeed, this is one of the key concepts in MG.

While the mere sharing of volatile compounds did not explain the most used recipes and ingredients, Ahn et al found that this aspect varies according to the geographic location considered, *e.g.* in North American recipes, the more volatile compounds are shared by two ingredients; the more likely they are used in recipes. On the contrary, for East Asian cuisine the opposite was found. It should also be considered that a few outliers contributed strongly to the food pairing effect, as these few ingredients are frequently used in many recipes. In conclusion, some patterns were found, but there is still a need of obtaining a wider database of food components, as well as to fill the gap for texture, due to the fact that many ingredients are used not just for their flavour but also to provide a desired texture to the final dish (Kort, Nijssen, van Ingen-Visscher and Donders, 2010).

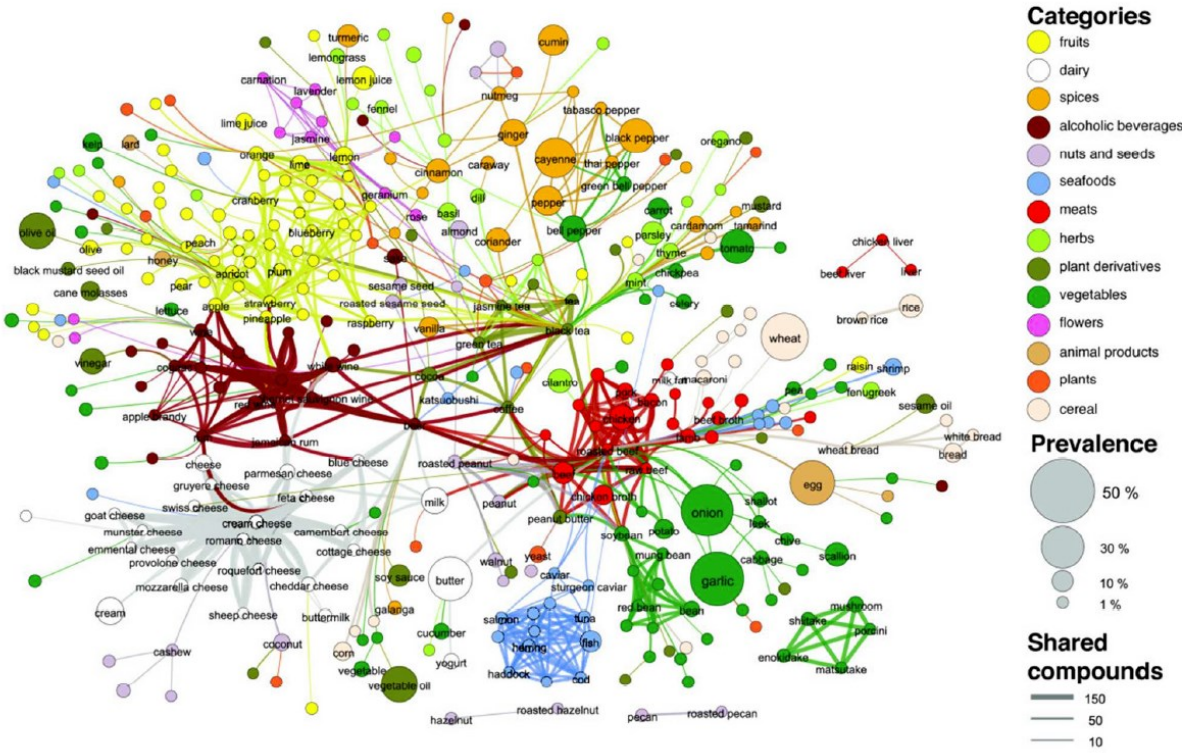

Figure 1.3. Flavour network published by Ahn et al. (2010), showing the links among ingredients based on their shared flavour compounds. Each node denotes an ingredient, node colour indicates food category, and node size reflects the ingredient prevalence in recipes. Ingredients are connected if they share a significant number of flavour compounds (Ahn et al., 2010).

More recently, Traynor et al. (2013) from an experimental work evaluated new food pairing combinations. They reported that it would be difficult to hypothesize successful food pairings due to this sharing of common volatiles, mainly because of the complex synergistic or antagonistic interactions between food matrices and volatile compounds, which influence the hedonistic rating of the new combination. It appeared that creating certain flavour balances in the volatile profiles of food pairings is important for a positive hedonic response. The authors paired banana with each of the following basmati rice, bacon and extra virgin olive oil, and applied the theory of food pairing. They evaluated both the volatile composition of the food ingredients and the sensory scores (hedonistic) obtained by a panel. Data seem to suggest the rejection of the hypothesis that the success of food pairings is based on a mere volatile compound sharing. As the foods chosen by Traynor et al (2013) shared the majority of volatile compounds, according to the theory, the score of dishes' sensory appreciation had to result very high. It should be highlighted that the authors used GC/MS analysis of volatile compounds for the foods they tested, and not just a database, which was the approach reported by Ahn et al. (2011) and Kort et al. (2010).

In fact, it is well known among flavour scientists that not all volatile compounds in a food are of interest from a sensory point of view, as other factors rather than their headspace concentration should be considered. For example, there are volatile molecules that are perceived at very low concentrations, *e.g.* few parts per million (ppm), while for others the odour ppm threshold is higher, perhaps hundreds or thousands. To further complicate this, apart from the concentration of a particular volatile compound, its odour description (which can also vary according to its concentration in the headspace) and its odour activity threshold, perception is also altered whether odour and taste are separated, as well as whether the odour molecules reach the olfactory epithelium though the orthonasal and retronasal routes. During eating, we perceive volatile compounds through the retronasal path, and due to the interaction with saliva and other factors, its final perception can be different (Caporaso, Genovese, Canela, Civitella and Sacchi, 2014).The interaction between volatile compounds and the matrix, with particular emphasis on olive oil oxidation during cooking, has been recently studied by Caporaso et al., (Caporaso, Panariello and Sacchi, 2015) who reported the influence of the type of oil in the traditional cooking of the typical Neapolitan pizza.

Information about the changes in oxidation indices, phenolic compounds and volatile molecules in the headspace were also reported. Other relevant studies from this research group evaluate the volatile release and perception in different type of foods, *e.g*. olive oil phenolic compounds (Genovese, Caporaso, Villani, Paduano and Sacchi, 2015),the effect of pairing between olive oil and whey proteins, (Genovese, Caporaso, De Luca, Paduano and Sacchi, 2015) and the impact of different home preparation of coffee brews on their composition and aroma release (Caporaso, Genovese, Canela, Civitella and Sacchi, 2014).

For other studies about volatile release, the reader can refer to Guichard (Guichard, 2002), where the interactions between flavour compounds and food ingredients were reviewed in detail, with a deep discussion about their influence on flavour perception. The author presented the impact of protein binding toward volatiles, the flavour release from emulsions, the impact of the matrix, hydrocolloids, and the influence of fat content on flavour release and perception.

New culinary trends and future of MG and molecular cooking

It has been recently published that the latest emerging trend under the influence of MG has being molecular mixology. It takes knowledge adapted from MG, more correctly from molecular cooking, to experience new ingredients for the production of cocktails (Ivanovic, Mikinac and Perman, 2011). Its purpose is to manipulate the state of aggregation of the liquid in order to create new aromas, flavours, textures and appearance that increase the attractiveness of drink and make the experience of consumption of these types of cocktails much more interesting (Ivanovic, Mikinac and Perman, 2011). "Mixology" is viewed as the new trend in molecular cooking, as it applies techniques such as the conversion of liquids into gels, foams and solids, and spherification to create cocktails and other fancy and surprising drinks.

More recently, according to This (2014), the new culinary trend is known as "note-by-note cuisine", which consists of cooking with pure compounds, *i.e.* those found in a food research laboratory or used by industry for specific purposes, obtained both from chemical synthesis or extraction, instead of usual ingredients. Now, only a few years after its appearance, molecular cooking has been declared dead, and substituted by the note-by-note cuisine (Everts, 2012). There are few examples of famous restaurants or research institutes where this type of cooking was applied or tried, like the French chef Pierre Gagnier who proposed a note-by-note meal. However, this new trend seems not yet to be appreciated by chefs or the general public, whereas some restaurants seem to have positive outcomes. The favourable arguments about, for example, the reduction of energy costs related to food production and transport by using "pure compounds" (purified compounds that are used in scientific laboratories like ethanol, anthocyanins, pure volatile compounds and other compounds generally referred as "chemicals") instead of 'common' foods (plant and animal derived food) could be easily countered (Svejenova et al., 2007).Therefore, this trend seems more a fancy and innovative way to play with tastes and flavour, or to provoke and amuse the consumer, than a serious proposal in food design. Nevertheless, some researchers argued that whereas the evolution of note-by-note cooking has being slow, many groups worldwide are interested and started working with it and consequently future developments are expected.

Concerns about the future of MG have no clear answers, because its influence is wide and includes aspects from several research areas. This scientific discipline has opened a new direct connection between kitchens and scientific laboratories. This is then applied at practical level for the creation of new flavours, shapes and textures. Molecular cooking may also be seen as the application of science in everyday cooking, as the technology and techniques applied are based on the knowledge obtained from the studies about the chemical and physical processes involved in cooking. The introduction of molecular cooking in restaurants implied changes in the method of interacting with guests particularly in the way of conceiving the meal: the number of servings and portions of each dish are very small, the dish is presented as an artistic result, the concept of menus disappears, while the duration of the meal are very long. This is because people are living an experience and not simply eating.

Other authors have asked rhetorically where MG will lead, and what will be its future, stating that a clear answer still does not exist (Barham et al., 2010; Ivanovic, Mikinac and Perman, 2011). They suggested that perhaps the most important goal of the MG should be to outline the basic principles that underlie the pleasure of eating, by elucidating the conditions when a dish becomes "delicious", and then predict whether a dish or a meal will be appreciated by consumers (Barham et al., 2010). At the moment, it seems that the

general public, chefs and scientists are divided into at least two factions. One group is enthusiastic, very active in the diffusion of knowledge, concepts and applications of MG. The other group consists of people who criticize the "chemicals in the plate" considering them harmful, regarding MG as an attack to the traditional gastronomy of their country, or just a fancy application of new techniques to create uncommon dishes. There is also concern or rejection from a more specialist public, *e.g*. one of the fathers of the principles of molecular cooking, Ferran Adrià, rejected the use of the term MG by using that of "deconstructive cooking" (Adriá et al., 2010). Lastly, whereas many scientists consider MG as a new scientific discipline and argue that it will acquire more scientific importance in the future, there are very few scientific publications that could be directly considered as MG studies. Most of them are just opinions, reviews, or applications of well-known phenomena already studied by food scientists. Therefore, studies about MG seem more useful to open a debate about food preparations than a serious "revolution" in the field. As some applications of MG sometimes seem non-substantial, more efforts are needed both from the perspective of the general public, to make them rationally understand the safety or nutritional issues related to food preparation and consumption, but also to further persuade other scientists to research into these topics. Nevertheless, the greatest merit and outcome of this discipline has probably been the practical application of concepts, knowledge and techniques into restaurants kitchens, as well as develop the interest from the general public about science and food.

The approach of Molecular Gastronomy in food science might be of great interest to study the interaction of ingredients also in model systems or in emulsions, which in turn represent a wide range of food products and can be applied at several levels, both at food industry of by chefs and restaurants to create new combinations, new flavours and to maximise the stability and aroma release of dispersions/emulsions.

Food colloids and emulsions

An emulsion is a mixture of two immiscible liquids, which are part of a class called "colloids" (McClements, 2005). Both water droplets can be present in a water bulk phase, or liquid oil droplets can be present in a continuous phase made by water. In the first case, the emulsion is called "water-in-oil" (W/O) emulsion, while the second one is called "oil-in-water" (O/W) emulsions.

Many food products are multiphasic systems, representing a complex chemical and physical mixture (Barham et al., 2010; Dickinson, 2006), and therefore there is the simultaneous presence of a diverse range of components dispersed in mixed solutions (Ettelaie, 2003). The dispersed phases can derive from natural food products such as globular proteins in milk, or be artificially created via food processing, e.g. during emulsification (**Table 1.1**). Many natural and processed foods exist either partly or wholly as emulsions (salad dressings, mayonnaise, cream liqueurs, etc.) or have been in an emulsified state at some time during their existence (breads, soft drinks, etc.) (Charcosset, 2009; Dalgleish, 2006). They can also be ingredients which participate in the formation of more complex products such as yoghurts, ice creams and whipped products (Leal-Calderon et al., 2007; Traynor, 2013).

These systems are commonly referred as "colloids", and complex chemical and physical interactions take place in these products (Dickinson, 2006). The dispersed phases can derive from natural food products such as globular proteins in milk, or be artificially created via food processing such as oil droplets in mayonnaise (Aguilera & Stanley, 1999).Next to these structures of the dispersed phase, food contains smaller molecular species, like salts, sugars, polyols and phospholipids, which moderate the properties of the continuous or dispersed phases, or their interfaces by serving as surface active molecules (surfactants), plasticisers and humectants (van der Sman & van der Goot, 2009).

An important class of food dispersions are emulsions (Schramm, 2005). The culinary industry is one of many industries that heavily rely on the use of emulsions and emulsifiers (Guzey & McClements, 2006). Emulsions have wide applications for only in the food sector, but also in cosmetic industry, as lotions, creams, moisturizers, and shower gels. The main functions of emulsion in cosmetic industry are moisturizing and occlusion to prevent the loss of water from the skin (Somasundaran et al., 2007; Sakai et al., 2006). In the pharmaceutical industry, emulsions act as a carrier for active ingredients or drugs. For this application, high stability is required both for long storage at relatively high temperatures, with droplets size usually much lower than those applied at food industry level. Emulsions are also applicable in other sectors of industries, such as agriculture, paint, paper coating, lubrications, petroleum extraction, bitumen emulsion etc. (Sakai et al., 2006).

Table 1.1. Classification of dispersed systems, with examples of food products.

Common name	Disperse phase	Continuous phase	Examples
Foam	Gas	Liquid	Whipped cream
Solid foam	Gas	Solid	Bread, meringue
Emulsion	Liquid	Liquid	Milk, mayonnaise
Gel	Liquid	Solid	Jelly
Sol	Solid	Liquid	Egg white
Aerosol	Solid/liquid	Gas	Flavoured "airs"
Solid dispersion	Solid	Solid	Chocolate
Powder	Solid	Gas	Flour

Source: Traynor (2013)

Emulsion stability

Stability can be defined as the capability of emulsions to keep their physical stability over time. Creaming, flocculation, coalescence, partial coalescence, phase inversion and Ostwald ripening are examples of physical instability. In general, food emulsions are termodynamically unfavourable systems due to the different density of the two immiscible liquids, and the unfavourable contact between oil and water. Due to their thermodynamic instability, emulsions will always breakdown over time. This fact bring to the needs of

using stabilizers, such as emulsifiers, thickening agents, or weighting agents. The instability of emulsions due to gravitational separation is referred to as creaming (upwards) or sedimentation (downwards).

The rate of separation can be calculated by Stokes' law, as follows:

$$v_{Stokes} = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1}$$

in which r is the particle radius; ρ_2 is the density of disperse phase and ρ_1 is the density of continuous phase; η_1 is the viscosity of continuous phase; g is the gravity force.

Flocculation and coalescence are the main reasons of droplet aggregation in emulsions. The flocculation is the process of association of neighbouring droplets but each one remains as an individual particle. In a diluted systems, flocculation accelerates gravitational separation due to an increase in particle size (Dickinson and Stainsby, 1982), but in concentrated systems it retards the separation due to network formation (Geigenmuller and Mazur, 1986). Coalescence is the process where two or more particles merge into one larger particle due to the breakage of the thin film between them.

As emulsions are instable systems, there is a significant influence on their shelf life and texture (Dickinson & McClements, 1995). The density of the dispersed phase is one of the main factor which might be controlled (Robins *et al.*, 2002). **Fig. 1.4** reports the most common processes of emulsion destabilization: droplet-droplet coalescence, flocculation, creaming and sedimentation (Tcholakova *et al.,* 2006). In fact, the stability of emulsions is dependent upon the characteristics of emulsions droplets (radius and density) and continuous phase (viscosity and density), as reported by the Strokes' equation above. Thus, emulsions with large mean particle size have lower physical stability than, for example, nanoemulsions. Also, a large difference in density can accelerate the separation of emulsion system which indicates that difference in density is an effective mean to stabilize the emulsions. The addition of thickening agents increases the viscosity of continuous phase, thereby inhibiting creaming (McClements and Decker, 2007; McClements, 2005; Chanamai and McClements, 2007).

The destabilisation processes greatly influence shelf life and texture of emulsions (Dickinson & McClements, 1995; Phillips *et al.,* 1994). A major coalescence mechanism which leads to a gradual coarsening of emulsion droplets is Ostwald ripening, which by and large has a detrimental effect on the shelf life of these types of food products (Ettelaie, 2003). The flocculation of emulsions is commonly via the depletion interactions, which are important in many food systems (Manoj *et al.,* 2000).Depletion flocculation is an entropic effective attraction in which non-adsorbed species (polysaccharide molecules, protein aggregates and surfactant micelles) are excluded from a small region between two droplets (Dickinson, 2002). The osmotic pressure due to the surfactant molecules in the continuous phase exerts a pressure driving the droplets toget (Robins *et al.*, 2002).

The perceived quality of emulsion-based food products is strongly influenced by their stability, rheology and appearance (Mirhosseini *et al.,* 2008a). An indicator of loss of stability is the increase in emulsion droplet mean diameter, and the growth rate of the droplets can reveal the mechanism responsible for this change (Silva *et al.*, 2010). Therefore a possible way of contrast emulsion instability could be the reduction of the droplet size (McClements, 2005).

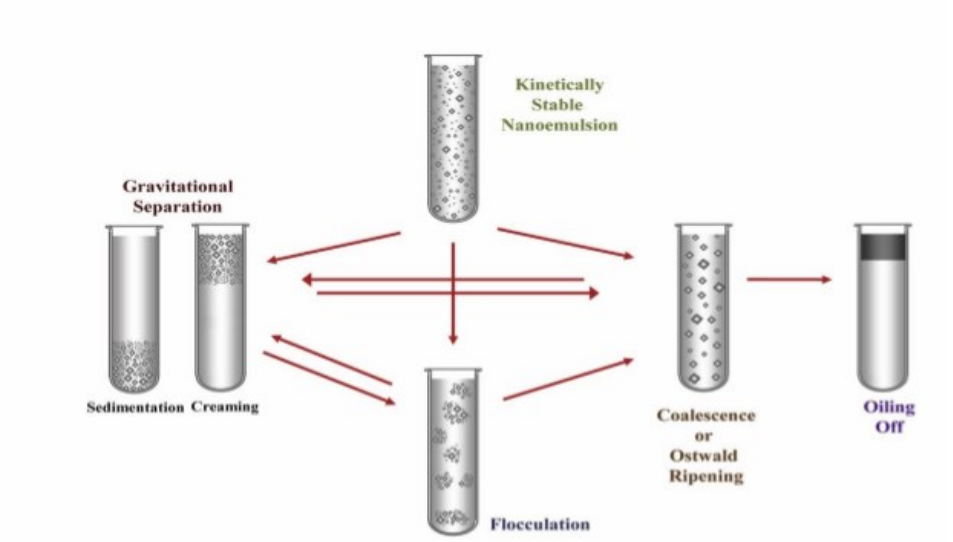

Figure 1.4. Representation of different types of O/W emulsions destabilisation (from Piorkowski & McClements, 2013).

In synthesis, sedimentation/creaming of emulsion droplets is caused by the density difference between the two phases and are forms of gravitational separation. Flocculation phenomenon is based on collision of droplets and the consequent formation of permanent flocs, while coalescence is a process whereby two or more droplets merge together to form a single larger droplet. Ostwald ripening is a diffusive transfer of disperse phase from smaller droplets to larger droplets, which occurs especially in a polydisperse system. The presence of micelle in the continuous phase promotes this mechanism. However, the rate of destabilizations can decrease by having small emulsion droplet size, increasing the viscosity of the disperse medium, lowering the difference in density between the two phases or/and creating an energy barrier at the oil and water interface (Somasundaran et al., 2007). (**Fig. 1.5**).

Creaming is the separation of oil droplets as a layer at the top of an oil-in-water emulsion due to a density contrast between the continuous and dispersed phases (McClements, 2004). Creaming is reversible, i.e. the droplets can be re-dispersed uniformly by gentle mixing (Dickinson and Stainsby, 1988). The rate of creaming in a highly dilute emulsion can be determined using Stokes' law previously reported. According to this physical law, when a particle moves at a constant velocity through a surrounding liquid the force acting on it due to gravity should be equal to the hydrodynamic frictional force acting against it. From this law, it results that creaming rate can be effectively reduced by increasing the aqueous phase viscosity, decreasing droplet size and matching the density of the two phases (Dickinson 1992). However, Stokes' law does not consider the influence of Brownian motion of emulsions droplets, as this may retard creaming for emulsions with droplet size lower than 0.1 μm (McClements, 2004), whereas this is only the case of very fine emulsions, e.g. those used at pharmaceutical industry of in food industry for special applications.

Das & Kinsella (1990) reported that forming a kinetically stable emulsion can be achieved through the addition of surfactants. Surfactants such as monoglycerides and phospholipids are common molecules in foods because they are used to stabilise interfaces. They can be charged or neutral, but in both cases the enthalpy of mixing of their hydrophilic and the hydrophobic parts is very high, which confers their typical amphiphilic nature (Mezzenga *et al.*, 2005). Emulsifiers are surfactants which lower surface tension and prevent droplet flocculation by absorption on the droplet surfaces (Krstonosic *et al.,* 2009).Legislators classify it as generally recognised these compounds as safe (GRAS), and most of them are commonly accepted as natural ingredient by consumers (Bylaite *et al.,* 2001).

Polysaccharides are surfactants employed as thickeners of emulsions which are commonly added to the aqueous phase of oil-in-water emulsions to confer emulsion stability (Quintana et al., 2002). Viscosity modification or gelation in the aqueous continuous phase is the main way of stabilisation (Dickinson, 2003). Xanthan gum has the ability to increase the viscosity of the aqueous continuous phase at relatively low concentrations, therefore it is one of the most employed thickeners to stabilise dispersed oil droplets in food emulsions such as salad dressings and sauces (Hemar et al., 2001).Dickinson (2009) found that at low concentrations of added hydrocolloids (such as xanthan gum) can have a destabilising influence on emulsion

stability due to a mechanism known as depletion flocculation. Hemar et al. (2001) observed that although increases in xanthan gum content caused more extensive flocculation of droplets. Other works confirmed this behaviour, indeed it was reported that when a critical concentration of xanthan gum is present, a destabilising effect from xanthan gum can be prevalent (Traynor, 2013). This information is particularly important for the optimisation of ingredient concentrations and specifically to define the amount of xanthan gum to be added as stabiliser in a food preparation based on emulsions.

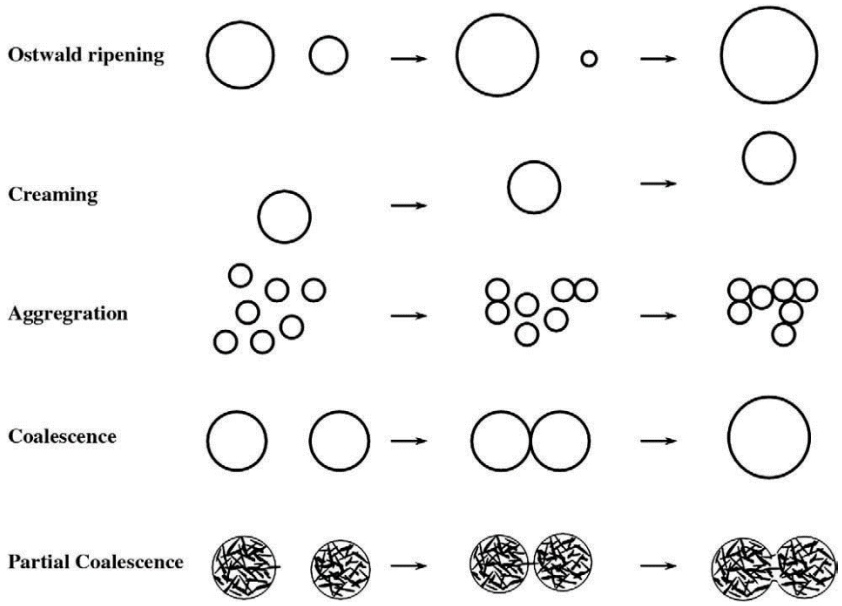

Figure 1.5. Schematic representation of potential instability mechanisms in oil-in-water emulsions, by showing the changes in the droplet characteristics over storage (Walstra, 2003).

About coalescence, further details are given due to the complexity of this phenomenon. There are many factors that can influence partial coalescences such as shear, fat volume fractions, colloidal repulsion and rheology of continuous phase, as reported below.

- Shear: the extent of partial coalescence increases as the collision frequency increases. O/W Emulsions containing fat crystals are destabilised once shear is applied. Shear increases the collision rate and decreases the minimum separation distance between droplets (increase collision efficiency). The shear force also brings droplets closer together, thus it increases the probability of protruding crystals to pierce the film (Darling, 1982; Vanapalli and Coupland, 2001).
- Fat volume fractions: emulsions with a high fat volume fraction are more susceptible to partial coalescence than those with a low fat content. When more crystals are available in the system the possibility of protruding fat crystals piercing adjacent droplet's interface will increase (Dickinson, 1992).
- Colloidal repulsion: colloidal repulsion strongly stabilises emulsions against partial coalescence. Two droplets would easily come close to each other, when the repulsion is weak, which permits fat crystals to penetrate. The magnitude of this repulsion force between droplets depends on the types of emulsifier used to stabilise the oil-in-water emulsions. It has been reported that droplets stabilised with protein are more stable against partial coalescence than those stabilised with small molecular surfactants (Granger et al., 2005; Ojijo et al., 2004).
- Rheology of continuous phase: as the viscosity of the continuous phase increases the collision frequency of the droplets decreases, thus decreasing the rate of partial coalescence. The presence of polysaccharides (Xanthan gum, guar gum and locust bean gum) in the system can be an effective approach to increase the partial coalescence stability by providing a sufficiently thick continuous phase and/or by supplying a protective coating around the globule (Fredrick et al., 2010).

Study of emulsion instability through rheology

The word rheology comes from the Greek words which carry the meaning of flow for "rheo" and "-ology" for study the flow behaviour of materials, including food products. According to the response of silk threads and elastic fluid, foods were classified as typical viscoelastic ones and non-Newtonian materials. Rheology can give a good indication of the behaviour of a material, and is widely used as a tool to test the texture and flow behaviour of industrial products especially in the processing industries such as foods.

Flow properties of commercial emulsions are very relevant for the industry and this parameter should be therefore assessed. The dependence of the viscosity to the shear rate indicates that the emulsions have typical non-Newtonian in behaviour. A viscoelastic material will recover after being subjected to small deformation showing the elasticity of the material, while a non-Newtonian material has a time and stress dependent viscosity providing information on the continuous flowability (Wei, 2009).

Is it known that oil concentration in an emulsion is another factor affecting the flow behaviour of the system (Chanamai and McClements, 2000; Akhtar et al., 2005). There is also an effect of lower droplet size on the rheological behaviour. In fact, lowering droplet dimensions increases the total droplet surface area and therefore the strength of the attractive force. For this reason, greater stress is required to initiate flow resulting in high viscosity and yield stress. The shear thinning region begins right after the yield stress of the emulsions is exceeded where the viscosity of the emulsion decreased with shear rate is observed (**Fig. 1.6**).

Emulsions steady state behaviour is important in order to monitor the texture of the product, and also link it to the consumer acceptability of the final products. The flow properties of emulsions can provide information on product processing, handling, storage and mechanical behaviour. Viscosity is the preferred parameter for the chemists especially those with cosmetic, foods, and pharmaceutical industries to evaluate the emulsion stability. The interdroplet forces are an important factor influencing the viscosity of the emulsions, and in turn it is related to the mean droplet size, polydispersity of droplets, and the presence of additives such as thickener and/or polysaccharides, the surfactant concentration, oil concentration and the age of the emulsions can influence the viscosity of emulsion (Radford, 2004; Sun and Gunasekaran, 2010). The viscoelastic spectrum can be divided into five regions, which are the terminal, transitions, plateau and glassy regions. As the frequency increases, the magnitude of G' increases and become dominant against the G", which is the plateau zone. Before this plateau zone, there is a transition zone where the two G' and G" curves crossover. This crossover point represented the relaxation time of the tested sample.

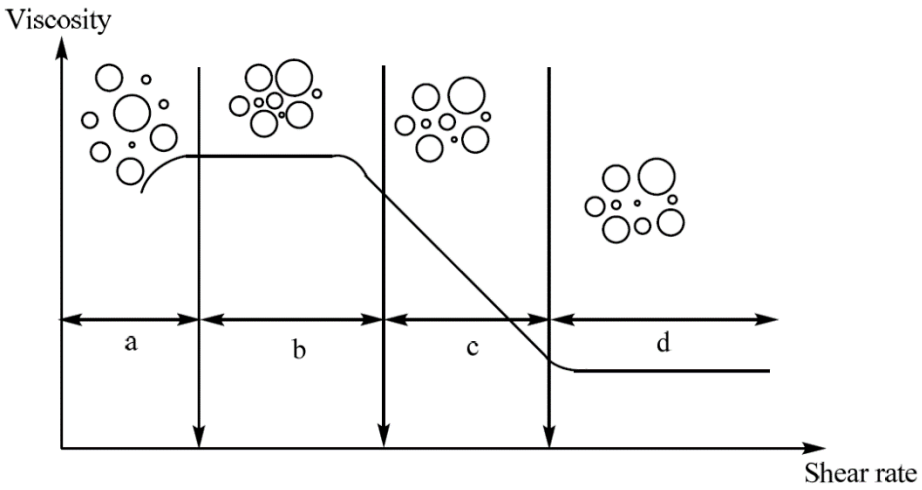

Figure 1.6. Schematic representation of the structural change when shear is applied to O/W emulsions. (a) Shear thickening region, (b) First Newtonian region, (c) shear thinning region, and (d) Second Newtonian region (Pal, 1996).

Usually the first and indicative analysis after the creaming rate observation is the viscosity measurement, possibly in combination with the analysis of mean droplet size and/or droplet size distribution (McClements, 2004).

Surfactants and hydrocolloids used a stabilisers: polysaccharides and proteins

Surfactant are amphiphilic molecules that contain both the hydrophilic and hydrophobic parts, and for this molecular structure they are able to reduce the surface or interfacial tension (Quintana *et al*., 2002).Polysaccharides (e.g. gum Arabic, modified starches) and proteins (e.g. caseins, whey protein concentrate, gelatin, egg protein) are the two most important classes of biopolymers used in food emulsions. Several natural hydrocolloids are commonly applied at food industry level to improve the stability or to give other functional properties to the final food product. Hydrocolloids such as gum Arabic, acacia gum, maltodextrins and xanthan gum are able to form viscous solutions, emulsify suspensions and bind with other macromolecules (Dickinson, 2003).

Xanthan gum has the ability to increase the viscosity of the aqueous continuous phase at relatively low concentrations, therefore it is one of the most employed thickeners to stabilise dispersed oil droplets in food emulsions (Hemar *et al.*, 2001). Xanthan gum is a bacterial polysaccharide from *Xanthonomas campestris*, with a complex structure, varying in its acetylation and puyruvylation. Its main structure is a cellulose (poly β(1→4) glyucopyranose) backbone with β(3→1) D-mannopyranose, β(2→1) D-glucuronic acid, and β(4→1) D-mannopyranose trisaccharide branches.

Proteins are of particular interest in terms of their emulsifying properties due to their amphiphilic nature (Foegeding & Davis, 2011), whereas they tend to diffuse at a much slower rate than other hydrocolloids (McClements, 2005). Emulsion capacity of proteins tends to be lower than with small molecular weight molecules (Surh *et al.*, 2006), however it depends on their physicochemical properties which play an important role in determining their emulsifying abilities (Papalamprou *et al.*, 2010). Once the viscoelastic film is formed, droplets can assume a negative or positive charge depending on whether the emulsion pH is above or below the protein's isoelectric point (and low ionic strength), respectively. High electrostatic repulsion between oil droplets tends to lead to greater emulsion stability, whereas under pH conditions close to the protein's isoelectric point droplet flocculation/aggregation dominates, with consequent coalescence and instability phemomena (McClements, 2005). The presence of protein within the continuous phase is also known to have slight effects on emulsion viscosity, reducing the mobility and diffusing of oil droplets within the emulsion (Jafari *et al.*, 2012), as they tend to cover the droplets surface (**Fig. 1.7**).

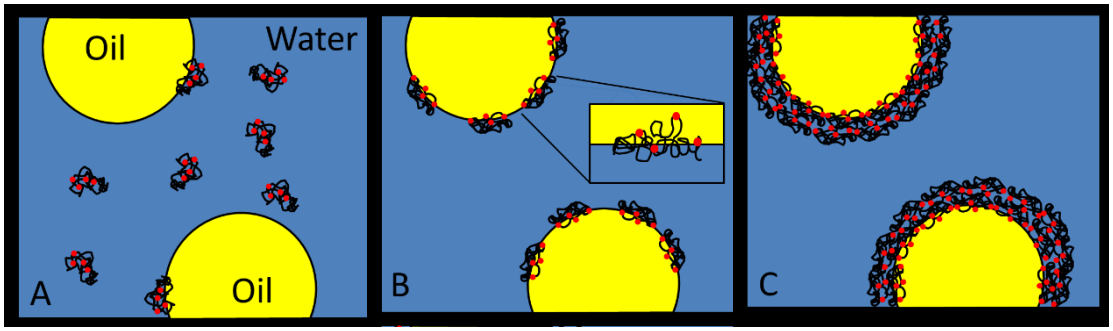

Figure 1.7. Scheme of globular proteins migrating to the water-oil interface (A) followed by reorientation (B) and viscoelastic film formation (C). The red dots represent hydrophobic moieties found in protein (Ram, 2014).

Effect of polysaccharides on protein-stabilized emulsions

The addition of anionic polysaccharides to protein-stabilized emulsions has been reported to have either positive or negative effects on emulsion stability. The level of protein-polysaccharide interactions depend on a large number of factors, e.g. biopolymer characteristics such as size, conformation, type and distribution of reactive sites, the solvent conditions and especially the pH, salts concentration and temperature, as well as the emulsion preparation method (Neirynck *et al.*, 2004; Padala *et al.*, 2009).

Polysaccharides can be electrostatically associated with proteins forming a coacervate (Liu *et al.*, 2009), and the emulsions made with mixtures of protein and polysaccharide can be less susceptible to destabilization by flocculation compared to those formed by multi-layer deposition (Jourdain *et al.*, 2008).

Polysaccharides can coat the proteins to prevent protein-protein interactions between two droplets, e.g. in O/W emulsions made using soy protein isolate (Tran & Rousseau, 2013). In some cases, the polysaccharide-protein interaction can lead to significant effects on texture, for example the different behaviours reported for locust bean gum, guar gum, carboxymethyl cellulose and xanthan gum on the shear thinning behaviour (Hayati *et al*., 2009). Due to the electrostatic nature of this complexation, emulsions stabilized by protein-polysaccharides are sensitive to changes in pH and ionic strength (Jourdain *et al*., 2008). For instance, bridging flocculation of a polysaccharide molecule electrostatically complexes to a protein-film surround more than one oil droplet leading to aggregation (**Fig 1.8**) (Blijdenstein *et al.*, 2004). Depletion flocculation can also occur where a non-adsorbing polysaccharide in solution causes flocculation, when the polysaccharide concentration is above a critical value (Chanamai & McClements, 2006). Depletion flocculation occurs when both the protein and polysaccharide are similarly charged and is characterized by an increased stability below and decreased stability above the respective critical concentration (Chanamai & McClements, 2006).

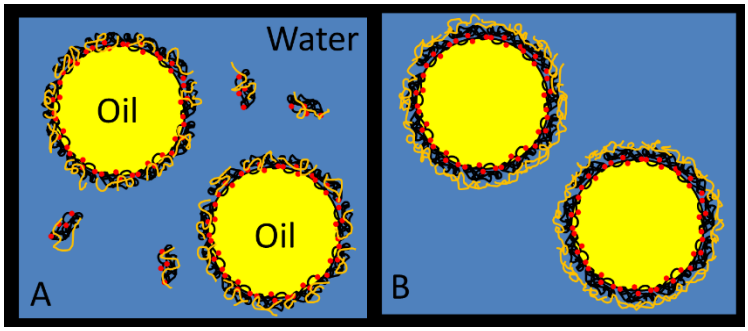

Figure 1.8. Representations of (A) homogenous mixed polysaccharide-protein emulsion and (B) emulsion formed using a layer-by-layer deposition technique. The red dots represent hydrophobic moieties found in proteins whereas orange strands represent polysaccharides (Ram, 2014).

In fact, emulsions are micellar systems and several theories have been proposed to describe their behaviour. It is known that during emulsion preparation, energy is applied to break down the oil layer into small oil droplets, which increases the oil-water interfacial area and therefore more surfactant monomers are required to stabilize these oil droplets against destabilization process. The amount of surfactant used in emulsification has to be well above the critical point, in order to maintain the equilibrium between micelles and monomers in the interfacial layer and bulk phase (**Fig. 1.9**). As a result, the oil droplets are not well covered by the surfactant, increasing the interfacial tension and eventually the oil droplet size increases (Chanamai and McClements, 2002).

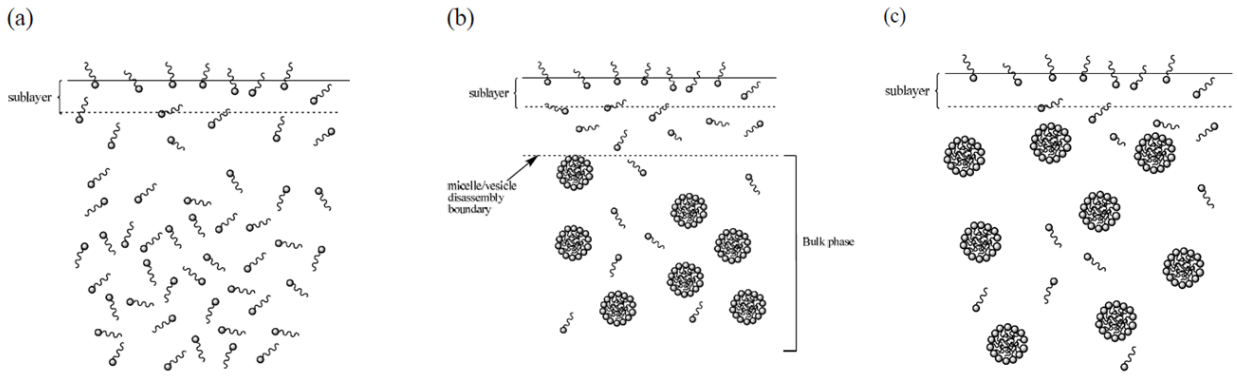

Figure 1.9. Schematic illustration of the effect of bulk concentration to micellisation process. (a) Surfactant concentration below a critical concentration point, with very dilute solution where the kinetic diffusion of the monomer is fairly important and the diffusion rate is proportional to the concentration gradient. b) The micelle concentration is small, a dissemble boundary is found, where there were two potential regimes, the micelle zone and the micelle free zone. (c) Bulk concentration is above the critical point, a large diffusion flux drives the micelle directly to the sublayer (Charoen et al., 2011).

Proteins are used in a broad range of food products as emulsifier agents in order to improve the emulsion stability and the nutritional value of the product (**Table 1.2**). Proteins can be absorbed to the surfaces of oil droplets if they are soluble, otherwise they tend to form large aggregates.The formation of an interfacial layer is achieved in three steps. Initially, soluble protein diffuses at the droplet interface during the emulsification process. As whey proteins are smaller than other proteins, they diffuse quickly to the interface. Once the protein is absorbed, its structure alters in a way that hydrophobic amino acids are in contact with the oil-water interface (McClements, 2005; McClements, 2004; Dickinson, 2009).

Milk proteins are widely used in the food industry due to their functional properties, such as emulsification, thickening, gelling and flavour binding (**Table 1.2**). Milk proteins can be classified into two main groups: caseins, which consist of αs1-casein, αs2-casein, β-casein and κ-casein; and whey proteins β-lactoglobulin, bovine serum albumin, α-lactalbumin and immunoglobulins (Singh, 2011). Caseins are known for their great emulsifying properties due to their hydrophilic and hydrophobic characteristics. Amongst the four major fractions of casein, β-casein is the most effective emulsifier as it can lower the interfacial tension more significantly than the other caseins, and they are also insensensitive to heat and stable against thermal denaturation due to their structure.

Whey protein isolate (WPI) are economically important by-products of the cheese manufacturing process, used as an ingredient by the food industry because of its high nutritional value and functional attributes. Whey proteins comprise of a mixture of proteins, dominated by β-lactoglobulin and α-lactalbumin with minor amounts of proteose-peptones, serum albumin and immunoglobulins (Walstra & Jenness). WPI has high solubility and has good foaming, gelling and emulsifying properties. The relationship between WPI structure and its functional properties has been widely studied in literature, also in relation to their aggregation in emulsions (*e.g.*, hydrophobic interactions, hydrogen bonding, electrostatic interactions and thiol-disulfide exchange reactions) (O'Kennedy, 2007). These interactions are dependent upon the physicochemical properties of the WPI, but they can also change with the temperature and pH (Hussain *et al.*, 2012). The use of WPI in foods is wide, as they are used in infant formulas, sports nutrition foods, meats, seafood, bakery, confections, snack foods, beverages and other food products, to improve their functional properties (Walzem *et al.* 2002).

Table 1.2. Review of the plant and animal proteins used as emulsifiers (adapted from Mao, 2013).

Protein	Factors Investigated	Measuring Parameters	Reference
Soy/Wheat Gluten	Protein-type, pH, temperature, alteration to pH post-emulsification	Rheology, droplet size	Bengoechea *et al.*, 2010
Wheat (gliadins)	Hydrolysis, pH, ionic strength	Surface hydrophobicity, emulsion activity index, emulsion stability index, foam capacity, foam drainage	Agyare *et al.*, 2009
Wheat (Gluten), BSA, Casein	Hydrolysis, ionic strength, pH	Foam stability, foam capacity, creaming kinetics, droplet size, resistance to coalescence	Popineau *et al.*, 1999
Soy and maize germ protein	Protein type (soy isolate vs. flour vs. concentrate), pH, temperature	Emulsifying capacity, emulsion stability	Wang & Zayas, 1992
Soy	Temperature	Protein aggregate size, droplet size, calorimetry, interfacial composition, emulsion viscosity, TEM	Keerati-u-rai & Corredig, 2009
Egg	Protein-type (egg yolk vs. egg white), addition of xanthan gum, storage time	Droplet size, creaming test, optical microscopy, emulsion viscosity, SDS-PAGE	Drakos & Kiosseoglou, 2006
Egg	Addition of polysaccharide, pH	Surface charge, protein adsorption, droplet size	Padala *et al.*, 2009
Milk (whey)	Oil concentration, protein concentration, emulsion viscosity, addition of salt, heat treatments	Accelerated creaming test, emulsion viscosity, droplet size	Djordjevic *et al.*, 2004
Milk	Protein-type (casein vs. whey), pH modification post-emulsification, addition of salt, heat treatment	Droplet size	Hunt & Dalgleish, 1995
Milk (caseinate)	Preparation method (single step vs. two-step), addition of	Creaming stability, emulsion activity index, droplet size	Einhorn-Stoll *et al.*, 2002

	surfactant		
Pea, Fava Bean, Cowpea, French Bean, & Soybean	Protein type, protein concentration, ionic strength, comparison of subunits	Thermal stability, SDS-Page, hydrophobicity, protein solubility, emulsion stability	Kimura *et al.*, 2008
Soy, Pea, Lentil, Chickpea, & Faba bean	Protein-type, extraction (isoelectric vs. salt extraction)	Surface charge, surface hydrophobicity, protein solubility, interfacial tension, emulsion capacity, emulsion activity index, emulsion stability index, droplet size, creaming stability	Can Karaca et al., 2011a
Milk (whey), Canola & flaxseed	Protein-type, extraction (isoelectric vs. salt extraction)	Emulsion capacity, emulsion stability, creaming stability, emulsion activity index, surface charge, surface hydrophobicity, interfacial tension, protein solubility, droplet size	Can Karaca et al., 2011b
Gelatin	Hydrolysis, oil polarity, addition of surfactant, pH	Surface tension, interfacial tension, surface charge, emulsion stability	Olijve et al., 2001
Gelatin and Milk (whey)	Protein content (alone and in mixtures), pH, protein concentration	Surface charge, droplet size, surface protein concentration, surface protein content, emulsion stability, emulsion rheology, optical microscopy, oil oxidative stability	Taherian et al., 2011

Polyphenols interactions in food systems and protein-polyphenols binding

Polyphenols are composed of either a single phenolic ring, or a more complex two- or three-ring structure which is substituted with components such as hydroxyl and methyl groups, sugars, carboxylic acids or other chemical moieties. These structural modifications result in differences in their biological functionality. The presence of complex mixtures of phenolics within a plant extract is of functional significance: bioactivity of phytochemicals is often the result of synergic interactions within a mixture rather than the action of a pure compound. Polyphenols can form hydrophobic interaction complexes with other phytochemicals in food systems. As the phenolic ring is a proton donor, these complexes are stabilised by hydrogen bonds. Complexation masks reactive groups on the anthocyanin molecule and increases its stability (Haslam et al., 2000).

Polyphenolic molecules can also form oligomers and polymers constructed from one type of phenolic ring or from mixed phenolic types, to give procyanidins and tannins. A significant additional component of polysaccharide, such as pectin or arabinogalactan, stabilises the polyphenol-protein complex and prevents aggregation and precipitation. The reactivity of phenolics and polyphenolics is due to the formation of highly reactive quinone species from hydroxyl groups that are adjacent (*ortho*-quinones), or opposite each other (*para*-quinones) on the phenolic ring. Quinones on phenolic rings react to form permanent covalent bonds with compounds such as anthocyanins, proteins, polysaccharides, sulphur-containing compounds and reducing agents such as ascorbic acid, depending on the reaction conditions. They can also act as oxidising agents, promoting further reactions with other compounds. These types of reactions are important effectors of quality during the processing of fruit and vegetable products: e.g. enzymatic browning during fruit product manufacture; condensation of grape phenolics with sulphite or anthocyanins resulting in colour changes on gradual oxidation of wine during maturation. During the early stages of fruit and vegetable processing, oxidative action of endogenous polyphenoloxidases catalyses rapid formation of quinones from phenolics. This leads to browning of the material as the quinones react further. These types of compounds can also be involved in complex multi-component interactions, e.g. grape phenolics and anthocyanins interact together with other components in wine such as polysaccharides and sulphite (Schwarz et al., 2003).

Interactions between proteins and polyphenols can be reversible associations (via hydrogenbonding, hydrophobic interactions and van der Waals forces), or can be permanent modifications of proteins. Reversible associations may or may not result in protein precipitation, dependent on factors such as ionic composition of solution and pH. Both simple phenolic compounds and higher polymeric polyphenols can react to give permanent modification of proteins. Interaction of proteins with phenolics is enhanced at a pH close to the pI of the protein. Complexation of tannin with proteins is achieved via a different mechanism to that of *o*-quinone- mediated covalent bond formation. Particularly susceptible to interactions with tannins are proline-rich proteins with an open structure, e.g. proline-rich salivary proteins or gelatin. Caseins have a considerable amount of proline, but also human saliva has been reported to have considerably high fractions

of proline-rich proteins, which was taken into consideration in the Chapter 5 (Beidler, 2995; Bennick, 2002). An initial hydrophobic attraction between proline and the phenolic group is stabilised by H-bond formation between phenolic ring groups and the bis-alkyl substituted amide nitrogen of the proline imino group. The interaction is a complex one, with water also likely to play a part (Luck et al. 1994).

It has been reported that when milk is added to black tea, which contains polyphenols (catechins) responsible for the reddish colour and astringency, this astringency is reduced due to the formation of a complex between black tea polyphenols and α- and β-casein of milk (Siebert et al., 1996). Precipitation of tannin-protein complexes can be enhanced by reducing the amount of free water for solvation by salting out, or by addition of phytochemicals such as saponins. Protein aggregation is reduced in the presence of bile salts, caseins and sodium caseinate (competitively bind polyphenolics to form soluble complexes), or specific polyelectrolyte polysaccharides (trap hydrophobic protein-polyphenol complexes in a hydrophobic gel and limit aggregation) (Haslam et al., 2000). Covalent binding to proteins may not affect the functionality of polyphenolics if they are sufficiently large, as in the case of epicatechin: being this molecule relatively large, its antioxidant activity is maintained also after complexation with bovine serum albumin or gelatin. Caseins were reported as the most important contributor to the free radical scavenging capacity in low-fat milk, and that the capacity increased with increasing fat content of the milk (Chen et al., 2003). These authors suggested the effect from fat was due to the interference of lipids and reactivity of lipid-soluble antioxidants and fat-globule membrane proteins.

The ability of some phenolic compounds under specific conditions to act as radical sinks makes them antioxidants, both in the aqueous phase and in lipophilic systems such as oils. Interaction with ions such as Fe^{3+}, Al^{3+} and Cu^{2+} also contribute to the antioxidant activity. Protection against oxidative processes in foods needs to encompass both aqueous and hydrophobic environments. Many plant phytochemicals have the capacity to prevent or neutralise negative oxidative reactions in foods via a range of mechanisms. There are also antioxidant phytochemicals having lipophilic, ampiphilic and hydrophobic solubilities. These have some or all of the following features: (a) can inhibit chain-breaking functions or decompose hydroperoxides to non-radical species; (b) can readily accept electrons/be oxidised in preference to other substrates or accept charge from oxidised compounds (e.g. lipids); (c) have metal-chelating function; (d) are readily reactivated after oxidation. Antioxidative phytochemicals include carotenoids, phenolic compounds, protease inhibitors, sulfides, and phytoestrogens (Tomás-Barberán and Espín, 2001). Phenolic phytochemicals are abundant micronutrients in fruits and vegetables, and there are several research papers reporting evidencies on their beneficial role in the health related to plant foods. Natural antioxidants from plant sources include tocopherols, olive and olive oil compounds and phospholipids (oils and oilseeds); lignin-derived compounds from rice bran and oats; ascorbic acid, hydroxycarboxylic acid derivatives (which act as metal chelators and help neutralize hydroperoxides), phenolic and terpene compounds, flavonoids and carotenoids from fruits, vegetables and herbs. The thermodynamic properties of free radicals was used by Buettner (1993) to predict the order of antioxidant ability. Breakdown products of polyphenolics can be active in their own right as antioxidants, e.g. protocatechuic acid, a breakdown product of quercetin, is an effective antioxidant. Therefore processing of a food product may not necessarily decrease overall antioxidant capacity. Also, non-phenolic components of plants produced by heat breakdown of acid citrate, tartrate and malate have antioxidant activity, as do Maillard products formed when plant sugars react with protein on heating.

The interactions between proteins and phenolic compounds have received considerable scientific attention in recent years. Interest in phenolic compounds is related to their dual role as substrates for oxidative browning reactions and as antioxidants, underlining their impact on organoleptic and nutritional qualities of fruits and vegetables; their role in plant growth and metabolism, and their physiological activity in humans (Rawel *et al.* 2007). As also reported in previous paragraphs, phenolic compounds can interact with proteins in many ways: with food proteins during food processing; with storage and physiological active proteins in the plant; with food proteins or enzymes in the course of digestion in the gastrointestinal tract; with blood plasma proteins; and, finally, with proteins in tissues of the human body (Ali, 2013).

The interactions between phenolic compounds and proteins play a role in haze formation in several foods and beverages. Emulsifying properties of proteins can change after the addition of phenolic compounds, it was in fact reported a lower stability and higher oil droplet size in presence of some phenolic compounds (Aewsiri *et al.* 2009). These interactions have potentials as new emerging techniques, for example the application of hetero-cross-linking to improve the quality of bread, emulsions or foams in food processing (Ali, 2013).

Lipid Oxidation and emulsions chemical stability

Fatty acids can be classified according to their chain length, carbon bond and the presence of essential and nonessential fatty acids. Addition of fat as ingredient in other foods is not a straightforward task as some of them can easily undergo lipid oxidation. This results in food products that become chemically unstable and lose their appearance, taste, texture, shelf-life and nutritional profile (McClements and Decker, 2002). Lipid oxidation can normally occur through different mechanism such as autooxidation, photo-oxidation, thermal oxidation and enzymatic oxidation. Auto-oxidation is the most common process in food products, and it involves a free radical chain reaction with the three following following steps:

- Initiation (formation of free radicals): free radicals (R°) known as the alkyl radical are formed when hydrogen atom is removed from unsaturated lipids (RH) in the presence of initiators, such as transition metals;
- Propagation (free-radical chain reaction): alkyl radical reacts rapidly with the oxygen to form peroxyl radicals (ROO°), which can abstract a hydrogen atom from another adjacent unsaturated fatty acid, leading to a lipid hydroperoxide (ROOH) and other radicals;
- Termination (formation of non-radical species): the free radicals begin to react with each other to form a more stable, non-radical product.

Although hydroperoxides are the primary product of lipid oxidation, it is not the main cause of off-flavour and rancid flavour appearance of food products, as they usually do not have sensory impact. The lipid hydroperoxides decompose via β-scission reaction to alkoxyl radicals. Alkoxyl radicals then tend to decompose into a wide variety of volatile, low molecules weight components known as secondary lipid oxidation products, such as aldehydes, ketones, alcohols, and hydrocarbons. These secondary products are responsible for off-odour and off-flavour (Frankel, 1987; McClements, 2008; McClements and Decker, 2000). Several parameters influence the stability of the lipids toward oxidation, briefly reported as follows:

- Type of lipid (especially the unsaturation rate)
- Storage temperature
- Presence of pro-oxidants (e. g. transition metals)
- Presence of antioxidants

In relation to the latter point, there are several approaches to incorporate either synthesis or natural antioxidants into the food products. Antioxidants can be classified as either primary or secondary antioxidants based on their mechanism of action.

The rate of lipid oxidation in emulsion is influenced by several factors including the molecular structure of lipids, heat, light, physical characteristics of emulsion droplets, processing conditions and the presence of antioxidants or pro-oxidant (transition metals such as, iron) compounds in the system (Kiokias et al., 2009). One of the major mechanisms that causes lipid oxidation in emulsions is the interaction between lipid hydroperoxides - at the droplet surface - and transition metals in the aqueous phase. The breakdown of lipid hydroperoxide into free highly reactive radicals is therefore accelerated, such as alkoxyl and peroxyl radicals, which is the main causes for the off-flavour and off-odour generation in foods (Matthaus, 2010).

Antioxidants have the ability to inhibit mechanisms such as free radical scavenging, that quenches reactive radicals and metal chelating agents which convert transition metals (pro-oxidants) into stable products. Currently, most of antioxidants used in the food industry are synthetic including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ehylenediaminetetraacetic acid (EDTA). In recent years, there has been an increase in the food safety issues surrounding the use of synthetic antioxidants, and therefore for a consumers' preferences there is a need to find alternatives as the inclusion of bioactive ingredients can have several health benefits (Matthaus, 2010).

Many researchers have improved the lipid oxidation stability of oil-in-water emulsions by using different types of emulsifiers to form multi-layered membranes around the oil droplets (Klinkesorn et al., 2005) However, this technique has some limitations, mainly the high tendency for the droplets to aggregate and flocculate when preparing samples (Guzey and McClements, 2006).As an example, Diaz et al. (2003) reported the use of caseinophosphopeptides as natural antioxidants in O/W emulsions and they found that in oil-in-water emulsions the iron binding by phosphoseryl residues would most likely inhibit iron promoted lipid oxidation, while more acidic pH can prevent metal binding and therefore reduce antioxidant activity.

Casein hydrolysates were more effective inhibitors of lipid oxidation than the enriched caseinophosphopeptides at equal phosphorus content. Thus, antioxidant properties might not be uniquely attributed to chelating metals by phosphoseryl residues but also by scavenging free radicals. They also suggest the use of casein and less purified sources of phosphopeptides also inhibited lipid oxidation (**Fig. 1.10**) (Diaz et al., 2003).

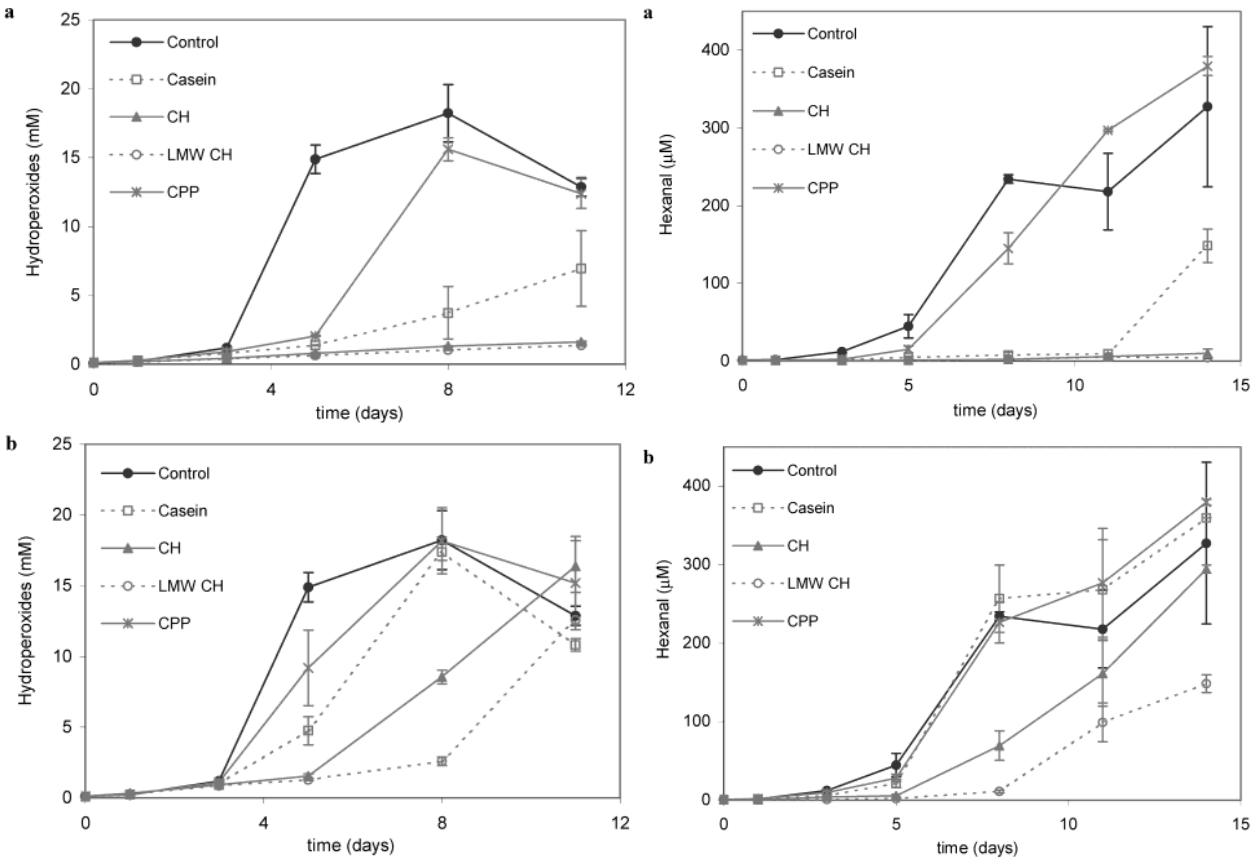

Figure 1.10. Lipid hydroperoxide (left column) and hexanal (right column) formation in maize oil-in-water emulsions at pH 7.0 stored at 37 °C in the presence of casein, casein hydrolysates (CH), low molecular weight casein hydrolysates (LMW CH), or enriched caseinophosphopeptides (CPP) at a concentration of (**a**) 78 µM or (**b**) 15 µM (Diaz et al., 2003).

The effect of several phenolic compounds, mainly catechins, was reported by Di Mattia et al. (2010), and they found that the effect of pure polyphenols is not straighfoward toward the production of primary and secondary oxidation products in model O/W emulsions (**Fig. 1.11**). Each phenolic compound also shown pecularities in terms of its power toward lipix oxidation.

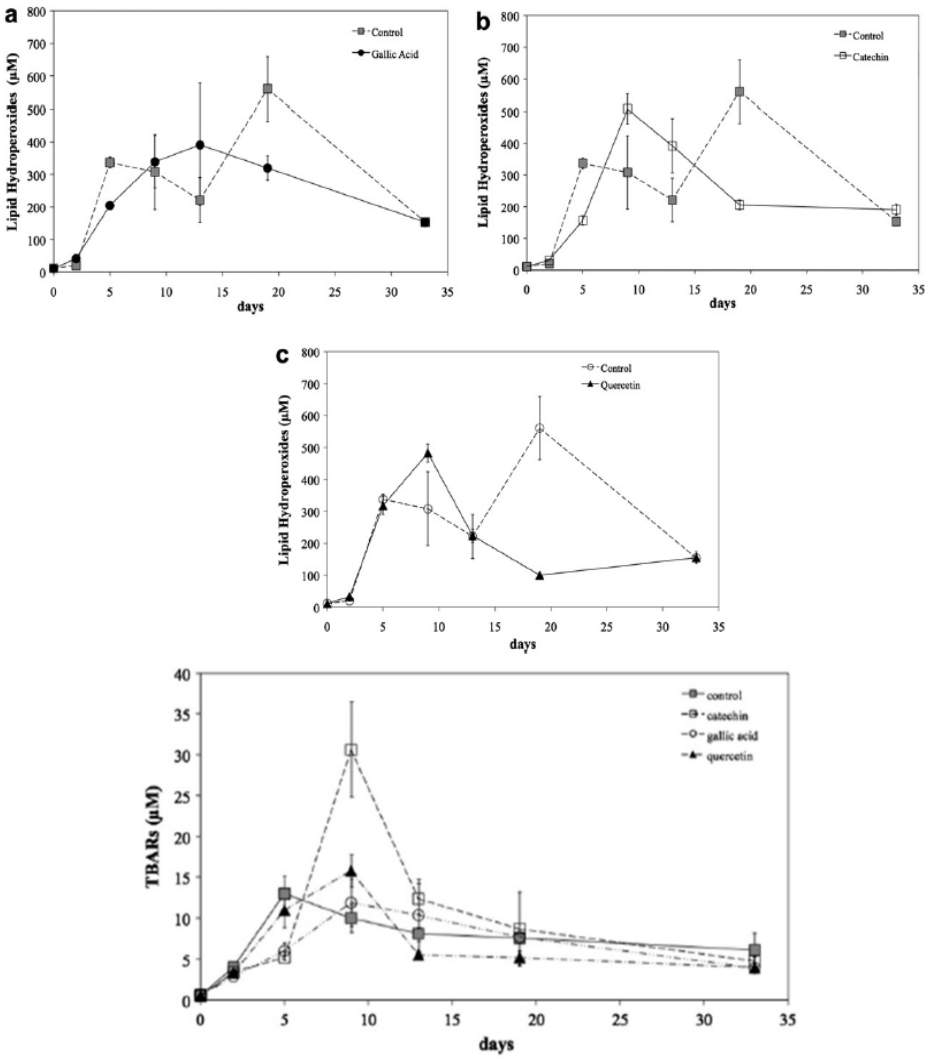

Fig. 1.11. Lipid hydroperoxides and TBARS formation over storage time of emulsions with added gallic acid (a), catechin (b) and quercetin (c) at 10^{-4} M.

Measurement of Lipid Oxidation

Primary Lipid Oxidation Products. The most common method used to measure the hydroperoxide formation in the food emulsion is the *peroxide value* (PV). Several measurement techniques are applied to measure PV, e.g. idometric titration and ferric thiocyanate methods. For measuring PV value based on idometric titration method, potassium iodide is used to react with hydroperoxides. The concentration of the peroxide in the system is related to the amount of released iodine. However, this method has several disadvantages such as time consuming, requires a large amount of sample and lack of sensitivity. The ferric thiocyanate method is an alternative approach to determine peroxide values. This method is based on the oxidation of ferrous iron (Fe^{2+}) to ferric ions (Fe^{3+}) when is in contact with lipid hydroperoxides, which is determined colorimetrically as ferric thiocyanate. Initially, hydroperoxide react with Fe^{2+} to produce Fe^{3+}. Ferric ions then react with thiocyanate (as a reactant) to form ferric thiocyanate (red-violate complex) which can be determined spectrometrically. This method requires small amounts of sample, is more sensitive and less time consuming than idometric titration method (Frankel, 1987).

Secondary Lipid Oxidation Products. Carbonyl compounds such as aldehydes are among the main secondary lipid oxidation products. The *p*-Anisidine value test can be used to determine the content of α and β- unsaturated aldehydes produced during the decomposition of hydroperoxides during oxidation. The thiobarbituric acid (TBA) assay is alternative method to determine degree of secondary oxidation products. During lipid oxidation, malonaldehyde, a minor component of fatty acids is formed as a result of the degradation of polyunsaturated fatty acids.In this assay, one mole of malonaldehyde is reacted with two

moles of thiobarbituric acid (TBA) to form a pink malonaldehyde-TBA complex that is measured spectrophotometrically at its absorption maximum at 530–535 nm. Many other substances may react with the TBA reagent and contribute to absorption, causing an overestimation of the intensity of colour complex. Furthermore, the presence of barbituric acid impurities in the TBA reagent may produce TBA-malonaldehyde -barbituric acid and malonaldehyde -barbituric acid adducts that absorb at 513 nm and 490 nm, respectively.

***Primary Antioxidants*:** primary antioxidants or "chain breaking" antioxidants can hinder lipid oxidation by delaying the initiation step or interfering with the propagation step. Primary antioxidants are able to react with lipid radicals (peroxyl and alkoxyl radicals) by rapidly donating a hydrogen atom to a lipid radical and subsequently converting them to more stable radicals. Thus, the mechanism of primary antioxidants is based on scavenging free radicals.The effectiveness of these antioxidants is dependent on their physical location in the system and their chemical properties. Commonly, antioxidants can be physically classified into hydrophilic (polar) and lipophillic (non-polar) group. According to the "polar paradox" theory polar antioxidants are more effective in bulk oils as they accumulate at the air-oil interface (where lipid oxidation is most dominant). However, non-polar antioxidants are more effective in emulsion because they concentrate on the oil-water interface where interactions between hydroperoxides at the droplet surface and pro-oxidants originating in the aqueous phase take place. Thus, based on this phenomenon, antioxidants that are effective at hindering lipid oxidation in emulsified oils may not be as effective in the bulk oil. It has been shown that non-polar α-tocopherol and propyl gallate were more efficient antioxidants in an oil-in-water emulsion compared to bulk oil, whereas polar Trolox and gallic acid showed the opposite behaviour (Frankel et al., 1987). The most efficient free radical scavengers which can enhance oxidative stability are compounds that contain hydroxyl (phenolics), sulfhydryl (cysteine) and amino groups (proteins).

Secondary Antioxidant: chelators are secondary antioxidants components that can inhibit lipid oxidation by various mechanisms including prevention of metal redox cycling, formation of insoluble metal complexes and steric hindrance of metal-lipid interactions. It should be noted that none of these mechanisms convert free radicals to more stable products (McClements and Decker, 2000; Chaiyasit et al., 2007).

One potential approach to enhance the lipid oxidation stability is to inhibit prooxidants ability by using metal chelators to alter physical location of pro-oxidant in the continuous phase (prevent pro-oxidant from the close contact with the droplet surface). The most common food chelators are phosphoric acid and ethylenediaminetetraacetic acid (EDTA). EDTA is capable of reducing lipid oxidation rate by removing the transition metals from the surface of oil droplets. It has been demonstrated that EDTA reduces the rate of lipid oxidation in corn oil-in-water emulsions at both pH 3 and 7 conditions (Cho et al., 2003). It it important to highlight that the concentration of EDTA in the system plays an important role on its antioxidant performance.

The demand for using 'natural' antioxidants has increased due to the potential hazardous effects of synthetic antioxidants (Waraho et al., 2010). The chelating ability of different types of protein (whey protein isolate, soy protein isolate and sodium caseinate) was investigated by several authors, also in emulsions. For example, it is known that sodium caseinate has the greatest ironbinding ability due to its phosphorylated serine residues and whey protein isolate is less effective at chelating iron (Faraji *et al*., 2004). In addition to proteins, polysaccharides have also been found to enhance the oxidative stability through chelating pro-oxidants and increasing the viscosity of continuous phase (Chen et al., 2010). For instance, xanthan gum was found to chelate metal ions through its negatively charged pyruvate sites (Matsumura et al., 2003).

Effect of emulsion ingredients on Lipid Oxidation in Oil-in-Water Emulsion

In oil-in-water emulsions, lipid droplets are dispersed in an aqueous phase. The transition metals (pro-oxidants) such as iron or copper are mostly located in the aqueous phase, whereas hydroperoxide can be found at O/W interface, as they are surface-active compounds (**Fig. 1.12**). Lipid oxidation takes place at the droplet interface, where the pro-oxidant in the continuous are in contact with the hydroperoxide, and there is a hydroperoxide breakdown into free highly reactive radicals such as alkoxyl and peroxyl radicals (Waraho, 2011).

The interaction of lipid hydroperoxides (ROOH) with iron can produce highly reactive peroxyl (ROO°) and alkoxyl (RO°) radicals which are able to react with another unsaturated lipid or promote β-scission reactions (**Fig. 1.13**). Subsequently, the presence of pro-oxidants compounds in the continuous phase is a major factor in accelerating lipid oxidation (Waraho *et al., 2010;* Kellerby et al., 2006).

Some natural ingredients of emulsions can have protective effects toward lipid oxidation in emuslions (**Table 1.3**), for example some proteins, for which the antioxidant activity has been determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) which measures secondary lipid oxidation products. TBARS also measures other than secondary lipid oxidation products, and therefore other measurements, such as hexanal and propanal, would be more applicable (Díaz et al., 2003; Hu et al., 2004). As stated in the previous chapter, it is of interest to measure both primary (hydroperoxides) and secondary lipid oxidation products, since they both affect significantly to the oxidative stability of foods.

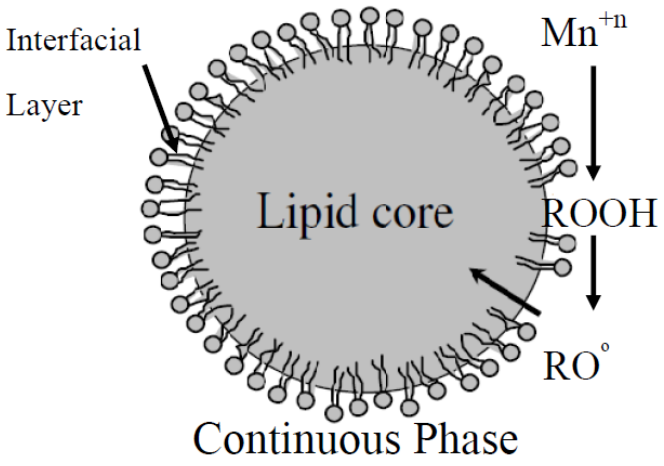

Figure 1.12. Schematic representation of lipid oxidation in oil-in-water emulsions. Mn+ is pro-oxidants in the continuous phase, ROOH is hydroperoxides at droplets interface and RO° is an alkoxyl radical (Decker et al., 2002).

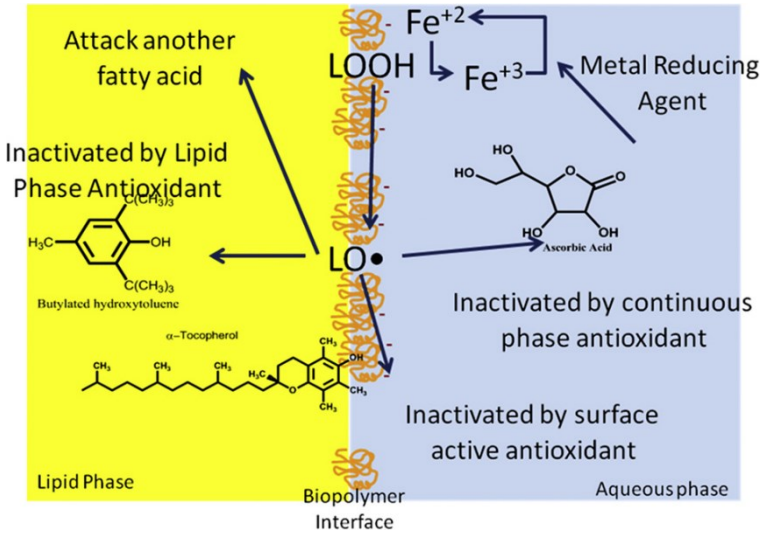

Figure 1.13. Potential reactions schemes for a lipid alkoxyl radical (LO°) in an emulsions (Waraho et al., 2011).

Table 1.3. Scheme of the factors capable of inhibiting lipid oxidation in O/W emulsions (Waraho et al., 2011).

Characteristic	Property	Factors
Lipid Phase	Composition	• Degree of unsaturation • Prooxidant impurities, e.g., free fatty acids, hydroperoxides • Inherent antioxidants, e.g., free radical scavengers and chelators • Added antioxidants e.g., free radical scavengers and chelators
	Physical state – solid fat content and crystal properties	• Solubility, partitioning and diffusion of antioxidants and prooxidants
	Physical properties	• Rheology determines diffusion of antioxidants and prooxidants • Polarity determines partition coefficients
Aqueous Phase	Composition – pH, ionic strength, solutes	• Prooxidant impurities, e.g., transition metals, photosensitizers, enzymes • Inherent antioxidants, e.g., chelators, free radical scavengers • Added antioxidants e.g., chelators, free radical scavengers • Micelles may alter location of antioxidants and prooxidants • Reducing agents that can redox cycle prooxidant metals
	Physical state – ice crystal structure and location	• Solubility, partitioning and diffusion of reactants and products
	Physical properties	• Rheology determines diffusion of antioxidants and prooxidants • Polarity determines partition coefficients
Interfacial Phase	Composition	• Anti-/prooxidant activity • Impurities (hydroperoxides)
	Thickness	• Steric hindrance of interactions between water- and oil-soluble components
	Charge	• Electrostatic attraction/repulsion of antioxidants and prooxidants
	Permeability	• Diffusion of antioxidants and prooxidants in lipid and aqueous phase
Structural Organization	Emulsion	• Droplet concentration • Droplet size distribution (surface area and light scattering)
	Spray dried powder	• Porosity • Exposed lipid levels • Emulsion droplet characteristics upon rehydration
	Hydrogel particles	• Hydrogel composition, structure and properties

The interaction of phenolic compounds with proteins, for example salivary proteins or globular proteins such as whey proteins has been reported to cause lowering in the oxidation rate, in presence of oxidised lipids, with respect to some other proteins such as caseins (Sharma et al., 2002). Protein oxidation process can occur via non-covalent complex formation by both electrostatic and hydrophobic attractions between lipid hydroperoxide (ROOH) and secondary lipid oxidation products.In the covalent interaction one or more secondary lipid oxidation products can react with protein molecules present at the interface of the oil droplets in the oil-in-water emulsion (Leaver et al., 1999). Emulsification itself can promote autoxidation in foods. The reaction between amino acids and secondary lipid oxidation products leads to formation of carbonyl groups in the proteins (Zamora et al., 1999). This interaction can also cause changes of the primary structure of the proteins.

In oil-in-water emulsions, the hydrophobic interactions are in a key role in the adsorption behaviour of protein at the oil interface (Meng et al., 2005).Some antioxidant activity was reported for proteins and amino acids in food models, which was attributed to their ability to (1) form cationic charges on the surface of emulsion droplets; (2) form thick viscoelastic films at the emulsion droplet interfaces, which retard lipid hydroperoxide-transition metal interactions; (3) chelate prooxidative metals; and (4) inactivate free radicals through their sulfhydryl groups. Casein, whey and soy proteins are known to inhibit lipid oxidation in oil-in-water emulsions, whereas the effectiveness can be dramatically different depending on the specific phenolic compound (Hu et al., 2003). For example, being whey proteins surface-active proteins, they inhibit lipid oxidation through preventing prooxidant metal-lipid interactions (Wharao et al., 2010; Wharao et al., 2011).

Thickness of the emulsion interface also affect lipid oxidation rate, but it depends on the specific protein used (Hu et al., 2003). In emulsion, the decrease in lipid oxidation rates may be due to the electrical repulsion of metal ions away from the emulsion droplets, which reduces contact between prooxidants and the oxidation substrate (Hu et al., 2003). However, the inhibition of lipid oxidation by different proteins seems to beconcentration-dependent, as for some proteins at higher concentration, inhibited lipid oxidation more than lower concentrations in O/W emulsion (Hu et al., 2003). Usually protein-stabilised emulsions have droplets with negativecharge at pH above proteins isoelectric point, while they are positively charged at pH below isoelectric point, and it was noticed that negatively charged droplets tend to attract iron and accelerate oxidation (Hu et al., 2003).

Some of these effects on the lipid oxidation status have been linked to the physical localisation of the protein and polyphenols in dispersed systems of emulsions, particularly when a complexation is formed and it might lead to precipitation (**Fig. 1.14**).

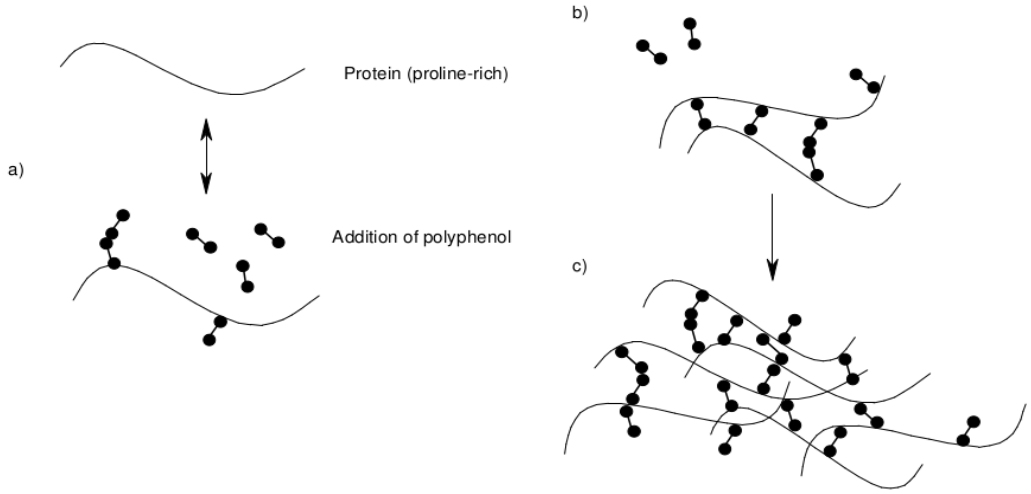

Figure 1.14. Protein-polyphenol interaction leading to the complex formation and precipitation: a) reversible hydrophobic interaction between protein and polyphenol; b) adding more phenolics, peptides are cross-linked and the complex becomes insoluble; c) further aggregation (phase separation) of the insoluble complexes (Charlton et al., 2002).

The covalent interaction between oxidized phenolic compounds and amino acid residues of the proteins allows the formed complexesthat have antioxidants capacity, when they present free phenolic hydroxyl groups in one of the phenolic rings (Almajano and Gordon, 2004; Rohn et al., 2004). There is however a certain masking of the antioxidant activity depending on the type of polyphenols and proteins, and covalent bounding of phenolic compounds which cause cross-linking and polymerization is one of the main cause of the loss of this antioxidant activity. Phenolic compounds may inhibit protein oxidation by binding to the proteins or the formed complexes may act as radical scavengers and radicals.

Different milk proteins are known to inhibit lipid oxidation in liposome model systems, whereas their activity depends on the specific milk protein. The modified amino acids formed in interaction between oxidized lipids and amino acids of proteins may play a significant role in foods, since they are produced naturally during processing and they provide antioxidant activity. In the case of low-fat food products, the fat is usually replaced with protein, and in such systems the oxidation can be focused from the lipid phase to the protein phase (Charlton et al., 2002).

Multiple Regression Models and RSM for the study of emulsion behaviour

Designs for Fitting Second-Order Model

Multiple regression models are needed understand the possible correlation among a set of (independent) variables which are contemporary changed in a system. This statistical approach allows to define whether the observed changes from an experimental dataset vary in dependence on, for instance, the concentration of a particular ingredient or chemical compound, when there is a second factor influencing it. The relationship between a set of independent variables and the response y is determined by a mathematical model called regression model. When there are more than two independent variables the regression model is called multiple-regression model. In general, a multiple-regression model with q independent variable takes the form of

$$y_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \ldots\ldots\ldots + \beta_q x_{iq} + \varepsilon_i \qquad (i = 1, 2, \ldots\ldots, N)$$

$$= \beta_0 + \sum_{j=1}^{q} \beta_j x_{ij} + \varepsilon_i \qquad (j = 1, 2, \ldots\ldots\ldots, q)$$

where $n > q$. The parameter βj measures the expected change in response y per unit increase in xi when the other independent variables are held constant. The i^{th} observation and j^{th} level of independent variable is denoted by x_{ij}.

First-order model is used to describe the flat surfaces, and therefore it is not suitable for analysing maximum, minimum and ridge lines. Other models have to be needed to better describe the behaviour of a particular response in a system, as described as follows.

The orthogonal first-order designs minimize the variance of the regression coefficients. A first-order design is orthogonal whether the off-diagonal elements of the matrix are all zero (Montgomery 2005). When the columns of the matrix are mutually orthogonal, the levels of the corresponding variables are linearly independent. A good estimated regression model can explain the variation (or a considerable part of it) of the dependent variable in the sample. The tests of hypotheses are useful to measure the effectiveness of the model, but in this case the error term should be normally and independently distributed. When the degrees of freedom available for estimating the experimental error is small, the formal F tests conducted in the ANOVA table may not have much power, so that smaller effects will not appear to be significant. Another way to estimate which effects are significant and which are probably just the result of noise or error, is the use of the "Normal Probability Plot of Effects". Once the normality assumption is considered satisfied, therefore the test for the significance of the regression can be applied to determine if the relationship between the dependent variable y and independent variables $x_1, x_2, \ldots, x_q$, exists. The statistic F is compared to the critical F values, and if observed F-value is greater than the critical F, then H_0will be rejected. Equivalently, $H_0$is rejected when P-value for the statistic F is less than significant level a. The so-called "significance of regression" is evaluated by the ratio between the media of the square of regression and the media of the square of residuals and by comparing these variation sources using the Fisher distribution (F test), taking into account the degrees of freedom associated to the regression and residual variances (Box et al., 2005).

Second-order model describes quadratic surfaces, and this kind of surface can take many shapes. Contour plot is a helpful visualization of the surface when the factors are no more than three.

There are many designs available for fitting a second-order model, being the Central Composite Design (CCD) one of the most applied by food scientists. It was proposed by Box and Wilson and consists of factorial point s (from a 2_qdesign and 2_{q-k}fractional factorial design), central points, and axial points. The following is the representation of 2_qaxial points:

x_1	x_2	…	x_q
-a	0	…	0
a	0	…	0
0	-a	…	0
0	a	…	0
.	.	…	.
.	.	…	.
.	.	…	.
0	0	…	-a
0	0	…	a

When a first-order model shows an evidence of "lack of fit", axial points can be added to the quadratic terms with more centre points to develop CCD. The number of centre points n_c at the origin and the distance a of the axial runs from the design centre are two parameters in the CCD design. The centre runs contain information about the curvature of the surface, if the curvature is significant, the additional axial points allow for the experimenter to obtain an efficient estimation of the quadratic terms (**Fig. 1.15**).

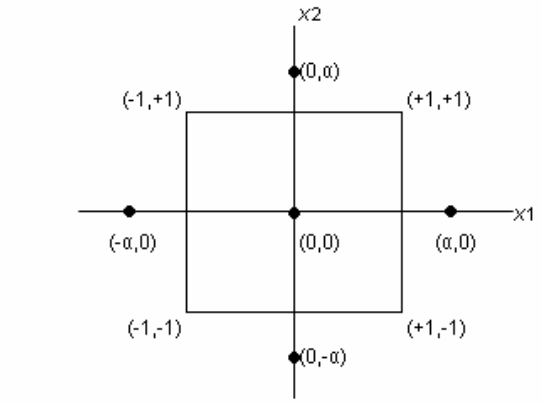

Figure 1.15. Graph presenting the levels points for the Central Composite Design for two independent variables (x_1 and x_2) (Oehlert, 2000).

CCD can run in incomplete blocks, a block is a set of relatively homogeneous experimental conditions so that an experimenter divides the observations into groups that are run in each block. An incomplete block design may be conducted when all treatment combinations cannot be run in each block. If the precision of the estimated response surface at some point x depends only on the distance from x to the origin, not on the direction, then the design is said to be rotatable (Oehlert, 2000). The use of a rotatable design that provides equal precision of estimation of the surface in all directions is justified. Therefore, when the first-order model shows a significant lack of fit, then an experimenter can use a second-order model to describe the response surface.

Response Surface Methodology (RMS) comprises a body of methods for exploring for optimum operating conditions through experimental methods. It involves using the results of one experiment to provide direction around a different set of conditions. Some may be categorical (e.g., the supplier of raw material) and others may be quantitative (feed rates, temperatures, and such). Categorical variables must be handled separately by comparing our best operating conditions with respect to the quantitative variables across different combinations of the categorical ones. Response-surface analysis is not simply a regression problem but it uses different follow-up analyses that are used depending on what type of model is fitted, and the importance of visualizing the response surface.

When treatments are from a continuous range of values, RSM is useful for developing, improving, and optimizing the response variable. In this case, the response variable y is chosen, and the function can be expressed as:

$$y = f(x_1, x_2) + e$$

The variables $x_1$and $x_2$are independent variables where the response y depends on them. The dependent variable y is a function of x_1, x_2, and the experimental error term, denoted as e. The error term e represents any measurement error on the response, as well as other type of variations not counted in f. In most RSM problems, the true response function f is unknown. In order to develop a proper approximation for f, the experimenter usually starts with a low-order polynomial in some small region. If the response can be defined by a linear function of independent variables, then the approximating function is a first-order model. A first-order model with 2 independent variables can be expressed as:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon$$

If there is a curvature in the response surface, then a higher degree polynomial should be used. The approximating function with two variables is called a second-order model:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_{11}^2 + \beta_{22} x_{22}^2 + \beta_{12} x_1 x_2 + \varepsilon$$

In general RSM use either one or the mixture of the both of these models. In each model, the levels of each factor are independent of the levels of other factors. In order to get the most efficient result in the approximation of polynomials the proper experimental design must be used to collect data. Once the data are collected, the method of Least Square is used to estimate the parameters in the polynomials. The response surface analysis is performed by using the fitted surface. The response surface designs are types of designs for fitting response surface. Therefore, the objective of studying RSM can be accomplish by: i) understanding the topography of the response surface (local maximum, local minimum, ridge lines), and ii) finding the region where the optimal response occurs.

Several models have been developed during the past few decades for the RSM. The orthogonal design was proposed by Box and Wilson (1951) in the case of the first-order model. For the second-order models, many subject- matter scientists and engineers have a working knowledge of the central composite designs (CCD) and three-level designs by Box and Behnken (1960). Hartley (1959) proposed a more economical or small composite design. The first goal for Response Surface Method is to find the optimum response. When there is more than one response then it is important to find the compromise optimum that does not optimize only one response (Oehlert 2000). The second goal is to understand how the response changes in a given direction by adjusting the design variables. In general, the response surface can be visualized graphically. The graph is helpful to visualise the shape of a response surface (Lenth, 2012).

Central Composite Design

A central composite design is an experimental design, useful in response surface methodology, for building a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment. The design consists of three distinct sets of experimental runs: i) a factorial (perhaps fractional) design in the factors studied, each having two levels; ii) a set of centre points, experimental runs whose values of each factor are the medians of the values used in the factorial portion. This point is often replicated in order to improve the precision of the experiment; iii) a set of axial points, experimental runs identical to the centre points except for one factor, which will take on values both below and above the median of the two factorial levels, and typically both outside their range (**Fig. 1.16**).

The design matrix for a central composite design experiment involving k factors is derived from a matrix, d, containing the following three different parts corresponding to the three types of experimental runs:

1. The matrix **F** obtained from the factorial experiment. The factor levels are scaled so that its entries are coded as +1 and −1;
2. The matrix **C** from the centre points;
3. A matrix **E** from the axial points, with $2k$ rows. Each factor is sequentially placed at ±α and all other factors are at zero. The value of α is determined by the designer. While arbitrary, some values may give the design desirable properties. This part can be expressed as follows:

$$\mathbf{E} = \begin{bmatrix} \alpha & 0 & 0 & \cdots & \cdots & \cdots & 0 \\ -\alpha & 0 & 0 & \cdots & \cdots & \cdots & 0 \\ 0 & \alpha & 0 & \cdots & \cdots & \cdots & 0 \\ 0 & -\alpha & 0 & \cdots & \cdots & \cdots & 0 \\ \vdots & & & & & & \vdots \\ 0 & 0 & 0 & 0 & \cdots & \cdots & \alpha \\ 0 & 0 & 0 & 0 & \cdots & \cdots & -\alpha \end{bmatrix}.$$

Then **d** is the vertical concatenation:

$$\mathbf{d} = \begin{bmatrix} \mathbf{F} \\ \mathbf{C} \\ \mathbf{E} \end{bmatrix}.$$

The design matrix **X** used in linear regression is the horizontal concatenation of a column of 1s (intercept), **d**, and all elementwise products of a pair of columns of **d**:

$$\mathbf{X} = \begin{bmatrix} \mathbf{1} & \mathbf{d} & \mathbf{d}(1) \times \mathbf{d}(2) & \mathbf{d}(1) \times \mathbf{d}(3) & \cdots & \mathbf{d}(k-1) \times \mathbf{d}(k) & \mathbf{d}(1)^2 & \mathbf{d}(2)^2 & \cdots & \mathbf{d}(k)^2 \end{bmatrix},$$

where **d**(*i*) represents the i^{th} column in **d**.

There are several methods to select a useful value of α. If*F* is the number of points due to the factorial design and $T = 2k + n$, the number of additional points, where *n* is the number of central points in the design. Common values are as follows (Myers, 1971):

1. ***Orthogonal design*:** $\alpha = (Q \times F/4)^{1/4}$, where $Q = (\sqrt{F+T} - \sqrt{F})^2$;
2. ***Rotatable design***: $\alpha = F^{1/4}$

The visualisation of the results from the ANOVA when the model is created, is done by using the Pareto graphs. From these graphs, the length of each bar is proportional to the value of a t-statistic calculated for the corresponding effect. Any bars beyond the vertical line are statistically significant at the selected significance level, set by default at 5%. The most complicated model that can be fit is the two-factor interaction should be used, as defined by:

$$Y = \begin{matrix} \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 \\ + \beta_{15} x_1 x_5 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{45} x_4 x_5 \end{matrix}$$

It consists of each experimental factor by itself (the main effects) and terms involving each pair of factors (two-factor interactions).

In the present research thesis, an experimental work was carried out to test the effects of the independent variables on the emulsion stability of an oil-in-water emulsion and their interaction were analysed using polynomial regression analysis. A second order polynomial equation for the three dependent variables (x_1-x_3) was established to fit the experimental data. The regression coefficients for the linear, quadratic and interaction effect terms was evaluated, both with the error term corresponding to an independent normal distribution. Estimation of the regression parameters and their standard errors was done using the least-square technique (Myers *et al*., 2009).

Rotatable central composite designs present the following characteristics: i) require an experiment number according to $N = k2 + 2k + cp$, where *k* is the factor number and (*cp*) is the replicate number of the central point; ii) α-values depend on the number of variables and can be calculated by $\alpha = 2^{(k-p)/4}$. For two, three, and four variables, they are, respectively, 1.41, 1.68, and 2.00; iii) all factors are studied in five levels ($-\alpha, -1, 0, +1, +\alpha$).

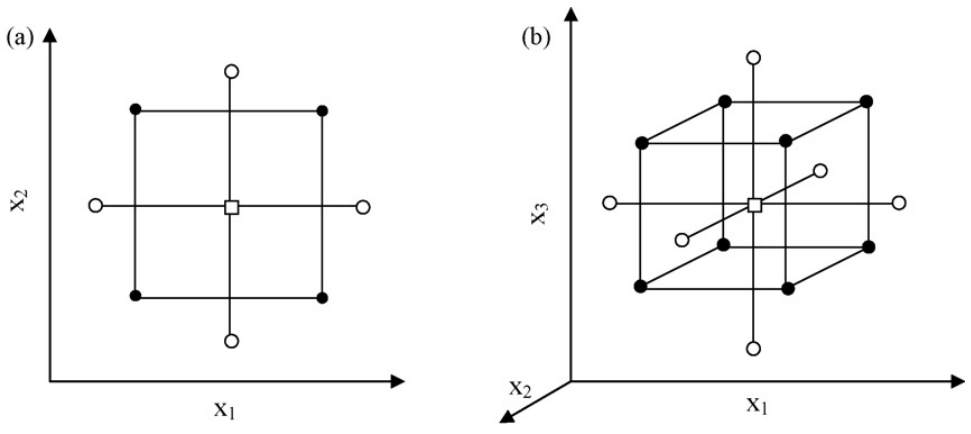

Figure 1.16. Central composite designs for the optimization of: (a) two variables (α= 1.41) and (b) three variables (α = 1.68). (black circles) Points of factorial design, (white circles) axial points and (square) central point (Oehlert, 2000).

Model optimisation: determination of the optimal conditions and multiple responses optimisation in food science

Optimizing refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. The term optimization has been commonly used in analytical chemistry as a means of discovering conditions at which to apply a procedure that produces the best possible response multivariate statistic techniques is a much better technique to optimize the formulation of a product.

As reported in previous paragraphs, before applying the RSM methodology, it is first necessary to choose an experimental design that will define which experiments should be carried out in the experimental region being studied. There are some experimental matrices for this purpose. Experimental designs for first-order models (e.g., factorial designs) can be used when the data set does not present curvature. However, to approximate a response function to experimental data that cannot be described by linear functions, experimental designs for quadratic response surfaces should be used, such as three level factorial, Box–Behnken, central composite, and Doehlert designs (Bezerra et al 2008).

The surfaces generated by linear models can be used to indicate the direction in which the original design must be displaced in order to attain the optimal conditions. However, if the experimental region cannot be displaced due to physical or instrumental reasons, the research must find the best operational condition inside the studied experimental condition by visual inspection.

For quadratic models, the critical point can be characterized as maximum, minimum, or saddle. It is possible to calculate the coordinates of the critical point through the first derivate of the mathematical function, which describes the response surface and equates it to zero. The quadratic function obtained for two variables as described below is used to illustrate the example

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$$

$$\frac{\partial y}{\partial x_1} = b_1 + 2b_{11}x_1 + b_{12}x_2 = 0$$

$$\frac{\partial y}{\partial x_2} = b_2 + 2b_{22}x_2 + b_{12}x_1 = 0$$

Thus, to calculate the coordinate of the critical point, it is necessary to solve the first grade system formed by the equations shown above to find the x_1 and x_2 values. The visualization of the predicted model equation can be obtained by the surface response plot. This graphical representation is an n-dimensional surface in the $(n + 1)$-dimensional space. Usually, a two-dimensional representation of a three-dimensional plot can be drawn. Thus, if there are three or more variables, the plot visualization is possible only if one or more variables are set to a constant value.

It is relatively simple to find the optimal conditions for a single response using surface response designs. However, it is of interest in the food science research to optimize several responses simultaneously. If the numbers of response are not very large, the surfaces can be overlapped to enable finding the experimental region that can satisfy all the responses studied. In case the optimal values for each response are localized in different regions, it will be more difficult to find the conditions that simultaneously satisfy all responses. It is not rare to encounter cases where all surfaces found do not present its optimum under the same set of experimental conditions. Thus, changes in the level of a factor can improve one specific response and have a very negative effect on another. An approach for solving the problem of the optimization of several responses is the use of a multicriteria methodology. This methodology is applied when various responses have to be considered at the same time and it is necessary to find optimal compromises between the total numbers of responses taken into account. The Derringer function or desirability function is the most important and most currently used multicriteria methodology in the optimization of analytical procedures. This methodology is initially based on constructing a desirability function for each individual response. The measured properties related to each response are transformed into a dimensionless individual desirability (di) scale. The scale of the individual desirability function ranges between $d = 0$, for a completely undesirable response, and $d = 1$, for a fully desired response. This transformation makes it possible to combine the results obtained for properties measured on different orders of magnitude. With the individual desirability, it is then possible to obtain the overall desirability (D). The overall desirability function D is defined as the weighted geometric average of the individual desirability (di).

Multiple response optimization using desirability functions has so far its limited utilization to the chromatographic field, its related techniques, and to electrochemical methods. However, its principles can be applied to the development of procedures using various analytical techniques, which demand a search for optimal conditions for a set of responses simultaneously. As an alternative to classical modelling, an adaptive learning technique that utilizes neural networks combined with experimental design, can be employed to model a dependence relation. This approach has demonstrated a superior accuracy in data learning and prediction over the traditional RSM. The application of desirability functions in analytical chemistry brings advantages as efficiency, economy, and objectivity in the optimization of multiple response procedures. Despite the obvious advantages of this methodology in the optimization of analytical procedures, there are still few applications found in the literature. Derringer functions have been more applied for optimization in chromatographic and related techniques principally because they can establish conditions for the best resolution among several peaks simultaneously (Bezerra et al., 2008).

Application of RSM in the optimization of analytical procedures is today largely diffused and consolidated principally because of its advantages to classical one-variable-a-time optimization, such as the generation of large amounts of information from a small number of experiments and the possibility of evaluating the interaction effect between the variables on the response. In order to employ this methodology in experimental optimization, it is necessary to choose an experimental design, to fit an adequate mathematical function, and to evaluate the quality of the fitted model and its accuracy to make previsions in relation to the experimental data obtained. The central composite design is still the symmetrical second order experimental design most utilized for the development of analytical procedures. The application of three-level factorial designs is not frequent, and the use of this design has been limited to the optimization of two variables because its efficiency is very low for higher numbers of variables. However, the Box–Behnken and Doehlert designs present more efficient matrices and have increased the number of published works in recent years.

It is of interest to study contemporary the effect of emulsions ingredients to assess possible interactive effects on the physical and chemical stability of olive O/W emulsions. The Response Surface Methodology (RSM) is therefore a valid statistical method to explore the operating conditions and is considered as the preferred experimental design for fitting polynomial model when the aim is to analyse the response surface of multi-variable combinations. It comprises great advantages in terms of numbers of experimental runs and further possibility of statistical validation, also for the optimization of the best conditions (Lenth, 2012).

Biosynthesis of aroma compounds in virgin olive oil

Virgin olive oil has unique characteristics which results from its fatty acid composition and the presence of minor compounds including volatile and phenolic compounds. Some of these are biosynthesized during the olive fruit ripening, especially during the climateric period. Some volatile compounds are still present in the fruit belonging from the lipids or aminoacids metabolism (Conde et al., 2008; Kalua et al., 2007). However, the majority of the olive aroma derives from enzymatic oxidation of the fatty acids linoleic and linolenic (Angerosa, 2002) (**Fig. 1.17**).

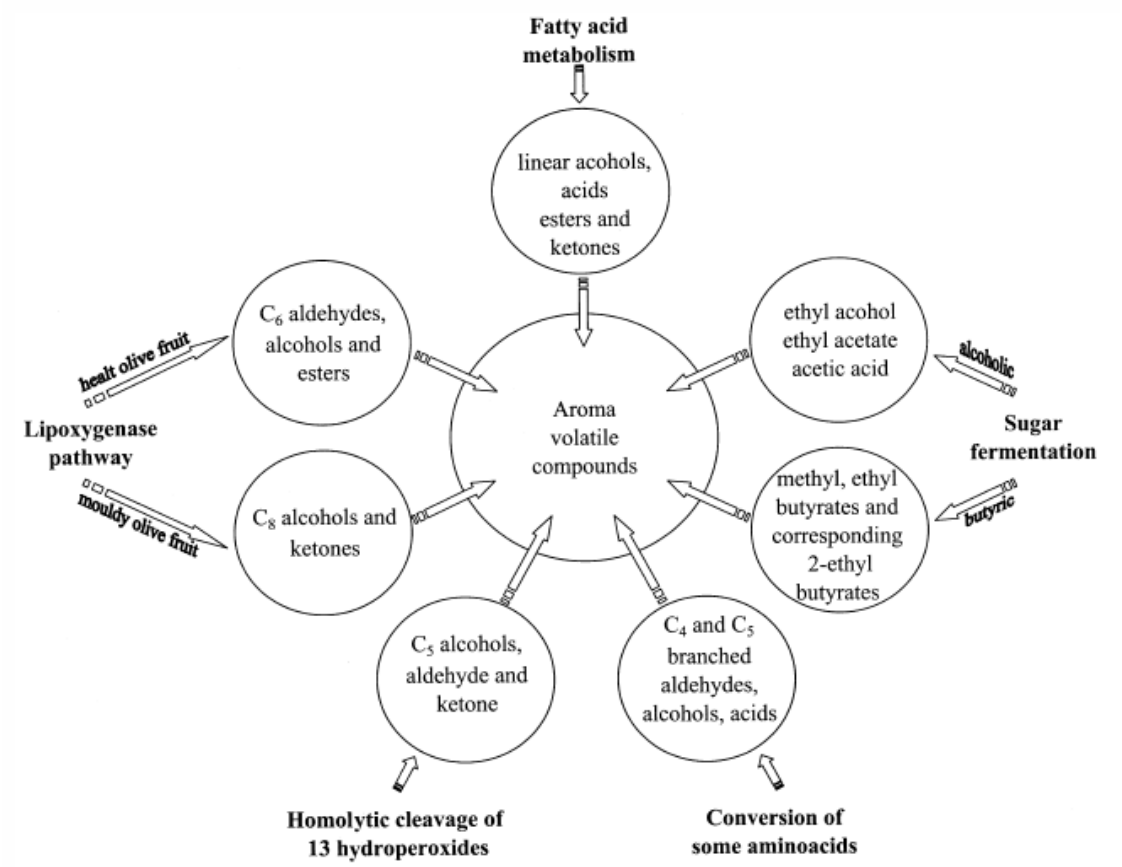

Figure. 1.17. The main pathways involved in the production of the volatile compounds of virgin olive oil aromas (from Angerosa, 2002).

Saturated aldehydes having Carbon number comprised between 7 and 12 are the major responsible of VOO aroma. This class of volatile compounds is usually present at concentrations of 50 and 75% of the total volatiles in unripe and fully mature olive drupes, respectively (Kiritsakis, 1998).

Aliphatic and aromatic hydrocarbons, aliphatic and triterpenic alcohols, aldehydes, ketones, ethers, esters and furan derivatives are present in the VOO aroma. The most abundant individual compounds are hexanal, trans-2-hexenal, hexanol and 3-methylbutanol (Kiritsakis, 1998; Angerosa, 2002). Among volatile compounds, those attributed to the sensory note described as "green" are C6 aliphatic compounds, hexanal, cis-3-hexenal, trans-2-hexenal, hexanol, cis-3-hexenol, trans-2-hexen-1-ol and the corresponding esters (Olias et al., 1993).

The following volatile compounds account to circa 80% of the total:

- C6 aldehydes (hexanal, cis-3-hexanal, trans-2-hexanal);
- alcohols (hexanol, cis-3-hexenol, trans-2-hexenol);
- esters (hexyl acetate, cis-3-hexenyl acetate (Sanchez e Harwood, 2002; Conde et al., 2008; Angerosa, 2002).

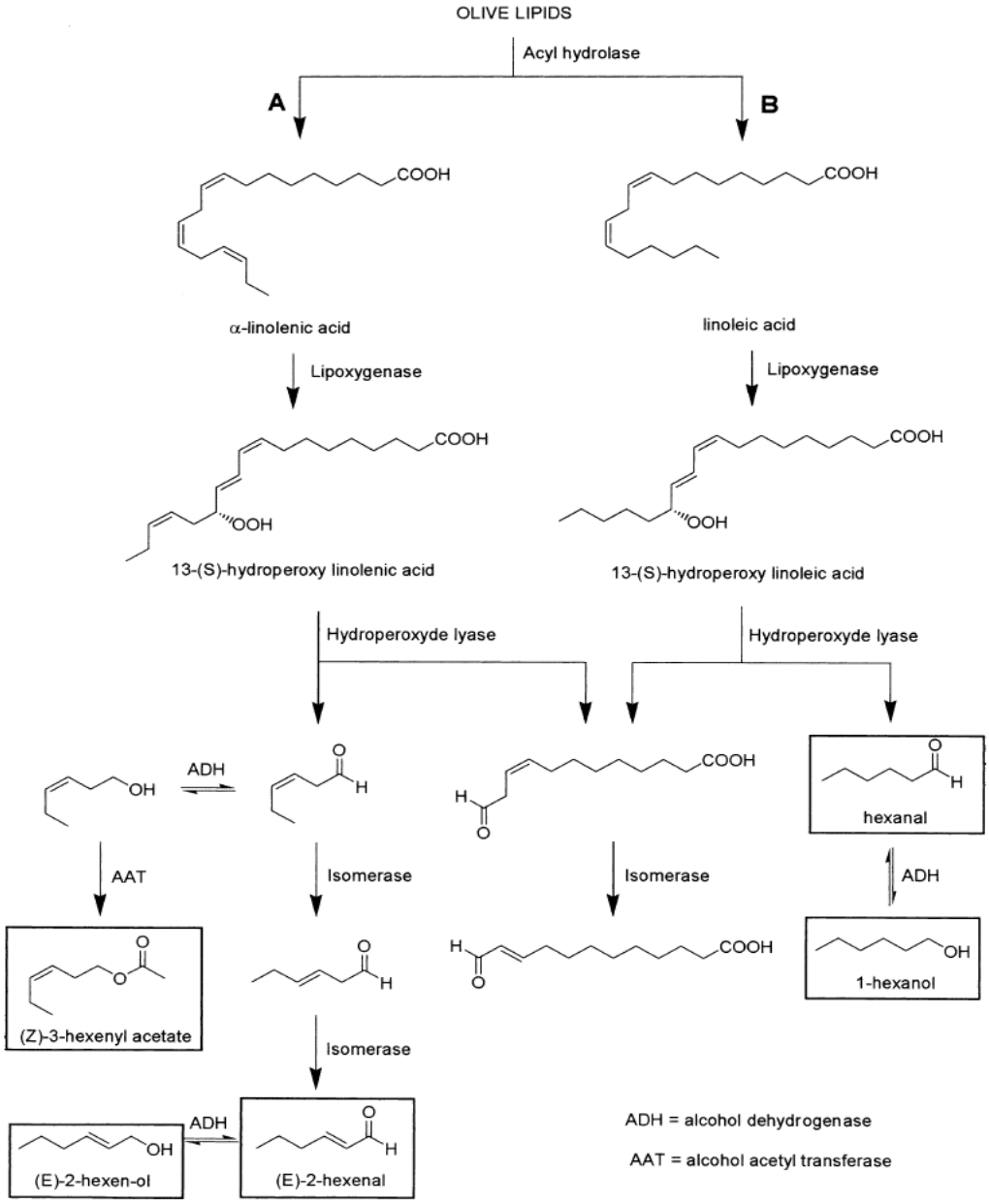

Figure 1.18. Lipoxigenase pathway for olive oil aroma generation (from Benincasa et al., 2003).

The Lipoxygenase (LOX) pathway is a prominent enzymatic route for olive oil aroma generation, and involves a variety of enzymes acting on unsaturated fatty acids. LOX is activated when the olive drupe is crushed, which is the first step of olive oil extraction process. During olive crushing and the following malaxation phase, the typical olive oil aroma is produced (Sanchez e Harwood, 2002). The LOX pathway involves the activity of four enzymes, namely lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase and alcohol acyltransferase (Olias et al., 1993; Conde et al., 2008; Sanchez e Harwood, 2002) (**Fig. 1.18**).

When the integrity of olive drupe is compromised, the linoleic and linolenic acids are attacked in their position 9 and 13 on the carbonyl chain, with a well-defined ratio depending on the genetic characteristics of the olive cultivar, e.g. 65:35 and 55:45, respectively (Olias et al., 1993; Angerosa et al., 1999). The hydroperoxide lyase produces aldehydes, subsequently reduced in alcohols by the alcohol dehydrogenase. These compounds are then esterified by the alcohol acetyltransferase to produce esters (Sanchez e Harwood, 2002; Conde et al., 2008).

The 13-hydroperoxide undergoes an enzymatic scission by the hydroperoxide lyase, with the subsequent production of hexanal and cis-3-hexenal. The hydroperoxide lyase found in the olive pulp is highly specific for 13-hydroperoxides (Salas e Sanchez, 1999a; Conde et al., 2008). The 13-hydroperoxide formed from linolenic acid is rapidly reduced to cis-3-hexenol, from which cis-3-hexenyl acetate is produced. Alternatively, it is isomerised to form trans-2-hexenal, which is then reduced to trans-2-hexenal by following a different biosynthetic route (Angerosa et al., 1999).

Pentanal would be generated through an additional branch of the LOX pathway that would involve the production of a 13-alcoxyl radical by LOX as demonstrated in soybean seeds. This radical would undergo subsequent nonenzymatic ß-scission in a homolytic way to form a 1,3-pentene allylic radical that could be chemically dimerized to form pentene dimers (PD) or react with an hydroxyl radical to form C5 alcohols. The latter would be the origin of C5 carbonyl compounds present in the volatile fraction of olive oil through an enzymatic oxidation by ADH as suggested to occur in soybean leaves (Sanchez-Ortiz et al., 2013) **(Fig. 1.19)**.

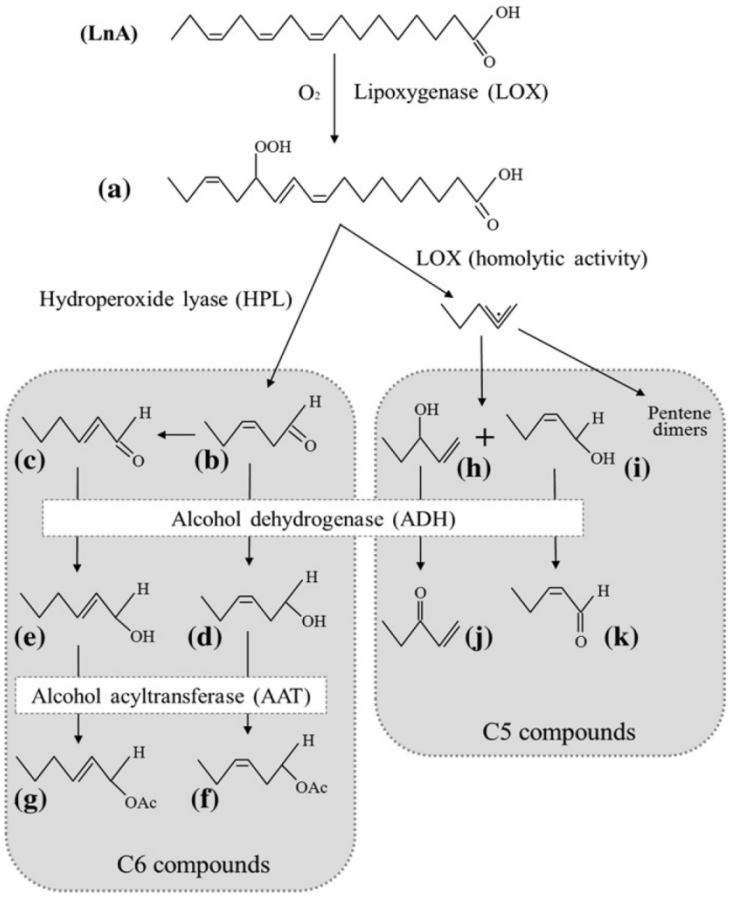

Figure 1.19. Synthesis of aroma compounds of virgin olive oil: Significance of the cleavage of polyunsaturated fatty acid hydroperoxides during the oil extraction process (from Sanchez-Ortiz et al., 2013).

Influence of the olive oil extraction process on olive flavour

Olive milling. The majority of VOO aroma compounds are produced during after the crushing phase, when the enzymatic oxidation of linoleic and linolenic acid takes place (Baccouri et al., 2008). The type of mill influences the final oil quality, by changing the quali-quantitative composition of some minor constituents, with consequent influence on the organoleptic properties and product stability.

There are two major types or olive crushers: granite stone mill and metallic crusher. This letter can be divided into two types, i.e. disc and hammer crushers. The hammer crushers has stronger effects and cause a strong emulsification on the olive paste and an increase of its temperature, due to friction phenomena, with

disadvantages on the yield and on the oil quality. It should be also noted that the stone mill is a discontinuous system, while the metallic crusher system are used in continuous and therefore their performance in terms of processed olive is much higher.

VOO obtained using stone crusher is more aromatic and harmonic than oils obtained using the other two types of crushers. The disc crusher, however, leads to the formation of oil richer in phenolic compounds, more bitter and stable to lipid oxidation over storage (Angerosa and Di Giacinto, 1995).Olias et al. (1993), on the contrary, reported that stone mill leads to higher concentration of volatile compounds than metallic crushers, e.g. trans-2-hexenal, hexanal and cis-3-hexenol. For cv. Coratina and Oliarola, Servili et al. (2002) reported that disc crusher cause higher concentrations of C6 aldehydes and some esters such as hexyl acetate, 3-hexenyl acetate and cis-4-hexenyl acetate, than hammer crusher.

Malaxation. The malaxation is a fundamental stage in VOO extraction, which involves the continuous slowly mixing of the olive paste to aggregate the oil/water emulsion. Malaxation time and temperature are parameters that are usually controlled by the industry during VOO extraction process, which can potentially affect the sensory profile and composition of the final product (Kalua et al., 2007)

The factor “malaxation time” was reported to be positively correlated to the total content of volatile compounds, but negatively correlated with the concentration of total phenolics (Ranalli et al., 2001). Longer malaxation times promote the accumulation of alcohols and C5 other compounds, particularly hexanal. The temperature increase speeds up the activity of oxidative enzymes such as polyphenol oxidase, lipoxygenase and peroxidase (Angerosa, 2002).However, there is a loss of volatile compounds when high malaxation temperatures are applied, attributed to the enzymes deactivation, especially hydroperoxide lyase (Sanchez e Harwood, 2002). Indeed, the increase of malaxation temperature causes a considerable decrease of C6 compounds, cis-3-hexenol and C5 metabolites, as well as an increase in hexanol and trans-2-hexen-1-ol and a loss of the bitter-pungent notes (Angerosa et al., 2001).

Angerosa et al. (2001) studied the combined effect of malaxation temperature and time on the VOO aroma from cv. Coratina and Frantoio, and highlighting that malaxation negatively affects the total amount of secoiridoids. Some phenolic compounds, such as tyrosol, seem not undergo major changes as affected by the malaxation time, but they are only affected by the temperature adopted. The opposite trend was reported for the phenolic compound 3,4-DHPEA-DEDA, for which the maximum concentration was obtained at short malaxation time. The loss of phenolic compounds during malaxation is due to the enzymatic oxidation of the secoiridoid derivatives, catalyzed by polyphenol oxidase and peroxidase (Servili et al., 2008).

The hydrolysis of complex phenols (oleuropein, dimethyl oleuropein, ligstroside, verbascoside, rutin and luteolin-7-glucoside) by the endogenous glucosidase results in the production of aglycones and simple phenols, which dissolve appreciably in the oil phase, while the repartition of unhydrolysed glycosidic phenols is more toward the aqueous phase (Ranalli et al., 2004).The following compounds were proposed as markers for malaxation temperature: 1-penten-3-ol, cis-3-hexenal and octane, which were proposed as markers to discriminate malaxation temperatures of 15, 30 and 35 C, respectively (Kalua et al., 2006). The concentration of oxygen in the paste headspace during the malaxation process can be manipulated to achieve significant modification of the VOO aroma, in relation to the desired characteristics and industrial needs (Servili et al., 2008).

Extraction phase. The separation of the liquid phase and solid particles from the olive paste is usually performed using two major systems, i.e. pressure or centrifugation. The subsequent step involves the separation of the oil from the oil-water mixture, which is performed by centrifugation. When separation systems based on pressure area applied, the VOOs tend to be higher in the fruity sensory score and having a higher concentration of volatile alcohol, but possible fermentation and/or degradation phenomena can take place, with consequent sensory defects (Angerosa, 2002).Differences in phenolic content in the oils was also observed between the conventional centrifugal ("three-phase") and the "two-phase". The latter does not involves the addition of water (or very little amounts), which results in a lower dilution of hydrophilic phenolic compounds such as ortho-diphenols and hydroxytyrosol.

The use of two phases centrifuge, compared to the three-phase one, allows the production of VOOs with higher concentrations of trans-2-hexenal and greater total aromatic content, but with lower

concentration of pigments, aliphatic and triterpene alcohols, sterols and waxes (Aparicio and Moon, 2002). On the contrary, the use of the three-phases centrifuge causes decrease in the content of C6 aldehydes, hexanol and trans-2-hexenol compared with the pressure extraction, probably because of the addition of hot water (Angerosa et al., 2004). Incorrect management and improper management of the pressure extraction system may lead to olive paste fermentation that and the subsequent off-flavours (Di Gioacchino et al., 1994).

Filtration and storage. VOO filtration may have important effects on its sensory properties and shelf life. Unfiltered oils contain a certain amount of water (from 2 g/kg to 4 g/kg) dispersed in micro-droplets present in dispersion as part of the colloidal system of the fruit. The filtration might influence the bitter-pungent sensory note, caused by the hydrolysis of phenolic compounds.It has been recently reported that the industrial-scale filtration of highly bitter-pungent extra VOO has influence on the release of key aroma compounds in the product after storage, and therefore the filtration process should be also regarded as one of the possible parameter influencing VOO flavour (Sacchi et al., 2015).

VOO quality, similarly to other vegetable oils, is affected by the storage conditions. Oxygen and light exposure, high temperatures and presence of metal ions as trace can accelerate the lipid oxidation and then shorten the shelf life of the product. The stability of VOO depends on its fatty acid composition, in particular by the ratio of the oleic to linoleic acid, and presence of minor compounds such as α-tocopherol, carotenoids, squalene and phenolics (Skevin et al., 2003). Among the saturated aldehydes, nonanal and hexanal suffer a sharp increase as oxidation rate increases. Some authors suggested the relationship hexanal/nonanal as an indicator of the oxidation status, while others reported that trans-2-heptenal is associated with the perception of rancid defect. trans-2-Hexenal is the most abundant volatile compound present in VOOs, and it undergoes changes similar to that of phenolic compounds. The presence of phenolics during oil storage depends on the hydrolytic processes of complex forms and the oxidation of ortho-diphenols (Angerosa et al., 2004). The type of container where the oil is stored was also reported to influence the lipid oxidation status and the chemical composition of VOOs under normal retail storage conditions (Savarese et al., 2013), and therefore this parameter should be carefully considered to avoid quality loss over storage.

Sensory description of VOO aroma compounds

The most abundant volatile compounds in VOO are aldehydes and C6 alcohols, which were related to the sensory note of sweetness. Aldehydes and C5 alcohols contribute to other positive attributes of VOOs. The odour threshold of VOO volatiles is dramatically different depending on their chemical structure and physico-chemical characteristics. For example, cis-3-hexenal, which gives the typical odour of freshly cut grass and despite its low concentration, greatly contributes to the aroma as it has a very low odour threshold. On the contrary, trans-2-hexenal has much higher perception threshold and it contributes minimally to the final VOO aroma, despite of being the most abundant volatile in the oil (Kalua et al., 2007).

trans-2-Hexenal and trans-2-hexenol have been associated to VOOs obtained from olives in good conditions. The following compounds contribute mostly to the "green note": cis-3-hexenal; cis-3-hexenol (grass and banana); cis-3-hexenyl acetate (fruity and green leaves). Important in defining the complex flavour are also trans-2-hexenal, hexanol and trans-3-hexenol. Hexyl acetate contributes to perceptions of fruity and sweet, while hexanal is responsible for the "green" and apple note. This latter compound is not only produced during the lipoxygenase pathway, but is also generated from lipid auto-oxidation, together with other oxidation products (Ranalli et al., 2001).

cis-3-Hexenol has been associated to the bitter note, as well as the attributes of "apple", "tomato", "vegetable bitter", "grass" and "fruity olive oil", along with some C5 alcohols (cis-2-penten-1-ol and 1-penten-3-ol) and trans-2-hexenal (Caporale et al., 2004). Other compounds such as toluene, octane, octene and 3-methyl butanol arise from different routes from the LOX pathway, and are not related to positive sensory attributes of VOO.

The "fruity" note of VOO was positively correlated with the cis-3-hexenol and negatively correlated with 3-pentanone. The "ripe fruit" sensory description is correlated with 3-pentanone, while "leaf" is

correlates positively with hexyl acetate, 1-penten-3-ol, cis-2-penten-1-ol and negatively correlated with hexanol, associated with the "grass" note. The note of "almond" correlates positively with the hexyl acetate, 1-penten-3-ol and cis-2-penten-1-ol and negatively with the hexanol; cis-3-hexenol correlates positively with the scent of "tomato" and "bitter vegetable"; 3-pentanone correlates negatively with the attributes of "bitter", "spicy", "tomato" and "vegetable bitter".

The attributes of bitter and pungent in olive oil are due to the presence of phenolic compounds. The bitter taste has been attributed to aglycone compounds, particularly the dialdehydic form of the decarboxymethyl oleuropein, and other forms of the oleuropein aglycone; the “pungent” note has been attributed to the aglycone form the dialdehydic of the decarboxymethyl ligstroside (Servili et al., 2009).

The sensory defect of "winey-vinegary" is associated with fermentative processes especially from Lactobacillus, when olives are left on the ground for prolonged times. The consequent production of acetic acid is a marker of olives collected from the ground. A high concentration of acetic acid and octane was correlated to the defect of "reheating", a consequence of the activity of Enterobacteriaceae the genus Aerobacter and Escherichia during the first days the permanence of the olives to the ground. The genera Pseudomonas, Clostridium and Serratia appear if the olives are left in bags for a long time after harvesting. The activity of these microorganisms results in the presence of some volatile compounds at high concentrations. The defect of "mould-humidity" has been correlated to the presence of 3-methyl-1-butanol.

Other compounds that are important in defining the overall aroma oil, were reported in low concentrations in the olive paste: the hexyl acetate, characterized by the smell fruity and marker of good quality oils, it does not contribute to the aroma of the paste due to its low perception threshold (~ 1:04 mg/kg) and low concentration in the paste (maximum at 1.00 mg/kg) (Garcia-Gonzalez et al., 2007).

Perception of aroma compounds and factors affecting aroma release

When food is consumed, the compounds responsible for the flavour can be perceived through a series of events involving the release and transfer to sensory receptors.

The olfactory receptors are present in a small area (about 2.5 cm^2 containing approximately 50 million receptors) in the upper part of the nasal cavity. The mucus acts as a solvent in respect of the substances responsible of smells allowing contact with the receptors (Kalua et al., 2007). However, there are two routes for volatile compounds to reach the olfactory receptors, i.e. through the direct olfaction, so-called "orthonasal" route, or through the nasal pharynx while the food is consumed, called "retronasal" route, which have been shown in **Fig. 1.20**.

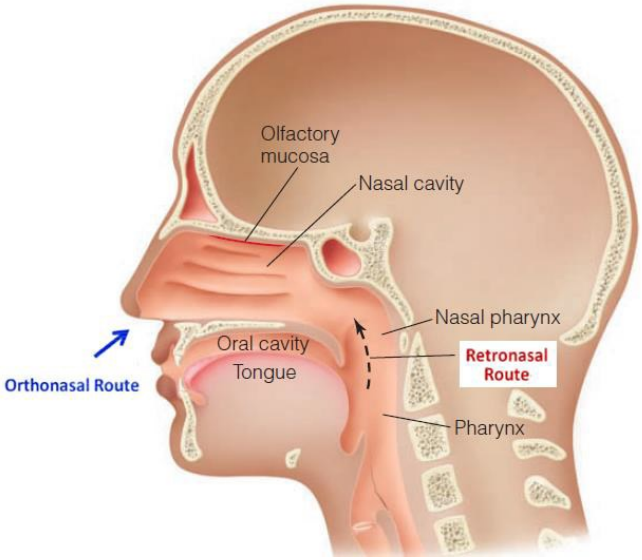

Figure 1.20 Representation of olfactory perception via orthonasal route and retronasal route (Traynor, 2013).

In this phase, the molecules that stimulate these receptors have to be dissolved in the aqueous phase to reach the olfactory receptor taste cells. Simultaneously, through the breath, the transfer of aromatic molecules to the factory receptors takes place. The orthonasal and retronasal perceptions are different, even if they involve the same mechanisms (Burdach et al., 1984; Linforth et al., 2002; Voirol & Daget, 1986, 1989). In fact, the in-mouth perception may significantly increase or decrease the odour detection of the same odour compounds in the orthonasal route. When olive oil is consumed, it is heated to 37 °C (body temperature) and the compounds responsible for the flavour can be detected through a series of events that provide for the release and transfer to the sensory receptors. The release of volatile compounds from the food is influenced by his physical state, by chewing and saliva. Various effects of breakdown, determine the concentration of aromatic compounds in different phases generated in the mouth during food consumption. For the volatile compounds, these processes can be summarized in terms of:

- initial release from the food to the saliva, in the mouth;
- passage from saliva/food to headspace;
- dilution and transport into the mouth from the headspace to the nose cavity (Solms et al., 1973).

The breakdown of the various phases depends on the physicochemical properties of the volatile compounds. The nature and intensity of the aromatic stimulus and taste depend not only on the presence and concentration of volatile compounds in the oral cavity and in the nasopharyngeal tract, but is also related to other factors influencing their release.

The release of volatile aroma compounds before or during food consumption is one of the key factors in perception of food. Lipids, being a large reservoir of aroma compounds, significantly influence aroma release from food emulsions. Also, lipid-soluble volatile compound act differently on the aroma release than water-soluble ones (**Fig. 1.21**), as better described in the following sections.

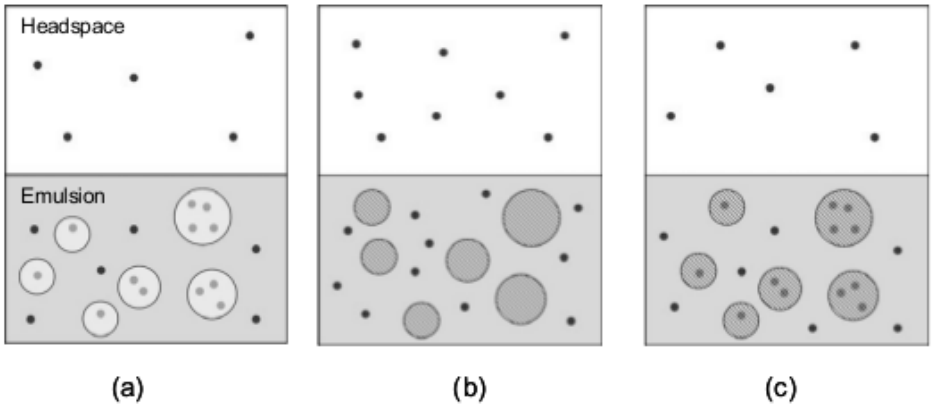

Figure 1.21. Scheme of the headspace release of aroma compounds (black points), in O/W emulsions, to emphasise the difference of aroma release when the volatile are mainly hydrophobic (b) and hydrophilic (c) (Traynor, 2013).

When a food is consumed, for instance olive oil which is the object of this study, the compounds responsible for its flavour (stimuli) can be perceived through a series of events that provide for the release and transfer to the sensory receptors. In this phase, the molecules that stimulate these receptors must be dissolved in the aqueous phase to reach the olfactory receptor cells of taste. Simultaneously, through the breath, the volatile compounds are transferred to the olfactory receptors. At this stage, an equilibrium in reached to determine the distribution of volatile compounds between the different phases generated in the mouth during food consumption. The processes of distribution of volatile compounds (VC) can be summarized as follows:

VC (matrix) ↔ VC (emulsion) ↔ VC (air) ↔ VC (mucosa) ↔ VC (receptor)

When the olive oil is paired to a food product, e.g. ricotta cheese, this equilibrium is further complicated by the interaction between the two food ingredients:

VC (lipid matrix at liquid state) ↔ VC (water matrix at semi-solid state) ↔ VC (bolus) ↔ VC (emulsion) ↔ VC (air) ↔ VC (mucosa) ↔ VC (receptor)

The ability to reach the olfactory receptor depends on such allocations and, therefore, how the equilibrium is shifted towards the right side of balance. The nature and intensity of the aromatic stimulus and taste depend not only on the presence and concentration of the stimulus in the mouth and in the nasopharyngeal tract, but also by all oral processes that modify their release and transfer. These include the structure and the composition of the food, the chemical nature and physical-chemical properties of the different stimuli and physiological factors such as the temperature and pH of the mouth, saliva, salivary flow, chewing and the bolus size (Piggott & Schaschke, 2001). In addition, breathing and dilution with saliva causes a continuous change in volume, composition and viscosity of the food when introduced into the oral cavity.

Influence of the food matrix on aroma release

The physico-chemical changes of a food can influence its flavour, especially when the volatile compounds are generated by enzymatic reactions that develop during the degradation (breakdown) of the food; or when their release depends on hydration that is performed by saliva during chewing; or even when its fat content is able to modify the solubility of the volatile compounds (Overbosch, 1991). The nature of the non-volatile constituents such as proteins, lipids, carbohydrates and salts has a major impact on the retention of aromatic compounds in food matrices. In fact, the chemical-physical interactions between the aroma compounds and the food constituents. These interactions can alter the equilibrium of volatile compounds in food, thus affecting the flavour (De Roos, 1997; Fischer & Widder, 1997; Lubbers et al., 1998; Guichard, 2002; van Ruth et al., 2002). Among the macromolecular components of a food, it was reported that the proteins are capable of influencing the volatility of the aroma compounds by physical reversible adsorption (van der Waals interactions) and/or by irreversible covalent bonds (as in the case of aldehydes, the group

NH_2 and SH groups of proteins) (van Ruth et al., 1995; van Ruth et al., 2000; Reineccius, 2006). The interactions that can take place are influenced by the type and amount of protein (amino acid composition), types of flavouring compounds, the presence of other food components, ionic strength (salts), pH, temperature and time (O'Neil, 1996; Fischer & Widder, 1997). As the length of the carbon chain changes, there might also be a higher release of volatile compounds that have hydrophobic interactions, because of the type of interaction which take place in this case. The loss of the release of aroma compounds occurs when these react with functional groups of proteins (for example, -OH, -NH_2, or -SH) (Schirle-Keller, 1995). The pH can have considerable influence in interactions with proteins, through different modes, as it might cause the change of the tertiary structure of a protein, its ionic form (amino groups), and its reactivity base (for example, formation of Schiff bases). Each of these changes may lead to a different interaction between proteins and aroma compounds. Protein denaturation caused by chemical or thermal processing can promote the opening of the protein structure, thus making the hydrophobic conformation of the protein which is now more accessible to the formation of bonds with aroma compounds (O'Neil, 1996). Proteins are often used as food ingredients for their paramount role in human nutrition, but they are also used for their functional roles, e.g. the ability as emulsifiers and stabilizers, especially milk proteins for their wide range of applicability. One of the most studied protein among milk protein is β-lactoglobulin, the main whey protein milk in quantitative terms. This protein interacts with many volatile compounds, such as aldehydes, ketones and esters and is used in various conditions that induce changes in its ability to bond. In many cases the interactions between proteins and aroma compounds are reversible, involving hydrophilic or hydrophobic bonds (Lubbers et al., 1998). It has been described that aliphatic aldehydes can bind to β-lactoglobulin by a covalent irreversible bounding. It was demonstrated that the affinity of compounds for β-lactoglobulin is higher when the hydrophobicity of the chain or the total hydrophobicity (expressed as log P), except for the terpenes. Even α-lactalbumin was reported to bind aldehydes and ketones but with a more limited binding capacity (Guichard, 2006).

Carbohydrates in foods have several technological functions: they can be used as sweeteners, stabilizers, thickeners, emulsifiers, probiotics, etc. Mono- and disaccharides exhibit a so-called “salting out” effect, i.e. the relative volatility of the aromatic compounds related to water, while the polysaccharides can suppress the volatility of the compounds as a result of non-specific molecular interactions (Reineccius, 2006). Several studies have reported that polysaccharides have the effect of increasing the density of aqueous solutions, leading to a substantial reduction in the release of flavour from the food (Naknean & Meenune, 2010).

The lipid fraction of food is known to have the most important effects on the distribution of aroma compounds between the product and the vapour phase (Brauss et al., 1999). Lipids adsorb and dissolve most of the organic aroma compounds, strongly influencing their perception. The volatile compounds are divided between the product and the gaseous phase in function of their physical properties. Therefore, they are generally mainly concentrated in the lipid phase, because of their hydrophobic nature, and their breakdown in the gas phase or aqueous phase is greatly reduced, with a consequent increase of the perception threshold (van Ruth et al., 2000). Lipids alter the thermodynamic and kinetic conditions in food systems, influencing the volatility and the mass transfer of flavouring compounds. Furthermore, fat can also act as precursor for flavour compounds, as a solvent for aroma compounds and modulator of the flavour release (Brauss et al., 1999; Haar et al., 2000).

Literature data have also reported about the interaction of polyphenols and volatile components through a non-covalently and that these interactions may have effects on the release of aroma (Pozo-Bayon & Reineccius, 2009). Studies conducted by King and Solms (1982) documented the interactions between phenolic compounds and aroma compounds in systems containing water. These studies have suggested that hydrophobic interactions between aroma compounds and phenolic compounds increase the solubility of the volatiles. Using the analysis of exponential dilutions and the nuclear magnetic resonance NMR technique, Dufour and Bayonove (1999) confirmed the existence of weak interactions between catechin and aromatic compounds in a model system and wine have also shown that the hydrophobicity is the driving force of this interaction. They also reported that different phenolic compounds, i.e. catechins or tannins, have different types of interaction, which also depends on the nature of the volatile compound.

The reactions of aldehydes (especially acetaldehyde) with wine polyphenols such as flavonols and anthocyanins have been reported previously (Timberlake & Bridle 1976; Dallas et al., 1996; Escribano-Bailon et al., 1996; Fulcrand et al., 1996; Saucier et al., 1997; Es-Safi et al., 1999), and it was suggested that

the anthocyanidins may form a bond from hydrogen with some aromatic compounds when present in high concentrations (Voilley et al., 1991). Some studies conducted using NMR (Jung et al., 2000) confirmed that the interaction between some phenolic compounds (gallic acid) and some aroma compounds (for example, ethyl hexanoate and 2-methylpyrazine) are due to the modification of the π-π ring of gallic acid with an aromatic ring in the volatile compounds. The hydrogen bonding between the functional groups of aromatic compounds and polyphenols stabilize the complex and helps to orient the molecules in a specific conformation.

The literature also indicates that polyphenols irreversibly interact with food proteins with digestive enzymes, which are transported in the body linked to plasma proteins (Brunet et al., 2002). Then, the digestive environment and the bloodstream significantly affect the biological activity of polyphenols (Papadopoulou et al., 2004). Other works shown that the polyphenols-proteins bond is hydrophobic and/or hydrophilic and is reversible. These links lead to the formation of soluble or insoluble aggregates, whose formation depends on various factors, such as pH and temperature of the system. The formation of the aggregates involves the presence of hydrophobic groups of the aromatic groups of the proteins and polyphenols, or interactions of the polyphenols hydroxyl group with lateral groups of proteins. In any case this bond causes the opening of the structure of proteins and affect the bioavailability of both compounds (Siebert et al., 1996; Bandyopadhyay et al., 2012.). Consequently, a change in the conformation of the proteins may result in a greater or weaker action on the aroma compounds and thus this finally affects the final food flavour.

Interactions of volatile compounds with food non-volatile molecules can be chemical of physical interactions. Chemical interaction may reduce the vapour pressure of the volatile compound thus reducing the force for its evaporation in the oral cavity and reducing its movement towards the olfactory receptors. Interactions of physical type include those in which the food matrix physically interferes with an aroma in reaching a sensory receptor, then the food matrix acts as a barrier to the movement of an aroma compound. The barrier may be small, e.g. food viscosity, which reduces the mixing or the surface of a food in the mouth and reduces the possibility of evaporation in the oral cavity or by contact with taste receptors. The barrier can be stronger, for example in low moisture products which dissolves very slowly during mastication, and in this case the flavour in the food matrix would remain trapped until the food matrix is broken, thereby releasing the aroma. The rate of aroma release also depends on the physical state in which the food is located (liquid, semisolid, solid). In liquid food, the release after eating and swallowing depend on the interaction of aromatic compounds with the other components of the food during dilution with the saliva. Dilution with saliva of liquid foods containing lipids will change the distribution of the aroma and will change the release kinetics (van Ruth et al., 2001). The diffusion of volatiles between the lipid phase and the aqueous phase in a liquid food is extremely rapid (Harrison & Hills, 1997). For semi-solid food such as gels, which have a melting point lower than the body temperature, the driving force of the release of the flavour is the speed at which heat can diffuse in the gel matrix and the initial melting. For the gels with higher viscosity, with a melting point above the temperature of the mouth, the diffusion of the sucrose from the surface of the gel phase in the saliva is the limiting step for the release of flavour, due to the lower melting temperature of the surface (van Ruth & Roozen, 2010).

Finally, for solid foods the release of the flavour is much more complex. The release of volatile compounds in the phase gas implies three phases, being the saliva the intermediate one. The limiting step for the flavour release is the transfer of volatiles through the food-saliva interface (Harrison *et al.,* 1998; De Roos, 2000).The initial rate of the flavour release from solid food is not very sensitive to the frequency of mastication and saliva flow, but it is extremely dependent on the mechanical breakage of the food and the coefficient of mass transfer. This implies that the structure and composition of the food determines the speed and the maximum level of release of aromatic compounds. The modifications that may suffer the food during consumption are more or less significant depending on the nature of the food itself. This can, in fact, be in different physical states: from liquid (for example, a beverage) to complex heterogeneous matrices (Taylor, 2002).

Almost no food is consumed as "pure", but the majority of food products are consumed in a combination, i.e. as a mixture of ingredients, both cooked or not. The food pairing can lead, for example, to the formation of emulsions or dispersions. An example could be the combination of olive oil with dairy products. During swallowing and mastication, the emulsions/dispersion undergo a series of processes such as mixing with the saliva, change of ionic strength, pH and temperature. In particular, the release of volatile

compounds present in the emulsion is strongly influenced by the presence of saliva (van Ruth et al., 2001; Buettner, 2002a-b; Hansson et al., 2003). It has been demonstrated that saliva causes a change of the headspace concentration of volatiles due to the dilution, the salting out and the interactions between the volatile compounds and salivary proteins. The dilution effect of the saliva in the emulsions is the result of different solubility and affinity of the compounds for saliva and for the oil (Doyenette et al., 2011).

Amylase and mucin, which are proteins present in saliva, together with the shear force due to the movement of the tongue, have an important role in the phenomena of flocculation and coalescence which take place during the consumption of oil in water emulsions. These changes in the structure of the emulsion have an impact on the perception of the product (Feron & Poette, 2013). When other food ingredients are added to the oil, they form an emulsion or a dispersion, causing the odorous components lipophilic to move into the oil phase and their concentration in the continuous phase decreases considerably, thus lowering their concentration in the gaseous phase. Thus, the presence of oil in food pairing tends to have a significant influence on the release of aroma in the gas phase and on its global perception of the aroma.

Impact of volatiles physico-chemical properties on their release kinetics

The relative volatility of a volatile compound is its ability to reach the gaseous phase, when it is introduced in a solution or in a mixture (van Ruth et al., 2000), and it is expressed as the ratio between the concentration of the volatile in the headspace and in the food system considered, under equilibrium conditions:

$$P_{ap} = C_a / C_p$$

where P_{ap} indicates the repartition coefficient between the air and the product, while C_a e C_p are the concentration (g L^{-1}) of the volatile compound in the headspace and in the product, respectively.

The air-product partition coefficient is function of the composition of the food and the temperature, but it is not influenced by the texture and by the structure (De Roos, 2003). The rate of release of volatile compounds, also depends on the physical state in which there is the food (liquid, semisolid, solid). The volatility of the aroma compounds depends on the physico-chemical properties of the molecule itself as well as on the characteristics and structure of food matrix. For example, the molecular weight, the functional groups and the molecular size are responsible for the interactions with both the receptors of the olfactory epithelium and the food matrix (Naknean & Meenune, 2010). Properties such as molecular weight, vapour pressure, boiling point, octanol-water are important, and the latter one is used to indicate the hydrophobicity (positive value)/hydrophilicity (negative value) of a compound, which is relevant for the prediction of the volatility of volatile compounds (Kinsella, 1988; Roberts & Acree 1996; van Ruth et al., 2000).

Among these properties, molecular weight is particularly important, as the higher the molecular weight, the slower is the release of volatile compounds from the food matrix. Haar et al. (2000) verified that the release of aldehydes and of diacetyl in a model system oil-in-water is affected by the length and degree of unsaturation of the carbonyl chain, apart from the general characteristics of the model system. The correlation between molecular weight of the volatile compound and its retention probably derives from the greater ability of the compounds with lower molecular weights to diffuse through the matrix. In fact, seen that the molecule is not linear, weight and molecular size are closely connected to each other: the higher the molecular weight, the larger the size of the molecule and, therefore, the slower is its rate of diffusion. As a consequence of this, the aromatic compound does not rapidly reach the surface of the matrix. However, the form of the aromatic compound, and in particular the position and the nature of its functional groups, seem to have a very significant effect on the nature and strength of the interactions that are established with the matrix. It is not sufficient to consider only the functional groups of the aromatic compounds (also because an aromatic compound may have more than one functional group), but it is also necessary to consider the polarity of the compound. Ultimately, De Roos (2003) suggests that there are two main factors that regulate the rate of release of aromatic compounds from a food, the volatility (thermodynamic factor) and the resistance to mass transfer from the product to air (kinetic factor). Several mathematical models have been reported in literature to describe the kinetics of aroma release from foods (**Fig. 1.22**). The structure, i.e. the arrangement and size of the phases, is only really important as it introduces an additional “interphase” with different thermodynamic properties that alter the affinity of the food for the flavour. As food structure is changing rapidly inside the mouth due to mastication and mixing with saliva, this effect must be considered to create a valid mathematical model.

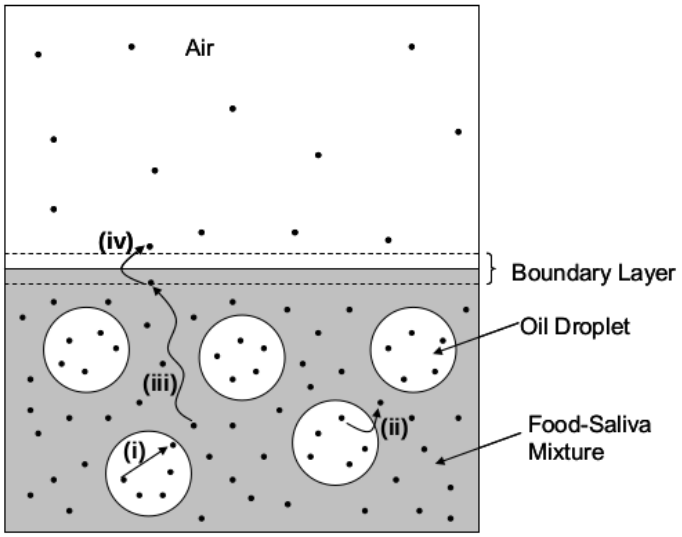

Figure 1.22. Schematic diagram showing potential rate limiting steps that may affect the kinetics of aroma release from a food to the surrounding headspace (from Ghosh, 2007).

The perceived aroma of a food depends both on the total concentration of volatiles in the product but also the extent to which they are bound by the food. For a system at equilibrium, the distribution of a given volatile between the food and the headspace gas is given by its partition coefficient:

$$K_{ge} = \frac{c_g}{c_e}$$

where c_g and c_e are the concentrations of the volatile compounds in the headspace and the food respectively.

In a multiphase food, the volatiles will distribute between all of the available phases according to the relevant partition coefficients and these can be combined into an effective partition coefficient for the food; for example in a food emulsion the effective partition coefficient (K_{ge}) is related to both the volume fraction of oil (φ_o) and the gas-oil (K_{go}) and gas-water (K_{gw}) partition coefficients respectively:

$$\frac{1}{K_{ge}} = \frac{\phi_o}{K_{go}} + \frac{(1-\phi_o)}{K_{gw}}$$

In an O/W emulsion, volatile aroma compounds distribute themselves between the oil droplets, aqueous phase and the surface of the droplets according to the equilibrium partition coefficients.

The overall partition coefficient of aroma compound from an emulsion to the gas phase above it can also be expressed as a function of individual partition coefficients according to the following mass balance equation (Ghosh, Peterson and Coupland 2006a):

$$\frac{1}{K_{ge}} = \frac{\phi_o}{K_{ow}} + \frac{1-\phi_o}{K_{gw}} + \frac{K^*_{iw}.A_s}{K_{gw}}$$

where, K_{ge} , K ow and K_{gw} are the overall gas-emulsion, oil-water and gas-water partition coefficients, respectively, φ_o is the oil volume fraction in emulsion and A_s is the interfacial area per unit volume of emulsion. K^*_{iw} is the surface binding coefficient defined as the ratio of surface excess concentration, i.e., volume of aroma compound per unit interfacial area, to aqueous concentration (Ghosh, Peterson and Coupland 2006a).

Influence of saliva on the release of aroma compounds

As mentioned in the previous paragraph, among other physiological factors, saliva plays an important role in the retronasal perception of food aroma (Roberts & Acree, 1995; van Ruth & Roozen, 2000a,b; van Ruth et al., 2001; Buettner, 2002a, 2002b). Saliva is the first digestive juice that foods encounters in the digestive tract; his action is closely correlated with chewing and swallowing. Salivary secretion in humans ranges from 0.5 ml/min (without stimulation) to more than 2 ml/min (with meals) (Edgar, 1990). The total quantity reaches 1-1.5 litres in 24h, including the phases digestive where is greater (Humphrey & Williamson, 2001). Even olfactory signals contribute to the salivary reflection through complex pathways that include the olfactory bulbs and tracts. There are three types of salivary glands with their peculiarities. The major glands produce about 90% of the saliva, the remaining 10% is produced by the accessory glands (Chen, 2009). The salivary glands are made up of two types of secretory cells: mucous cells and serous cells. The first produces a viscous secretion rich in mucus; whereas the latter produce a secreted fluid, rich in water, mineral salts and enzyme. The secretion of saliva from the salivary glands occurs under control sympathetic and parasympathetic (van Ruth & Roozen, 2000a). The latter has a control over the volume of saliva product, while the former has a great control on proteins released (Beidler, 1995). The composition of saliva depends on the speed with which it is secreted, the type of salivary gland that is preferentially activated and the intensity of the stimulation of glands. The saliva possesses, in fact, the important characteristic of changing its composition depending on the speed of production: the more the salivary secretion increases (as during mastication), the more increases the water component of the saliva itself, and diminishes the osmotic pressure, promoting a better dilution of the food, albeit at the expense of the amount of active ingredients.

It has been estimated that over 200 different proteins and peptides are present in the saliva of men (Bedler, 1995). The flow rate and composition of saliva varies between subjects and each subject may vary within a day (Chen, 2009). The factors responsible for this great variability may be the degree of hydration, body position, exposure to light, smell, smoke, stimulation previous, age, climatic conditions, and heart rhythms and respiratory (Wisniewiski et al., 1992) Saliva is a hypotonic fluid containing about 98% water and 2% of organic and inorganic substances dissolved in it as electrolytes, mucus, glycoproteins, proteins, antibacterial compounds and enzymes, with a pH ranging between 6.2 to 7.4 (Chen, 2009). In addition to salts, saliva is composed primarily of proteins and glycoproteins that are synthesized, stored and secreted by cells of the terminal segments. These include: amylase (ptyalin), which hydrolyse the starch hydrolysis and a mucin glycoprotein that is secreted by the sublingual glands responsible for the viscosity of saliva (Friel & Taylor 2001; Genovese et al., 2009). Amylase and glycoproteins constitute the bulk of the organic secretion of terminating segments. In addition to the salivary amylase, human saliva contains a number of other enzymes that can also be present in the salivary glands. They may be salivary lipase enzymes that are dominant in human saliva, esterase, enzymes with transfer properties of hydrogen and oxidase (Van Ruth & Roozen, 2000a; Genovese et al., 2009).

Mucin is a group of macromolecules found in mucus, constituted by heterogeneous glucoproteins found in many species. It is produced by the stomach epithelium and most of the peptide backbone is glycosylated, having O-glycosidic bonds to threonine and serine residues. The polydispers state of these proteins are given by the enzyme-controlled production of the glycosylation. The residues present on the proteins are responsible of the interactions with other macromolecules, e.g. chitosan, glycosaminoglycans, etc. (Xu et al., 1996). Mucin is an O-glycosylated protein linked with many oligosaccharides by covalent bonds to serine residues or threonine, and has a molecular weight that varies from 0.5 to 20 MDa, is formed for 80% from carbohydrates, primarily by N-acetylgalactosamine, N acetylglucosamine, fucose, galactose, sialic acid and traces of mannose and sulphate (Bansil- Turner, 2006). It is a viscous, filamentous substance and gives the saliva its typical viscosity. The presence of glycoproteins are fundamental for giving saliva its central role in making the bolus suitable for swallowing and in protecting the oral mucosa (Chen, 2009).

In the presence of saliva, it has been reported that the area of the GC peaks obtained from the headspace of foods analysed is much lower, which indicates a significantly decreased of volatile compounds by the presence of saliva. The flavour-mucin interactions are particularly significant in the case of aldehydes, with which it can form Schiff bases, by means of reversible or irreversible bonds with proteins (Van Ruth et al., 1995; van Ruth & Roozen, 2000; Friel & Taylor, 2001). Several other studies have revealed interactions between polyphenols and salivary proteins. In particular, polyphenols such as flavan-3-ols, catechins and flavanoids interact through non-covalent bonds with saliva proteins rich in proline (Jobstl et al., 2004;

Ferruzzi et al., 2012). It was also shown that certain compounds such as benzaldehyde, diacetyl, heptyl-acetate and ethyl-hexanoate are influenced by interactions between mucin and the type of solute in the matrix (Friel & Taylor, 2001). In fact, since mucin has binding sites, preferentially occupied by sugars, it is able to easily trap these molecules (Friel & Taylor, 2001). Evidence of this type was described for esters and thiols, which are subject to hydrolysis and the first to the second oxidation, by esterases and oxidases saliva, respectively (Hussein et al., 1983; Buettner, 2002a).

Therefore, polyphenols and human saliva might interact, with a consequent effect on the release of aroma compound. This effect was shown recently in the research published by our research group, for olive oil phenolic compounds. Also in olive oil model systems, i.e. without the presence of saliva, the phenolic compounds in olive oil have been shown to affect the release of olive key volatiles (Genovese et al., 2015). However, in other model systems this effect has not been studied or reported yet, and this was therefore further investigated in the present thesis, as reported in Chapter 5.

Analytical techniques used to study volatile compounds

The study of food flavour is not a simple matter. So far, the attention of the scientific community has focused on the determination of the volatile fraction, as it is considered as the main responsible for the overall flavour of a food, and it is also easily detectable through the analytical tools currently available, such as the gas-chromatography coupled to mass spectrometry (GC/MS) or gas chromatography-olfactometry (GC/O) (Taylor & Linforth, 1996).

Several chemical methods exist for the characterisation of food flavour, e.g. distillation and extraction with following analysis of the headspace volatiles to identify and quantify the volatile compounds as potential stimuli. The time-intensity methods are among the dynamic sensory measurements applied. This technique has been developed to assess the persistence of taste as sweet, bitter and astringent (Pineau et al., 2009). The result of the measures are represented by a curve that show how the intensity of each feeling change as a function of time during the food tasting. The dynamic chemical methods include the static or dynamic analysis of the headspace, which provides the results more similar to the real process that occurs during the consumption of a food. The headspace analysis has been widely used to determine the factors which influence the distribution of the volatile compounds between the product and the gaseous phase.

To obtain information on the behaviour of volatile compounds at the level of the olfactory receptors, during consumption, and to study the release of volatile components from simple to complex food systems in relation to food volatiles physical-chemical parameters, many models have been proposed in the literature. The variations on the dynamic headspace concentration is usually done on an extract containing the volatile components actually present during the consumption of the food. Van Ruth et al. (1995) evaluated the release of the volatile compounds from vegetable rehydrated in three mouth model systems, and compared the flavour released directly from the mouth of 12 volunteers. By using the technique called “oral vapour” (OV) and applying the analysis of variance, no statistical difference was reported between the dynamic headspace with mastication (DHM) and OV. In fact, the researcher compared the Purge and Trap (PT) technique with DHM and OV.

The flavour profile is directly related to the human perception of the food. The techniques of simple headspace sampling without mastication have often been used to determine the profiles of the food volatile compounds. Since it has been observed that significant differences were identified between DH and OV and between PT and OV, which means that such techniques could not be used to simulate the release of flavour in the mouth. Subsequently, starting from these results van Ruth & Roozen (2000) studied the influence of three physiological parameters of the oral process, such as saliva composition, chewing and the volume on the release of volatile compounds of black pepper and rehydrated French beans. For liquid foods, Nassl et al. (1995) developed an artificial mouth to study the release of volatile components from an oil-water emulsion. Following the transfer of the volatile compounds in the gas phase a GC analysis was performed on a series of gaseous samples collected in small intervals of time. Other researchers have highlighted other aspects of the system as a better control of the temperature at 37 °C, using artificial saliva and a magnet that creates agitation to simulate chewing. Roberts & Acree (1995; 1996) developed a system which simulates the conditions of the mouth called Retronasal Aroma Simulator (RAS), which consists of a one-litre mixer, in a system similar to the Purge-and-Trap, where artificial saliva is added under controlled temperature (37 °C)

and other defined conditions (**Fig. 1.23**). Volatile compounds were collected from a trap in fused silica and quantified by a GC/FID and GC/MS with high precision and sensitivity. Through the RAS system they studied the effects of artificial saliva, temperature, mixing speed and the viscosity of the food matrix on the release of a range of volatile compounds with different physico-chemical properties. Various modifications to this system have led to obtain a volume similar to the mouth, and a control over salivation and mastication. While the study of the release of volatile compounds *in vivo* shows the limitations related to the reproducibility as due to the dependence of human factors cannot be controlled (Laing & Jinks, 2001), the *in vitro* study allows to individually verify the specific parameters that influence the release of volatile compounds (Margomenou et al., 2000).

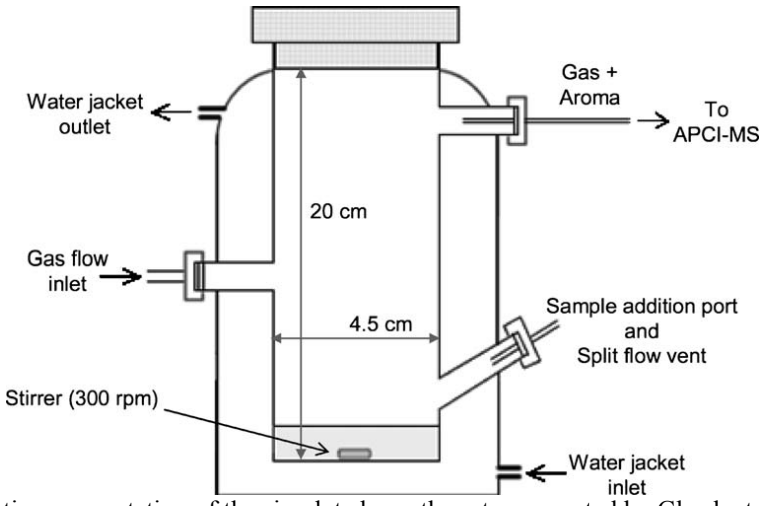

Figure 1.23. Schematic representation of the simulated mouth system reported by Ghosh et al. (2008).

Other authors have performed the analysis of the headspace concentration of aroma compounds, by extracting and analysing the volatiles directly in the mass spectrometer (Elmore & Langley, 1996; Roberts et al., 1996). The release of volatiles from food systems was reported from several vegetable, vegetable oils, sauces and emulsions has been studied using this model system (van Ruth et al., 1996; Odake et al., 1998; van Ruth & Roozen, 2000). These studies focused on the effects of the composition and volume of saliva, chewing and composition of the volatile release. In this mouth model system, the release of volatile compounds was studied as a function of time (release time). Odake et al., (1998) studied the release of the aroma from a dressing emulsion using a model of dynamic chewing headspace and reported that such a model could provide information on the release of volatile compounds better than the other models. Other variants have been proposed, with the intention to optimize the conditions of model systems and mouth simulator (Springett et al., 1999; Margomenou et al., 2000). For solid foods, a crushing step is necessary, and different systems were reported (Roberts & Acree, 1996; van Ruth et al., 1996). Roberts & Acree (1996) studied the release of volatile components using a RAS system, in combination with GC-O to study the aroma of raspberry. This technique was compared with the analysis of solvent extraction, in order to assess the instrument sensitivity. Other system have been reported in literature to simulate the mouth and chewing mechanisms and provide information on the food behaviour during its tasted, but they do not provide information on the processes that occur in the mouth. All systems are designed to sample the headspace from the nose or mouth assuming that this would better reflect the perception of the volatile components free (Taylor, 1996; Taylor & Linforth, 1996). Linforth & Taylor (1993) reported different methods for sampling volatile compounds from the mouth, and found that the direct introduction of the volatile components in the mass spectrometer was not adequately sensitive.

The cold trapping of volatile compounds using capillary columns in fused silica, followed by GC or GC/MS analysis and the use of Tenax traps allowed satisfactory results in the past. A satisfactory design was developed to solve the problems related to the direct mass injection and it was applied to many food model systems (Brauss et al., 1999; Linforth et al., 1999). However, using the techniques of rapid headspace analysis such as trapping by Tenax (Roozen & Legger-Huysmans, 1994; Delahunty et al., 1996a; O 'Riordan et al., 1998), it was possible to reduce the resolution time (Delahunty et al., 1996b). Van Ruth et al. (2000) studied the release of seven compounds from an oil-water system a model system simulating the mouth, and using a Tenax trap to adsorb the volatile components released in the headspace above the sample. They

observed the influence of mastication and how it influence on the aroma release. A comparison between the oil and water matrices was observed, as the release of hydrophobic compounds from the oil decreased while the hydrophilic components increased. A parallel application was performed using sensors in the mouth to follow the action of the salts and the change of the pH during foods consumption. Jack et al. (1995), they have used a system of conductivity in the mouth to indirectly measure the release of the salts from the mouth as a result of the rupture of the cheese, and Davidson et al. (1998), they have followed the release of the salts and of the acids from a variety of foods. These techniques provide information on the aromatic content of the food and clarify the reason why foods with the same composition can have a different flavour. In parallel with the development of the most relevant and the most sensitive analytical methods discussed previously, there has been a development corresponding to the knowledge of the chemical and physical characteristics of the compounds of the flavour and their behaviour in a food. The allocation mechanism of the volatile components in a mixture consisting of oil and water, or between the liquid phase and the gaseous phase in the mouth is responsible for the final aroma of the food (Taylor, 1998). Many studies have provided information regarding the interactions between the components responsible for the aroma volatile and non-volatile food matrix. Most of them took simple food or model systems, considering both the food structure and the initial distribution of aroma compounds in the product (Draux & Volley, 1997).

The oil-water systems have been previously studied in the literature, and the transfer of volatile compounds between the two phases and in the headspace is known. Other systems that have provided information on the links of the volatile compounds in the β-lactoglobulin (Seuvre et al., 2001; Benjamin et al., 2013), the release of volatile components from a gel solution (Boland et al., 2004), bonds between the volatile compounds and the starch and other carbohydrates (Naknean & Meenune, 2010).

Hills & Harrison (1995) developed a theoretical model considering the release of the volatile compounds from a homogeneous solid food, using a theory of two-layers to study the in-mouth mass transfer of the flavour from a food to the saliva. The release of the flavour from a gelatin solution shown the dependence and the concentration of sugar from the melting point of the gelatin. At low concentrations of sugar the limiting step was the heat transfer followed by gel fusion, but at high concentrations of sugar resulted in a reduction of the melting point (Harrison & Hills, 1996). A computerized simulation was developed (Harrison et al., 1998) to simulate a model of release from solid foods, and this simulation shown that an initial release of the flavour depends on the mass transfer and the breaking characteristic of the food, while a subsequent release depended on the velocity of the flow of saliva, by chewing and the mixing speed. The release of the flavour from a liquid emulsion (Harrison et al., 1997) showed dependence on a mass transfer at the interface of the emulsion. This model has been widely used to assess the effects of the flow velocity of air (Harrison & Hills, 1997b) and the flow of saliva (Harrison, 1998), and the latter was considered influential on the release of volatile components in the gas phase.

Rabe et al. (2004) studied the influences of shear rate (surface extension), airflow, in-mouth headspace volume, synthetic saliva and human epithelial cells (modelling mucosa) on the initial dynamic flavour release from liquids. A mouth model apparatus was used to analyse the aroma release from flavoured waters, and the authors reported that neither addition of saliva alone nor the combination of saliva and mucosa showed significant influence on in-mouth flavour release from liquids in the model mouth (Rabe et al., 2004). In another paper, the authors demonstrated that in case of O/W emulsions, the emulsion droplet diameter has no significant influence on the dynamic flavour release, while there is a statistically significant influence of the chemical composition of the lipids (Rabe et al., 2003a). Indeed, a significant influence of lipid molarity on liquid/liquid partitioning and release of some flavour compounds was reported, whereas it was shown that volatile compounds with low hydrophobicity were not affected by the presence of lipids. On the contrary, for hydrophobic compounds a positive correlation between was reported between their release and decreasing molarity of the lipid phase, in O/W emulsions (Rabe et al., 2003b). The same research group has carried out several other experiments reported in a wide range of model systems and further details were reported in the discussion sector on the chapter dealing with aroma release.

Mechanism of volatile release from emulsions

As stated in the previous paragraphs, an emulsion is formed from two immiscible liquids with one of the liquid dispersed in the form of small particles (droplets) in a continuous phase (Mc Clements, 2005). A system comprising the oil phase dispersed in an aqueous phase is called oil-in-water emulsion, while a system consisting of droplets of water dispersed in the oil phase is called water-in-oil emulsion. In many foods, the particles diameters of fine emulsions are between 0.1 and 0.01 μm, although during storage the mean particle size increases, determining the physical instability and thus the phase separation. The process that converts two immiscible liquids in an emulsion is known as homogenization. Many food emulsions are formed by three regions which have different physico-chemical properties: the inner part of the particles, the continuous phase and the interface. The non-polar molecules tend to localize in the oil phase, polar molecules in the aqueous phase, and amphiphilic molecules at the interface (McClements, 1999). The particles concentration in emulsions is described in terms of volume fraction in the dispersed phase, which is equal to the volume of the particles in the emulsion divided by the total volume of the emulsion, and this is a very important property because it affects the appearance, texture, flavour and stability of emulsions. When all the emulsion particles have the same magnitude the emulsion is called monodisperse, otherwise in case of different sizes they are referred as polydisperse emulsion (McClements, 1999). In the mouth during consumption, the emulsions undergo a series of processes such as mixing with the saliva, changes in ionic strength, pH and temperature. Therefore, the release of volatile components in the emulsion appears to be strongly influenced by the presence of saliva (van Ruth et al., 2001; Buettner, 2002; Hansson et al., 2003). It has been shown that saliva change the headspace concentration of the volatiles due to dilution, salting out and the interactions between the volatile compounds and salivary proteins.

The dilution effect of the saliva in the emulsions is the result of different solubility and affinity of the compounds for saliva and for the oil phase (Doyenette et al., 2011). The amylase and mucin together with the shear force due by the movement of the tongue, play an important role in the phenomena of flocculation and coalescence which take place during the consumption of O/W emulsions. The changes in the structure of the emulsion have consequently an impact on the perception of the food product (Feron & Poette, 2013). When oil is added to a food to form an emulsion or a dispersion, the odorous components lipophilic tend to move into the oil phase and their concentration in the continuous phase decreases considerably, thus lowering their concentration in the gaseous phase. Thus, the presence of oil in a food tends to have a significant effect on the release of aroma in the gaseous phase and so on his perception. Therefore, that the composition and structure of the food have a strong influence on the release of aromatic compounds, which can be dissolved, adsorbed, bound, trapped, encapsulated or limited in circulation by members of the food matrix. The relative importance of each of these mechanisms varies also according to the chemical-physical properties of the volatile compounds of the food (Kinsella, 1988). The orthonasal and retronasal odour differ in the level of perception, even if they involve the same mechanisms (Burdach et al., 1984; Voirol & Daget, 1986, 1989; Kuo et al., 1993; Linforth et al., 2002). In fact, the changes of food during consumption affect the profile volatile and therefore the sensory markedly. These differences are due to the fact that salivation, chewing and temperature are factors that can modify the sensory properties of the food, when it enters the mouth (Taylor, 1996; Buettner & Schieberle, 2000; van Ruth & Roozen, 2000a, 2000b; van Ruth & Buhr, 2004). Overall, the difference between perception orthonasal and retronasal depends on the nature of the volatile components and by individuals (Marie et al., 1987).

In conclusion, when an extra virgin olive oil (EVOO) is combined with a milk product, the overall retronasal aroma could vary in relation to the interaction of non-volatile matrix of the food, for the presence of the whey proteins and/or olive oil polyphenols, the nature and/or physical-chemical properties of the olfactory stimuli and to the various physiological factors (salivation, temperature, chewing etc.). For example, the protein fraction of milk has a strong influence on the volatile profile, both for their chemical interactions and physical entrapment (Reineccius, 2006). In addition, when the food is introduced in the mouth and comes into contact with saliva a further emulsion is created, so that the aroma compounds tend to move in the lipophilic oil phase and their concentration in the continuous phase decreases significantly; on the contrary, the hydrophilic compounds tend to move in the aqueous phase. In addition, amylase and mucin, the most important salivary proteins, along with the shearing force due to the tongue movements, play an important role in the phenomena of flocculation and coalescence which take place during their consumption (Van Anken, 2004; Vingerhoeds et al., 2005; Vingerhoeds et al., 2007). These changes in the structure of the emulsion have a significant impact on the sensory properties (Vingerhoeds et al., 2005; Dresselhuis et al.,

2008; Vingerhoeds et al., 2008; Benjamins et al., 2009). Several techniques have been applied to monitor the release of aromatic compounds during the consumption of a food such as the trapping of compounds of exhaled material adsorbents (Delahunty et al., 1996) or real-time measurements followed by techniques of mass spectrometry. Unfortunately, although these *in vivo* approaches experimental data acquired are closer to the site of perception, it is difficult to control the many physiological variables of the oral cavity (Laing & Jinks, 2001). For this reason other systems have been developed to simulate mouth conditions, and they are known by the acronym RAS (Retronasal aroma simulator). These simulators make the technique simpler to use and with very similar results to those obtained with *in vivo* techniques.

To date, several studies have been aimed at understanding the retronasal perception of model solutions in bulk oil and emulsions, prepared exclusively by seed oils (van Ruth & Roozen, 2000b; van Ruth et al., 2000a; van Ruth et al., 2000; van Ruth et al., 2001; van Ruth et al., 2002; Malone et al., 2003; Arancibia et al., 2011; Frank et al., 2011). However, Mestres et al. (2006) have studied the release of ethyl butanoate after interaction between the protein serum and saliva. However, only one study has verified the retronasal aroma of a model solution in soybean oil and in the presence of β-lactoglobulin (Benjamin et al., 2013). In addition, most of the *in vitro* studies included the use of artificial saliva (Malone et al., 2003; Mestres et al., 2006; Arancibia et al., 2011; Frank et al., 2011) without being able to then test the effect of several enzymes and proteins present in human saliva (Humphrey-Williamson, 2001; Chen, 2009). Since the sensory aspect plays a vital role in addressing the preference of consumers, it is important to know and to better characterize these interactions that can change the overall aroma in the case of combination of two different foodstuffs, trying to avoid the actual sensory test but creating an analytical method to describe the possible perfection of aroma compounds. For this reason, since human saliva plays an important role in the perception retronasal food (Roberts & Acree, 1995; van Ruth & Roozen, 2000a, 2000b; Friel & Taylor, 2001; van Ruth et al., 2001; Buettner, 2002; 2002b; Genovese et al., 2009), it was used in the present study to assess the headspace aroma release under simulated mouth conditions, i.e. retronasal aroma, as due to ingredients interaction and particularly olive biophenols and proteins.

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Motivation of the study

Chapter 2

The investigation of the chemical and physical interactions among food constituents is of great importance for the assessment of the global quality and consumer acceptance, but also for the development of new food products with specific functional properties. Being olive oil the top product among vegetable oils, it has particularly high importance for food scientists, food industry and producers, but also for consumers, due to its unique sensory and health properties.

Phenolic compounds are very relevant in this context, as they provide the typical bitter and pungent note in virgin olive oils, but they also represent a major problem in producing countries, due to their high pollution charge being present in high concentrations in the olive mill wastewater (OMW). For this reason, the use of OMW phenolic extracts in food products can be advantageous in terms of improving the nutritional value of the food and creating a functional food.

Emulsions, and especially oil-in-water (O/W) emulsions have wide applications in food industry and they can be used in a number of foods, as well as food ingredients. For this reason, in the present study O/W emulsions formulated using olive oil as the sole fat phase. To simulate an extra virgin olive oil (EVOO), the phenolic compounds and the key aroma compounds typically found in good EVOOs were added to the model, especially for the study of aroma release and interaction.

The main objectives of the present work were the followings:

- Investigate the formation and stability of a food dispersion model system in relation to the presence and concentration of olive phenolic compounds and hydrocolloids used as stabilisers;
- To verify the effect of OMW biophenol extracts and whey protein isolate on the stability of olive O/W emulsions stabilised by hydrocolloids;
- To create a mathematical model by response surface methodology for the description of emulsions behaviour (physical and chemical stability) depending on emulsion ingredients;
- To study the aroma release of olive oil key aroma volatiles by assessing the effect of biophenols and whey proteins interactions in a *vitro* system to simulate the retronasal aroma without involving human subjects;
- To assess the effect of olive phenolics and whey proteins on the simulated retronasal aroma release, also in the comparison to the measured orthonasal aroma release;
- To propose the use of olive mill wastewater extracts as a promising food ingredient in food emulsions and dispersions based on the models studied.

Topic 1

Chapter 3

Physical and oxidative stability of functional olive oil-in-water emulsions formulated using olive mill wastewater biophenols and whey proteins

Introduction

As described in the introduction chapter, olive oil is one of the most appreciated fats both for its composition and sensory properties. The presence of high proportion of the monounsaturated oleic acid has been associated to many health benefits. In case of virgin olive oil, the presence of phenolic compounds gives further benefits in terms of antioxidant properties in vivo and in vitro (Owen et al., 2000).

Olive oil production technology implies major environmental problems in the countries where its production is mainly localized, i.e. the Mediterranean area, as industry produces a high output of liquid by-products represented by the olive mill wastewater. Due to the high concentration in phenolic compounds, this waste can be conveniently converted into a valuable source of antioxidant compounds, which can be added in a variety of foods to design functional product with better nutritional properties. A characterisation of the olive mill wastewater (OMWW) has been previously reported (De Marco et al., 2007), and its biological activity has been reviewed by others (Obied et al., 2005). Olive mill wastewater contains a wide range of phenolic compounds, mainly secoiridoid derivatives, such as hydroxytyrosol and the dialdehydic form of decarboxymethyl oleuropein aglycone along with tyrosol and verbascoside (Servili et al., 1999).

During the past few years, new technologies have been tested and applied for the extraction of phenolic compounds from wastewater, particularly membrane processes which implies ultrafiltration in combination with nanofiltration and reverse osmosis (Paraskeva & Diamadopoulos, 2006). Spray-drying of the extract obtained from membrane filtration could be theoretically applied in many foods, while a problem still remains for its inclusion into liquid foods and in particular in vegetable oils. The addition of phenolic compounds in olive oil, i.e. a refined product that has very little phenolic compounds due to the refined process, could lead to higher stability toward lipid oxidation during storage.

However, being these phenolic extract highly hydrophilic, a different approach should be tried apart from their simple addition to the oil, i.e. the production of O/W emulsions. This approach is also applicable at industrial level for the production of a wide range of food products, like mayonnaise, creams, sauces and other spreads. Moreover, emulsions can be also used as part of more complex food products such as yoghurts, ice creams and whipped products (Leal-Calderon et al., 2007), which could have further nutritional benefits.

Emulsions are kinetically instable systems, and their instability is due to many mechanisms, including creaming, coalescence and flocculation (Dickinson, 2009; McClements, 2004). Therefore, stabilizers and emulsifiers are needed to provide physical stability toward the natural separation phase. Thickening agents are mainly polysaccharides used to increase viscosity of the continuous phase (Dickinson, 2009; Paraskevopoulou et al., 2005). Xanthan gum, maltodextrin, galactomannans, intact or modified starches, propylene glycol alginate, pectin and carboxymethyl cellulose are the main stabilizers used in food industry, for their rheological properties and versatility (Dickinson, 2009). When amphiphilic polysaccharides are used, e.g. gum arabic or propylene glycol alginate, they adsorb onto the surface of the droplets and prevent aggregation by steric and/or electrostatic forces, which in turn gives better stability against flocculation and coalescence (Paraskevopoulou et al., 2005). Maltodextrin was previously applied in emulsions as an encapsulating material, i.e. to encapsulate flavour or functional ingredients (O'Regan and Mulvihill, 2010). Maltodextrin are hydrolysis products of starch consisting of α-(1,4) linked D-glucose oligomers and/or polymers, and they are used in food emulsions as stabilisers (Klinkesorn et al., 2004). Xanthan gum has appreciated characteristics related to its viscoelastic properties and its chemical properties, in particular the water solubility and pH stability (Logaraj et al., 2008; McClements, 2004). Many other stabilizers are commonly studied in O/W emulsions, e.g. pectin and carboxymethyl cellulose, and the physical stability, turbidity, cloudiness and flavour release are the most usual parameters assessed in O/W emulsions (Mirhosseini et al., 2008). Visual observation, ultrasound profiling, microscopy, droplet size distribution, small deformation rheology, nuclear magnetic resonance, confocal

microscopy, diffusing wave spectroscopy and turbiscan are the methods applied for the characterisation of emulsions stability and behaviour (Huck-Iriart et al., 2011).

Among the hydrocolloids showing thickening and emulsifying properties, milk proteins (caseinate and whey proteins) are used being naturally found in many food systems, and for their good solubility and their behaviour under heating conditions (Huck-Iriart et al., 2011). Whey protein isolate (WPI) contains both hydrophobic and hydrophilic regions and can be rapidly adsorbed on the oil–water interface in the form of a protective film (Djordjevic et al., 2004; Sun, Gunasekaran, and Richards, 2007). WPI can form a monolayer on the oil droplets, being these proteins surface-active (Sun & Gunasekaran, 2009). Proteins are usually less effective emulsifiers than synthetic surfactants, but their use in food industry has being increasing due to the trend to use "clean label" ingredients or "natural" products (Klein et al., 2010).

Many factors have been considered for the characterisation of O/W emulsions, including the effect of pH (Ratjika Chanamai et al., 2002; Sørensen et al., 2008), oil phase concentration (Dapčević Hadnađev et al., 2013), the composition of the oil phase used (Hayati et al., 2007) and the effect of stabilizers, emulsifiers, droplet size and droplet size distribution (Dapčević Hadnađev et al., 2013; Lethuaut, Métro and Genot, 2002; Ogawa et al., 2003; Silva et al., 2010) and the presence of metals and phenolic compounds on O/W emulsion (Paiva-Martins et al., 2006; Sørensen et al., 2008). The effect of environmental stresses, such as heating, chilling, freezing and drying, was also considered, as it is significant in determining the behaviour of protein-stabilized emulsions (McClements, 2004).

The amount of polysaccharide has also dramatic influence on the microstructure and large deformation properties of mixed gels and gel-like emulsions (de Jong and van de Velde, 2007). Moschakis et al. (2010) evaluated the behaviour of protein-stabilized O/W emulsions using chitosan and gum Arabic as stabilizers, demonstrating that emulsion behaviour and microstructure are strictly dependent upon the precise stabilizer concentration. Indeed, when higher polysaccharides ratio are used in WPI-stabilized emulsions, they observed a rapid serum separation and increase in mean droplet diameter (Moschakis et al., 2010). Other researchers reported on the interactions occurring between WPI and xanthan gum when forming electrostatic conjugates in aqueous medium. A synergism between WPI and xanthan gum was shown, as they form hybrids that exhibit significant effects on the surface tension at specific levels (Benichou et al., 2007).

Food emulsions are often multiphase systems containing mixtures of proteins and polysaccharides, and hence the importance of studying the behaviour of emulsions containing more than one biopolymer is necessary in order to better understand their properties and interactions. E.g., Traynor et al. (2012) studied the stability of O/W emulsions produced using sunflower oil (10-20%), stabilised by soy lecithin (1-5%) and xanthan gum (0.01-3%). Emulsions were studied under 14 days of accelerated storage condition and the conditions to obtain the maximum emulsion stability were reported (Traynor et al., 2013).

Olive oil has been used by various researchers to formulate O/W emulsions (Bylaite et al., 2001; Protonotariou et al., 2013). E.g., Bylaite et al. (2001) compared the effect of olive oil to soybean phosphatidylcholine on the stability of emulsions stabilized using β-lactoglobulin. Paraskevopoulou et al. (2005) reported on an olive O/W emulsion formulated with lemon juice to produce a salad dressing, and they reported about the creaming behaviour and rheological parameters as affected by the stabilisers, such as propylene glycol alginate, xanthan and gum Arabic (Paraskevopoulou et al., 2005). Nikovska (2012) investigated the effect of lipid concentration, constituted by olive oil, and compared soy protein isolate and WPI as stabilizers. The author confirmed that lipid oxidation in emulsion is more rapid than in bulk oil, and that WPI was less effective in retarding oxidation than soy protein isolate (Nikovska, 2012). It was shown that olive oil has better physical stability in low-fat emulsion than other oils like sesame, and WPI and xanthan gum were used as stabilizer agents (Protonotariou et al., 2013).

Olive oil was also used by others to formulate model O/W emulsions (Di Mattia et al., 2009; Di Mattia, et al., 2010; Sotiroudis et al., 2005). The stability of olive O/W emulsions as affected by the presence and concentration of synthetic surfactants and β-lactoglobulin, and the better stabilisation behaviour of whey proteins than Tween 20 was also demonstrated (Di Mattia et al., 2010).

In emulsions, the presence of the aqueous phase has the ability to decrease the activity of antioxidant compounds because hydrogen-bonded complexes formed with water are ineffective in scavenging lipid radicals by hydrogen donation (Paiva-Martins et al., 2006). Hydroxytyrosol, the most abundant phenolic compound in olive oil and OMWW was reported to exert the highest antioxidant activity within the olive phenolics, whereas a dramatic general lower effect was observed in the presence of copper ions (Paiva-Martins

et al., 2006). The strong antioxidant activity of polyphenols is due to their radical scavenging activity resulting from resonance stabilization of the resulting radical after oxidation (Kumamoto et al., 2001). The antioxidant activity of more polar phenolic compounds was reported to be reduced in emulsions compared to bulk oil, for the so-called "polar paradox", which explains the different effectiveness of antioxidant compounds in relation to their partitioning between water and lipid phases (Porter, 1993). Previous studied reported on the antioxidant activity, considering pure hydroxytyrosol, 3,4-DHPEA-EA, 3,4-DHPEA-EDA and oleuropein under different pH conditions, and the ranking of effectiveness in retarding the increase in conjugated dienes over storage was as follows: 3,4-DHPEA-EA>3,4-DHPEA-EDA>hydroxytyrosol>oleuropein (Paiva-Martins and Gordon, 2002).

Beside the known effect about the antioxidant properties of polyphenols and oxidation stability of both bulk systems and emulsions, little is known about the interactions with proteins in emulsions. In single proteins, e.g. β-lactoglobulin, BSA etc., the effect of polyphenols binding was previously reported by others (Almajano and Gordon, 2004), but there is a lack of information about the behaviour of these biopolymers in olive O/W emulsions and its effect on the stability. Indeed, natural or synthetic antioxidant compounds are usually added in O/W emulsions to longer their shelf life, as they inhibit lipid peroxidation. Whereas the effectiveness of some phenolic compounds has been proven so far in some studies, their final effect depends upon many factors, including their surface active properties and their ability to accumulate at the interface where the reactive species are likely to be formed (Frankel and Meyer, 2000). Also, Di Mattia et al. (2010) suggested that, being olive oil a complex mixture of minor compounds and triacylglycerols, a better stabilisation is obtained thanks to the interaction with minor compounds with whey proteins (Di Mattia et al., 2010).

The need of food industry to verify these effects on real food products and the need of knowledge about complex systems has to be satisfied by using food-grade products. The possibilities to find a valid application of phenolic extracts from by-products, particularly OMWW, could have great benefits on both the environmental aspects and the nutritional value of food products. Indeed, the possibility of using by-products from food industry, specifically OMWW, and converting them into a valuable ingredient for the creation of a functional food product has been investigating by using olive oil as a source of fat, and whey protein isolate and olive phenolic compounds in a model O/W emulsion system.

Therefore, the aim of the present experiment was to characterise the behaviour and properties of O/W emulsions formulated with 20% olive oil and functionalised using OMWW powder extract at two concentrations, also considering the possible interaction of WPI and xanthan gum added as emulsifiers and stabilizers.

Materials and methods

Olive oil sample, stabilizers and OMWW powder

Freshly refined olive oil was donated by I.O.B.M. srl (Montesarchio, BN, Italy). Xanthan gum from *Xanthomonas campestris* was purchased from Sigma-Aldrich (Darmstadt, Germany). WPI was 97.5 wt% protein, and lactose content was less than 1 wt%. A phosphate buffer solution at pH 7.0 was prepared using monosodium phosphate and sodium hydroxide (Darmstadt, Germany). The buffer was used to maintain constant pH, as this parameter can affect emulsion stability (Sorensen et al., 2008). All other chemicals were of analytical grade purity. Phenolic extract from olive mill wastewater OMW was kindly donated by LABS (Department of Agricultural Sciences, University of Naples Federico II, Italy). P-OMW production process has been reported by Troise et al.(Troise et al., 2014). The composition of the three main phenolic compounds analysed by HPLC-UV-Vis was as follows: OHTy 32±0.2 mg g-1, Ty 1.9±0.1 mg g-1, verbascoside 2.8±0.09 mg g^{-1} (Troise et al., 2014).

Emulsions preparation

Emulsions were prepared by dispersing spray-dried P-OMW powder at two concentrations (1 or 5 mM expressed as OHTy) and WPI (0.13 or 0.5% w/v) into a buffer solution (5mM phosphate buffer, pH 7). The aqueous phase was gently stirred for 2 h at room temperature to ensure dissolution, using a magnetic stirring bar and magnetic stirrer hotplate (Stuart CB162, Bibby-scientific, Staffordshire, UK). The pH was checked and adjusted to pH 7.0 using 1M HCl. Xanthan gum (0.06 or 0.2% w/v)

was added to the emulsions and gently stirred (100 rpm) overnight at room temperature to allow complete hydration. Emulsions were produced by blending 20% (v/v) refined olive oil in the solution previously prepared using a high-speed blender at 8,000 rpm for 2 min, after a pre-emulsification phase (Traynor et al., 2013). The emulsions were then transferred in glass tubes for the creaming stability analysis, and stored into an incubator at 40 °C ±1 °C for kinetic stability evaluation (**Fig. 3.1**).

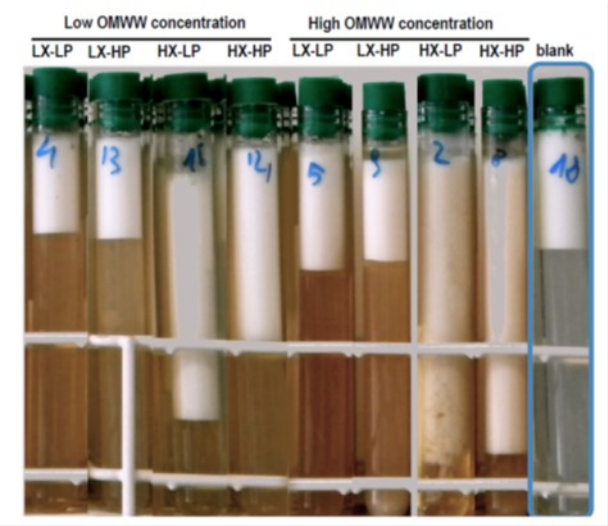

Figure 3.1. Olive O/W emulsions (20% olive oil) functionalised by adding olive mill wastewater (OMWW) extract, at day 7 of storage under accelerated storage (40 ºC), using low (L) or high (H) concentrations of stabilisers. X: xanthan gum; P: whey protein isolate

The concentrations of the stabilisers were chosen according to previous works (Di Mattia et al., 2009; Sun et al., 2007). Emulsions with low or high concentrations of stabilisers are named as follows: LX-LP, low xanthan (0.06% w/v) and low WPI (0.13% w/v); LX-HP, low xanthan and high WPI (0.5% w/v); HX-LP, high xanthan (0.2% w/v) and low WPI; HX-HP, high xanthan and high WPI. For each system, two concentrations of P-OMW were used, i.e. 1 and 5 mM as OHTy, corresponding to low and high average phenolic concentrations in commercial virgin olive oils (Caporaso et al., 2015). Blank system had no added P-OMW. In this case, WPI and xanthan gum concentration was 0.5% and 0.2% (w/v), respectively.

Creaming value

Creaming value was monitored visually according to literature.(Dickinson, Radford, & Golding, 2003) Duplicate samples of emulsions were stored in 75 mm x 12 mm sample tubes (York Glassware, UK) at 25 ±0.5 °C. Measurement of the serum layer (creaming index) was carried out manually using a 60% fiberglass Measy 2000 Typ 5921 (Baty, Switzerland). Stability was evaluated as percentage decrease from the initial height, using the following formula: Creaming Index=100 × (*H*S/*H*E); where *H*S is serum layer formed at the bottom of glass tubes, and *H*E is the total height of the emulsions in the tubes (Klinkesorn et al., 2004).

Mean particle size by image analysis through optical microscopy

Observation through the microscope is the direct way to determine the type of an emulsion. The different reflectance index of the water and oil phase allows the visual appreciation of the droplets. This microscopic technique is a more detail technique as compared to others, and it also allows a direct observation of the droplet shape and measurement of the droplet size (Fig. 3.2).

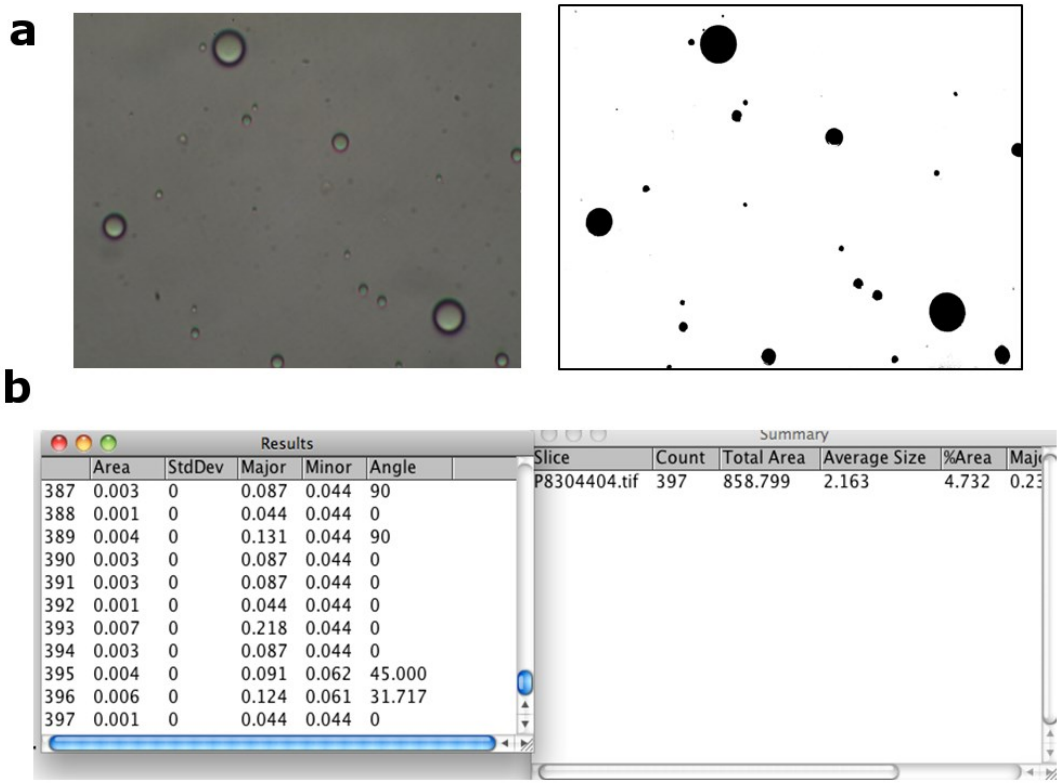

Figure. 3.2. Conversion to binary format (binarisation) and noise removal (a); analysis of emulsion particle size in Image (b).

For particle size determination by digital image analysis, emulsions were diluted 1:1000 using buffer solution to avoid droplet overlapping. A drop of emulsion was placed on a microscope slide and then covered with a cover slip. The microstructure of the emulsion was observed using an Olympus DP72 optical microscopy (Japan) at 40x and 400x magnification. Digital pictures were taken by using an Olympus E-620 digital camera mounted on the microscope. Mean droplet size was calculated by analysing the microscopic images with ImageJ 1.47t 64-bit software (National Institutes of Health, USA). The function Analyse Particles was used after colour threshold and using the following options: size 0.01-infinity; circularity: 0.00-1.00; exclude on edges; particle size >2 μm^2; options exclude droplets on edges and include holes. At least 20 pictures were taken at 100x magnification for each emulsion analysed. Image analysis was performed as described by previous workers, (Silva et al., 2010) and details are reported below.

The images were then transferred to a personal computer, and were processed following the method sequence of other studies involving binarisation and droplet quantification (Silva et al., 2010; Trindade et al., 2008; Freire et al., 2005). The Image J software (Version 1.451, U.S National Institute of Health, Bethesda, MD, USA) and a stage micrometre (OB-M stage micrometre, Olympus Imaging and Audio Ltd., Essex, UK) of known size were used for calibration of the droplet diameter.

In a more detailed way, the image analysis performed followed a three-step sequence: image binarisation, droplet quantification, and evaluation of statistical parameters. The binarisation consisted in the conversion of the captured image to black and white and in the removal of noise. The sequence of the operations performed can be divided into four primordial parts: (a) creation of a binary image from an intensity image based on a luminance threshold; (b) use of a median filtering to remove noise; (c) suppression of the structures connected to image border; and (d) erosion of the binary image and morphological reconstruction using a mask previously created. This first step is the vital part of the image analysis process, since all the measurable data are going to be taken from it. The second step quantifies the droplets in the image, yielding parameters such as droplet diameter, droplet volume, droplet area, and particle sharpening. During the last step a statistical analysis of the data acquired

from several images were performed in order to evaluate the total number of analysed objects, the average droplet diameter and its standard deviation. It also provides the particle roundness, useful in detecting if objects other than droplets are being analysed, as well as the particle size distribution. The use of this automatized procedure allowed the analysis of a larger number of objects with higher precision and accuracy compared to manual quantification. This image processing procedure was developed using a program developed ImageJ. A microscopic rule was used for calibration of the droplet size. For each sample an average of 20 different images were analysed providing several hundred of droplets for each data point. In the end of the experiment.

Light scattering techniques and emulsion droplet size distribution

Large particles in solution can scatter visible light and the amount of scattering gives information about the particle dimension. There are several techniques based on light scattering to measure particle size in solution and particularly in emulsion.

The course of the scattered intensity as a function of the detector angle depends on size and structure of the particles. Light scattering techniques have been used to measure the size of dairy components including fat globules, lactose crystals and casein micelles in suspension or in composite media. Consequently, a suspension of particles forms an angular pattern of scattered light characteristic of its particle size distribution (PSD). SLS tries to find a PSD whose theoretical scattering pattern best fits the experimentally- observed scattering pattern.

Based on the theory, the primary scattering response observed from the surface of the particle can predict its size, with the intensity predicted by the refractive index difference between the particle and the dispersion medium. It also predicts how the effect on the secondary scattering signal, which is important for particles below 50 μm in diameter. It predicts scattering intensities for all particles, small or large, transparent or opaque within the following assumptions:

- the particles being measured are spherical;
- the suspension is dilute so that the scattered light is measured before it is re-scattered by other particles;
- the optical properties of the particles and the medium surrounding them is known;
- the particles are homogeneous.

Since the model assumes spherical particles, the particle size of irregular particles is expressed in terms of a spherical equivalent diameter. In the case of laser diffraction, the diameter of the sphere that would produce an equivalent light scattering pattern to the measured particle is reported.

Droplet-size distributions of the emulsions were determined by using a Mastersizer 2000 Hydro 2000S (Malvern Instruments, UK), which gives measurement based on light scattering under high dilution conditions by dispersing the samples in distilled water (Dickinson et al., 2003; Lethuaut et al., 2002). To avoid multiple scattering effects, the freshly prepared emulsions were diluted to reach an obscuration rate of about 3. The refractive indices of water and refined olive oil were 1.330 and 1.418, respectively. Average droplet sizes were characterized in terms of the volume mean diameter $d_{4,3} = \Sigma_i \cdot n_i \cdot d_{i4} / \Sigma_i \cdot n_i \cdot d_{i3}$, where ni is the number of droplets and of diameter di. The $d_{4,3}$ parameter is a useful mean diameter value, sensitive to small changes in droplet-size distribution (Moschakis et al., 2010). All measurements were made at room temperature and four measurements were obtained for each sample. A bimodal particle-size distribution was taken to be indicative of non-reversible flocculation (Dickinson et al., 2003; Lethuaut et al., 2002).

Cloudiness and turbidity measurements

Cloudiness measurement, also called turbidity or opacity, was carried out according to previously published methods for O/W emulsions (Mirhosseini et al., 2008). Samples were taken after 24 h to their preparation and analysed over the storage period. In the case of separated emulsions, the supernatant was sampled, while in the absence of evident phase separation, the upper part was sampled as well. Emulsions were diluted (2.5:1000) and cloudiness was expressed from the absorbance at 660 nm.

Rheological properties of emulsions

Rheological measurements were carried out in accordance to previous papers reporting on O/W emulsions stabilized by WPI and xanthan gum (Sun and Gunasekaran, 2009). Steady shear viscosity and small-amplitude oscillatory shear tests were conducted using a Bohlin C-VOR dynamic rheometer (Malvern Instruments Inc., Southborough, MA). Emulsion viscosity was measured at 25°C, over a shear rate range of 0.01–100 s^{-1} with cone-plate geometry (CP 40/4°). All measurements were performed within 24 h from emulsion preparation. A logarithmic progression was applied, and sweep time was 120 s. Oscillatory tests were performed by pouring emulsion samples (typically 1-1.5 mL) directly on the holding stage and samples were covered with thin paraffin oil layer preventing water evaporation. In oscillatory experiments the storage (G') and loss (G'') moduli were recorded versus frequency (0.1–10 Hz) at constant strain, with increasing of logarithmic scale. The linear viscoelastic region was previously determined selecting a strain of 0.5 Pa, recording G' and G'' versus shear stress (0.01–100 Pa) at constant frequency.

The strain sweep profile gives information on the critical strain and gives information about the elastic component of the emulsions. It was reported that the interdroplet interaction strength increases as the surfactant and oil concentration increase, as reported in the following equation:

$$E_c = \frac{1}{2} G' \gamma_c^2$$

These interdroplet interactions can be represented as the cohesive force, Ec which can be estimated using the elastic modulus (G') and γ c of the emulsions. The cohesive energy is related to the structure of the emulsion system which correlated to the droplet size and number of contact area between the droplets. The droplet concentration and the packing of the droplets directly influence the strength of the cohesive force.

Oxidative stability: lipid hydroperoxides

Lipid hydroperoxides were measured according to previous works (Di Mattia et al., 2009). Emulsions (0.3 mL) were mixed with 1.5 mL of isooctane/2-propanol (2:1, v/v), vortexing three times for 30 s and centrifuging for 2 min at 2000 x g (Hettich Rotanta 460R centrifuge). The supernatant (200 µL) was collected and 2.8 mL of a methanol:1-buthanol solution (3:1, v/v) were added, followed by 15 µL of 3.94 M ammonium thiocyanate and 15 µL ferrous iron solution (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, absorbance was measured at 510 nm using a Lambda Bio 20 spectrophotometer (Perkin Elmer, Boston, MA). Hydroperoxides concentration was determined using a calibration curve prepared with hydrogen peroxide.

Oxidative stability: Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARs) were determined according to previously published methods (Di Mattia et al., 2010). Emulsions (0.1-1 mL) were mixed with 2.0 mL of TBA reagent (15% w/v tricholoracetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 minutes and then centrifuged (2000 g using a Hettich Rotanta 460R centrifuge) for 15 min at 20 °C. After 10 minutes, the absorbance was measured at 532 nm. TBARs concentration was determined by a standard curve prepared using 1,1,3,3-tetramethoxypropane.

Statistical analysis

In order to better understand the influence of polyphenols, WPI and xanthan gum, as well as their interactions, a multifactor ANOVA with second-order interactions was performed. XLStat (2009.3.02), add-in software package for Microsoft Excel (Addinsoft Corp., Paris, France), was used for data elaboration. Cluster analysis was used to construct hierarchical dendrograms, searching the

natural groupings among the samples. The sample similarities were calculated on the basis of the squared Euclidean distance, using the Unscrambler X software v 10.3 (CAMO Software, Trondheim, Norway).

Results and discussion

Creaming rate

The creaming rate of olive O/W emulsions added with P-OMW and stabilized by WPI and xanthan gum is reported in (**Fig. 3.3**). Different behaviour was observed depending on xanthan gum levels. This hydrocolloid is a stabiliser, it was expected to delay creaming when higher concentrations are used. Indeed, xanthan gum is widely applied for its good viscoelastic and chemical properties, e.g. water solubility and pH stability.(McClements, 2004) The control sample contained a high concentration of xanthan gum (0.2% w/v). The creaming rate observed was lower in the first storage period, i.e. up to 8 days, from which a creaming similar to the samples containing low concentration was measured.

The addition of P-OMW did not cause dramatic changes in the creaming rate between low and high concentrations of xanthan gum. In the systems with low P-OMW level (**Fig. 3.3a**), a difference in the creaming rate was observed due to the concentrations of WPI. While in the emulsions with low xanthan concentration a significant statistical difference was not reached, samples with high xanthan concentration showed higher creaming rate when higher WPI levels was used.
Two different creaming processes exist, i.e. the creaming of individual particle, with particle size around 10 μm, and the migration of flocculates, which is observed at lower particle sizes (Huck-Iriart et al., 2011). Creaming occurs when the density of the droplets is less than that of the continuous phase (Dickinson and Golding, 1997), and stabilizers act in increasing emulsion viscosity and therefore they retard droplets movement.

A possible effect of WPI on creaming rate was reported by Sun and Gunasekoran (2009), as increasing WPI concentrations caused a slight decrease in creaming index. Indeed, it has been reported that 0.2% of WPI was not enough to cover the entire droplet surface to stabilize the emulsions containing 20 to 40% oil (sun and Gunasekara, 2009). The effect was explained by the unabsorbed WPI in the aqueous phase, while the presence of xanthan increased the amount of protein unabsorbed at the interface. The different behaviour observed in our emulsions with low OMWW and high concentration of xanthan (HX) is likely to be due to this phenomenon. The possible protein-polyphenols interaction seems to be of little extent, explained by the simple chemical structure of olive phenolics and their molecular size, as well as by the presence of hydrocolloids which could interfere with the binding, particularly xanthan gum, representing a barrier for the physical interaction and consequently the chemical binding. Indeed, P-OMW phenolics have a relatively simple structure and lower molecular weight than other classes of phenolics for which the protein-polyphenols effect was reported, i.e. tannins. Previous papers have shown that olive phenolics have binding affinity with caseinate and whey proteins, whereas OHTy and Ty did not interact significantly with pure BSA (Pripp et al., 2005). In our previous study, (Genovese et al., 2015) the effect of the interaction between olive phenolics and WPI on headspace release of volatile compounds in emulsion was reported.

Particularly, a significant increase of some key odour compounds was found when phenolic compounds were added to the emulsion, probably due to the polyphenol−protein interaction influencing the binding effect of volatile compounds by WPI.

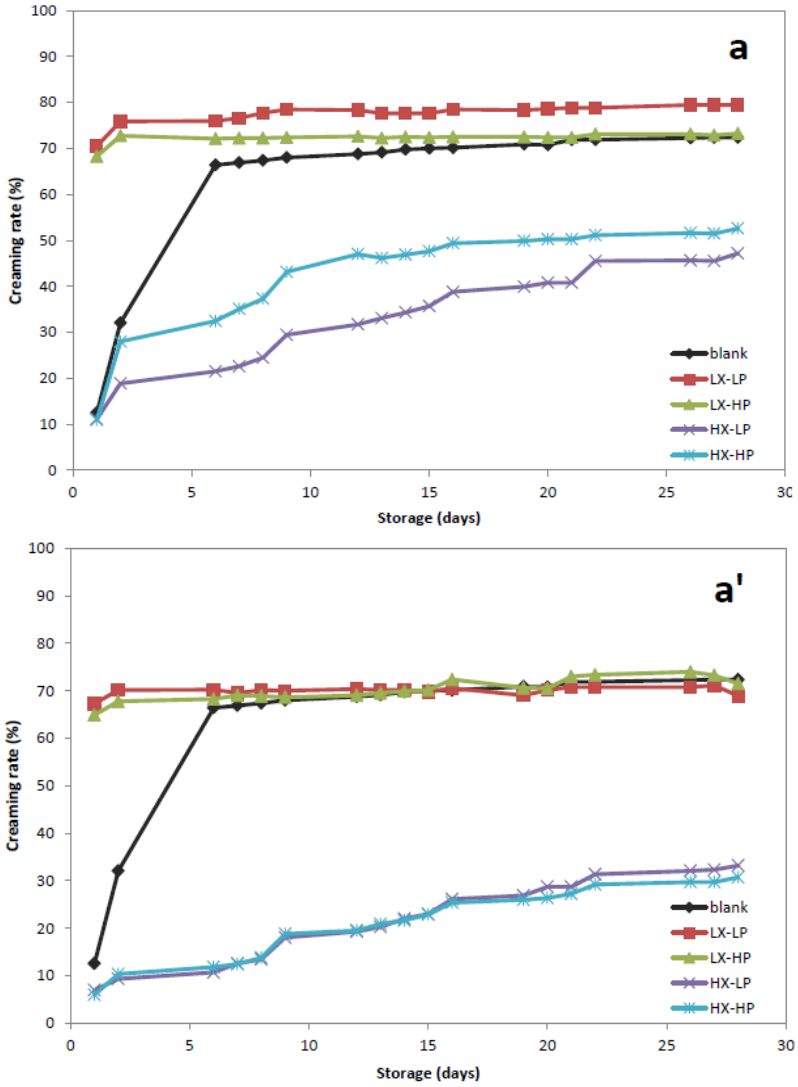

Figure 3.3. Creaming stability of 20% (v/v) olive oil-in-water emulsions stabilized by WPI and xanthan gum, and added with low (**a**) and high (**a'**) concentration of OMWW, under accelerated storage conditions (40 ºC).

Table 3.1 Two-way ANOVA analysis with interactions considering the storage time, level of OMW phenolic compounds, WPI and xanthan gum, on different parameters analysed to assess emulsion stability.

	Creaming		**Droplet size**		**Cloudiness**		**Hydroperoxides**		**TBARS**	
Variable	F ratio	*p* value	F ratio	*p* value	F ratio	*p* value	F ratio	*p* value	F ratio	*p* value
Time	283.631	**< 0.0001**	224.42	**< 0.0001**	18.162	**< 0.0001**	46.710	**< 0.0001**	9.459	**0.002**
OMW	105.837	**< 0.0001**	1.711	0.191	6.653	**0.012**	0.000	0.999	8.118	**0.005**
WPI	23.503	**< 0.0001**	0.728	0.394	6.080	**0.016**	0.049	0.825	0.094	0.760
Xanthan gum	20.277	**< 0.0001**	8.822	**0.003**	0.815	0.370	0.132	0.717	4.907	**0.028**
Time*OMW	0.140	0.708	5.154	**0.023**	5.462	**0.023**	0.208	0.649	1.851	0.175
Time*WPI	0.956	0.329	0.710	0.400	2.310	0.134	0.007	0.933	0.714	0.399
Time*Xanthan	0.823	0.365	17.075	**< 0.0001**	0.218	0.642	0.002	0.964	3.286	0.072
OMW*WPI	6620.019	**< 0.0001**	4.437	**0.035**	5.741	**0.020**	0.255	0.614	11.817	**0.001**
OMW*Xanthan	55.185	**< 0.0001**	2.695	0.101	39.212	**< 0.0001**	0.298	0.585	0.102	0.750
WPI*Xanthan	2.570	0.110	2.278	0.132	1.069	0.305	0.989	0.321	2.813	0.095

OMW: olive mill wastewater phenolic extract; WPI: whey protein isolate. A value of P<0.05 was considered as statistically significant.

A multifactorial ANOVA analysis was carried out to evaluate the influence of polyphenols, WPI, xanthan, and their interactions over storage time (**Table 3.1**). The results showed that creaming is influenced by all variables and by interaction between polyphenols-WPI and polyphenol-xanthan. Moreover, the statistical analysis shown that storage influenced all the other parameters considered.

For creaming rate, a significant effect of the interaction between P-OMW and WPI was found, as well as P-OMW and xanthan gum.

Mean droplet size by image analysis and droplet size distribution

Droplet size distribution in freshly-prepared emulsions was assessed by measuring both $d_{3,2}$ and $d_{4,3}$ using a Mastersizer (**Fig. 3.4**). Being $d_{4,3}$ more sensitive to the presence of large particles in emulsions than $d_{3,2}$, it is often more sensitive to phenomenon such as coalescence (McClements, 2004). A bimodal distribution of particle size was observed, which also implies a high standard deviation of the average particle size.

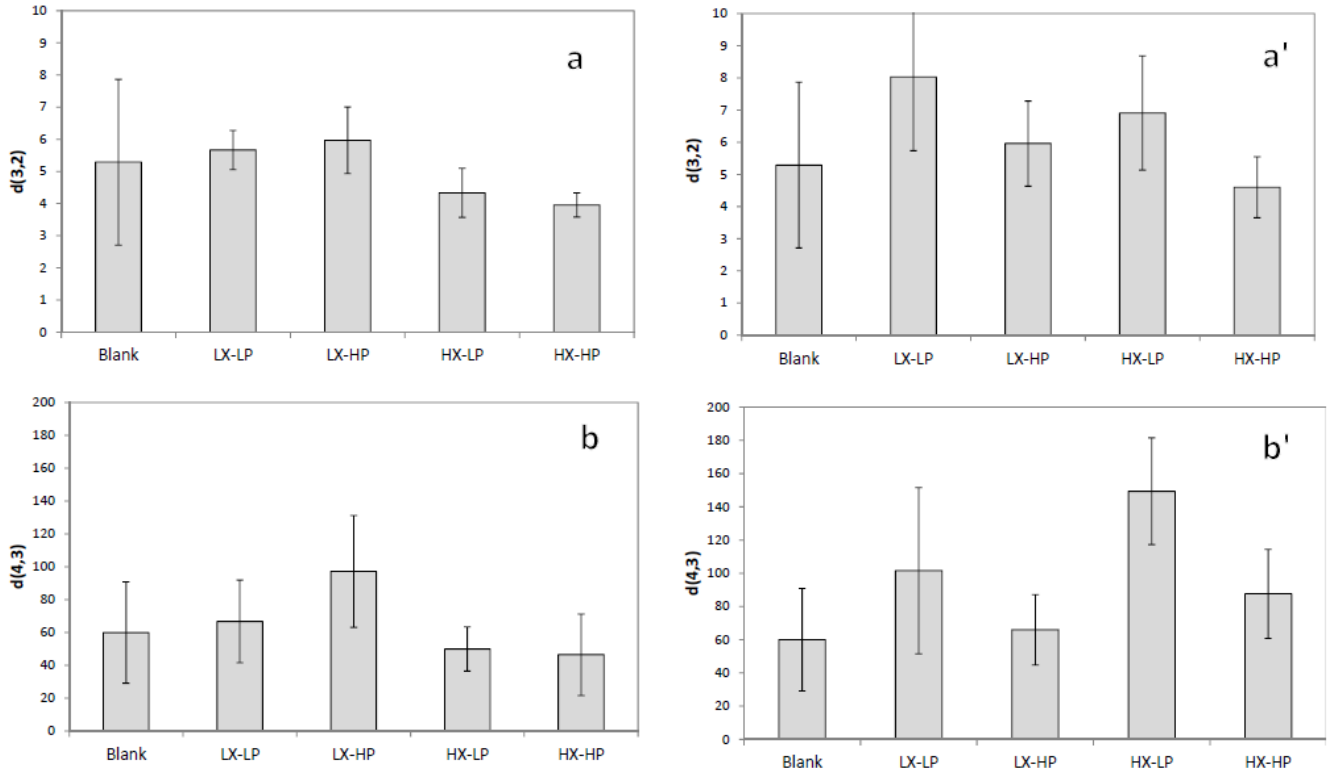

Fig 3.4. Mean droplet size, surface-weighted mean diameter ($d_{3,2}$) and volume-weighted mean diameter ($d_{4,3}$) in O/W emulsions with low (**a**) and high (**a**') concentrations of OMWW, stabilized by xanthan gum (HX/LX) and whey protein isolate (HP/LP).

Particle size in O/W emulsions is known to be influenced by WPI level (Yuan et al., 2013), as higher WPI concentrations were reported to cause lower particle size diameter (Sun and Gunasekaran, 2009). However, the authors applied a high pressure homogenizer to reach a considerable lower mean particle size, while in our case the absence of dramatic differences are likely to be due to the lower exposed surface area, as the diameter of the particle size in our samples was circa ten times higher. The authors explained the lower particle size was due to a larger area stabilized by the higher amount of added protein and to a better coverage of the droplet surface (Yuan et al., 2013).

In our emulsions, $d_{3,2}$ was influenced by high xanthan concentration (**Fig. 3.4a**), as at higher levels a lower average particle size was measured, probably due to the effect of this hydrocolloid on the stabilization after a few hours from emulsion production. Some emulsifier agents have been reported to have no significant effect on the mean droplet diameter, but xanthan gum was reported to be a critical factor (Hemar et al., 2001). The effect of xanthan gum on droplet size of emulsions is controversial, as some paper indicated a decrease in mean droplet diameter at increased xanthan concentration (Krstonosic et al., 2009), while others reported an increase in droplet size attributed to the flocculation caused by the hydrocolloid (Hemar et al., 2001; Ye et al., 2004). At lower concentrations, it was reported that droplet size of O/W emulsions is unaffected by the presence of

xanthan in the range 0-0.15% (w/v) (Moschakis et al., 2005). A more obvious difference was observed between the systems containing low and high P-OMW concentration, with generally higher $d_{4,3}$ in the second case, with the only exception of LX-HP (**Fig. 3.4b**). This result suggests a possible destabilisation effect of olive oil phenolics, which caused greater droplet dimensions.

The change in mean droplet size was also assessed by digital image analysis of micrographs, over storage (**Fig. 3.5**). A general increasing trend in the particle diameter was measured up to 23 days of storage, particularly evident at the last period of incubation. This information could appear in contrast with the creaming observation, as the main changes were observed during the first week, while a *plateau* was observed later. Previous works suggest that there could be an increase in droplet size without any direct effect on creaming rate when emulsions are stabilized by thickening agents (Lethuaut et al., 2002). The mean droplet sizes assessed by image analysis were bigger than the value obtained by light scattering technique, i.e. Mastersizer analysis. This effect was explained by the impossibility of detecting the smallest droplets in the micrographs. From our micrographs, it was observed that the main driver affecting the structure of the O/W emulsions was xanthan gum, as the presence of some aggregates was observed at high xanthan gum concentrations (data not shown), which in accordance with previous findings (Moschakis et al., 2005).

No dramatic influence of P-OMW extract on mean droplet size was observed, except at the last sampling time. At day 23, droplet size increased in the presence of phenolics by a factor of two as compared to the control. It was noted, moreover, that in samples with high xanthan concentration, the particle size was significantly ($P<0.05$) higher than in emulsions with lower level. Xanthan gum level had statistically significant effect on droplet size. Interestingly, the interaction of phenolics during time, and phenolics with WPI was statistically significant (**Table 3.1**). Xanthan gum effect is explained by the way in which this compound stabilises emulsions, i.e. acting as a thickener and therefore retarding the phase separation, whereas they do not act directly in the maintenance of small particle size diameters. In fact, both the presence of insufficient emulsifier and its excess in the aqueous solution could lead to different droplet size of the obtained emulsions (McClements, 2004).

Previous literature described a general increase in droplet size during accelerated storage of olive O/W emulsions, also depending on the type of phenolic compounds used, and their possible interference on protein rearrangements was shown (Di Mattia et al., 2010). However, in our case, OMWW contains phenolic compounds which are less reactive toward protein-protein interaction and therefore a more limited effect was expected depending on their chemical structure.

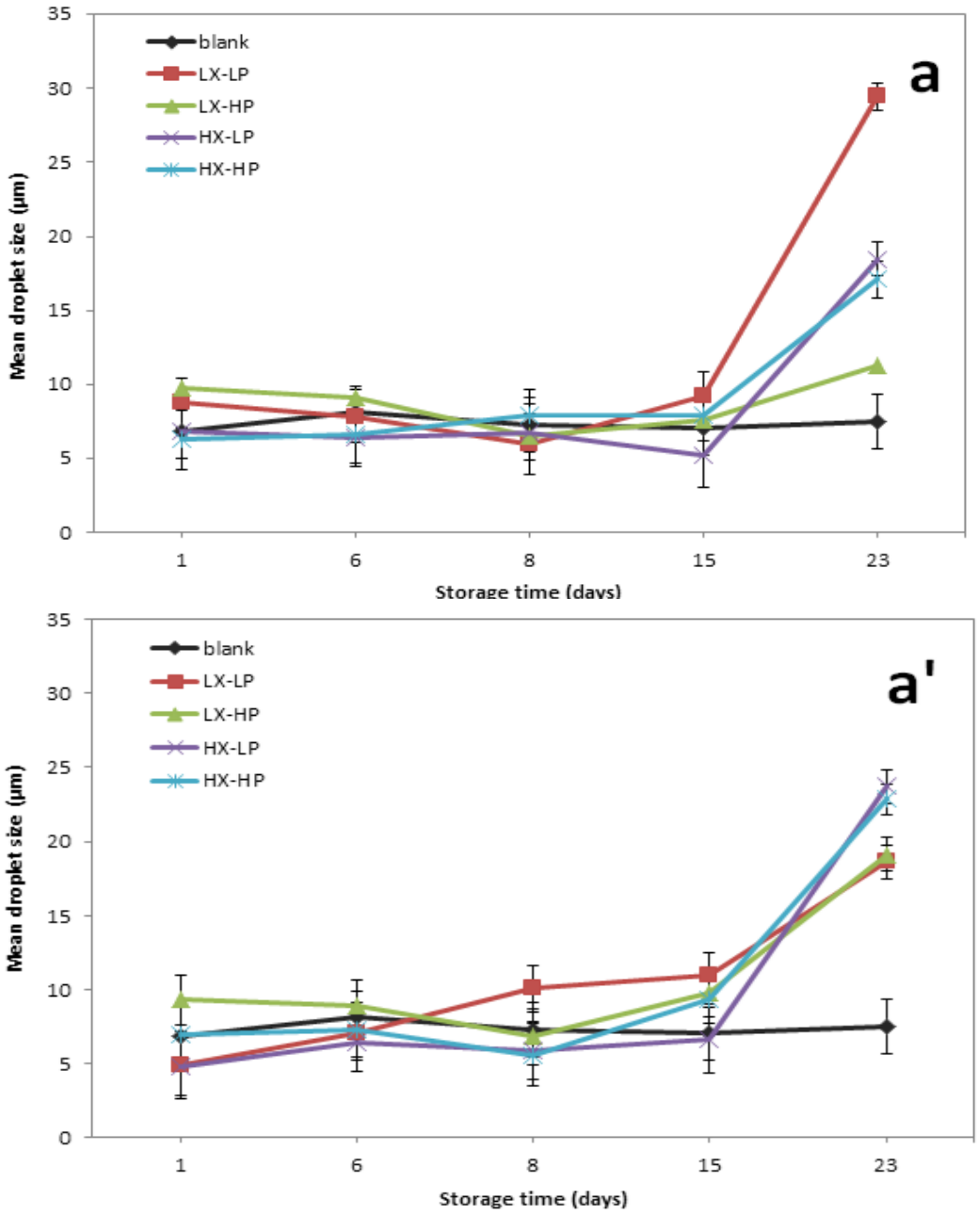

Figure 3.5. Mean droplet size analysed by digital image analysis over storage time, in samples added with OMWW at low (a) and high (a') concentrations. Each data point represents the weighted average from at least 20 pictures containing 400-1800 droplets

Cloudiness

Cloudiness of the emulsions under accelerated conditions showed a general decreasing trend over storage time (**Fig. 3.6**). Cloudiness is dependent upon the type and concentrations of hydrocolloids, and the variation in its value obtained in our experiment is in accordance with previous findings (Mirhosseini et al., 2008). It was explained by the changes in average droplet size induced by the aggregation of oil droplets as well as the changes in the refractive index of oil phase and aqueous phase. Turbidity loss is known to be linked to mechanisms such flocculation, coalescence and aggregation, which are responsible for the turbidity loss over storage (McClements, 2004). Higher level of xanthan caused higher cloudiness values especially during the first day of storage.

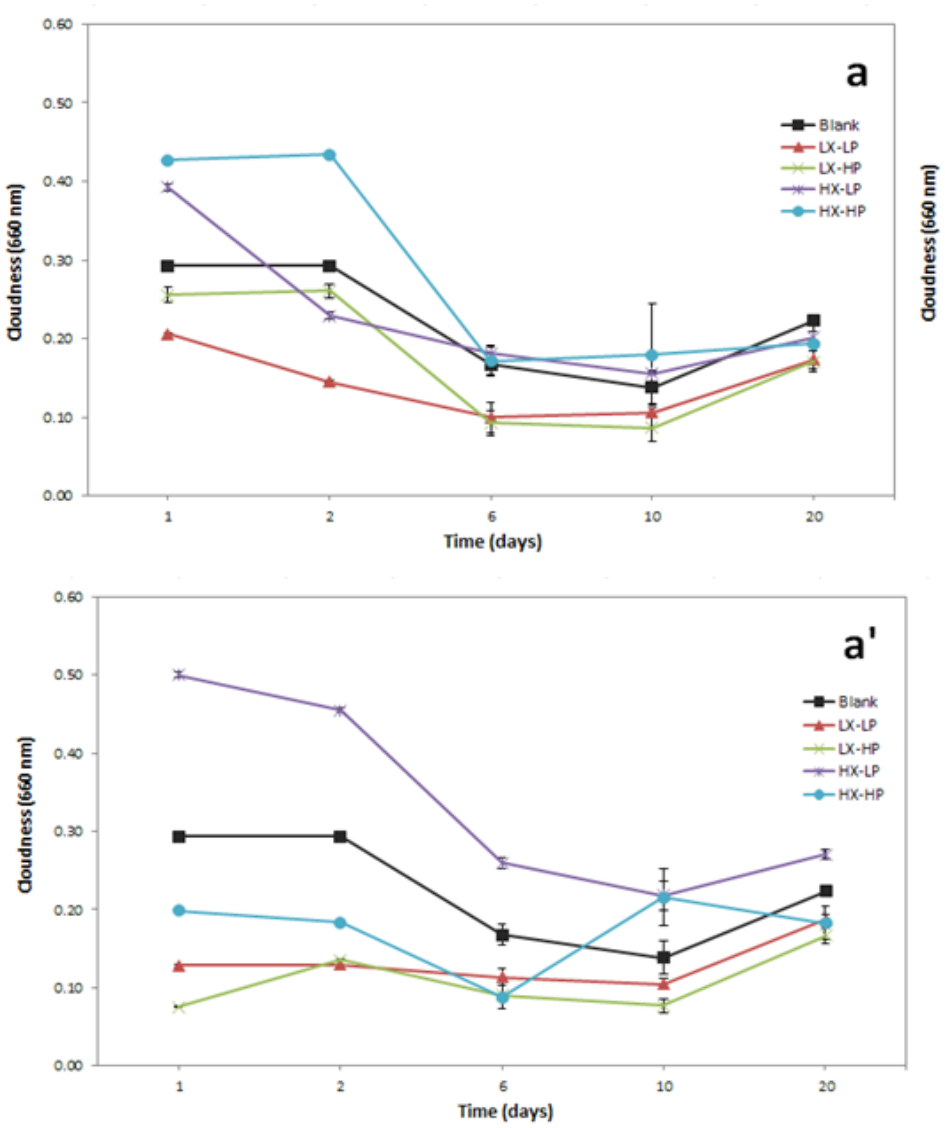

Figure 3.6. Cloudiness index in functionalised O/W emulsions with OMWW at low (a) and high (a') concentrations.

P-OMW resulted in significant effects on emulsion cloudiness (**Table 3.1**), as the measured value was significant lower when higher concentrations were added. At low P-OMW concentrations (**Fig. 3.6a**), higher levels of WPI caused greater cloudiness values, while the opposite effect was found at high P-OMW concentrations (**Fig. 3.6a'**). This phenomenon could support the idea of protein-polyphenols interactions occurring in such a system as the hydroxyl group interact with protein, in turn leading to

a lower turbid emulsion. The interaction of P-OMW with xanthan gum was statistically significant for creaming and cloudiness, while its interaction with WPI was significant for all the following parameters: creaming, droplet size, cloudiness and TBARS (**Table 3.1**). The general decrease in turbidity could be explained by emulsion phase separation. The serum and cream layers of the separated emulsions were separately analysed to verify this hypothesis, as shown in **Fig. 3.7**. The absorbance of serum was significantly higher than that of the cream layer, due to the higher presence of phenolic extract in the water phase, as these phenolic compounds are highly hydro-soluble. A possible influence of the xanthan gum concentration was also observed. This was expected due to the optical properties of the phenolic extracts. Also, the oxidation status of the oil might affect this parameter, as it was suggested that linoleic acid oxidation affect the structural organisation of the micellar/emulsion system, leading to a turbidity increase (Sotiroudis et al., 2005).

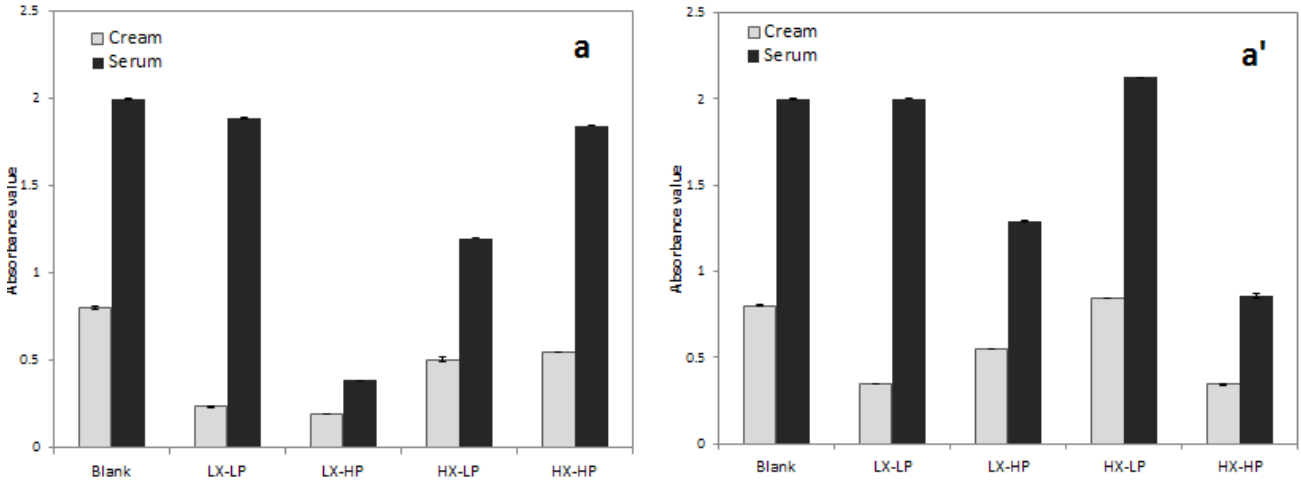

Figure 3.7. Absorbance at 600 nm of the cream and serum layer of emulsions with low (a) and high (a') OMWW levels, xanthan gum (HX/LX) and WPI (HP/LP) at day 7 of storage (40 ºC).

Emulsions viscosity and rheological behaviour

As shown in **Fig. 3.8**, the most influencing factor for emulsion viscosity and rheological property was xanthan gum concentration, which caused a sharp increase in the measured viscosity. A significant effect was found for xanthan gum concentration on O/W emulsion viscosity, and the shear-thinning behaviour found in the samples is in accordance with previous researches (Moschakis et al., 2005; Sun et al., 2007). The presence of P-OMW caused a further increase in viscosity than the control, probably due to the presence of maltodextrin as coating agent of the phenolic powder.

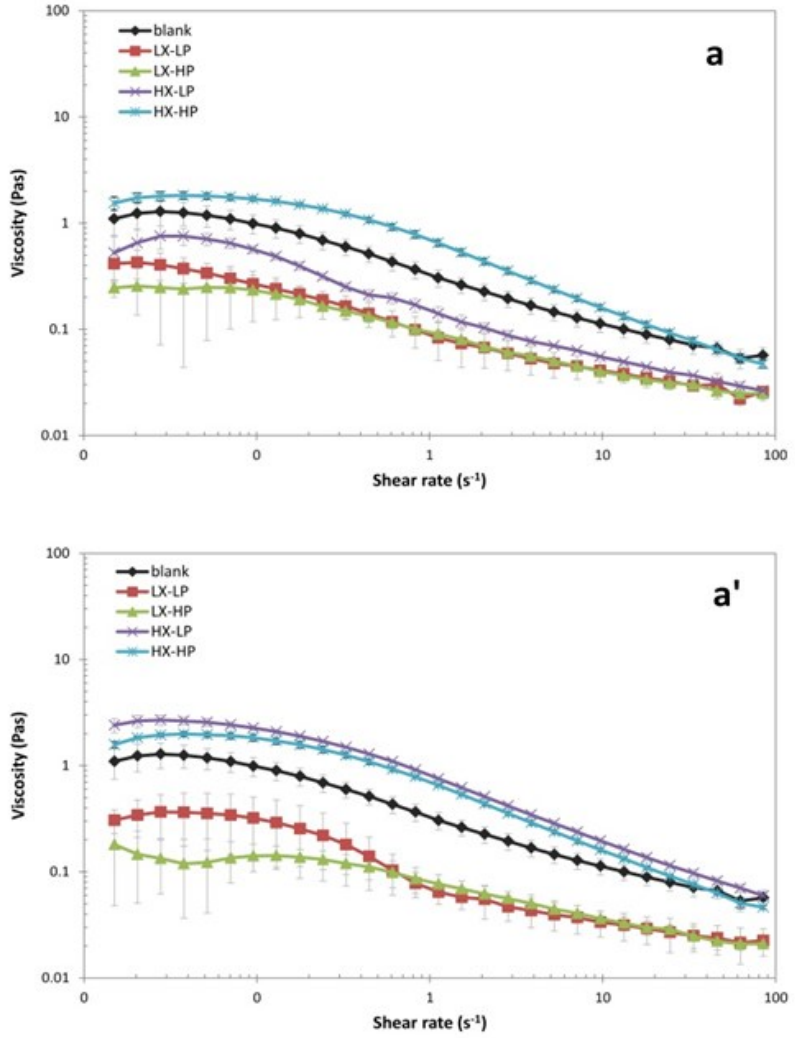

Figure 3.8. Shear-rate dependence of the apparent viscosity of O/W emulsions with low (a) and high (a') concentration of OMW.

The viscoelastic region of the emulsions was determined by amplitude sweep test, and both levels of xanthan gum resulted in linear viscoelastic region (data not shown), in accordance with others (Krstonosic et al., 2009). Small amplitude oscillatory shear measurement to define the oscillatory sweep properties of the emulsions was shown in **Fig. 3.9**. In all systems, the value of both moduli increased in the range 0.01-2 Hz (**Fig. 3.9a**). The storage and loss moduli (G'-G'') were unaffected by protein level, which is in accordance with previous findings (Sun and Gunasekaran, 2009). The only exception was represented by the emulsion with high P-OMW level and high xanthan concentration. In this case, the addition of higher WPI concentration caused a significant decrease in shear stress. The increase in G' in time was attributed to the formation of strong droplet flocculation, and the gel-like rheological behaviour of protein-stabilized emulsions was attributed to the network structure formed by protein coating (Dickinson and Golding, 1997). As the viscous modulus (G'') was higher than the elastic one (G'), it indicates pseudo-plastic behaviour. The shear stress over the shear rate of the samples showed that xanthan gum was the main driving factor, while a significant difference was observed between low and high WPI concentrations at high OMWW level (**Fig. 3.9b**). This phenomenon is likely due to the weak interactions occurring among WPI and P-OMW.

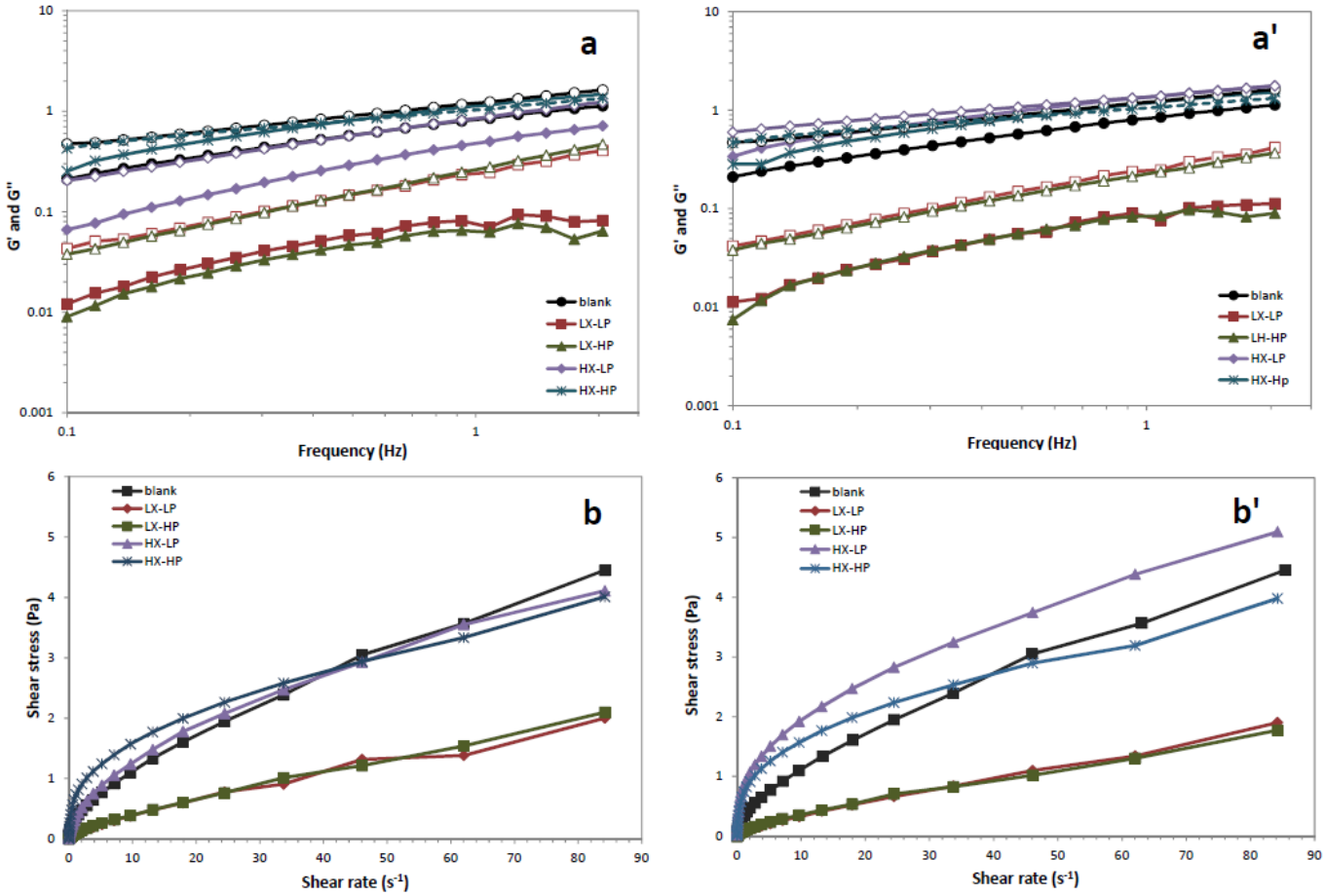

Figure 3.9. Viscous (G', filled) and elastic (G'', unfilled) moduli of emulsions at low (a) and high (a') OMWW concentrations. Rheograms obtained on freshly prepared 20% olive O/W emulsions added with low (b) and high (b') OMWW concentrations, at 25 ºC.

Oscillatory rheological measurements of storage modulus and loss modulus can indicate whether the emulsion system is strongly or weakly associated. Xanthan gum influenced emulsion yield stress, as low xanthan systems were obviously differentiated from those with high xanthan concentration, while both the presence of WPI and P-OMW did not significantly influence emulsion rheological behaviour. Based on the theory of droplet flocculation, the viscoelasticity of the flocculated emulsions should increase with increasing xanthan gum concentration, whereas the thickening effect of this polysaccharide causes a delay of this phenomenon (Moschakis et al., 2005).

Lipid oxidation in emulsion

The results for lipid oxidation test are reported in **Fig. 3.10**. An increase in lipid hydroperoxides concentration was observed up to circa 1 week upon storage in both systems (low and high P-OMW), with a limited extent in those with high WPI concentration (**Fig. 3.10a**). These results indicate a possible antioxidant effect of WPI, as suggested by other workers (Hu et al., 2003).

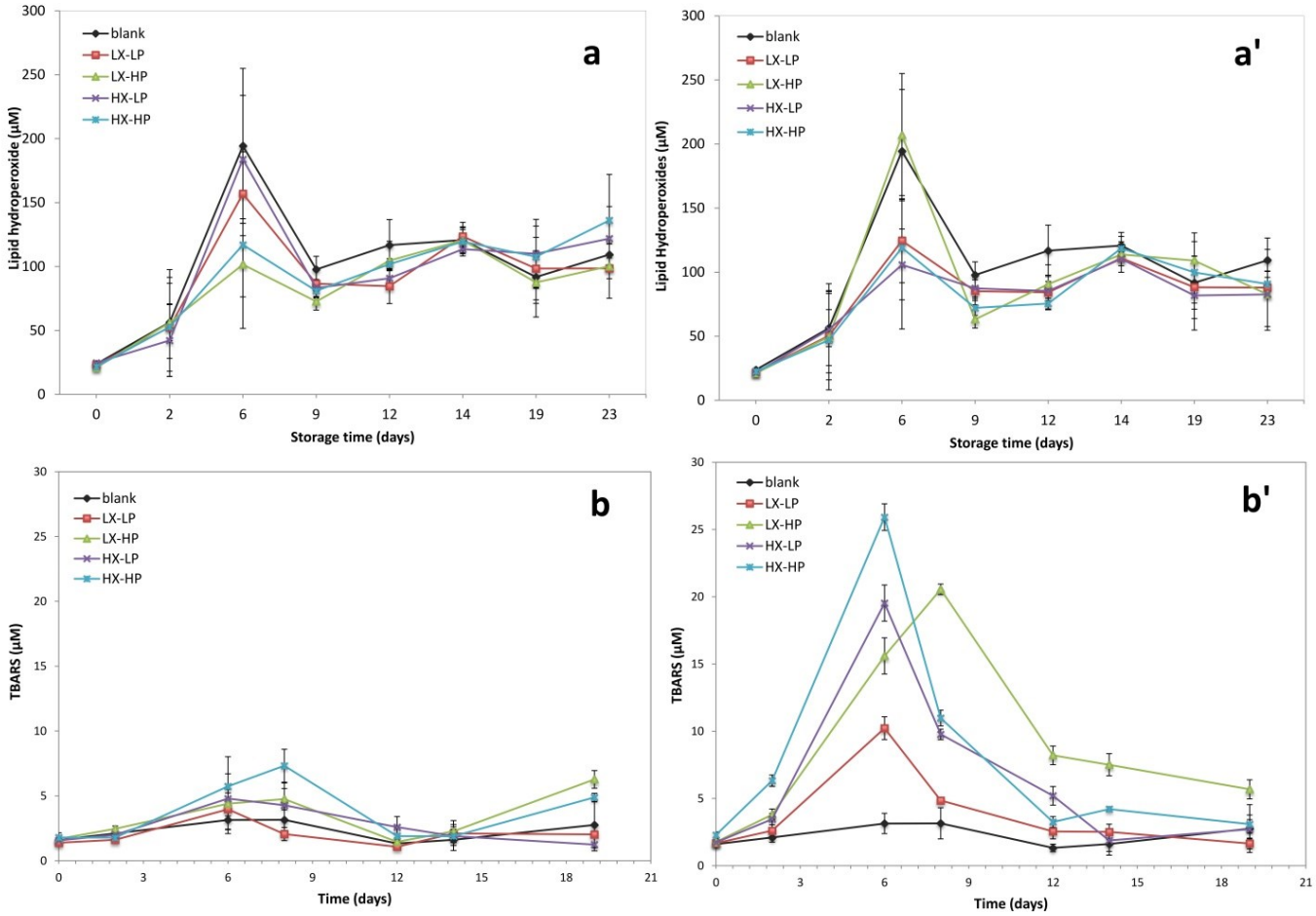

Figure 3.10. Lipid hydroperoxides (a) and secondary oxidation products TBARS (b) upon storage of emulsions. Phenolic compounds concentration was 150 (a-b) and 750 (a'-b') mg kg-1 expressed as OHTy.

The concentration of primary oxidation products followed a stabilisation period in the final storage period. In this latter period, a slight increase was observed in the system with low P-OMW, while in the high P-OMW one the oxidation products were lower than the control. Hydroperoxides concentration was dramatically lower in emulsions with phenolics, being LX-HP the only exception. However, a clear statistically significance was not reached for primary oxidation products depending on emulsion formulation, except for storage time (**Table 3.1**).

The results obtained for TBARS were quite different (**Fig. 3.10b**), as their concentration in systems with high P-OMW was circa 3 fold higher than low P-OMW. Also in this case, the highest concentration was obtained at about 1 week storage. Generally, high WPI concentration was associated with higher TBARS production, especially when olive phenolics were present at high concentration. In this latter case TBARS value was always higher than the control. WPI and xanthan gum significantly ($p<0.05$) influenced TBARS concentration, and the interaction of WPI with P-OMW also resulted statistically significant (**Table 3.1**).

The higher TBARS concentrations are related to the decomposition of primary oxidation products, and therefore the formation of aldehydes and other products leading to off-flavour in the final product. In accordance with Di Mattia et al.,(Di Mattia et al., 2009; Di Mattia 2010) the presence of phenolic extracts in systems with low P-OMW had only limited effects on lipid hydroperoxides formation. On the contrary, high P-OMW concentration was associated with higher oxidation level in the system with high WPI (HX-HP). Accordingly, certain phenolic compounds, e.g. catechin, were reported to cause higher TBARS concentration and they are not able to delay the formation of primary oxidation products, showing pro-oxidant activity (Di Mattia et al., 2009; Di Mattia 2010).

Some phenolic compounds can exert the opposite effects on the chemical stability, e.g. caffeic acid retarded the production of peroxide while promoting at the same time higher concentration of secondary oxidation products (Sorensen et al., 2008). Zhou and Elias (Zhou and Elias, 2013) confirmed the potential of some phenolic compounds to act as antioxidants or pro-oxidants in O/W emulsions, being strongly influenced by the pH and their concentration. The authors reported that epigallocatechin-gallate had higher TBARS concentration (in the range pH 2-4) in the range 1-100 μM. Up to 500 μM, the observed effect was lower, and the authors explained by the competition between antioxidant and pro-oxidant effects (Zhou and Elias, 2013). In the present paper we used directly a complex phenolic extract, which is obviously different from pure laboratory phenolics and therefore their effect on lipid oxidation is not straightforward. Moreover, the possible presence of metallic ions as traces in our phenolic extract might have affected the antioxidant activity of the phenolics, influencing lipid oxidation. Metallic ions affect O/W emulsion oxidation and a pro-oxidant effect was demonstrated for pure hydroxytyrosol and oleuropein in the presence of ferric ion, depending on the pH (Paiva-Martins and Gordon, 2002). This demonstrates the importance of further studying lipid oxidation in O/W emulsions considering the effects of phenolics and proteins, as well as the presence of other stabilizers.

The interaction between olive oil phenolic compounds and food proteins was studied previously using other techniques (Pripp et al., 2005). However, sodium caseinate, bovine serum albumin, β-lactoglobulin and gelatine were used, in comparison to gallic acid and tannic acid, which are chemically different from the compounds used in the present paper. A relatively weak binding capacity was shown, as both OHTy and Ty had little or no binding. However, previous studies show that free phenols in the aqueous phase can bind to proteins, both in bulk systems and emulsions, while the phenomenon was more limited in this latter case (Pripp et al., 2005). Milk proteins can be considered as antioxidant compounds, e.g. casein hydrolysates and caseinophosphopeptides, being capable of binding transitional metals and limit lipid oxidation in O/W emulsions (Diaz et al., 2003). However, the effectiveness of WPI in emulsions was assessed not considering OMWW or olive phenolics in such system (Sun and Gunasekaran, 2009).

Whereas P-OMW seem not have positive effects on hydroperoxides or TBARS, their effectiveness in vivo is likely to be possible, as the polysaccharide coating could be broken during the digestion and therefore its phenolic content may be available to exert its antioxidant effects.

A cluster analysis was carried out to understand the similarity among the samples, based on creaming index, mean droplet size, apparent viscosity, peroxide value and TBARS parameters after 2 weeks storage. As shown in **Fig. 3.11**, the clustering resulted into three main groups, mainly differentiated by xanthan gum, which was in fact the main driver for emulsions behaviour, and olive phenolic extract in the system with high level of xanthan gum. Finally, the blank sample with no added phenolic compounds was clustered at a short distance to LX-HP sample. This result confirmed the important effect of polyphenols in an emulsion system, as previously discussed.

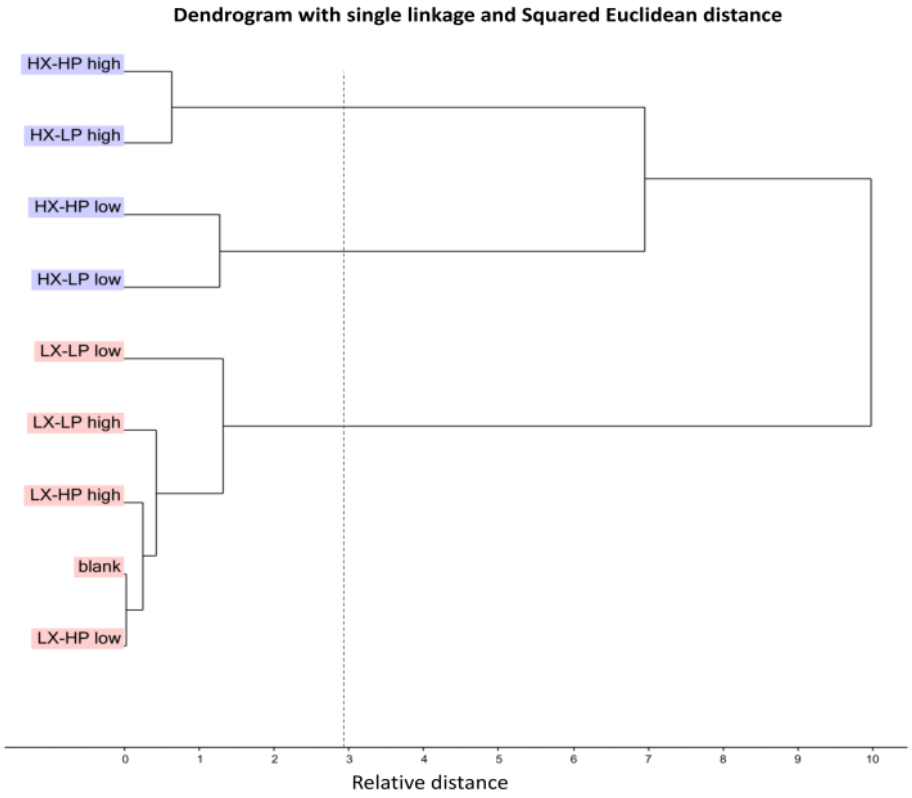

Figure 3.11. Cluster dendrogram of olive O/W emulsions formulated with high or low concentrations of olive mill wastewater phenolic extracts, and stabilized using xanthan gum (X) and whey protein isolate (P).

Conclusions for topic 1

This paper reported on the stability of the model oil-in-water emulsions functionalized with polyphenols by addition of olive mill wastewater. The presence of P-OMW can lead to higher creaming rate and physical instability; however it was shown that its influence on the rheological behaviour was limited. At high concentrations of P-OMW and WPI, both the physical and chemical stability was negatively affected, showing particularly high TBARS values. The addition of phenolic extracts is not straightforward in terms of oxidative stability, and their interaction with the hydrocolloids, mainly whey proteins, can exert a significant influence on physical stability, but can also exert a pro-oxidant effect depending on its level. These results suggest the importance of accurately choosing the concentration of stabilizers, and a practical consequence is the suggestion of using low WPI concentrations when P-OMW is added in food emulsions.

The knowledge of olive O/W emulsion stability is of great importance for the formulation of emulsion-based food products having enhanced health properties due to the widely known health benefit of this oil, and the antioxidant activity of olive phenolics. Moreover, the possibility of using by-products from olive oil extraction process with high environmentally impact is of interest for food industry, as well as for food scientists to incorporate hydrophilic phenolic compounds in fatty foods. This research has great potential in terms of applicability at industrial level, and it is hoped to contribute to the "hot topic" of environmental pollution reduction and for the creation of innovative functional foods. Further work is needed to better define the optimum concentrations of each hydrocolloid according to the final use of the product, as well as to understand the *in vivo* effect of the consumption of these functional emulsions.

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Topic 2

Chapter 4

Effect of olive mill wastewater phenolic extract, whey protein isolate and xanthan gum on the behaviour of olive O/W emulsions using Response Surface Methodology

Introduction

A wide range of foods are emulsions, e.g. mayonnaise, salad dressings, sauces, etc., and other food products include emulsions as an ingredient, e.g. yoghurts, ice creams and whipped products (Traynor, Burke, Frias, Gaston, & Barry-Ryan, 2013). Food emulsions are kinetically unstable systems as the lipid and water phases tend to separate over storage (McClements, 2004a; Mirhosseini, Tan, Hamid, Yusof, & Chern, 2009).

Many stabilisers are usually added to food emulsions to improve their stability and lead to a longer shelf life, or improve the appearance, rheology and other factors (Mirhosseini, Tan, Taherian, & Boo, 2009; Sun, Gunasekaran, & Richards, 2007). Polysaccharides, including xanthan gum, have been widely applied in the food industry, due to their characteristics related to its viscoelastic properties and its chemical properties, in particular the water solubility and pH stability (Sun et al., 2007). Proteins are also used in oil-in-water (O/W) emulsions to facilitate their formation, improve their stability and provide specific physicochemical properties. Milk proteins, i.e. whey protein isolate and caseinate, and soy proteins have been reported (McClements, 2004b). The ability of food manufacturers to formulate emulsion-based products with desirable and reproducible characteristics depends on knowledge of the relationship between the emulsion properties and matrix composition as well as microstructure (Mirhosseini, Tan, Hamid, et al., 2009). For this reason, it is of great interest to study the interaction between stabilisers and other compounds added to give specific functional properties in O/W emulsions.

Olive oil is one of the most appreciated vegetable fats, and is of paramount importance in the Mediterranean diet, which has been linked to human health benefits (López-Miranda et al., 2010). Olive oil has been described as a natural functional food, due to its composition and presence of valuable phytochemicals, including phenolic compounds, squalene and α-tocopherol (Stark & Madar, 2002).

However, the presence of phenolic compounds in refined olive oil, and generally in vegetable oils which undergo refining processes, is dramatically lower than virgin olive oils. A possible approach to overcome this issue, to improve the nutritional profile of the vegetable oils and high fat foods, could be the addition of olive phenolic compounds recovered from by-products of olive oil extraction, i.e. olive mill wastewater (OMW).

OMW leads to important environmental issues in olive oil producing countries because of its high pollution risks due to its high chemical oxygen demand. However, OMW also contains high concentrations of phenolic compound derivatives of oleuropein, e.g. hydroxytyrosol, tyrosol, caffeic acid, vanillic acid, verbascoside, luteolin-7-glucoside, etc. (De Marco, Savarese, Paduano, & Sacchi, 2007). Alternatives for its disposal and innovative ways for the application of the extracted phenolic compounds in food products are therefore important research questions (Garcia-Castello, Cassano, Criscuoli, Conidi, & Drioli, 2010; Schieber, Stintzing, & Carle, 2001).

Response surface methodology (RSM) is usefully applied as an effective tool for the optimization of a process when the independent variables can exert a combined effect on the desired response (Koocheki, Taherian, Razavi, & Bostan, 2009). RSM has been previously applied by other researchers to study the behaviour of food emulsions and the dependence of emulsion stability on their ingredients (Gharibzahedi, Mousavi, Hamedi, & Ghasemlou, 2012; Mirhosseini, Tan, Hamid, et al., 2009; Mirhosseini, Tan, Taherian, et al., 2009; Traynor et al., 2013). Among the advantages of its application, RMS allows the design of a comprehensive model describing the characteristics of a system, by minimizing the number of experiments required. Central Composite Design (CCD) is one of the most common forms of RSM applied to various food systems (Ahmed, Rico, Martin-Diana, & Barry-Ryan, 2011). This approach was therefore applied in the present research.

Whereas the characterisation of O/W emulsion behaviour is a widely researched topic, little is still known about olive O/W emulsions formulated with stabilisers like WPI and xanthan gum, with particular emphasis on the possible effects of olive biophenols. In this context, the use of these phenolic extracts for the development of a functional food is of great interest for the food industry and might help in the valorisation of olive by-products.

Therefore, the aim of the present paper was to evaluate the effect of concentration on the stability of O/W emulsions made by using olive oil and functionalised by the addition of olive phenolic extracts, using different levels of hydrocolloids, i.e. WPI and xanthan gum, and to build statistical models to describe the emulsion stability in terms of both the physical behaviour and oxidation status over storage.

Materials and methods

Olive oil sample, stabilisers and OMW extract

Freshly refined olive oil was donated by I.O.B.M. srl (Montesarchio, BN, Italy). Xanthan gum from *Xanthomonas campestris* was purchased from Sigma-Aldrich (Darmstadt, Germany). WPI was 97.5 wt% protein, and lactose content was less than 1 wt%. A phosphate buffer solution at pH 7.0 was prepared using monosodium phosphate and sodium hydroxide (SA, Darmstadt, Germany). The buffer was used to maintain constant pH, as this parameter can affect emulsion stability (Sørensen et al., 2008). All other chemicals were of analytical grade purity. Phenolic powder extract from OMW was donated by LABS (Department of Agriculture, University of Naples Federico II, Italy). P-OMW production process has been reported by Troise et al. (2014) and the emulsions formulated with these extracts were previously applied for other studies on O/W emulsions (Caporaso, Genovese, Burke, Barry-Ryan, & Sacchi, 2016). The composition of the three main phenolic compounds analysed by HPLC-UV-Vis was as follows: OHTy 32±0.2 mg g-1, Ty 1.9±0.1 mg g-1, verbascoside 2.8±0.09 mg g-1 (Troise et al., 2014).

Experimental design

RMS was applied in the present experiment to study the effect of P-OMW, WPI and xanthan gum on olive O/W emulsion properties and stability over storage. The effect of three independent variables, i.e. P-OMW (0.01-4.4 mM), xanthan gum (0.06-0.25 % w/w) and WPI (0.13-0.63 % w/w) were studied in relation to the emulsions physical and oxidative stability. In particular, the creaming index, mean droplet size, turbidity, lipid hydroperoxide formation and TBARS were assessed over the storage period.

The experimental design was based on central composite design (CCD), with three replicates of the central point, and data was fitted with a second order polynomial equation. ANOVA and regression surface analysis were used to determine the statistical significance of the model factors and responses and to calculate a regression equation based on the experimental data. The mathematical model used to describe the variation in the responses used the following equation:

$$Y = \beta 0 + \Sigma \beta ixi + \Sigma \beta iix2i + \Sigma \beta ijxixj + \varepsilon \qquad [1]$$

where Y is the response value predicted by the model; β0 is a offset value; βi, βii and βij are the main (linear), quadratic and interaction regression coefficients, respectively (Neter & Wasserman, 1992).

The software uses a quadratic model to build response surfaces. The complete design consisted of 17 experimental points (runs) including 3 replicates of the central point. The concentrations of the P-OMW, xanthan gum and WPI that were used in the 17 runs for the RSM are reported in Table 1.

The range of concentrations of the hydrocolloids and OMW phenolics were chosen according to previous works (Di Mattia, Sacchetti, Mastrocola, & Pittia, 2009; Sun et al., 2007). In particular, the minimum and maximum concentration of OMW biophenols were chosen based on previous literature regarding the natural content of olive oil biophenols (Caporaso et al., 2015), and calculations were made to mimic similar amounts expressed as hydroxytyrosol emulsions (Caporaso et al., 2016).

Emulsion preparation

Emulsions were prepared by dispersing different amounts of spray-dried P-OMW powder (0.9-4.4 mM expressed as OHTy) and WPI (0.13 or 0.5% w/v) into a buffer solution (5mM phosphate buffer, pH 7). The aqueous phase was gently stirred for 2 h at room temperature to ensure dissolution, using a magnetic stirring bar and magnetic stirrer hotplate (Stuart CB162, Bibby-scientific, Staffordshire, UK). The pH was checked and adjusted to pH 7.0 using 1M HCl. Xanthan gum (in the range 0.06 to 0.2% w/v) was added to the emulsions and gently stirred (100 rpm) overnight at room temperature to allow complete hydration. Emulsions were produced by blending 20% (v/v) refined olive oil in the solution previously prepared using a high-speed blender at 8,000 rpm for 2 min, after a pre-emulsification phase (Traynor et al., 2013). The emulsions were transferred into glass tubes for analyses, and stored in an incubator at 40 °C ±1 °C for kinetic stability evaluation.

Creaming value

Creaming value was monitored visually according to literature (Dickinson, Radford, & Golding, 2003). Duplicate samples of emulsions were stored in 75 mm x 12 mm sample tubes (York Glassware, UK) at 25 ±0.5 °C. Measurement of the serum layer (creaming index) was carried out manually using a 60% fiberglass Measy 2000 Typ 5921 (Baty, Switzerland). Stability was evaluated as percentage decrease from the initial height, using the following formula: Creaming Index=100 × (HS/HE); where HS is serum layer formed at the bottom of glass tubes, and HE is the total height of the emulsions in the tubes (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004).

Viscosity

Rheological measurements were carried out using a Bohlin C-VOR dynamic rheometer (Malvern Instruments Inc., Southborough, MA). Emulsion viscosity was measured at 25 °C, over a shear rate range of 0.01–100 s-1 with cone-plate geometry (CP 40/4°). All measurements were performed within 24 h from emulsion preparation. A logarithmic progression was applied, and sweep time was 120 s. Oscillatory tests were performed by pouring emulsion samples (typically 1-1.5 mL) directly on the holding stage and samples were covered with thin paraffin oil layer preventing water evaporation. In oscillatory experiments the storage (G') and loss (G'') moduli were recorded versus frequency (0.1–10 Hz) at constant strain, with increasing of logarithmic scale. The linear viscoelastic region was previously determined selecting a strain of 0.5 Pa, recording G' and G'' versus shear stress (0.01–100 Pa) at a constant frequency.

Mean particle size by image analysis (optical microscopy)

For particle size determination through digital image analysis, emulsions were diluted 1:1000 using the buffer solution to avoid droplet overlapping. A drop of emulsion was placed on a microscope slide and then covered with a cover slip. The microstructure of the emulsion was observed using an Olympus DP72 optical microscopy (Olympus, Tokyo, Japan) at 40x and 400x magnification. Digital pictures were taken by using an Olympus E-620 digital camera mounted on the microscope. Mean droplet size was calculated by analysing the microscopic images with ImageJ 1.47t 64-bit software (National Institutes of Health, USA). The function Analyse Particles was used after Colour Threshold and using the following options: size 0.01-infinity; circularity: 0.00-1.00; exclude on edges; particle size >2 μm2; options exclude droplets on edges and include holes. At least 20 pictures were taken at 100x magnification for each emulsion analysed. Image analysis was performed as described by previous workers (Silva, Rocha-Leão, & Coelho, 2010), where details of the method were reported.

Emulsion droplet size distribution (D[3,2])

Droplet-size distributions of the emulsions were determined by using a Mastersizer 2000 Hydro 2000S (Malvern Instruments, UK), which gives measurement based on light scattering under high dilution conditions by dispersing the samples in distilled water (Dickinson et al., 2003; Lethuaut, Métro, & Genot, 2002). To avoid multiple scattering effects, the freshly prepared emulsions were diluted to reach an obscuration rate of about 3. The refractive indices of water and refined olive oil were 1.330 and 1.418,

respectively. Average droplet sizes were characterized in terms of the volume mean diameter d4,3 = Σi · ni · di4 / Σi · ni · di3, where ni is the number of droplets and of diameter di. The d4,3 parameter is a useful mean diameter value, sensitive to small changes in droplet-size distribution (Moschakis, Murray, & Biliaderis, 2010). All measurements were made at room temperature and four measurements were obtained for each sample. A bimodal particle-size distribution was taken to be indicative of non-reversible flocculation (Dickinson et al., 2003; Lethuaut et al., 2002).

Cloudiness

Cloudiness measurement, also called turbidity or opacity, was carried out according to previously published methods for O/W emulsions (Mirhosseini et al., 2008). Samples were taken after 24 h to their preparation and analysed over the storage period. In the case of separated emulsions, the supernatant was sampled, while in the absence of evident phase separation, the upper part was sampled as well. Emulsions were diluted (1:1000) and cloudiness was expressed from the absorbance at 660 nm. High absorbance reading values correspond to the high emulsion cloudiness. Distilled water was used as a reference (Mirhosseini, Tan, Hamid, et al., 2009).

Emulsions viscosity

Rheological measurements were carried out in accordance to previous papers reporting on O/W emulsions stabilised by WPI and xanthan gum (Sun & Gunasekaran, 2009). Steady shear viscosity and small-amplitude oscillatory shear tests were conducted using a Bohlin C-VOR dynamic rheometer (Malvern Instruments Inc., Southborough, MA). Emulsion viscosity was measured at 25°C, over a shear rate range of 0.01–100 s-1 with cone-plate geometry (CP 40/4°). All measurements were performed within 24 h from emulsion preparation. A logarithmic progression was applied, and sweep time was 120 s.

Oxidative stability

Lipid hydroperoxides

Lipid hydroperoxides were measured according to literature (Di Mattia et al., 2009). Emulsions (0.3 mL) were mixed with 1.5 mL of isooctane/2-propanol (2:1, v/v), vortexing three times for 30 s and centrifuging for 2 min at 2000 x g (Hettich Rotanta 460R centrifuge). The supernatant (200 μL) was collected and 2.8 mL of a methanol:1-buthanol solution (3:1, v/v) were added, followed by 15 μL of 3.94 M ammonium thiocyanate and 15 μL ferrous iron solution (prepared by adding equal amounts of 0.132 M BaCl2 and 0.144 M FeSO4). After 20 min, absorbance was measured at 510 nm using a Lambda Bio 20 spectrophotometer (Perkin Elmer, Boston, MA). Hydroperoxides concentration was determined using a calibration curve prepared with hydrogen peroxide.

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARs) were determined according to previously published methods (Di Mattia, Sacchetti, Mastrocola, Sarker, & Pittia, 2010). Emulsions (0.1-1 mL) were mixed with 2.0 mL of TBA reagent (15% w/v tricholoracetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 minutes and then centrifuged (2000 g using a Hettich Rotanta 460R centrifuge) for 15 min at 20 °C. After 10 minutes, the absorbance was measured at 532 nm. TBARs concentration was determined by a standard curve prepared using 1,1,3,3-tetramethoxypropane.

Statistical analysis by RSM

The approach chosen in the present paper to analyse the contemporary effects of three variables on the emulsion stability was the Surface Response Methodology. The statistics was performed as previously reported by others (Ahmed et al., 2011; Traynor et al., 2013).

RSM was used to fit the experimental data to the quadratic polynomial equation to obtain coefficients of the equations. The model and statistical analyses and contour plots were analysed using Statgraphics Centurion XVI (Statistical Graphics Co., Rockville, USA) and Design-Expert 6.0.11 (Stat-Ease, Inc., Minneapolis, USA).

To verify whether the observed differences were statistically significant as due to the presence and level of P-OMW, WPI and xanthan gum, the analysis of variance (ANOVA) was performed for each response to assess whether the observed differences were statistically significant. The adequacy of the model was checked accounting for R2 and adjusted-R2. A level of $p<0.05$ was set as critical to consider the differences as significant in the model. The quality of fit was assessed by regression coefficient of determination along with an analysis of residuals. The fitting ability of the tested models was also evaluated by calculating the root mean squared error (RMSE) according to the literature (Neter & Wasserman, 1992). The analyses were performed at least in triplicate (with the exception for the digital image analysis, for which circa 20 pictures were taken per sample) and data was reported as the estimated parameter ± standard error (SE). The adequacy of the regression equations was also checked by comparing the experimental data with predicted values obtained from the equations.

Results and discussion

Model fitting and statistical analysis

The positive or negative effects of OMW biophenols, WPI and xanthan gum, considered as independent variables, on physical and chemical parameters related to emulsion quality and stability were investigated using RSM. Table 1 reported the concentrations of the independent variables of the model and the experimental data obtained on 20% olive O/W emulsions. The estimated regression coefficients of the response surface models, along with the corresponding R2 values and lack of fit tests are given in Table 2. In addition the adjusted R2 was calculated to check the model adequacy. The significance of the models was also studied using the F-ratio and p-value (Table 3).

The rotatable central composite design (CCD) with $\alpha = 1.682$ has been carried out on seventeen randomized runs (23 + (2 x 2) +3), i.e. using 2 replicates of factorial points and 3 replicates of the central points. CCD has been applied widely in similar experiments in the field of food science when several factors are under investigation, and it was reported to be particularly useful (Daneshvand, Ara, & Raofie, 2012; Gharibzahedi et al., 2012; Mirhosseini, Tan, Hamid, et al., 2009; Traynor et al., 2013).

From the normal probability graph and the residuals histogram, the data followed a normal distribution and therefore the model gives a good description of the experimental data. The design allowed for modelling the responses by fitting a second-order polynomial. The results of the regression analysis show that the models prediction capacity were very good for creaming and viscosity and were acceptable for PV and TBARS, while no statistically difference was found for the droplet size, both in case of image analysis on stored emulsions and D[3,2] on freshly prepared emulsions. The results from ANOVA are reported in Table 3, while Fig 1 shows the Pareto graphs to highlight the major effects of the variables and their possible interactive effects. The responses were therefore employed to predict the physical and chemical properties of the emulsions, excluding the responses for which the regression values were not adequate, i.e. emulsion droplet size. In fact, the models did not give significant results for the mean droplet size, as the P-value was unacceptably high. Consequently, this model was not applied for the prediction of droplet size and droplet size distribution.

The results of the R^2 for the statistically significant model had satisfactory or good R^2 values for the resulting independent variables, with the lowest being 0.847 for hydroperoxide concentration (PV), and the highest $R^2 = 0.939$ for viscosity. The adjusted R^2 is considered a better indicator for statistical model evaluation, and here it resulted in very good performances for creaming and viscosity, i.e. 0.90 and 0.94, respectively. RSM models with R2 values higher than 0.80 are considered as valid ones (Daneshvand et al., 2012; Joglekar, May, Graf, & Saguy, 1987). The parameter lack-of-fit is an indication of the adequacy of a model to describe the experimental factors and the response variable, considering the data not included in the regression or some variations that cannot be accounted for random error (Montgomery, 2001). The lack of fit illustrated in Table 2 did not result in significant p-value for the studied variables, therefore meaning that these models were sufficiently accurate for predicting the relevant responses.

From the obtained regression models, the response surface was calculated for each response separately. It has been previously suggested that the polynomial regression models and recommended optimum region should be considered as meaningful only in the studied independent variable ranges. Thus, it may not be true beyond the ranges of the factors (Montgomery, 2001), and for this reason the models were studied only in the specified regions as it was suggested to avoid extrapolating statistical models beyond these range (Mirhosseini, Tan, Taherian, et al., 2009).

The analysis of the results is shown for the interaction effects by using the Pareto chart, and setting a P value of 0.05, which was shown by the vertical line. Moreover, the positive or negative sign indicate whether a factor has a positive or negative effect on the studied response (Fig. 1), as better described in the following sections. The positive sign indicates that a specific ingredient

(independent variable) lead to higher values of the response set, i.e. creaming, viscosity, PV and TBARS, while the negative sign indicates an opposite effect. When an ingredient is shown twice, it indicates a quadratic effect of the variable, which might also indicate a stationary area (Gharibzahedi et al., 2012; Neter & Wasserman, 1992).

Creaming

As reported in Table 2 for the regression coefficients of the model, and in Fig. 1a to show the statistical significance, the creaming rate was negatively associated with all the three independent variables studied, with particularly higher values for xanthan gum. However, only the independent variables xanthan gum and OMW polyphenols were statistically significant ($p<0.05$), both with a quadratic effect of xanthan gum which indicates a stationary point. Therefore, this is clearly the critical parameter for creaming rate. As the aim of emulsion production is the lowering of creaming velocity, the lowest values of creaming in terms of RMS model are desired, i.e. statistically negative effects that indicate lower physical instability toward phase separation (McClements, 2004a). In fact, it was reported that O/W emulsions stability is strongly correlated with the presence of polysaccharides (Klinkesorn et al., 2004; Sun et al., 2007; Traynor et al., 2013).

Fig. 2 shows the response surface plots for the creaming index. The highest rate, i.e. lowest stability, was obtained at low WPI concentration, and high concentrations of both xanthan gum and OMW biophenols caused a reduction of the phase separation rate.

This result could be related to the emulsifying properties of some proteins and especially WPI, as previously reported (Hu, McClements, & Decker, 2003a; McClements, 2004b; Moschakis et al., 2010). However, in comparison to xanthan gum, WPI had more limited effects as shown in the surface plot, and particularly at low concentrations, suggesting that no further improvement are obtained once the emulsion droplets are covered by the proteins.

Interestingly, the plot of xanthan gum and OMW phenolics show a slight but important improvement of these compounds on emulsion physical stability, and for this parameter a high polyphenols concentration lead to longer stability. This result is likely not be an effect of the sole polyphenols but also due to the coating agent used for encapsulation.

Emulsifying agents are known to interact with several other components in emulsion systems, which lead to different effects on the stability of the emulsion, depending on the affinity of the molecules (Dickinson et al., 2003; Gharibzahedi et al., 2012; McClements, 2004a; Mirhosseini, Tan, Hamid, et al., 2009). The creaming index was expected to be directly influenced by xanthan concentration (Krstonošić, Dokić, Dokić, & Dapčević, 2009), whereas some authors reported that at certain concentration, stabilisers like xanthan gum induce a higher creaming rate by the mechanism of depletion flocculation (Chanamai & McClements, 2001). The results for creaming rate were in line with the findings reported by Sun and Gunasekoran (2009), as increasing WPI concentrations caused a slight decrease in the creaming index.

Table 4.1. Experimental design with composition of the olive O/W emulsions (concentration of Whey Protein Isolate (WPI), Olive Mill Wastewater phenolic extract (P-OMW) and xanthan gum), and results of the Central Composite Design, with 2 replicates of factorial points, star point (2^n+star) and 3 centre points.

Run	Independent variables			Response variables						
	WPI (% w/w)	P-OMW (mM)	Xanthan gum (% w/w)	Creaming (%)	Viscosity (Pa s^{-1})	Particle size (μm)	D[3,2]* (μm)	PV (μM)	TBARS (μM)	Cloudiness value
1	0.315	2.2	0.13	59.45	0.2889	6.761	0.289	85.25	2.415	0.110
2	0.13	3.5	0.2	33.21	0.7530	6.592	0.753	105.71	1.954	0.500
3	0.6261	2.2	0.13	55.72	0.3258	6.772	0.326	130.71	3.426	0.200
4	0.13	0.9	0.06	79.48	0.0825	9.247	0.083	156.94	0.412	0.207
5	0.13	3.5	0.06	68.91	0.0644	11.011	0.064	124.54	0.971	0.129
6	0.315	2.2	0.0123	71.77	0.0975	5.280	0.098	143.70	0.453	0.115
7	0.0039	2.2	0.13	74.63	0.2601	12.532	0.260	122.03	1.176	0.133
8	0.5	3.5	0.2	30.72	0.6537	9.309	0.654	119.10	2.196	0.199
9	0.5	3.5	0.06	71.52	0.0768	9.733	0.077	206.87	4.111	0.076
10	0.315	2.2	0.2477	1.12	0.9244	6.698	0.924	134.61	1.834	0.324
11	0.315	4.3863	0.13	47.39	0.2876	8.153	0.288	81.68	2.696	0.176
12	0.315	2.2	0.13	56.09	0.2250	6.206	0.225	97.59	2.617	0.220
13	0.5	0.9	0.06	73.26	0.0901	7.581	0.090	101.65	0.951	0.256
14	0.5	0.9	0.2	52.61	0.6503	7.970	0.650	116.99	1.465	0.427
15	0.315	2.2	0.13	50.00	0.3274	10.626	0.327	116.67	3.332	0.085
16	0.13	0.9	0.2	47.16	0.3262	5.232	0.326	183.89	0.856	0.393
17	0.315	0.0137	0.13	73.51	0.1406	8.854	0.141	143.05	0.671	0.410

* D[3,2]: Sautern mean diameter of oil droplet size analysed by Mastersizer; PV: Peroxide Value (primary oxidation products); TBARS: Thiobarbituric acid reactive substances (secondary oxidation products).

Table 4.2. Results of the regression analysis of the central composite rotatable design of the second-order polynomial model for the responses variables measured in O/W emulsions.

Regression coefficient	Creaming rate (%)	SE	Viscosity (Pa s^{-1})	SE	Droplet (µm)	SE	D[3,2] (µm)	SE	PV (µM)	SE	TBARS (µM)	SE	Cloudiness (A)	SE
β_0	54.976	3.784	0.281	0.037	7.846	1.312	6.132	0.792	99.063	11.164	2.790	0.322	0.135	0.044
β_1 (WPI)	-2.376	1.777	0.026	0.018	-0.525	0.616	-0.046	0.372	-0.868	5.243	0.609	0.151	-0.012	0.021
β_2 (Polyphenols)	-6.742	1.777	0.047	0.018	0.398	0.616	0.561	0.372	-7.796	5.243	0.656	0.151	-0.057	0.021
β_3 (Xanthan gum)	-18.182	1.777	0.253	0.018	-0.446	0.616	-0.596	0.372	-5.829	5.243	0.172	0.151	0.088	0.021
$\beta 1^2$	4.236	1.956	0.003	0.019	0.046	0.805	-0.538	0.486	12.028	5.771	-0.179	0.166	-0.055	0.027
β_2^2	2.564	1.956	-0.025	0.019	1.050	0.805	-0.116	0.486	7.073	5.770	-0.397	0.166	-0.033	0.027
β_3^2	-5.925	1.956	0.080	0.019	-0.152	0.805	0.109	0.486	16.554	5.772	-0.588	0.166	0.017	0.027
β_{12}	0.111	2.321	-0.052	0.023	0.693	0.678	-0.378	0.410	27.238	6.850	0.279	0.197	0.019	0.023
β_{13}	0.821	2.321	0.026	0.023	0.287	0.678	-0.023	0.409	-10.067	6.850	-0.353	0.197	0.064	0.023
β_{23}	-2.941	2.321	0.058	0.023	-0.602	0.678	-0.357	0.410	-18.612	6.850	-0.236	0.197	0.038	0.023
R^2	0.954	-	0.973	-	0.469	-	0.520	-	0.847	-	0.892	-	0.854	-
R^2 (adjusted)	0.895	-	0.939	-	-0.214	-	-0.098	-	0.650	-	0.752	-	0.667	-
Regression (*p*-value)	0.001a	-	0.000a	-	0.706b	-	0.605b	-	0.034a	-	0.012a	-	0.029a	-
Lack of Fit (F-value)	1.80	-	1.80	-	0.85	-	0.54	-	1.70	-	1.48	-	1.16	-
Lack of Fit (*p*-value)	0.394b	-	0.394b	-	0.618b	-	0.750b	-	0.411b	-	0.451b	-	0.523b	-

β_0 = intercept. β_i = estimated regression coefficient for the main linear effects. β_i^2 = estimated regression coefficient for the quadratic effects. β_{ij} = estimated regression coefficient for the interaction effects. SE = standard error. D[3,2] = volume-surface mean of droplet emulsions. PV = hydroperoxide value. TBARS = thiobarbituric acid reactive substances. a = statistically significant (p<0.5). b = not significant (p>0.5).

Figure 4.1. Standardised (p = 0.05) Pareto in the Central Composite Design (2^n+star with 3 central points replicates) for creaming rate (**a**), apparent viscosity (**b**), peroxide value (**c**) and TBARS. A: WPI; B: OMW biophenols; C: xanthan gum. The models for all these responses were statistically significant (P<0.05) based on ANOVA.

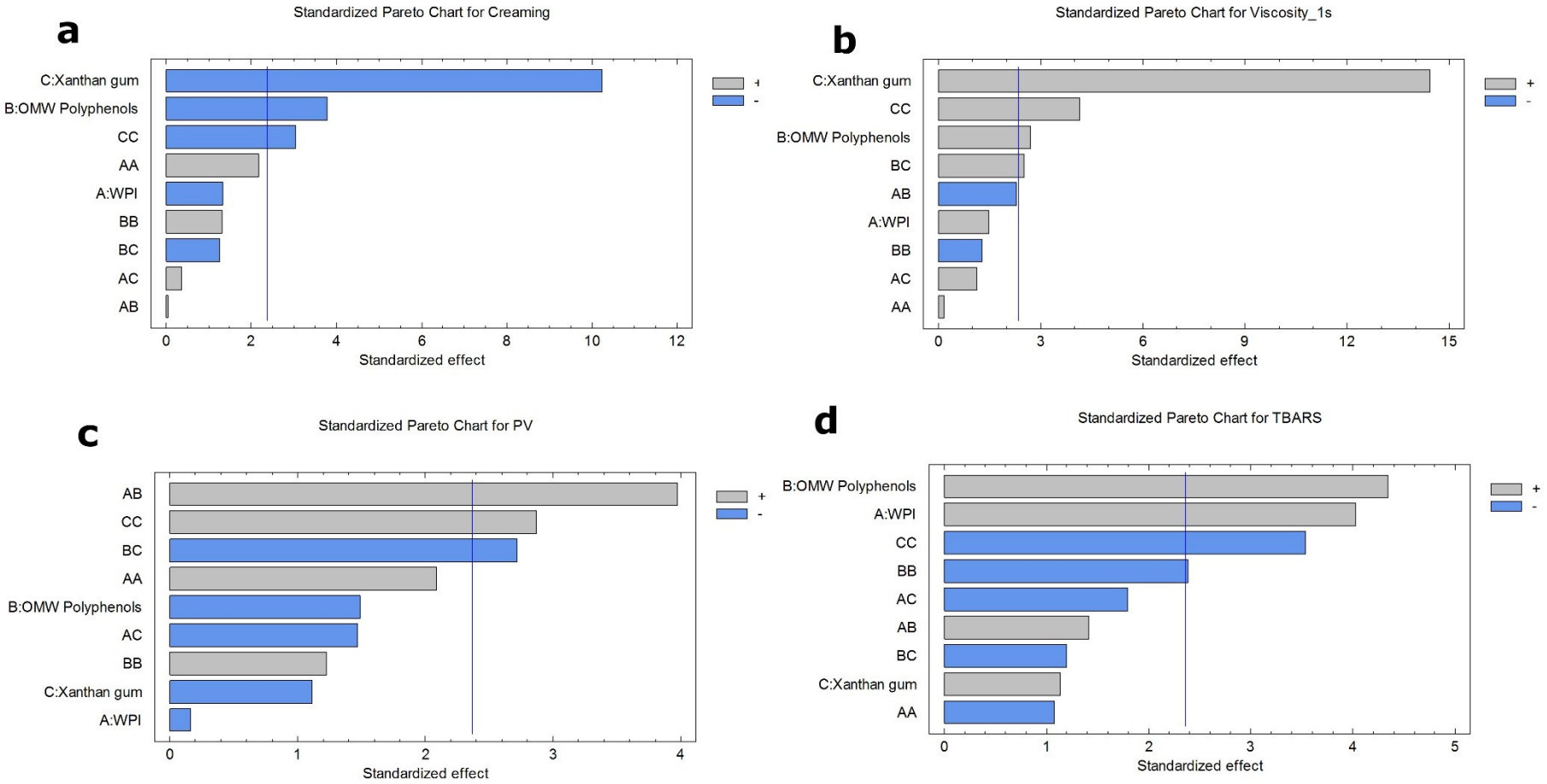


Figure 4.2. Response surface plots for emulsions creaming rate (%) showing the interaction effects between (a) WPI, (b) OMW phenolic compounds and (c) xanthan gum.

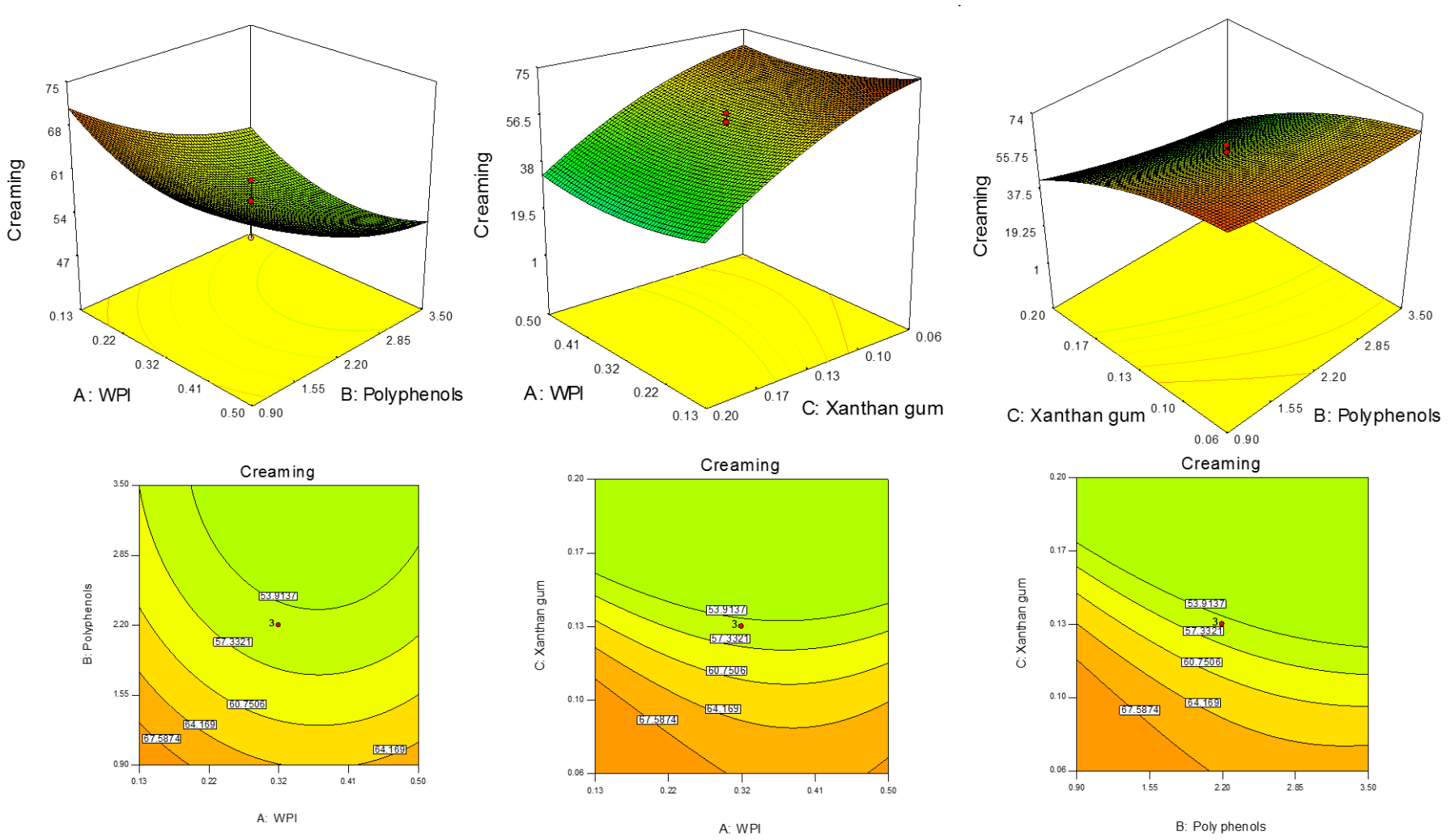


Figure 4.3. Response surface plot for emulsions apparent viscosity (Pa s^{-1}) at shear rate of 1 s^{-1} as affected by composition. The interaction between (A) WPI, (B) OMW phenolic compounds and (C) xanthan gum.

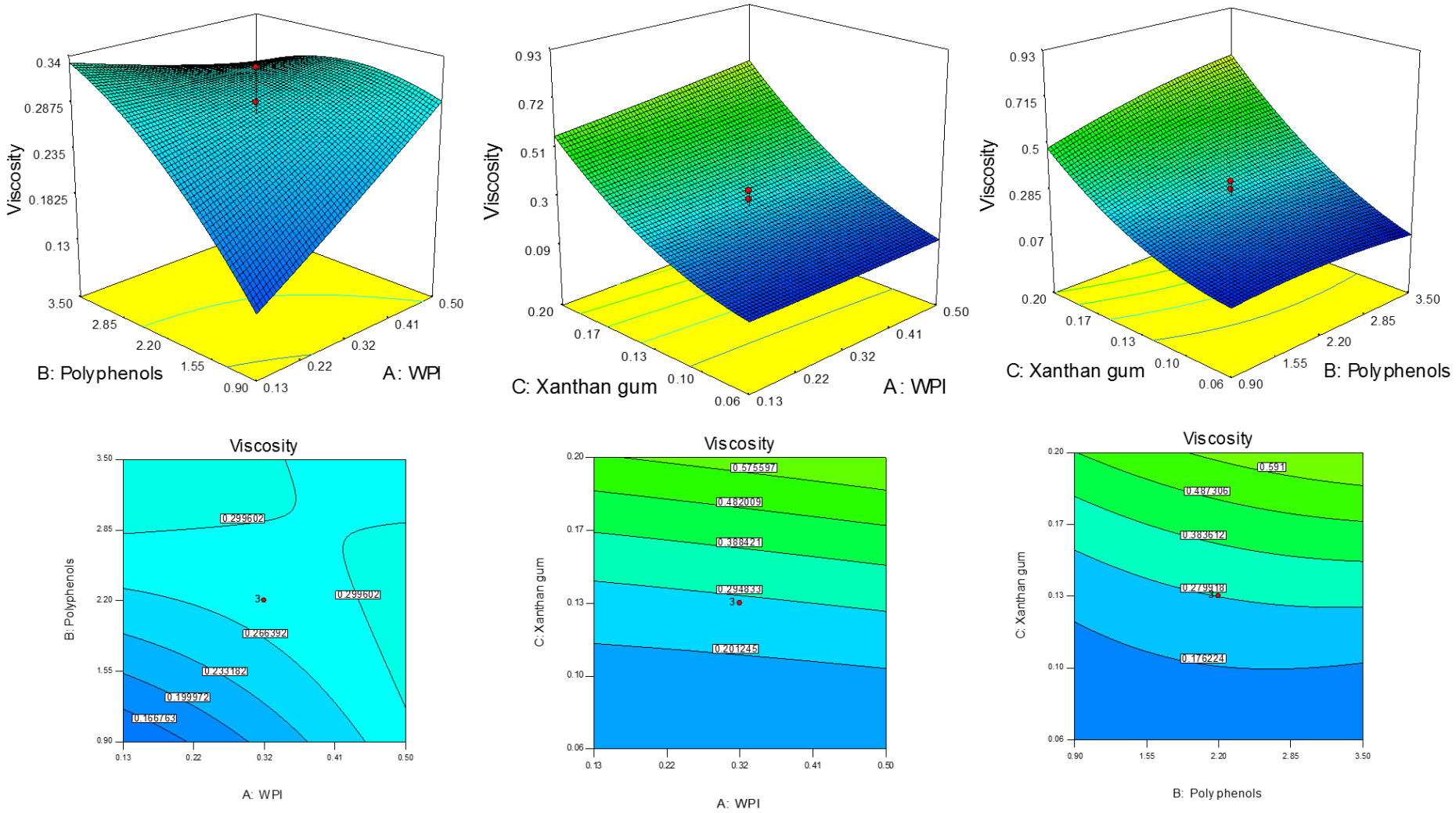


Table 4.3. Analysis of variance of model fits for the responses studied.

Source	Degree of freedom	Sum of squares	Mean square	F	*p*
Creaming					
Model	9	6237.613	693.068	16.075	0.001*
Lack of fit	5	255.909	51.182	2.230	0.338
Pure error	2	45.893	22.947		
Viscosity					
Model	9	1.073	0.119	28.336	0.000*
Lack of fit	5	0.024	0.005	1.802	0.394
Pure error	2	0.005	0.003		
Mean particle size					
Model	9	32.044	3.560	0.687	0.706
Lack of fit	5	24.675	4.935	0.851	0.618
Pure error	2	11.599	5.799		
D[3,2]					
Model	9	14.323	1.591	0.842	0.605
Lack of fit	5	7.604	1.521	0.540	0.750
Pure error	2	5.629	2.814		
PV					
Model	9	14542.733	1615.859	4.305	0.034*
Lack of fit	5	2126.314	425.263	1.697	0.411
Pure error	2	501.146	250.573		
TBARS					
Model	9	17.946	1.994	6.396	0.012*
Lack of fit	5	1.717	0.343	1.478	0.451
Pure error	2	0.465	0.232		
Cloudiness					
Model	9	0.237	0.026	4.568	0.029*
Lack of fit	5	0.030	0.006	1.163	0.523
Pure error	2	0.010	0.005		

*Statistically significant at $p<0.05$. D[3,2]: Sautern mean diameter of oil droplet size analysed by Mastersizer; PV: Peroxide Value (primary oxidation products); TBARS: Thiobarbituric acid reactive substances (secondary oxidation products).

Viscosity

The Pareto chart highlights that the main effect of xanthan gum and polyphenols was statistically significant, both with the second-order effect of xanthan gum and the interactive effects of polyphenols and xanthan gum (Fig. 1b). These independent variables positively affected the viscosity measured at strain of 1 s-1, meaning a higher apparent viscosity of the product. This result was expected for xanthan gum, as this hydrocolloid is mainly used as a stabiliser through its action on viscosity when added in water solutions. However, the influence of the olive biophenols extract was significant, also in its interaction with xanthan gum. This effect might be explained mainly by the coating material used for the spray-drying of the OMW phenolic extracts, namely maltodextrin, as this starch-derived polysaccharide can also affect the rheological properties of food products. Previous papers on O/W emulsion stability reported that xanthan gum had a main effect on viscosity value and viscosity ratio (Mirhosseini, Tan, Taherian, et al., 2009).

As shown in Fig. 3, when xanthan gum concentration is plotted against WPI or polyphenols, these latter factors had little influence on the apparent viscosity, with a slight effect at the highest concentrations tested for both ingredients. On the contrary, the protein-polyphenols plot show a dramatic decrease in viscosity at low concentrations of WPI and polyphenols.

Emulsion droplet size by image analysis and light scattering techniques

The statistical models for mean droplet size analysed by digital optical microscopy was not statistically significant at p=0.05 (Table 3). For this reason, the response surface plots were not shown and no further analysis was carried out. The absence of statistical significance was attributed to the high standard deviations of the measured mean particle sizes. In fact, a binomial distribution was observed both in fresh and stored emulsions.

Image analysis through optical microscopy was previously reported for the evaluation of aging mechanisms of olive O/W emulsions, and they stated that this technique is a valid approach (Silva et al., 2010), whereas there are objective issues in overcoming the high variability of this type of analysis. Similarly, the light scattering techniques using the Mastersizer has been widely applied for the characterisation of emulsion droplet distribution, and the volume-surface mean (D[3,2]) has been reported as a valid means for particle size distribution measurement (Hu, McClements, & Decker, 2003b; McClements, 2004a).

It should be also noted that previous papers reported that a narrow range of variations of particle diameters in O/W emulsions, as in the present model systems, may not markedly influence particle behaviour (Logaraj, Bhattacharya, Sankar, & Venkateswaran, 2008).

Lipid oxidation and cloudiness value

As shown by the Pareto graph, the level of lipid hydroperoxides (PV) formation in O/W emulsions was significantly ($p<0.05$) affected by the WPI-polyphenols interaction, by a second-order interaction for xanthan gum, and by the interaction between polyphenols and xanthan gum (Fig. 1c). A statistically negative effect resulted from the interaction between polyphenols and xanthan gum. For both oxidation indices PV and TBARS, a negative effect is desired, as this indicates a lower concentration of oxidation products in emulsions over storage, with consequently longer emulsion chemical stability.

As shown in Fig. 4a, the hydroperoxide concentration was highest at the lowest concentrations of OMW polyphenols and WPI. This result was expected as polyphenols are added to retard lipid oxidation in emulsions, but also some milk proteins are reported in literature to exert some antioxidant activity (Di Mattia et al., 2009; Sun & Gunasekaran, 2009; Tong, Sasaki, McClements, & Decker, 2000). Interestingly, an interactive effect was noticed at the highest concentrations of both compounds which caused an increase of PV value. This demonstrates that a pro-oxidant effect can be found at high concentrations of both olive phenolics and WPI. From the RSM plot, higher polyphenols concentration caused the lowest PV, while when it was associated with high WPI concentration, a deleterious effect was noticed.

Opposite results were found for TBARS concentration, as the lowest values were observed at low polyphenols and WPI levels (Fig. 4b). Therefore, the highest concentrations should be avoided to retard excessive production of secondary oxidation products. In fact, both polyphenols and WPI had statistically significant effects, shown by the Pareto chart (Fig. 1d), both with a second-order effect of xanthan gum. The first two independent variables had positive effects while xanthan gum resulted in a negative effect on TBARS, where lower values are desired. Xanthan gum at high concentrations was reported to accelerate lipid oxidation in menhaden O/W emulsions stabilised by WPI and xanthan gum; this was explained by the fact that it interacts with unabsorbed WPI in the continuous phase and prevents the WPI from acting as an antioxidant (Sun et al., 2007). However, in the present research the chosen concentrations of xanthan gum was similar, but the WPI was ten-fold lower and this might explain the different behaviour observed.

WPI alone were reported to decrease the formation of hydroperoxides in O/W emulsions (Sun & Gunasekaran, 2009). This phenomenon was observed only when the minimum amount of OMW phenolics were used, while in these experiments an opposite effect was observed.

The improvement in the oxidation indices of olive O/W emulsions as affected by the addition of individual phenolic compounds was previously reported to be not dramatic, both for primary and secondary oxidation products. In fact, for some phenolic compounds such as catechin, a higher production of TBARS was reported in such emulsions with respect to the control (Di Mattia et al., 2010). The primary lipid oxidation marker (hydroperoxides) did not correlate with the secondary oxidation marker (TBARS). This effect is in accordance with previous papers, where it also suggested that polyphenols in O/W emulsions could promote the degradation of primary oxidation products due to their ability to reduce transition metals to their catalytically active state (Mei et al., 1999).

Cloudiness surface plots show a clear positive dependence with xanthan gum concentration, but OMW polyphenols also influenced emulsion cloudiness (Fig. 4c). Whereas the colour of the phenolic powder caused a darker appearance of the final water dispersion, the lower cloudiness at increasing concentration of phenolics was not surprising due to their light absorption in the lower regions of the light spectrum, i.e. below 300 nm, while the cloudiness value was assessed as 660 nm in accordance with previous literature for diluted O/W emulsions (Mirhosseini, Tan, Hamid, et al., 2009). Mirhosseini et al. (2009) reported a positive effect of both Arabic gum and xanthan gum on the droplet concentration thereby increasing the cloudiness, as in theory the emulsion cloudiness is due largely to the suspended solid particles in the emulsion and dispersion system. Finally, it should be noted that a higher or lower cloudiness value itself cannot be considered as a positive or negative indicator, as this evaluation depends on the specific use of the food product and the desired characteristics.

Model optimisation

OMW biophenols, WPI and xanthan gum were considered for the prediction of the best experimental conditions, i.e. the highest desirability based on the responses which were statistically significantly from the model. The optimum conditions for 20% olive O/W emulsion stability were evaluated to obtain the minimum creaming rate, maximum viscosity, and minimise the PV, TBARS and cloudiness. These parameters were set up to optimise the model desirability. As the model for emulsion droplet size was not statistically valid, this was not considered in the optimisation model.

The range of WPI, polyphenols and xanthan gum set for the model optimisation calculations was kept in the range tested experimentally, as this model should not be used for higher and lower values than those used in the experiment. As shown in Fig. 5, the highest desirability was shown for high olive polyphenols extracts, but medium-high WPI concentrations. The interaction between xanthan gum and polyphenols had high desirability values at increased concentrations of both factors, and the lowest hydrocolloids concentrations resulted in the lowest values. It was also shown that high WPI concentrations should be avoided, especially at low xanthan gum concentrations. In fact, when medium-high WPI concentrations are used, the xanthan gum concentration in the final formulation should also be increased. However, the model overall desirability was 0.70 and therefore other concentrations should be tested in the future for better optimization, i.e. lower and/or higher factor concentrations. Also, other hydrocolloids might be tested to check whether better stabilisation can be obtained, especially considering the interaction with olive polyphenols.

Figure 4.4. Response surface plot for lipid hydroperoxides concentration (PV, µM) (**a**), secondary oxidation products (TBARS, µM) (**b**) and cloudiness (**c**) as affected by emulsion composition. Only the interaction between WPI and OMW phenolic compounds is shown, as these had the main effect on emulsion oxidation behaviour.

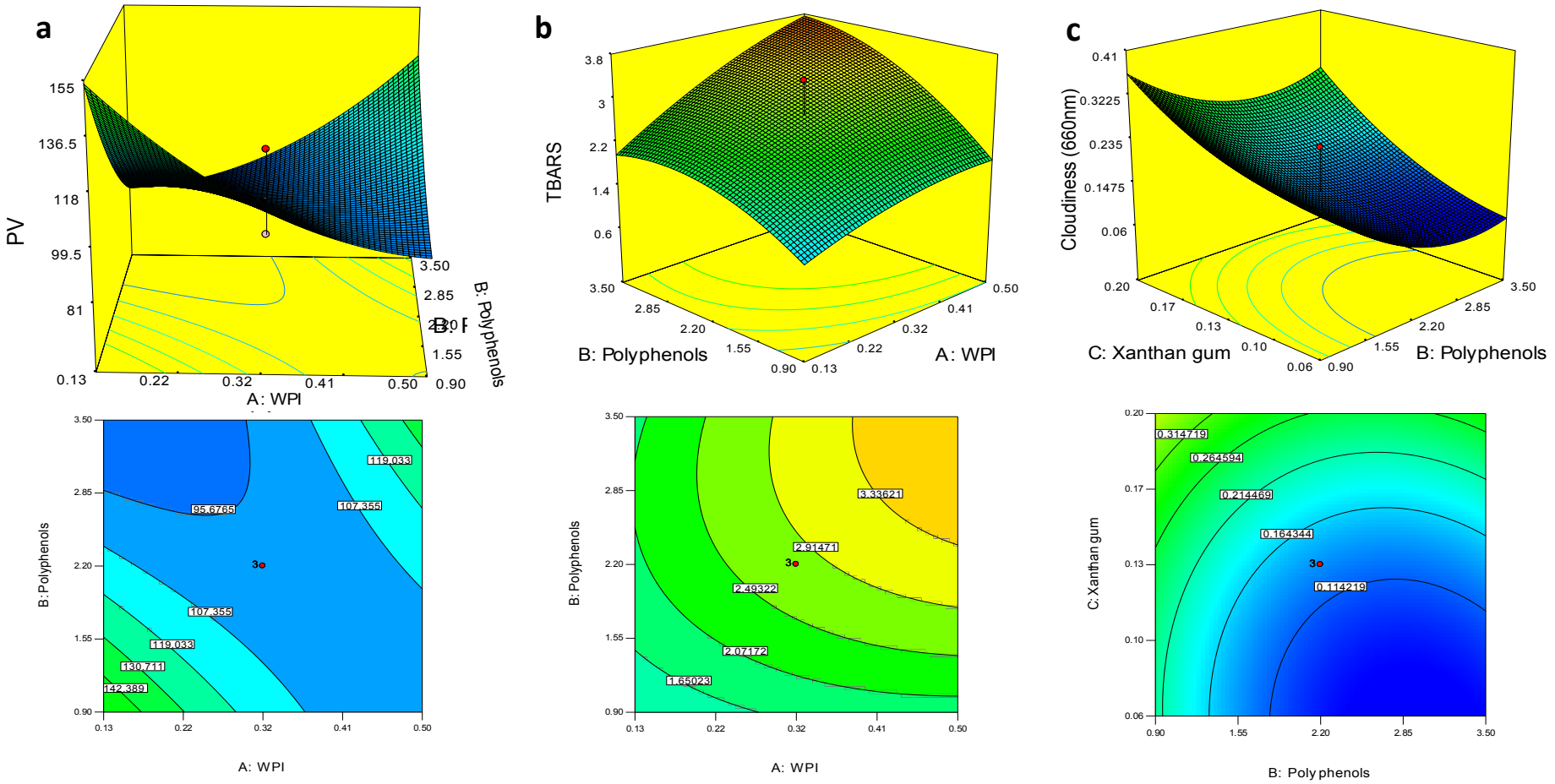
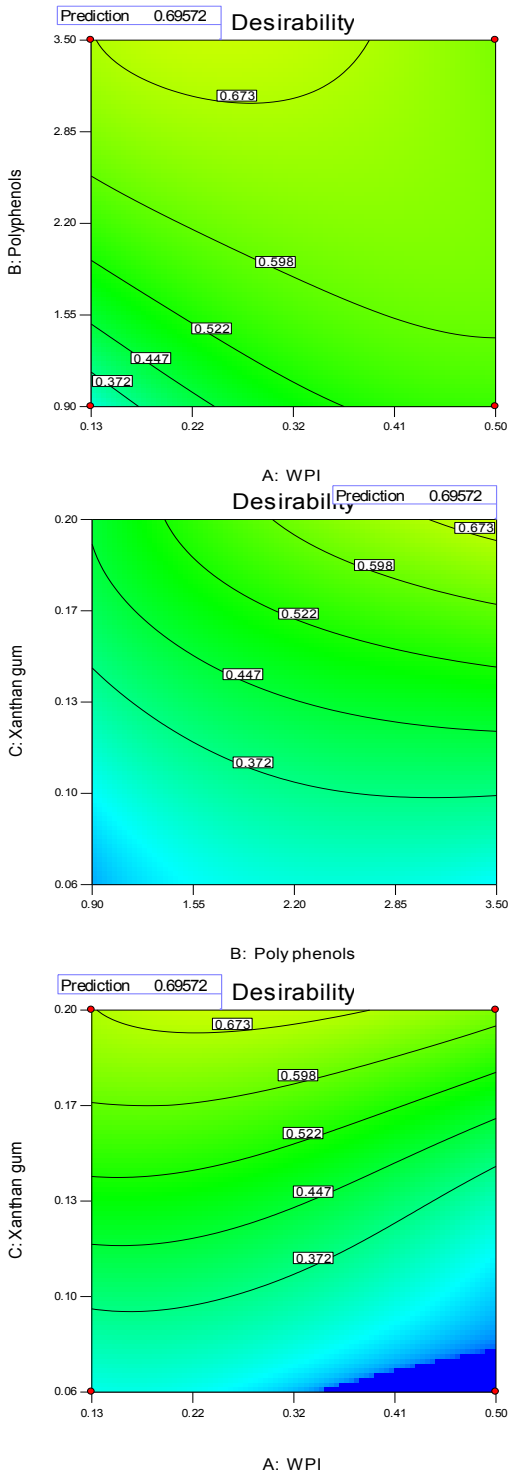


Figure 4.5. Model optimisation to show the highest degree of "desirability" of the emulsion ingredient concentration, by considering (A) WPI, (B) OMW biophenols and (C) xanthan gum, obtained by overlapping five responses (creaming rate, viscosity, PV, TBARS and cloudiness).



Conclusions for topic 2

Refined olive oil was used as fat source to produce model O/W emulsions, which were functionalised by adding olive mill wastewater phenolics. The emulsions were stabilised by using whey protein isolate and xanthan gum, and analysed over accelerated storage conditions. Our results showed that the effect of OMW phenolic, WPI and xanthan gum was statistically significant on emulsion creaming rate, viscosity, PV, TBARS and cloudiness. Second-order polynomial models were obtained for predicting these responses, resulting in good performances especially for the first two responses. It has been highlighted that the interactions between OMW phenolic extract and the hydrocolloids studied should be seriously considered for the formulation of these olive O/W emulsions, because of possibly unwanted negative effects on both physical and chemical stability. Whereas the addition of biophenols extracted from natural sources is of great interest for researchers and food industry, many variables should be taken into consideration, as they might improve or decline the emulsion stability, depending on the parameter analysed. The greater oxidative stability was lower than expected, and this was explained by the possible protein-polyphenols interactions taken place when WPI are present in the system. In addition, another positive effect of the presence of VOO biophenols in O/W emulsion is the extent of aroma release, probably due to the contrasting effects of binding of volatile compounds caused by whey proteins, for the polyphenol−protein interaction phenomenon (Genovese, Caporaso, De Luca, Paduano, & Sacchi, 2015). This chapter reported on the role of olive mill wastewater phenolics in a variety of emulsion-based food products considering the effect of the other hydrocolloids used as stabilisers or as constituent part of the food. Further investigation can deepen on the coating material used for OMW spray-drying to verify whether they can exert their full potential in terms of antioxidant effect during *in vivo* digestion.

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Topic 3

Chapter 5

Interactions of olive oil phenolic compounds and whey proteins in model dispersions: effect on the aroma release

Introduction

Olive oil is considered as one of the healthiest promoting nutritional habit worldwide, because of its high intake of monounsaturated fatty acids that showed to help in lowering cholesterol and heart disease (Estruche et al., 2013). Also, the peculiarities of virgin olive oil (VOO) are linked to its flavour and biophenol-rich polar fraction which gives rise to other benefits on human health and consumer acceptability (Tripoli et al., 2005; Frank et al., 2013). Therefore, an increase of VOO biophenol consumption, without increasing the fat intake, should be recommended by health authorities in order to encourage the general population to use it. The delicate flavour of the VOO is first perceived during inhalation, when the odorants are released into the headspace; they pass through the external nostrils, and stimulate the olfactory receptors in the nasal cavity (nasal route). Then the aroma is perceived during tasting, when the odorants interact with the receptors by migrating from the mouth to the nasal cavity via nasopharynx (retronasal route). Simultaneously other sensations take place, *e.g.* bitterness, sweetness, astringency, and pungency. VOO phenolic compounds are responsible for the astringency and pungency perceptions, the former acts by stimulating the taste receptors, while the latter sensation is perceived by the trigeminal nerve. On the other hand, volatile compounds are responsible for the odour and aroma by stimulating the olfactory receptors (Salles et al., 2011; Angerosa et al., 2004).

VOO is mainly employed in Mediterranean countries in many food preparations or as an ingredient in salad dressings. Generally, there are two distinct ways to pair VOO with food: a complementary or contrasting approach. A complementary flavour is obtained when two similar ingredients are blended. This will result in the enhancement of primary flavours, while a contrasting approach consists in tasting each ingredient separately. VOO paired with salads, vegetables, pesto, tomato sauces, etc., are examples of the first case. The latter example could be the case of pairing VOO and fresh mozzarella cheese in the famous and delicious “insalata Caprese.” In fact, the unique flavour of a "strong" VOO (bitter, pungent, and fruity), could contrast the delicate texture and taste of fresh mozzarella (sweet, acid, and milk note). Frequently, ricotta cheese is also used as an ingredient in many recipes with VOO to obtain a sweetening effect of bitter-pungent notes in strong VOOs. It is not surprisingly that renowned chefs pair VOO with many and different foods, in order to get new sensations and create new culinary experiences. When VOO is combined with dairy products, an oil-in-water emulsion or dispersion is produced. The presence of different phases and the level of volatile compounds affect their partitioning. In fact, lipophilic aroma compounds tend to move in the oil phase and their concentration considerably decreases in the continuous phase. On the contrary, hydrophilic compounds tend to move in the aqueous phase (van Ruth et al., 2000). In addition, non-volatile matrix components, like whey proteins, affect aroma release by interacting with the aroma compounds (Meynier et al., 2004; Kuhn et al., 2006; Guichard, 2006). Furthermore, it must be considered that volatile compounds analysed in foods per se could undergo significant differences when the food is consumed and, could lead to differences in the final aromatic perception. In fact, considerable physico-chemical changes which affect the release of volatile compounds from emulsion, occur in the mouth while eating. This is the reason for which the sensations of odour (the so-called nasal route) and aroma (retronasal route) could be perceived differently, even though the same olfactory sense is involved (Burdach et al., 1984; Linforth et al., 2002). In fact, salivation, mouth size, shear forces from tongue compression, breathing, pH, and temperature are factors able to change the food matrix structure and sensory properties (Chen, 2009). Among the physiological parameters, saliva was described in literature as the most important one. Its first effect is to dilute the oil-in-water emulsion when this latter is tasted. Then, the activity of salivary proteins (mucins, albumin, and proteins rich in proline), and enzymes (amylase, lipase, and lysozymes), are responsible of the emulsion destabilization (Vingerhoeds et al., 2005; Silletti et al., 2007). These changes in the structure of the

emulsion have also an impact on the final perception of the products (Dalgleish, 2006). Several studies aimed to understand the aroma release of volatile compounds in vegetable oil model solutions and emulsions (van Ruth et al., 2000; van Ruth and Roozen, 2000; van Ruth et al., 2001; van Ruth et al., 2002; Malone et al., 2003; Arancibia et al., 2011; Frank et al., 2011).

Only two published research papers reported about the release of only one volatile compound after the interaction between whey proteins and saliva by in vivo analysis. The first one studied the release of ethyl butanoate in gel, while the latter reported on 2-nonanone in aqueous solution (Mestres et al., 2006; Kuhn et al., 2009). Another study reported about the aroma release of emulsions obtained by soybean oil and β-lactoglobulin under simulated mouth conditions (Benjamin et al., 2013). Moreover, the majority of *in vitro* studies employed artificial saliva (Malone et al., 2003; Arancibia et al., 2011; Frank et al., 2011; Mestres et al., 2006). This could be a limit because the effect of numerous enzymes and proteins present in human saliva were not considered (Salles et al., 2011; Chen, 2009; Humphrey and Williamson, 2001). So far, only one published research investigated the interaction of phenolic compounds from olive oil with different food proteins, including β-lactoglobulin (Pripp et al., 2001). From a food technological point of view, this protein-polyphenols interaction is of interest as possible means to reduce bitterness, whereas the effects of such an interaction on the release of flavor compounds is still to be investigated. Up to now, no study reported about the aroma release of VOO after interaction with whey proteins and human saliva. The effect of these interactions could be important to understand the oral mechanisms of emulsion in relation to sensory perception. This information will also be useful to deepen into consumers' attraction towards foods or dishes made by pairing olive oil with dairy products. Therefore, it could be the basis of further studies in sensory science about consumers' preferences.

The present work aims to study the aroma release of volatile compounds in oil-in-water model dispersion by addition of refined olive oil spiked with VOO biophenols to a whey proteins solution (1:5 v/v). In particular, this research aims to verify the changes in headspace release of target volatile compounds due to the interactions between virgin olive oil biophenols extract and whey proteins, in presence of human saliva and simulating mouth conditions.

Material and Methods

Samples, standards, and reagents

Refined olive oil and extra virgin olive oil (EVOO) from Coratina cultivar were supplied by IOBM (Industria Olearia Biagio Mataluni, Montesarchio, Benevento, Italy). EVOO from Ravece cultivar was provided by APOOAT Soc.Coop a.r.l. (Avellino, Italy) in 250 mL green glass bottles. EVOO samples were stored in suitable condition avoiding light exposure and high temperatures in order to prevent oxidation and were used within eight months from their production (November 2013). The following chemicals were used for the analysis of volatile compounds: ethyl isobutyrate (99 %), ethyl butyrate (99 %), ethyl-2-methylbutyrate (99 %), hexyl acetate (99 %), *cis*-3-hexenylacetate (98 %), *trans*-2-pentenal (95 %), *trans*-2-hexenal (98 %), 1-hexanol (99 %), *cis*-3-hexen-1-ol (99 %), linalool (97 %), 1-penten-3-one (97 %) were supplied by Sigma-Aldrich (St. Louis, USA). The following reagents were used for the analysis: hexane (95 %), methanol (99.9 %), glacial acetic acid, trifluoroacetic acid, acetonitrile, diethyl ether, distilled water, supplied by Romil (Cambridge, England). Potassium iodide and sodium carbonate were provided by AppliChem (Darmstadt, Germany). Ammonium acetate, Folin-Ciocalteu solution and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide, phenolphthalein and starch were provided by Titolchimica spa (Rovigo, Italy). Sodium thiosulfate was supplied by Fluka (Buchs, Switzerland), and chloroform was supplied by LabScan (Dublin, Ireland).

Samples preparation

To study the effect of interactions between whey proteins isolate and olive oil, added or not, with biophenols from extra virgin olive oil on the aroma release, the experimental plan reported in **Fig. 5.1** was applied. Six model systems were set up in order to obtain known initial concentration of aroma compounds in olive oil added with phenolic compounds extract, and whey protein isolate (**Fig. 5.1A**). In order to verify our results, obtained by using model systems, a blank virgin olive oil was also analysed (**Fig. 5.1B**). Human saliva was added to the model systems and samples were subsequently analysed by using a dynamic headspace simulating mouth conditions by a Retronasal Aroma Simulator (RAS) device. In the systems without saliva, distilled water was added to obtain the same headspace volume in all the samples, with the same pH of saliva.

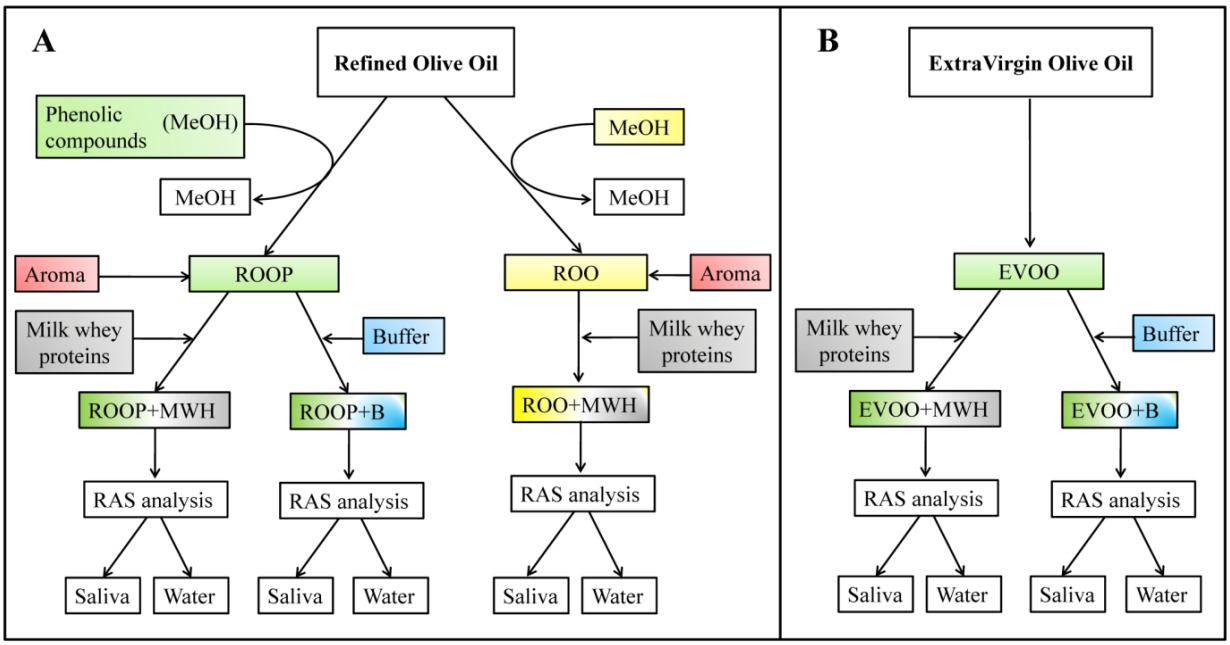

Figure 5.1. Experimental plan applied for the Retronasal Aroma Simulator (RAS) analysis of refined olive oil model (A) and extra virgin olive oil (B) systems.

Preparation of the refined olive oil sample with added virgin olive oil biophenols (ROOP)

The phenolic extract was obtained from extra virgin olive cv. Coratina, typically known for its high content of phenolic compounds. An aliquot of the oil sample (200 g) was dissolved in hexane (200 mL) and vigorously shaken for 10 s. A subsequent extraction was carried out using water/methanol mixture (40/60 v/v) in a separatory funnel. This step was repeated three times by using a total of 420 mL solvent. Subsequently, the obtained hydro-alcoholic extract was washed with hexane to remove any oil contamination and was centrifuged for 10 min at 3500 rpm (ALC International srl, PK-120, Milan, Italy). The organic phase was removed from the sample, and the hydro-alcoholic phase was collected in the flask and evaporated under vacuum in a rotary evaporator at 40 °C (Heidolph, VV 2000). The phenolic compounds were suspended using 40 mL methanol. An aliquot of the extract was used for the HPLC and Folin-Ciocalteau analyses. An amount of 2 mL polyphenol extract was adjusted to volume using refined olive oil in 100 mL volumetric flask and the oil mixture was treated in an ultrasonic bath for 15 min. Then, methanol was evaporated in vacuum evaporator (Heidolph VV 200) at 38 °C for 15 min (Garcia-Mesa et al., 2008). The polyphenol extract was added into the refined oil all at once and stored in a refrigerated environment. Before the analyses were carried out, the solution was brought to room temperature. The amount of phenolic compounds (334 mg kg^{-1}) added to refined olive oil was chosen on the basis of the average levels reported in literature for extra virgin olive oil, equivalent to about 260 mg kg^{-1} total phenolic compounds (Bayram et al., 2012). This level is between 220-340 mg kg^{-1}, *i.e.* it corresponds to a slight bitterness taste of VOO (Beltrán et al., 2007).

Preparation of the refined olive oil sample (ROO)

In the control sample phenolic extract was not added, 2 mL methanol was adjusted to volume using refined olive oil in 100 mL volumetric flask. Then, the oil phase was submitted to the same protocol previously described for phenolic compounds addition.

Preparation of the oil aroma solution

The most abundant and significant volatile compounds of virgin olive oils were considered in our study for preparing the solutions of aroma compounds, according to literature (Kalua et al., 2007), they included 5 esters, 3 aldehydes, 2 alcohols, 1 ketone and 1 terpene compound (**Table 5.1**). Volatile compounds were dissolved in 10 mL refined olive oil and homogeneously mixed. Aroma solution was obtained by diluting 4 mL of each volatile compound in oil to 100 mL refined oil. The aroma solution was added to oil sample 1h before the analysis, in order to allow its stabilization. The final concentration for each volatile compound in oil sample was reported in **Table 5.1**. The concentrations were chosen to stay within the range typically found in extra virgin olive oil (Angerosa et al., 2004; Kalua et al., 2007).

Preparation of the whey proteins solution (WP)

We considered the amount of whey proteins and the pH of the relative solution (11 % and 5.8-6.6, respectively) to simulate a real food product commonly used in many food preparations, i.e. the ricotta cheese (obtained from whey by adding sodium chloride and thermally treated to separate the upper phase). Whey protein isolate (95 %) was supplied by ACEF s.p.a. (Fiorenzuola d'Arda, PC, Italy). Therefore, a water solution at 11 % (WP) was prepared in ammonium acetate buffer solution. Buffer solution was added to 27.5 g whey proteins isolate. Ammonium acetate buffer solution 0.4% (Sigma-Aldrich, Germany) was acidified to pH 6 by using acetic acid solution (Romil, UK) by using few drops in 250 mL buffer solution. Whey proteins isolate were characterized by 96.7 % proteins, 2 % ashes, 0.3 % fat (dry weight), 4.7 % humidity.

Human saliva sampling

Mixed whole saliva (about 150 mL) was separately collected from 14 non-smoking subjects (8 males and 6 females, 23-48 years of age) from the Department of Agriculture, University of Naples Federico II (Buettner, 2002). Saliva was separated into aliquots of 2 mL and frozen at -20 ˚C. Before being used, samples were held in a thermal bath at 37 ˚C and shaken in order to dissolve any suspension.

Characterisation of the EVOO

EVOO Legal quality parameters

Olive oil acidity (% oleic acid per 100 g olive oil), peroxide value (meq O2 kg-1 oil) and UV determinations (K232, K270 and ΔK) were carried out according to the EC Reg. 2568/1991 and International Olive Council (IOC) standard methods. The parameters K232 and K270 are the oil absorbance at 232 and 270 nm, respectively, and DK was calculated from the absorbances at 262, 268 and 274 nm. Spectrophotometric determinations, K232, K232 and ΔK analyses were carried out using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Sensory analysis was carried out by eight assessors who were fully trained in the evaluation of VOO according to the official methods of the IOC (1996) and EC Reg. 2568/1991.

EVOO Fatty acid composition

GC analysis of the fatty acid methyl esters was performed as described by Christie (1982) with some modifications. The olive oil was diluted in hexane (1% oil) and 0.4 mL solution was added to 0.2 mL methanol solution with 2 N KOH. The mixture was shaken vigorously for 1 min and 1 mL of the hexane organic phase was collected for GC injection. A Shimadzu model GC-17A equipped with flame ionization detector (FID) (Shimadzu Italia, Milan, Italy) was used for the analysis. The acquisition software was Class-VP Chromatography data system version 4.6. (Shimadzu Italia, Milano, Italy). A FAME capillary column, 60 m, 0.25 mm i.d. with 0.25 mm 50% cyanopropyl-methyl phenyl silicone was used (Quadrex Corporation, New Heaven, CT, USA). The oven temperature was held at 170 8C for 20 min and then it increased at a rate of 10 °C min-1 until 220 8C, held for 5 min. Injector temperature and FID temperature: 250 °C. Carrier gas: Helium. Column flow: 2 mL min-1. Split ratio: 1/60. Injected volume: 1 mL. Peak identification was performed by comparing the retention times of the fatty acids with those of pure compounds (mixture of pure methyl esters of fatty acids; Larodan, Malmoe, Sweden) injected under the same conditions.

Table 5.1. Chemical standards, concentration added (Conc.), odour descriptor, physical-chemical properties, and MS fragments used for quantitative analysis.

Compound	Conc. mg/kg	Odour descriptor[a]	MW[b]	C[c]	log *P* o/w [d]	log *P* o/a [d]	log *P* a/w [d]	VP (Pa at 25 °C)[d]	IF[e] (m/z)	r^2	Calibration curve (mg/kg)	*n*
Ethyl esters												
Ethyl isobutyrate	0.141	Fruity	116	6	1.77	3.546	-1.78	3226	**71**-116-88	0.9998	0.0360-1.4061	5
Ethyl butyrate	0.129	Cheesy, fruity, sweet	116	6	1.85	3.637	-1.79	1946	**71**-88	0.9995	0.0329-1.2840	5
Ethyl-2-methylbutyrate	0.120	Fruity	130	7	2.26	3.912	-1.65	1071	**57**-102	0.9998	0.0306-1.1970	5
Hexyl acetate	0.219	Fruity	144	8	2.83	4.494	-1.66	193	**43**-56-84	0.9996	0.0224-2.1807	6
cis-3-Hexenylacetate	1.536	Banana-like, green , fruity, floral, ester	142	8	2.61	4.195	-1.59	152	**43**-67-82	0.9991	0.0627-15.2965	7
Aldehydes												
Hexanal	1.605	Green, green apple, grassy, cut grass	100	6	1.8	3.84	-2.06	1276	**56**-57-72	0.9996	0.0655-15.9858	7
trans-2-Pentenal	0.122	Green, apple, grassy, pleasant	84	5	1.09	3.607	-2.52	2466	**55**-84-83	0.9993	0.0123-1.2155	6
trans-2-Hexenal	6.112	Green, apple-like, bitter almond, grass	98	6	1.58	4.279	-2.70	629	**55**-69-83	0.9992	0.2493-60.8713	7
C_6-Alcohols												
1-Hexanol	0.207	Fruit, grass, floral, aromatic	102	6	1.82	5.185	-3.16	117	**56**-55-69	0.9988	0.0084-2.0589	7
cis-3-Hexen-1-ol	0.209	Leaf -like, green herbal, cut grass, banana, pungent	100	6	1.61	4.808	-3.20	125	**67**-82-55	0.9998	0.0213-2.0811	6
Others												
Linalool	0.086	Lilac, lavender	154	10	3.38	6.026	-3.06	11	**71**-93-121	0.9998	0.0220-0.8615	5
1-Penten-3-one	0.114	Green , pungent, sweet, strawberry, sharp , metallic	84	5	0.9	3.748	-2.85	5092	**55**-84	0.9983	0.0726-1.1340	4

n = Numbers of calibration points. r^2 = linear regression coefficient. [a] The odour descriptors were indicated as reported in literature. [b] Molecular weight. [c] Number of carbon atoms. [d] The logarithm of octanol/water, octanol/air and air/water partition coefficients (log *P*) and vapour pressure of the volatile compounds were calculated using EPI Suite v.4.1 software, U.S. Environmental Protection Agency and Syracuse Research Corp. [e] Bold numbers indicate quantifier ions. The sensory descriptors from the aroma compounds were taken from literature (particularly from Reiners and Grosch, 19988; and Kesen et al., 2014). The complete list of reference was reported in Genovese et al. (2014).

Extraction and analysis of phenolic compounds

The phenolic compounds were analysed both in ROO and EVOO Ravece samples. The extraction of phenolic compounds was performed according to Vásquez-Roncero (Vasquez-Roncero, 1978), but slightly modified, in order to minimize the volume of solvent used. 10 g of sample were dissolved in 10 mL hexane and vigorously shaken for 10 s. Then, the sample was extracted via a separating funnel by using 7 mL of water/methanol mixture (40/60 v/v) thrice. The obtained hydro-alcoholic extract was washed with hexane to remove any oil contamination and centrifuged (ALC International Ltd., mod. PK 120, Milan, Italy) for 10 min at 3500 rpm. The organic phase was removed from the extract. Therefore, the lower phase was collected in a flask and evaporated under vacuum in a rotary evaporator (Heidolph, VV 2000) at 40 ˚C. The obtained residue was dissolved in 2 mL methanol to obtain the final phenolic extract, and an aliquot of this solution was used for the analysis of phenolic compounds. All the extractions were performed in duplicate. The quantification of total phenolic compounds was carried out by using the Folin-Ciocalteau colorimetric method according to Gutfinger (Gutfinger, 1981). The samples were diluted in 1:50 and the solvent was completely removed by using a constant nitrogen flow. Distilled water (200 µL) was added to the polyphenol extract, 800 µL sodium carbonate 7.5 % (Na_2CO_3) and 1 mL Folin-Ciocalteau (2 N) previously diluted by using distilled water (1:10) were finally added to the solution. The samples were left to stay for 30 min in the dark at room temperature (Sacchetti et al., 2009). Absorbance was measured at 765 nm wavelength by using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank samples consisted of 200 µL distilled water and all the other reagents previously described. The analyses were carried out in replicate. The total phenolic content was determined by using a calibration curve constructed with caffeic acid and expressed as mg caffeic acid kg^{-1} of oil. The analyses were performed in triplicate for each extraction.

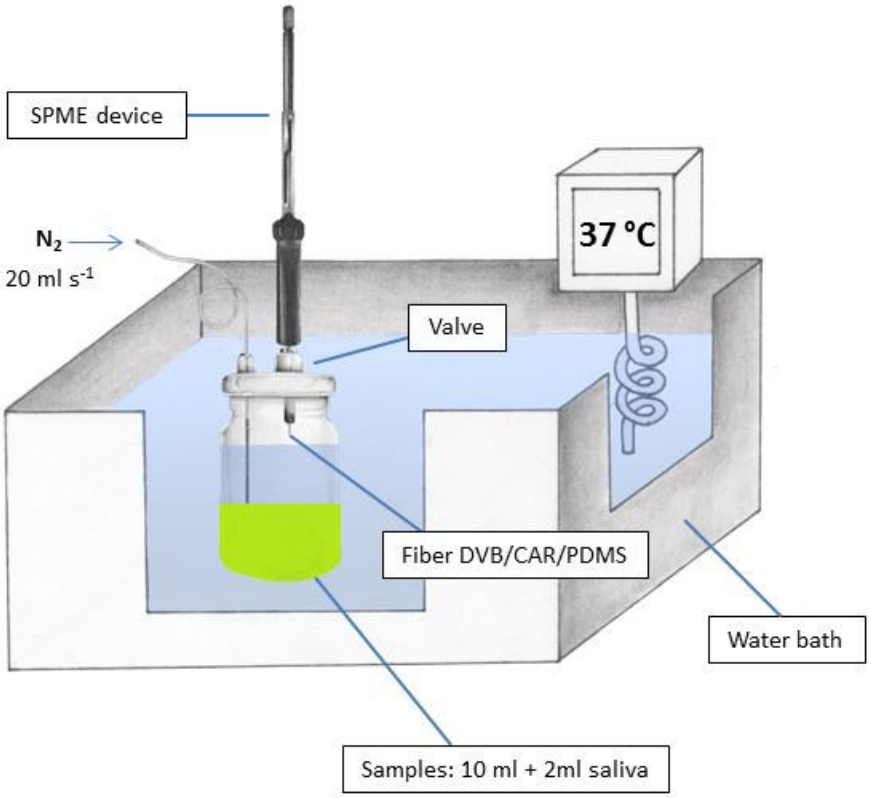

Fig. 5.2. Retronasal Aroma Simulator (RAS) used for the analysis of volatile compounds in olive oil.

Release of aroma compounds in model mouth system

The measurement of aroma compound headspace release was performed under dynamic conditions using an experimental RAS, as previously reported in literature (van Ruth et al., 2000; van Ruth and Roozen, 2000;

Genovese et al., 2009; Genovese et al., 2014). Ten µL aroma solutions were added to 1.66 mL oil sample and solution was left to react for 50 min. Subsequently, 8.33 mL buffer or whey protein solutions were added to the system and gently mixed by using a magnetic stirrer (200 rpm) for 10 min. After 1 h, 10 mL oil-in-water emulsion previously added with 10 µL internal standard (500 mg L^{-1} isobutyl acetate 99.8 % purity, Fluka, Buchs, Switzerland), was transferred into a cylindrical glass jar (100 mL) of the model mouth system. Then, human saliva or distilled water (2 mL), at the same pH of saliva, was added to the system, which was kept in a water bath (Analitica De Mori, Milano, Italy) at 37 °C. The SPME fibre was inserted through the Teflon septum in the RAS and exposed to sample headspace. Nitrogen flow (20 mL s^{-1}), passed through the sample for 4 min. During this time, the volatile compounds were trapped on the fibre. The exposure time was chosen according to data previously published by other authors (van Ruth et al., 2000; Mao et al., 2014). In addition, this exposure time could represent a realistic application to study the retronasal aroma perception of a food during the simulation of its consumption, as longer times would represent an unrealistic situation. A schematic representation of the RAS system is reported in **Fig. 5.2.**

Dynamic Headspace-Solid Phase Microextraction (SPME) and GC/MS analysis

The SPME device (Supelco Co., Bellefonte, USA) was equipped with a 50/30 µm thickness divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber coated with 2 cm length stationary phase. Volatile compounds were analyzed by GC coupled with a mass spectrometer using a GC/MS Shimadzu model QP5050A (Kyoto, Japan) equipped with a Supelcowax-10 capillary column (60 m x 0.32 mm i.d., with 0.5 µm thickness) (Supelco Co., Bellefonte, USA). Temperature was set at 40 for 4 min, followed by an increase of 3.5 °C min^{-1} up to 240 °C, and held for 3 min at maximum temperature. The injector was kept at 230 °C. Helium was used as carrier gas (1.4 mL min^{-1}). Volatile compounds thermal desorption was carried out by exposing SPME fibre in the injector for 10 min. Compound identification was performed by comparing retention times and mass spectra obtained by analysing pure reference compounds in the same conditions. Moreover, the identification was confirmed by comparing mass spectra with those of NIST database. Mass spectra were recorded at 70 eV. Source temperature was 200 °C and the interface temperature was 250 °C. Before using it, fibre was conditioned at 270 °C for 1 h for the analysis. A blank test was performed before each analysis to prevent the release of undesirable compounds. All the analyses were performed in triplicate.

Quantitative analysis

The quantitative analysis of olive oil volatile compounds was carried out using the selected ion monitoring (SIM). The selected ions for each volatile compound are listed in **Table 5.1**. Peak areas of each compound were normalized with respect to the area of the internal standard peak and interpolated on the calibration curve. A calibration curve for each molecule was constructed by preparing a solution containing known amounts of analyte in the oil and the internal standard. The oil was diluted in order to obtain 1-7 solutions with decreasing values of the concentration of analytes and each of these solutions was analysed by SPME-GC/MS in selected ion monitoring, by applying the same conditions previously reported. The concentration range considered for the calibration curve of each molecule (**Table 5.1**) was within the values typically found in virgin olive oil (Angerosa et al., 2004; Kalua et al., 2007). Peak areas were calculated by using Lab solutions acquisition system (GCMS solutions version 1.20; Shimadzu, Kyoto, Japan). The linear regression coefficient (r^2) for the studied volatile compounds was satisfactory, resulting higher than 0.9983 (**Table 5.1**).

Statistical analysis of data

Significant differences among the different model systems were determined for each compound by one-way ANOVA statistical analysis. Tukey's test was used to discriminate among the means of the variables. Differences with $p < 0.05$ were considered significant. In order to better understand the influence of saliva, biophenols and WP presence in the food model system, as well as their interactions on the volatile compounds concentration, a multifactor analysis of variance (ANOVA) with second-order interactions, was carried out. Data elaboration was carried out using XLStat (version 2009.3.02), an add-in software package for Microsoft Excel (Addinsoft Corp., Paris, France).

RESULTS AND DISCUSSION

Quality evaluation of the olive oil used for the experiment

The present study was carried out using three types of oil: a refined olive oil (RO), a filtered extra virgin olive oil (F) and an extra virgin olive oil, which was not subject to any filtration process (NF). Before using the oils in the experiment the samples were analysed in order to define the main quality parameters and compositional in terms of fatty acids and phenols. **Table 5.2** shows the results of the analyses performed on the different oil samples and the limits of the law, either to an oil which has undergone a process of rectification that for the extra virgin olive oils. The results obtained allow us to state that all oils are within the legal limits of the category. The filtered oil shows values of the indices of oxidation slightly above the non-filtrate (Hidalgo et al., 2000).

Table 5.2 - Legal quality indices for olive oil and total phenolic compounds by the Folin-Ciocalteu method for Refined Olive Oil (ROO) and Extra Virgin Olive Oil (EVOO) from Ravece cultivar.

	Free acidity	**Peroxide value**	**K_{232}**	**K_{270}**	**ΔK**	**Total phenolics (mg/kg)**
ROO	0.09±0.01	0.8±0.0	1.82±0.05	0.69±0.02	0.08±0.00	44.0±7.1
Ravece EVOO	0.21±0.03	11.8±0.4	2.18±0.01	0.16±0.00	-0.07±0.01	431.2±93.2
ROO spiked with phenolic extract						328.0± 59.1
EVOO accepted values	≤ 0.80	20.0	≤ 2.50	≤ 0.22	≤ 0.01	
ROO accepted values	≤ 0.30	≤ 5.0	-	≤ 1.10	≤ 0.16	

Acidity was expressed as oleic acid equivalent. Peroxide value was expressed as meq O_2 Kg^{-1} oil. Values are the average of three replicates (n=3).

Table 5.2 reports the free acidity, peroxide value, ultraviolet indices (K_{232}, K_{270}, ΔK), and total phenolic compounds in refined and extra virgin olive oils. Free acidity, peroxide value, K_{232}, K_{270}, and ΔK of all olive oil samples resulted within the legal limits of the category they belong to, *i.e.* extra virgin olive oil (EC 2568/91). VOO total phenolic compounds, quantified by the colorimetric method of Folin-Ciocalteau, resulted 431±93 mg kg^{-1}. Regarding the refined olive oil, the phenolic compounds were about 44±7 mg kg^{-1}, while in the refined olive oil added with an extract of VOO biophenols their level was 328±59 mg kg^{-1}.

T**able 5.3** shows the fatty acid composition obtained by means of gas-chromatographic analysis results of the determination of the fatty acid composition. From the table it is observed the presence of trans-isomers, this represents an index of the treatment of correction that results in an increase of the fatty acids to 18 carbon atoms with one, two or three unsaturations almost completely absent in the extra virgin olive oils.

It was also performed a quantitative determination by the method Folin-Ciocalteau and quali-quantitative by HPLC of phenolic substances of the oils (RO, F, NF) in order to verify the quantity and the composition of the polyphenols. Given that the experimental plan foresaw the addition of polyphenols, extracts oil from cultivar "Coratina", this analysis was also conducted on the sample of oil adjusted with the addition of polyphenols (ROP).

The phenolic compounds analysed by HPLC and by the colorimetric method is shown in the table. The total biophenols obtained by colorimetric method in F and NF extra virgin olive oil was 459.292 and 393.218 ppm, respectively. As regards the refined olive oil, it was 44.00 mg/kg, while the refined oil with added polyphenols resulted in a polyphenol concentration of 328.0 mg/kg of oil.

To verify the effect of the combined presence of whey proteins with olive oil on the release of aroma, several model systems were built, and some of them were constructed with the addition of olive phenolic compounds

at discrete concentrations. The concentration of each volatile compound added to olive oil adjusted by the addition of polyphenols (306 mg/kg) in an amount slightly higher than the average value reported for Italian EVOOs, i.e. 260 mg/kg (Bayram et al., 2012). The model systems studied simulated the combination of olive oil when paired with dairy products (in our case simulated with the presence of whey proteins in water solution). The ratio between the oil and whey protein was 1 to 5 for the emulsion formation. In such systems, there is a repartition of the volatile compounds between the two liquid phases (water and oil), and their release in the gas phase (air) was studied. This complex balance leads to a modification of release of aroma compared to the initial concentration of volatile compounds present in the oil alone.

Table 5.3. Fatty acid composition of refined olive oil, and the two extra virgin olive oils (EVOO) used for the present experiment.

Fatty acids		**Refined olive oil**			**EVOO – filtered**			**unfiltered**		
C 14:0	miristico	0,02	±	0,00	0,01	±	0,00	0,01	±	0,00
C 16:0	palmitico	14,12	±	0,00	13,17	±	0,01	13,31	±	0,00
C 16:1w9	palmitoleico	0,10	±	0,00	0,10	±	0,00	0,09	±	0,00
C 16:1w7	palmitoleico	1,37	±	0,00	0,77	±	0,01	0,71	±	0,01
C 17:0	eptadecanoico	0,08	±	0,01	0,05	±	0,00	0,06	±	0,01
C 17:1	eptadecanoico	0,11	±	0,00	0,06	±	0,00	0,06	±	0,00
C 18:0	stearico	2,47	±	0,02	3,38	±	0,01	3,56	±	0,01
C 18:1w9 t		0,05	±	0,01	0,01	±	0,00	0,01	±	0,00
C 18:1w9	oleico	64,85	±	0,09	65,54	±	0,03	65,05	±	0,06
C 18:1w7	vaccenico	4,74	±	0,05	3,70	±	0,03	3,60	±	0,06
C 18:2 c,t		0,05	±	0,00	0,01	±	0,00	0,01	±	0,00
C 18:2 t,c		0,04	±	0,01	0,00	±	0,00	0,00	±	0,00
C 18:2	linoleico	10,39	±	0,00	10,76	±	0,00	11,06	±	0,00
C 20:0	arachidico	0,42	±	0,01	0,45	±	0,01	0,46	±	0,00
C 18:3 c,c,t		0,04	±	0,01	0,00	±	0,00	0,00	±	0,00
C 18:3 t,c,c,		0,02	±	0,00	0,00	±	0,00	0,00	±	0,00
C 18:3	linolenico	0,50	±	0,01	0,71	±	0,01	0,70	±	0,01
C 20:1	eicosenoico	0,26	±	0,01	0,23	±	0,00	0,23	±	0,01
C 22:0	beenico	0,14	±	0,01	0,12	±	0,01	0,12	±	0,00
squalene		0,23	±	0,00	0,91	±	0,02	0,97	±	0,01
C 24:0	lignocerico	0,06	±	0,01	0,05	±	0,00	0,05	±	0,00

Effect of WP addition on VOO aroma release in presence of biophenols

To verify the effect of the interaction between WP and VOO on aroma release, several model systems were added to VOO phenolic compounds, to reach a final concentration of 328 mg kg^{-1}. Aroma compounds were set up and were kept to interact with WP (**Fig. 5 1A**). In these systems, VOO aroma compounds are partitioned between the two liquid phases (water and oil) and liquid and gas phase (air). In addition, by its emulsion destabilization effect (Vingerhoeds et al., 2005; Silletti et al., 2007), saliva could further affect the volatile compounds partition. This complex balance could lead to different aroma release compared to the initial level of volatile compounds in olive oil. In order to better detect these differences in headspace aroma release, a comparison was carried out by analysing the levels of added volatile compounds by using only

refined olive oil (ROO), *i.e.* without interaction of WP and saliva (it is possible to refer to it as orthonasal odour). In the systems without whey proteins (WP) a buffer solution (B) was added to obtain the same headspace volume in all the samples. The extent of simulated retronasal release of ethyl esters (**Fig. 5.3**) resulted to be similar within the three molecules belonging to this chemical class (ethyl isobutyrate, ethyl butyrate, and ethyl-2-methylbutyrate). Saliva seems to cause a significantly decrease in the release of the three esters analysed without whey proteins interaction (ROOP+B). This result could be explained by the binding effects of human saliva, as demonstrated by other researchers, who attributed both to the chemical binding or hydrolysis, and/or physical trapping of volatile compounds mainly caused by mucin (van Ruth and Roozen, 2000; Buettner, 2002; Genovese et al., 2009; Friel and Taylor, 2001). Also in whey proteins system (ROOP+WP) a significant and considerable decrease in the release of the ethyl esters was obtained, and this result is in accordance with literature,[9] but in the systems in which WP interacted with saliva, this decrease was lower. This behaviour could be explained by a possible interaction between human salivary constituents and whey proteins, with the result that their action on these volatile compounds could be limited. In fact, it was shown that mucins form aggregates with β-lactoglobulin in aqueous and in oil-in-water emulsion (Vardhanabhuti et al., 2011; Sarkar et al., 2009). These kinds of interactions, which occur *in vivo* during the consumption of dairy products, might be responsible for the sensory astringency (Vardhanabhuti et al., 2010). In fact, in agreement with the well-accepted astringency model of wine polyphenols (Kallithraka et al., 2001), this sensation is due to the loss of lubrication of saliva when mucin and lactoglobulin interact to form an aggregate which precipitates and leads to a dryness and roughness perception in the mouth. Therefore, as reported for red wine in the case of mucin and polyphenols (Genovese et al., 2009), in addition to sensory astringency, mucin and whey proteins interaction affect the extent of esters release. A similar behaviour was observed for acetates (hexyl acetate and *cis*-3-hexenyl acetate). In the case of this class of compounds, the level of aroma release was lower in presence of saliva in the buffer system (ROOP+B) with respect to distilled water (no saliva), while in the systems with WP and saliva the lower decrease of aroma compounds was observed only for *cis*-3-hexenyl acetate (**Fig. 5.3**). The result obtained for hexenyl acetate was in line with other acetates, whereas, in this case, statistical significance was not reached. In fact, it was demonstrated that within one chemical class, affinity for β-lactoglobulin increases with hydrophobic chain length or overall hydrophobicity (expressed as log $P_{o/w}$ values).[9] Hexenyl acetate, which has a log $P_{o/w}$ value of 2.83, showed a higher affinity to whey proteins than the other volatile compounds. Therefore, they resulted in a lower release than *cis*-3-hexenyl acetate (log $P_{o/w}$ 2.61), that had lower affinity and higher headspace release.

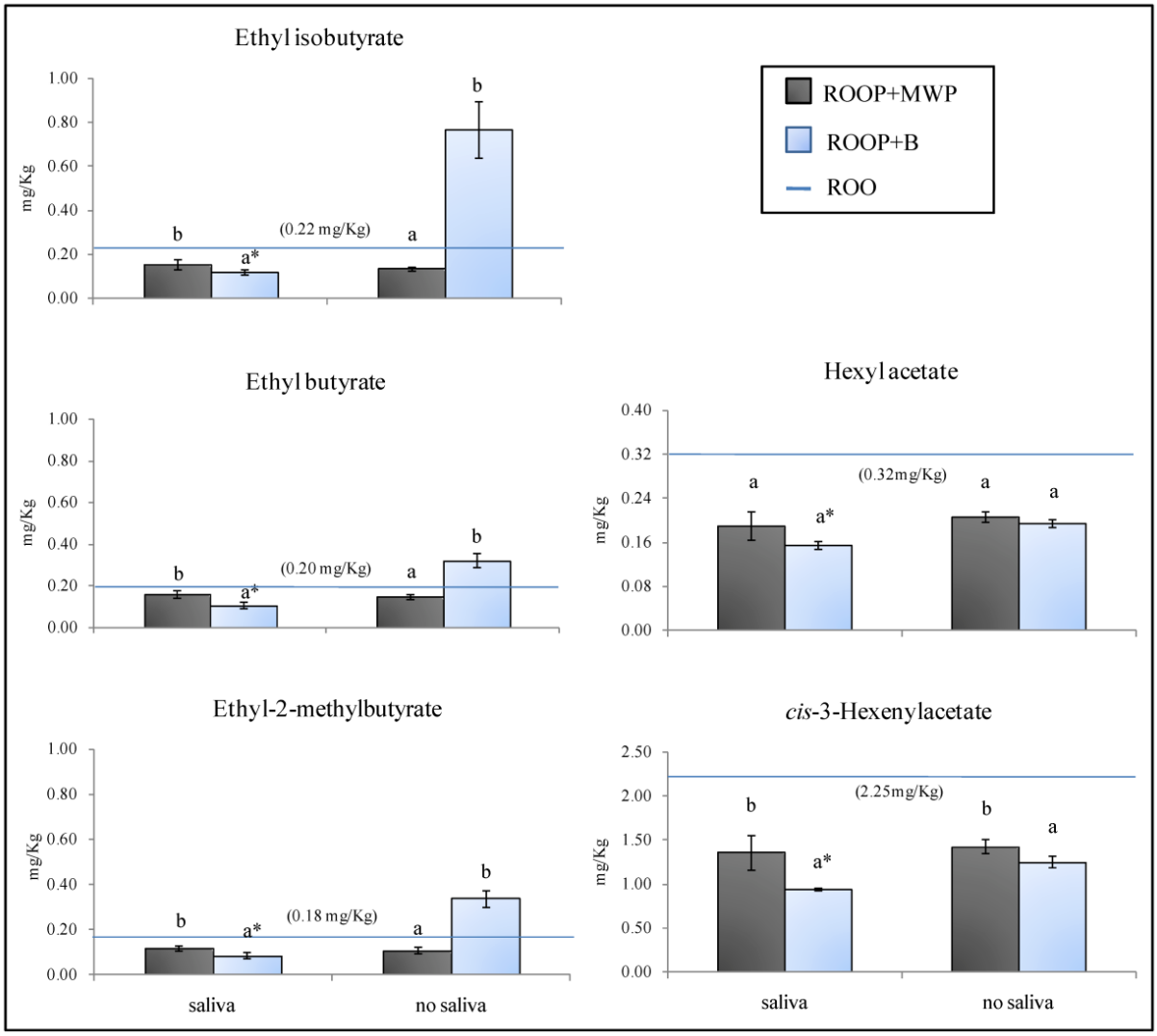

Figure 5.3. Headspace concentration of ethyl esters and acetates in Refined Olive Oil model system with the addition of VOO phenolic compounds (ROOP) in presence (WPI) and absence of Whey Protein Isolate (B) and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WPI and saliva. Different letters indicate significant differences (p<0.05) between WP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

In all the systems in which saliva or whey proteins were added, a lower release of ethyl esters was obtained under simulated retronasal conditions with respect to the orthonasal simulation assay, only for refined olive oil (ROO without addition of saliva and whey proteins). On the contrary, in the system without saliva and whey proteins, a higher release of ethyl esters was measured. This could indicate that aroma release is not only influenced by phase separation, but also by the increased viscosity of the emulsion could play an important role in the aroma release.

The behaviour obtained for aldehydes (hexanal, *trans*-2-pentenal, *trans*-2-hexenal) was quite different (**Fig. 5.4**). The saliva addition clearly resulted in a decrease of hexanal headspace concentration with respect to distilled water. When WP were added in the systems, a significant lower headspace concentration was observed, which indicates a possible further binding of hexanal by whey proteins. However, the behaviour was different to those discussed for esters, *i.e.* when WP interacted with saliva there was not a significant increase with respect to whey proteins or saliva alone, where a further decrease in headspace concentration was observed. Probably, this occurs because most of the aliphatic aldehydes are also linked to proteins by covalent irreversible bonds, as reported for β-lactoglobulin (Kuhn et al., 2006; Guichard, 2006) and mucin (Meynier et al., 2004; Friel and Taylor, 2001), to form Schiff bases. On the contrary, in the case of esters and acetates the interactions between protein and aroma compounds are reversible, involving only hydrophobic

binding sites. In the first case, when the bonds to proteins are covalent, *i.e*. irreversible, a cumulative effect of the individual reaction occurs. Otherwise, in the latter case reversible hydrophobic or hydrogen bonds occur, resulting in an antagonistic and not in a cumulative effect. Hexanal headspace concentration decreased in the presence of human saliva and WP, in accordance to Weel et al. (2003), where an *in vivo* study was reported. A dissimilar phenomenon was observed in the case of *trans*-2-hexenal and *trans*-2-pentenal. The first showed a lower headspace concentration only in the buffer system with saliva, while the latter in the presence of whey proteins. As discussed for acetates, this difference could be due to different hydrophobicity (expressed as log $P_{o/w}$ values) of aldehydes. In fact, hexanal, which has a log *P* value of 1.8, showed a lower release than *trans*-2-pentenal (log $P_{o/w}$ = 1.9), while *trans*-2-hexenal, characterized by an intermediate vale of log $P_{o/w}$ (1.58), showed an intermediate release. It is important to note that among the aldehydes, only hexanal showed a higher retronasal release with respect to the orthonasal simulation assay of only refined olive oil (ROO without addition of saliva and WP). This behaviour was also previously reported for fresh sunflower oil-in-water emulsions analysed by a mouth model system and related to hexanal generation by lipid oxidation reactions (van Ruth and Roozen, 2000).

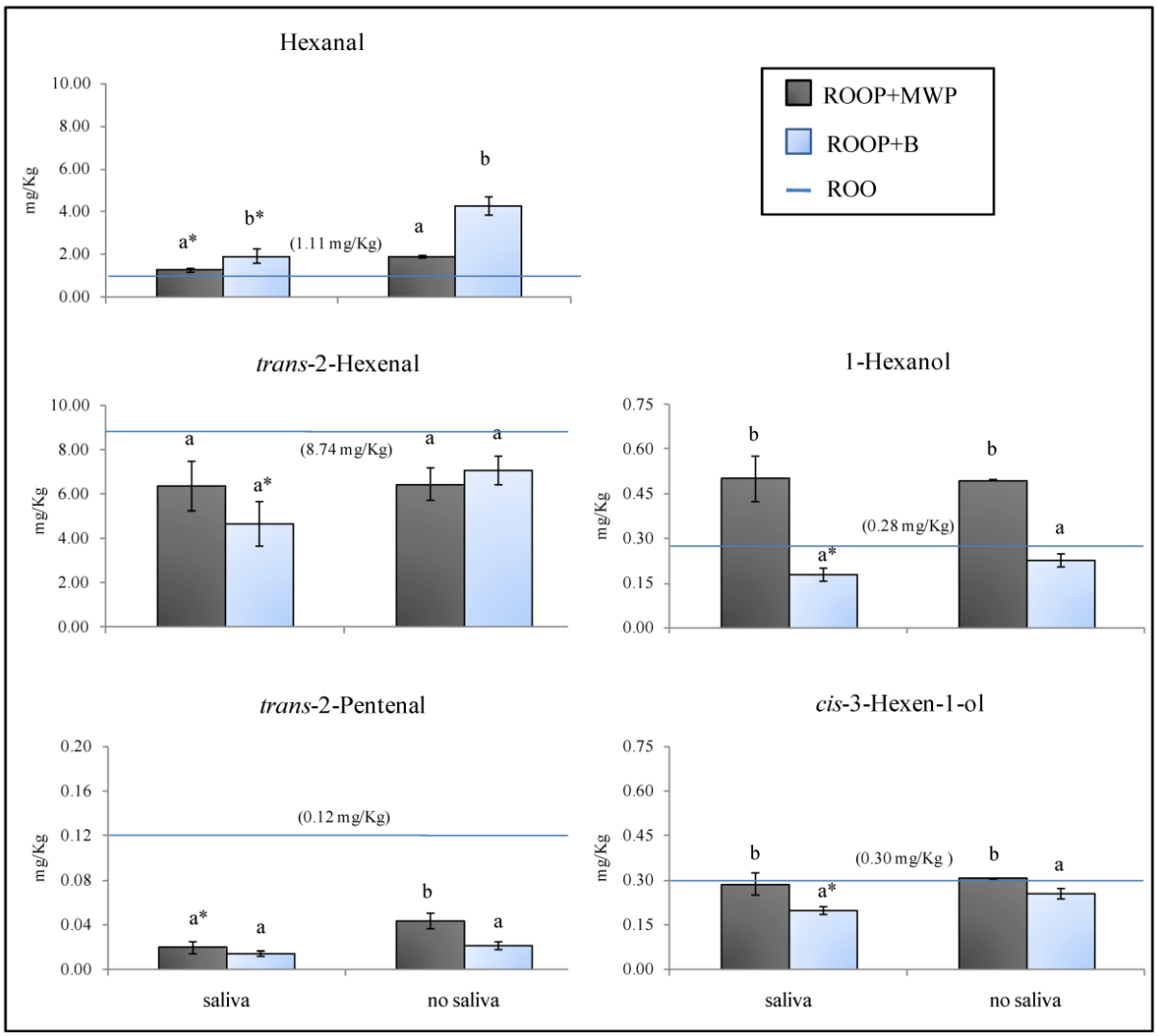

Figure 5.4. Headspace concentration of aldehydes and C_6-alcohols in Refined Olive Oil model system with the addition of VOO phenolic compounds (ROOP) in presence (WPI) and absence (B) of Whey Protein Isolate and saliva. Lines indicate the level of volatile headspace release in Refined Olive Oil (ROO) without interaction with WPI and saliva. Different letters indicate significant differences (p<0.05) between WPI and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

The two alcohols (1-hexenol and *cis*-3-hexen-1-ol) studied showed similar behaviour: when WP were not added to the system, saliva caused a decrease in headspace release (**Fig. 5.4**). When they were present, no

significant difference was observed in aroma release between the system with distilled water and those with human saliva. As discussed for ethyl esters, this phenomenon occurs by the interaction between mucin and whey proteins that limits their action on alcohols. Therefore, it finally results in a higher release of these compounds. The retronasal release of 1-hexanol in the system with whey proteins was dramatically higher with respect to the orthonasal simulation assay of only refined olive oil (ROO), and significantly higher with respect to buffer system. This so-called *salting-out* effect, accompanied by a decrease of about the same magnitude in hexanal headspace concentration, was reported by other authors (Weel et al., 2003) and was attributed to an enzymatic conversion of these two compounds. In **Fig. 5.5** the headspace release of 1-penten-3-one and linanool was shown. The retronasal concentration of 1-penten-3-one was significantly higher when distilled water was used instead of human saliva, but only in the buffer system solution. In fact, no statistical difference was observed between buffer solution and saliva in the system with whey proteins. This result suggests that the presence of whey proteins has a strong effect over the headspace release of some aroma compounds, resulting in a retention effect similar to that caused by human saliva. Whey proteins seem to have stronger binding effects on retronasal concentration than 1-penten-3-one, due to possible chemical binding, as reported for 2-nonanone, a similar hydrophobic volatile compound (Kuhn et al., 2007). On the contrary, in the system with saliva, this phenomenon was not observed, probably because the binding sites of volatile compound interacting with proteins are not available for further reaction with other proteins such as mucin. In fact, when whey proteins are added to such a system, covalent and irreversible interactions occur, as it was also reported for aldehydes (Meynier et al., 2004; Guichard, 2006).

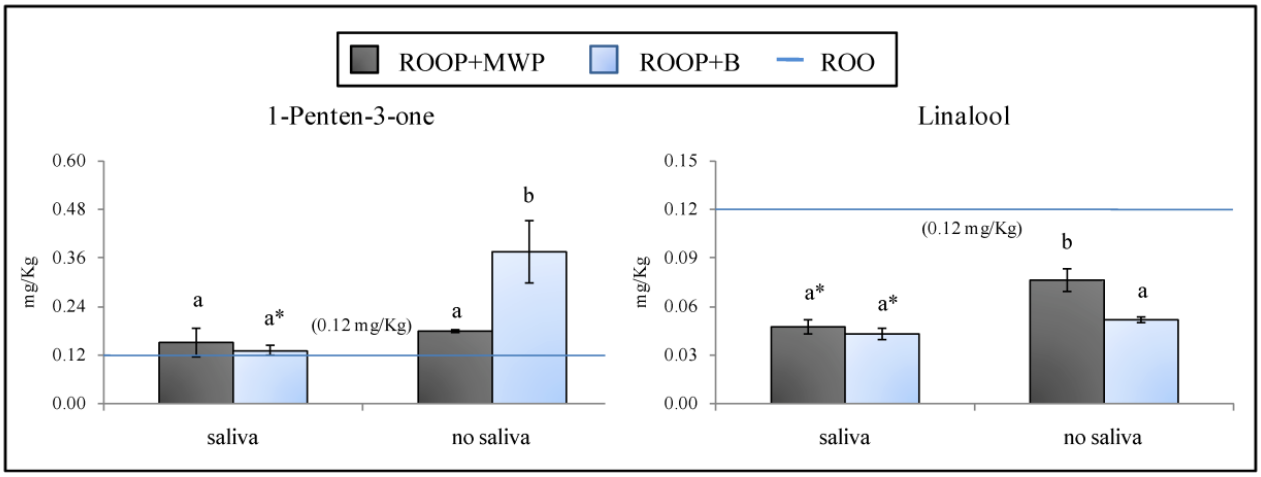

Figure 5.5. Headspace concentration of 1-penten-3-one and linalool in Refined Olive Oil model system with the addition of EVOO biophenols (ROOP) in presence (WPI) and absence (B) of Whey Protein Isolate and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WPI and saliva. Different letters indicate significant differences (p<0.05) between WPI and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

In the case of linalool, its headspace concentration in simulated retronasal aroma, was always lower than orthonasal simulation assay of ROO. Particularly, both in buffer and whey proteins systems, saliva caused a significantly lower concentration of linalool with respect to distilled water. In systems without saliva, higher release of linalool was measured in the headspace than buffer system when WP were added. In other words, also in this case the "salting out" effect was observed when whey proteins were added to the system with distilled water. This behaviour was previously reported in literature to be particularly affected at low pH values for other terpene compounds, such as limonene and myrcene (Jouenne and Crouzet, 2000). These compounds have a high hydrophobic properties (log $P_{o/w}$ = 4.38 and 4.17 respectively, EPI Suite v.4.1 software, U.S. Environmental Protection Agency and Syracuse Research Corp.) near to those of linalool (log $P_{o/w}$ = 3.38). This difference was not detected when human saliva was used and, this result was explained by the interaction between salivary components and whey proteins, as previously discussed.

In order to better understand the influence of saliva and whey proteins, as well as the interactions among these factors on the retronasal aroma release in olive oil, a multifactorial ANOVA analysis was carried out.

Table 5.4 shows the *F* ratio, *p* value, and the direction of the effect of the factors on the average relative recoveries of the aroma compounds. The *F* ratio represents the quotient between the variability due to the effect considered and the residual variance. The higher is the *F* ratio value, the more marked the effect of that factor with reference to a variable, at $p<0.05$ is. The sign of the effect shows the direction of the influence. Whey proteins seem to have a negative effect on the aroma release in the case of ethyl esters, hexanal and 1-penten-3-one, responsible of fruit, green, and pungent odours respectively (**Table 5.1**). On the contrary, they showed a positive effect on *trans*-2-pentenal (green), 1-hexanol (fruit) and linalool (floral). Saliva showed a negative effect on all volatile compounds concentration, except for 1-hexanol. The interaction between the two factors took place for the majority of the volatile compounds, except for both acetates and alcohols. The results herein described suggest that whey proteins affect the aroma release of VOO, but their interaction with saliva has an antagonistic effect on esters, acetates, and alcohols release while synergistic on aldehydes and ketones. This different effect may imply a higher perception of fruity notes of VOO and a lower green notes when VOO and dairy products are paired, when confirmed by further sensory studies.

Effect of whey proteins addition on EVOO aroma release

In the present section of our study a real sample of extra virgin olive oil was tested (**Fig.5 1B**). In order to compare this system (EVOO cv. Ravece) and the model systems (refined olive oil added with selected aroma compounds), a quantification of volatile compounds was performed. EVOO (**Fig. 5.6**) generally showed a slightly different behaviour compared to the "simulated VOO" (**Fig. 5.3-5.5**). Ethyl esters and linalool were not found in EVOO headspace, being mainly present at trace level. At low levels, ethyl esters contribute to olive oil flavour (Kalua et al., 2007), while at higher levels they are associated to fusty defect of virgin olive oil (Morales et al., 2005). Linalool, as well as all terpene compounds, could depend on their variety (Vichi et al., 2006). Generally, headspace release of volatile compounds was similar among their chemical classes, *e.g.* the release of aldehydes and ketones were lowered by the presence of WP, with no or slight effects of saliva addition. For acetate compounds, no dramatic differences were observed. Alcohols had a slight tendency to higher headspace release when WP were added, and also in this case saliva showed no statistically differences. Therefore, it is possible to state that WP affect the VOO aroma release more than our model systems simulating VOO, while human saliva on the contrary showed an opposite trend. In contrast to the model systems, the release of volatile compounds seems to be more in accordance with the chemical class they belong to. These differences are probably due to the chemical complexity of virgin olive oil in terms of both volatile and non-volatile compounds. This complexity leads to a higher competition in the interaction among volatile compounds, human saliva and WP which can affect their partition and release.

Table 5.4 Results of multifactor analysis of variance for volatile compounds of olive oil-in-water emulsion differing in whey proteins addition and saliva treatments.

Compound	**Whey proteins**			**Saliva**			**Whey proteins x Saliva**		
	F ratio	*p* value	Effect	*F* ratio	*p* value	Effect	*F* ratio	*p* value	Effect
Esters									
Ethyl isobutyrate	117.219	<0.0001	-	68.745	<0.0001	-	79.146	<0.0001	yes
Ethyl butyrate	100.671	<0.0001	-	65.589	<0.0001	-	80.540	<0.0001	yes
Ethyl-2-methylbutyrate	165.816	<0.0001	-	100.211	<0.0001	-	117.384	<0.0001	yes
Hexyl acetate	0.243	0.635		11.016	0.011	-	2.127	0.183	
cis-3-Hexenylacetate	0.088	0.774		8.771	0.018	-	3.416	0.102	
Aldehydes									
Hexanal	63.465	<0.0001	-	84.982	<0.0001	-	27.894	0.0001	yes
trans-2-Hexenal	5.130	0.053		7.883	0.023	-	7.096	0.029	yes
trans-2-Pentenal	20.362	0.002	+	29.733	0.001	-	9.511	0.015	yes
C_6-Alcohols									
1-Hexanol	9.979	0.013	+	0.472	0.511		0.891	0.373	
cis-3-Hexen-1-ol	0.002	0.962		11.613	0.009	-	3.090	0.117	
Others									
Linalool	28.796	0.001	+	53.019	<0.0001	-	14.292	0.005	yes
1-Penten-3-one	28.731	0.001	-	30.581	0.001	-	19.805	0.002	yes

Table 5.5. Results of multifactor analysis of variance for volatile compounds of olive oil-in-water emulsion differing in phenolic compounds addition and saliva treatments.

Compound	**Phenolic compounds**			**Saliva**			**Phenolic compounds x Saliva**		
	F ratio	*p* value	Effect	*F* ratio	*p* value	Effect	*F* ratio	*p* value	Effect
Esters									
Ethyl isobutyrate	0.119	0.739		4.260	0.073		0.377	0.556	
Ethyl butyrate	0.268	0.619		1.425	0.267		0.058	0.816	
Ethyl-2-methylbutyrate	0.043	0.840		1.281	0.291		0.251	0.630	
Hexyl acetate	7.068	0.029	+	0.065	0.805		1.960	0.199	
cis-3-Hexenylacetate	4.594	0.064	+	0.006	0.941		0.812	0.394	
Aldehydes									
Hexanal	146.648	<0.0001	+	161.752	<0.0001	-	14.387	0.005	yes
trans-2-Hexenal	1.394	0.272		2.615	0.145		2.095	0.186	
trans-2-Pentenal	47.657	0.000	+	30.042	0.001	-	14.633	0.005	yes
C_6-Alcohols									
1-Hexanol	3.759	0.088	+	0.159	0.701		0.022	0.885	
cis-3-Hexen-1-ol	10.501	0.012	+	0.007	0.937		3.475	0.099	
Others									
Linalool	38.807	0.000	+	21.722	0.002	-	25.323	0.001	yes
1-Penten-3-one	7.745	0.024	+	5.518	0.047	-	0.035	0.855	

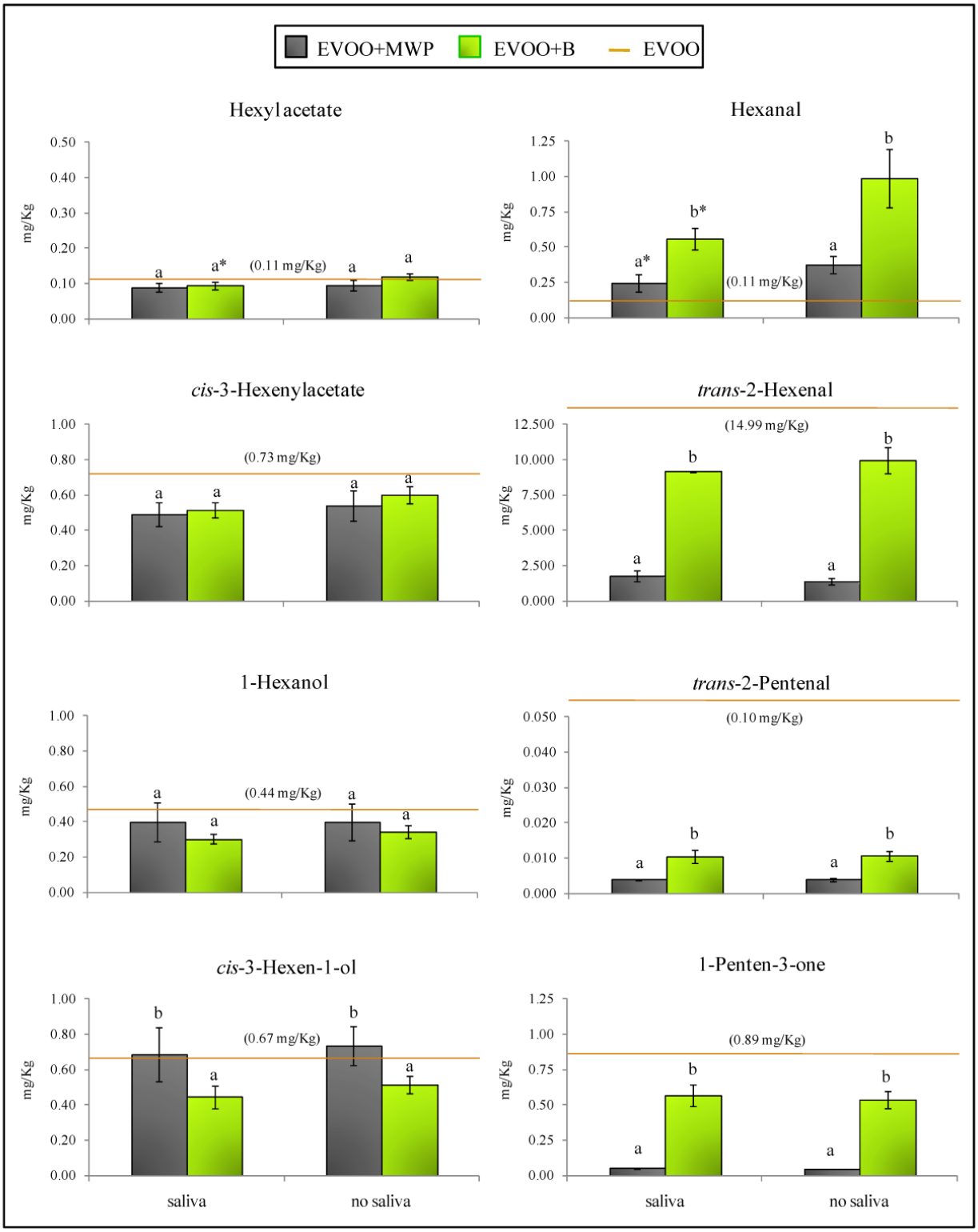

Figure 5.6. Headspace concentration of volatile compounds in EVOO model system in presence (WPI) and absence (B) of Whey Protein Isolate and saliva. Lines indicate the level of volatile headspace release in refined olive oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences (p<0.05) between WPI and blank samples. Asterisks indicate significant differences between saliva/no saliva runs

Effect of EVOO biophenols addition on aroma release in presence of whey proteins

In the previous paragraph an oil-in-water emulsion was studied, by considering a pairing between VOO and WP. It is known that VOO is naturally rich in phenolic compounds, which are important for human health. They are probably also involved in the *in vivo* aroma release during consumption of this food paired to other food products. For this reason, the specific effect of VOO biophenols presence was studied by comparing a system with refined olive oil and refined olive oil added with olive oil biophenols. All the model systems were allowed to interact with whey proteins (**Fig. 5 1A**). Also in this case, the effect of saliva was studied by comparing this system with that using distilled water. As shown in **Fig. 5.7**, no statistical difference was obtained for ethyl esters between the system with phenolic compounds and the blank (absence of phenolics). Biophenols did not influence the release either of ethyl esters or acetate compounds in the presence of saliva. Whereas in the system with distilled water a significantly higher level of acetate compounds were found in ROOP rather than ROO. This phenomenon could be probably due to a weak salting out effect, as it occurs for wine polyphenols (Lorrain et al., 2013). On the contrary, in presence of saliva it decreases for a possible action of the carboxylesterases, which would be able to hydrolyse these aroma compounds (Buettner, 2002). In all the systems, however, a lower release of volatile compounds was measured in simulated retronasal essay with respect to the orthonasal simulation assay of ROO.

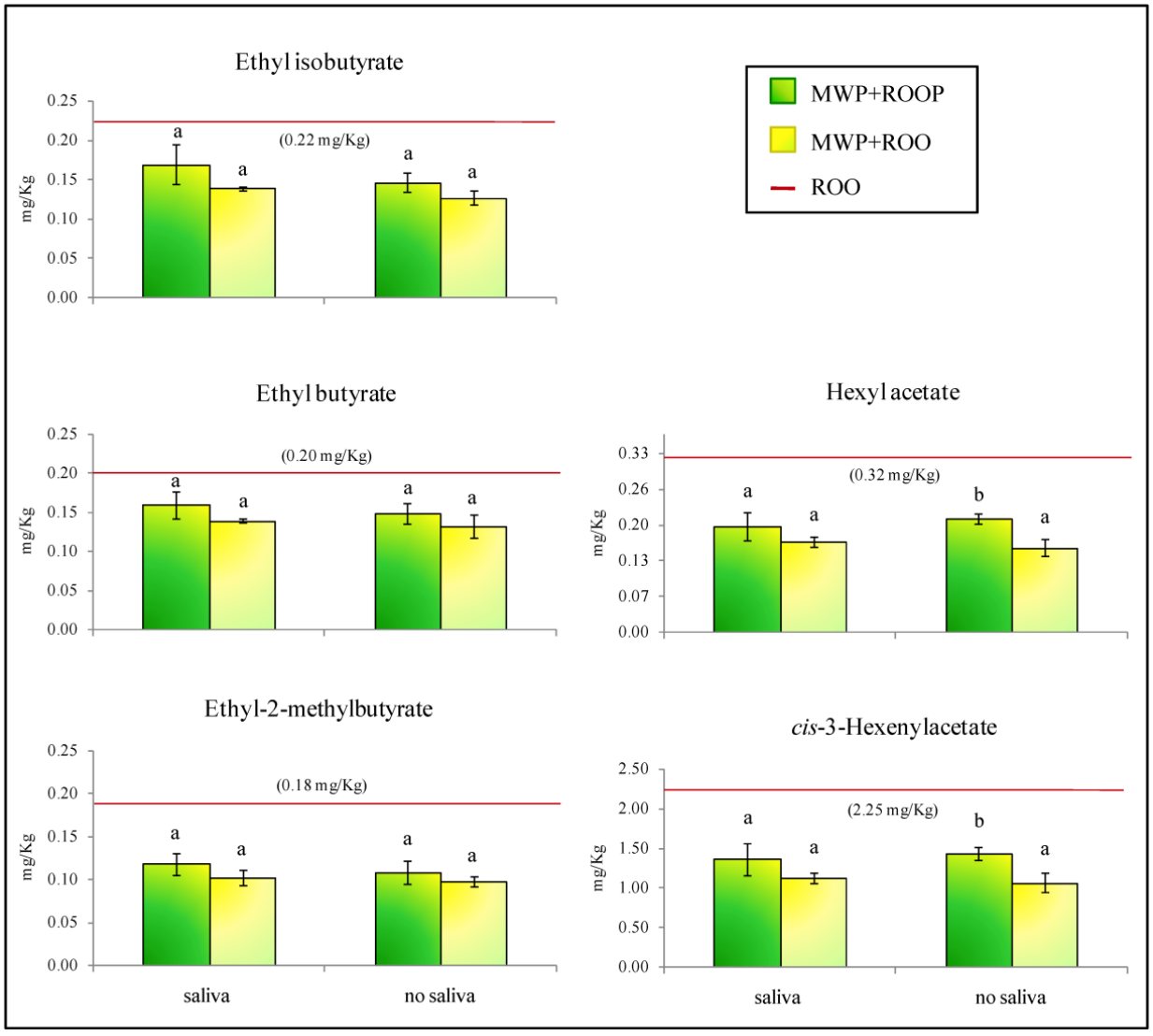

Figure 5.7. Headspace concentration of ethyl esters and acetates in refined olive oil model system after interaction with Whey Protein Isolate (WPI), in presence (ROOP) and absence (ROO) of VOO phenolic compounds and saliva. Lines indicate the level of volatile headspace release in Refined Olive Oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences (p<0.05) between ROOP and blank samples. Asterisks indicate significant differences between saliva/no saliva runs.

More interesting results were obtained for aldehydes (**Fig. 5.8**). In these systems higher release of 1-hexanol was measured in simulated retronasal essay with respect to the orthonasal simulation assay of only ROO. For hexanal, this occurs only for model systems with phenolic compounds addition. A significant effect of addition both of saliva and biophenols was observed. Particularly, saliva seems to decrease headspace release of aldehydes, while the presence of phenolic compounds seems to enhance their headspace release. For *trans*-2-hexenal, the concentration was always significantly higher when biophenols were added, but for this compound no difference was observed between distilled water and saliva, in the system with biophenols. This result strongly suggests that biophenols had a "salting out" effect, for a possible interaction between phenolic compounds and WP. In fact, in these model systems the addition of WP which are always responsible for both covalent irreversible and hydrophobic bonds with these volatile compounds (Kuhn et al., 2006) as previously discussed (3.1). When VOO biophenols were added in the systems, they weakly interacted with whey proteins (Pripp et al., 2001), with the result that the binding sites may be much less available for hydrophobic interactions with aldehydes. In the model system with saliva addition the release was lower than distilled water due to the mucin interaction (van Ruth et al., 2000; Friel and Taylor, 2001).

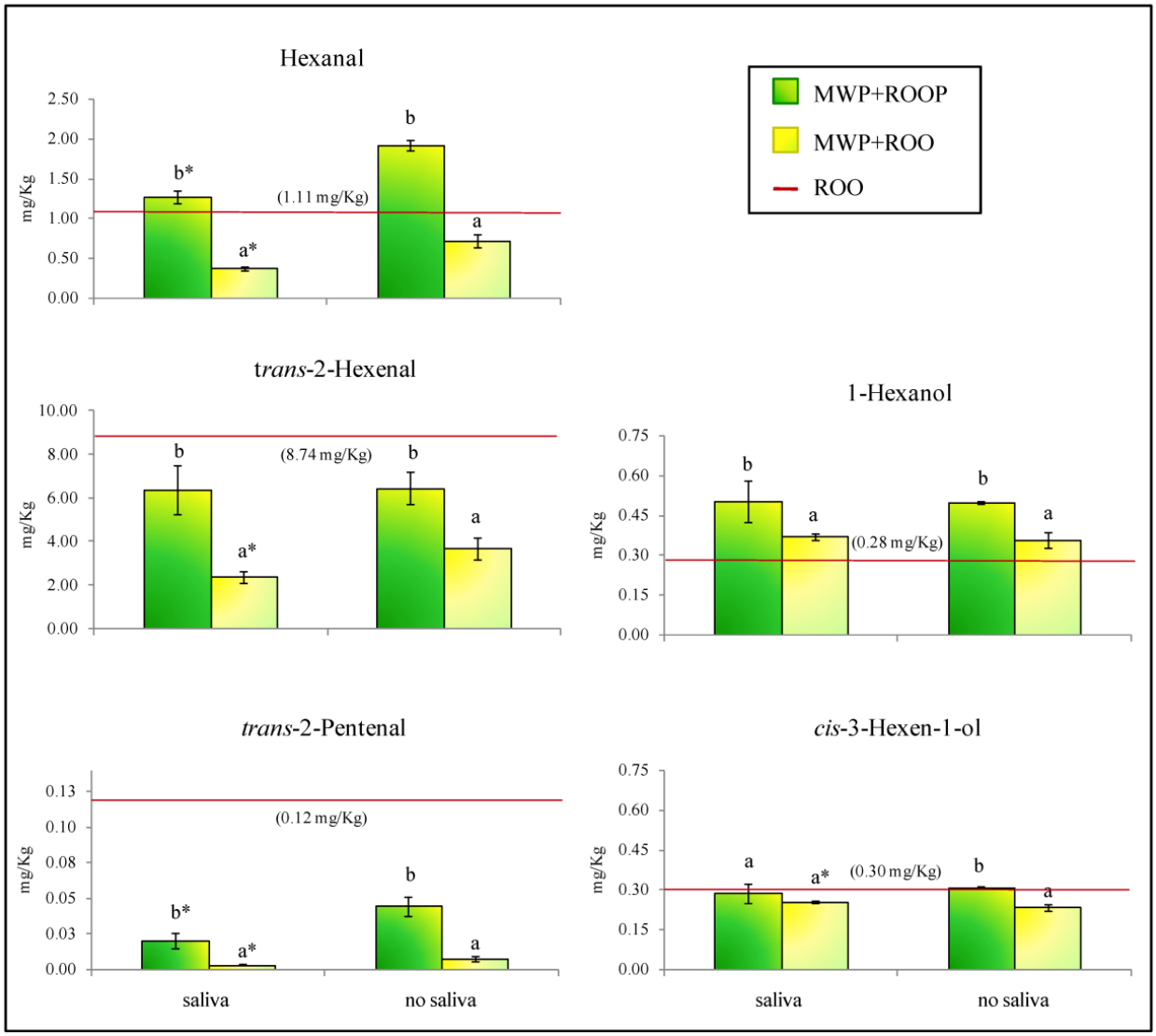

Figure 5.8. Headspace concentration of aldehydes and C6-alcohols in refined olive oil model system after interaction with Whey Protein Isolate (WPI), in presence (ROOP) and absence (ROO) of VOO phenolic compounds and saliva. Lines indicate the level of volatile headspace release in Refined Olive Oil (ROO) without interaction with WPI and saliva. Different letters indicate significant differences (p<0.05) between ROOP and blank samples (ROO). Asterisks indicate significant differences between saliva/no saliva runs.

A similar result was obtained for alcohols, which resulted in significantly higher headspace concentration in the system with phenolic compounds with no saliva addition. No difference was obtained between water and human saliva for 1-hexanol, while a significant but weak increase was observed for *cis*-3-hexen-1-ol (**Fig. 5.8**). As previously discussed, this behaviour could be due to WP-mucin interaction, which leads to significantly higher concentration of alcohols because it limits their action in the trap the alcohols, being the hydrophobic binding sites no longer available. Thus, for this class of compounds the presence of saliva seems to be of little or no extent, while the interaction with biophenols resulted to be however significant. The behaviour observed for 1-penten-3-one was similar to those reported for aldehydes and 1-hexanol, *i.e.* higher headspace release when biophenols were present, also even more than the orthonasal simulation assay of only refined olive oil (ROO without the addition of saliva and whey proteins) (**Fig. 5.9**). No significant difference was obtained between distilled water and the saliva system, due to the high variability obtained in this latter case. In general, the binding capacity of proteins increase from alcohols, to ketones and to aldehydes (Kuhn et al., 2006). For this reason, the salting out effect of VOO biophenols, inversely correlated to the binding capacity of whey proteins, resulted higher in aldehyde than ketones and alcohols. Linalool headspace concentration was affected by phenolic compounds presence only with distilled water (**Figure 5.9**). This result could be due to a possible linalool trapping during the interaction between biophenols and salivary constituents. In any case, its decrease was not due to mucin (Friel and Taylor, 2001). In order to better understand the influence of saliva and olive oil biophenols, as well as the interactions among these factors on the retronasal aroma release in olive oil, a multifactorial ANOVA analysis was carried out. **Table 5.6** shows the *F* ratio, *p* value, and the direction of the effect of the factors on the average relative recoveries of the aroma compounds. The VOO biophenols seems to have a strong positive effect on the aroma release in the case of the both acetates and alcohols, hexanal, *trans*-2-pentenal, linalool and 1-penten-3-one responsible of fruit, green, floral, and pungent odour (**Table 5.1**). Saliva showed a negative effect on hexanal, *trans*-2-pentenal, linalool and 1-penten-3-one concentrations; these compounds are responsible of green, floral, and pungent odours (**Table 5.1**). The interaction between the two factors took place only for hexanal, *trans*-2-pentenal, linalool. Therefore, our results suggest that VOO biophenols could enhance the extent of VOO aroma release of volatiles related to green, floral, and pungent notes, while saliva negatively affects this release.

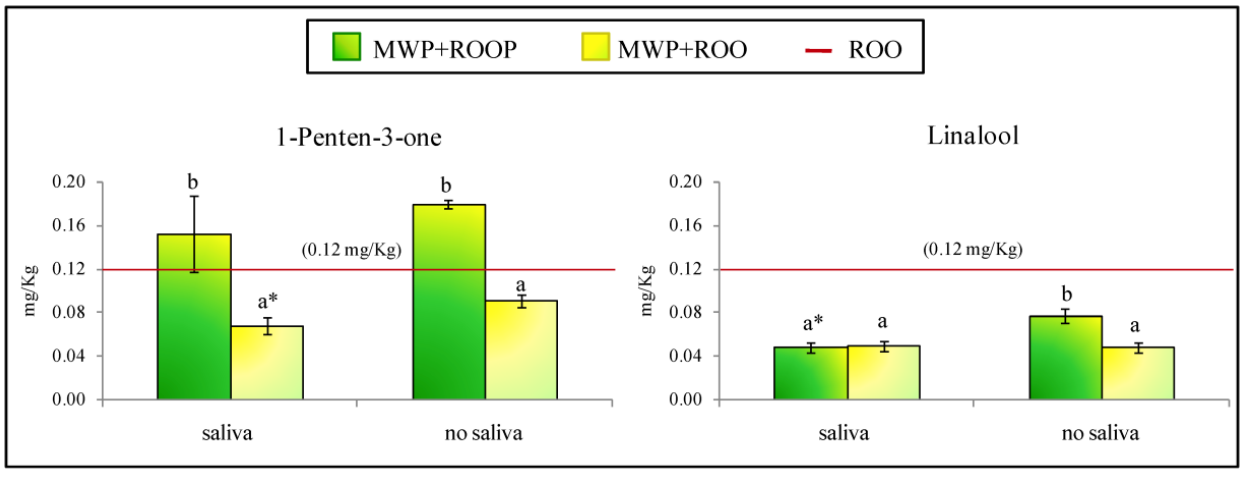

Figure 5.9. Headspace concentration of 1-penten-3-one and linalool in Refined Olive Oil model system after interaction with Whey Protein Isolate (WPI), in presence (ROOP) and absence (ROO) of VOO phenolic compounds and saliva addition. Lines indicate the level of volatile headspace release in Refined Olive Oil (ROO) without interaction with WP and saliva. Different letters indicate significant differences (p<0.05) between ROOP and blank samples (ROO). Asterisks indicate significant differences between saliva/no saliva runs.

In conclusion, our results indicated that whey proteins negatively affected the aroma release, whereas the interaction between proteins and human saliva led to a lower effect, particularly for some volatile compounds, such as acetates and alcohols. The presence of VOO biophenols positively affected the extent of aroma release, probably contrasting the binding of volatile compounds by whey proteins, for the polyphenols-proteins interaction phenomenon. Therefore, it is suggested that in the presence of higher level of biophenols, VOO would probably have higher retronasal release of green notes. Our results could be useful for the formulation of new functional foods to modulate flavour release, the consumer's acceptability, as well as to design new food products by using milk industry by-products and olive mill biophenols. In fact, being these products considered as waste and polluting substances, their use in functional foods could at least partially solve the problem of their disposal and could enhance the nutritional value of foods, for example in the case of whey protein added in beverages as dietary supplements.

Interactions of whey proteins and volatile compounds in two extra virgin olive oils

Table 5.6 shows the 34 volatile compounds identified in the headspace of two extra virgin olive oils, a filtered (F) and unfiltered (NF) samples. The concentrations of aldehydes (hexanal, trans-2-pentenale and trans-2-hexenal) and alcohols (1-hexanol and cis-3-esen-1-ol) shown similar concentrations in both samples.

The headspace release of volatile compounds in the RAS was studied in model systems with these two samples of EVOO. In both cases, no ethyl esters was detected and this absence could be due to the fact that these compounds are related to microbial fermentation and, therefore, it appear to be related to defects, absent in the EVOOs studied. In fact, during the storage and spontaneous decantation of suspended particles, especially in a non-filtered oil, a sediment may form through microbial fermentation which can give rise to undesirable compounds responsible for the characteristic flavour, i.e. muddy or sediment. High concentrations of ethyl isobutyrate and ethyl butyrate were found especially for UF, as the unfiltered oils are more easily prone to fermentation (Angerosa et al., 2004). However, in small concentrations these esters contribute to the overall EVOO flavour (Kaula et al., 2007; Angerosa et al. 2004). **Fig. 5.10** shows the concentration of acetate (hexyl acetate, cis-3-hexenyl acetate) in the headspace of the RAS device systems with filtered and (F) not filtered (NF) EVOO, with and without addition of whey proteins and human saliva. Acetates shown the same behaviour: there was no significant difference in both oil samples compared to the headspace release of these molecules. However, cis-3-hexenyl acetate showed a clear influence of WPI or saliva in the previously studied model systems, but it is no longer perceived as in extra virgin olive oils (**Fig. 5.10**).

Table 5.6. Headspace concentration of volatile compounds identified in filtered and non-filtered extra virgin olive oil (EVOO).

Compounds	Filtered EVOO	Not Filtered EVOO	F/UF (%)
Ethyl acetate	0,044 ± 0,002	0,040 ± 0,009	11
2-methyl-Butanal	0,108 ± 0,132	0,021 ± 0,009	411
3-Pentanone	0,148 ± 0,006	0,151 ± 0,015	-2
1-Penten-3-one	0,605 ± 0,057	0,616 ± 0,061	-2
Hexanal	0,894 ± 0,034	0,917 ± 0,209	-2
cis-2-Pentenal	0,023 ± 0,001	0,017 ± 0,002	42
trans-2-Pentenal	0,036 ± 0,007	0,039 ± 0,008	-7
1-Penten-3-ol	0,418 ± 0,034	0,288 ± 0,084	45
3-methyl-1-butanol	0,073 ± 0,006	0,056 ± 0,005	31
cis-2-hexenal	0,080 ± 0,009	0,080 ± 0,008	0
trans-2-hexenal	13,394 ± 0,513	13,328 ± 0,788	0
1-pentanol	0,017 ± 0,003	0,018 ± 0,001	-6
hexyl acetate	0,148 ± 0,009	0,179 ± 0,011	-17
octanal	0,046 ± 0,026	0,029 ± 0,008	63
3,3-dimetil-1,5-heptadiene	0,166 ± 0,012	0,160 ± 0,013	4
cis-2-penten-1-ol	0,053 ± 0,004	0,056 ± 0,002	-6
4-hexen-1-ol acetate	1,104 ± 0,059	1,279 ± 0,065	-14
cis-2-heptenal	0,065 ± 0,005	0,055 ± 0,005	18
6-methyl-5-hepten-2-one	0,034 ± 0,007	0,024 ± 0,001	44
1-hexanol	0,535 ± 0,022	0,564 ± 0,037	-5
cis-3-hexen-1-ol	0,015 ± 0,000	0,018 ± 0,003	-18
trans-3-hexen-1-ol	0,759 ± 0,031	0,781 ± 0,054	-3
nonanal	0,221 ± 0,099	0,209 ± 0,036	6
trans-2-hexen-1-ol	0,498 ± 0,078	0,646 ± 0,041	-23
2,4-hexadienal-(E,E)	0,011 ± 0,009	0,024 ± 0,003	-56
2,4-hexadienal-(E,E)	0,008 ± 0,007	0,021 ± 0,002	-60
1-octen-3-ol	0,014 ± 0,001	0,013 ± 0,002	8
2,4-heptadienal	0,037 ± 0,031	0,032 ± 0,002	16
benzaldehyde	0,026 ± 0,015	0,009 ± 0,008	195
1-octanol	0,022 ± 0,004	0,019 ± 0,002	17
1-nonanol	0,027 ± 0,026	0,007 ± 0,006	311
phenylethyl alcohol	0,030 ± 0,004	0,032 ± 0,005	-6

Hexanal concentration appeared to be influenced by the addition of WPI in both EVOO samples in the model system (**Fig. 5.11**). In both EVOO samples, hexanal was significantly lowered when saliva was present. For trans-2-pentenale, the headspace concentration in RAS was negatively affected by the addition of whey protein, and to a greater extent in the sample NF. This result was in contrast to what occurred in the model system. The concentration of trans-2-hexenal in sample F was positively influenced by the presence of WPI and saliva, while NF sample was negatively affected by the presence of WPI. These differences were not observed in the previous model system (**Fig. 5.6**). The concentration of hexanol (**Fig. 5.12**) did not change either in the system with filtered and non-filtered EVOOs. The only difference in the comparison between the two samples of oils was in the release of alcohol in the presence of buffer and saliva, which was higher than in the filtered oil sample. In the previous model system, this alcohol shown a major headspace release in the presence of WPI. Regarding cis-3-esen-1-ol (**Fig. 5.12**) the headspace release of alcohol in the system with filtered oil had no significant difference, while in the case of the not filtered (NF) oil the concentration was greater in the presence of whey protein. In this case, only the results of the oil NF agreed with the results obtained in the model system.

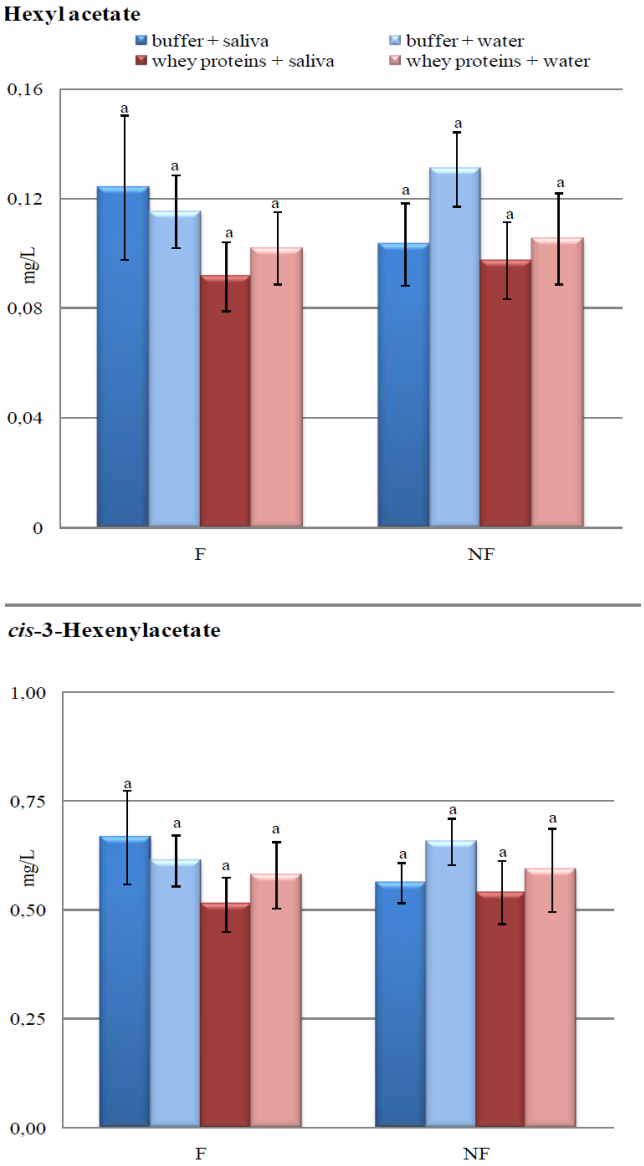

Figure 5.10. Headspace concentration of acetates in the systems with filtered (F) and non-filtered (NF) EVOO, with or without the addition of whey protein isolate. Different letters indicate statistically significant values ($p<0.05$).

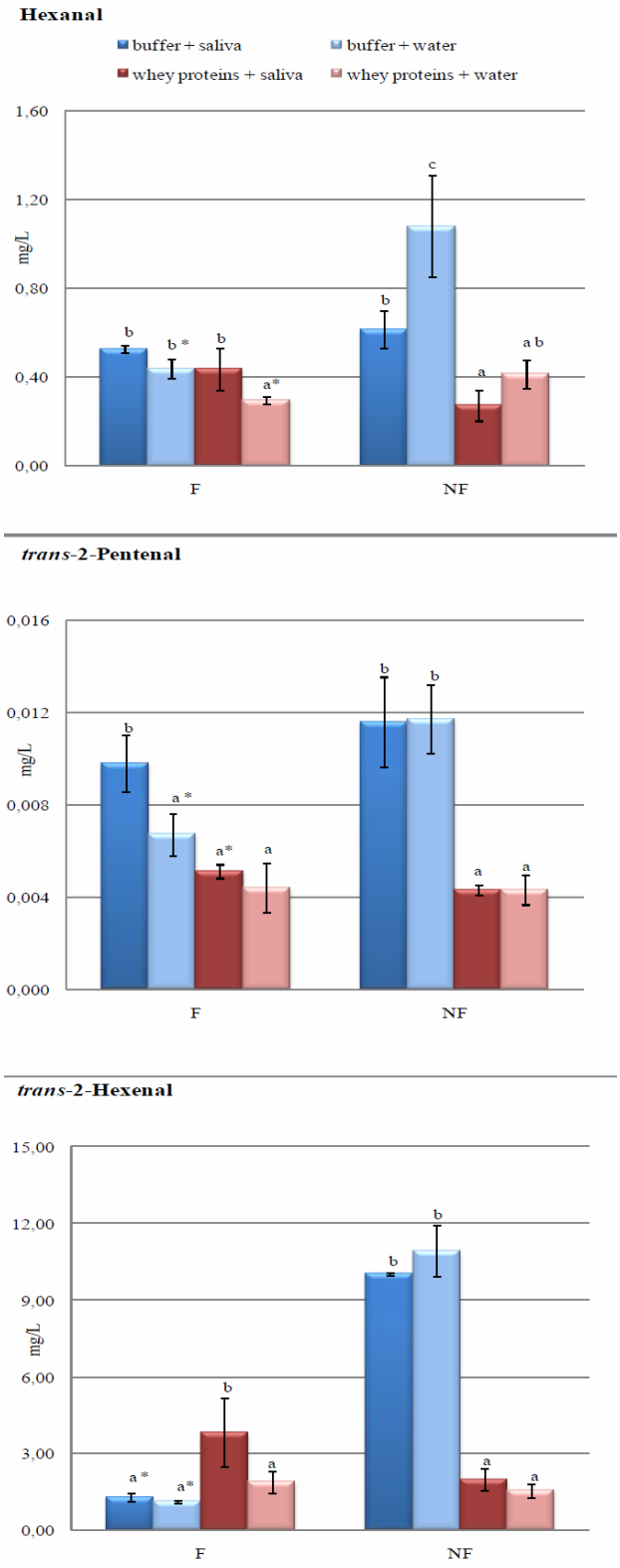

Figure 5.11. Headspace concentration of aldehydes in the systems with filtered (F) and non-filtered (NF) EVOO, with or without the addition of whey protein isolate. Different letters indicate statistically significant values ($p<0.05$).

For 1-penten-3-one (**Fig. 5.13**) is both EVOOs the concentration of the ketone was significantly lower in the presence of whey proteins, as also occurred in the previous model system. In the model system it was also highlighted a significant effect of the saliva for this compound which is not found in the real system.

The concentration of linalool in the headspace of F sample was lower in the presence of whey proteins, while in the NF sample increased (**Fig. 5.13**). NF was also negatively affected by the addition of saliva, but the overall behaviour was similar to that found in the previous model system.

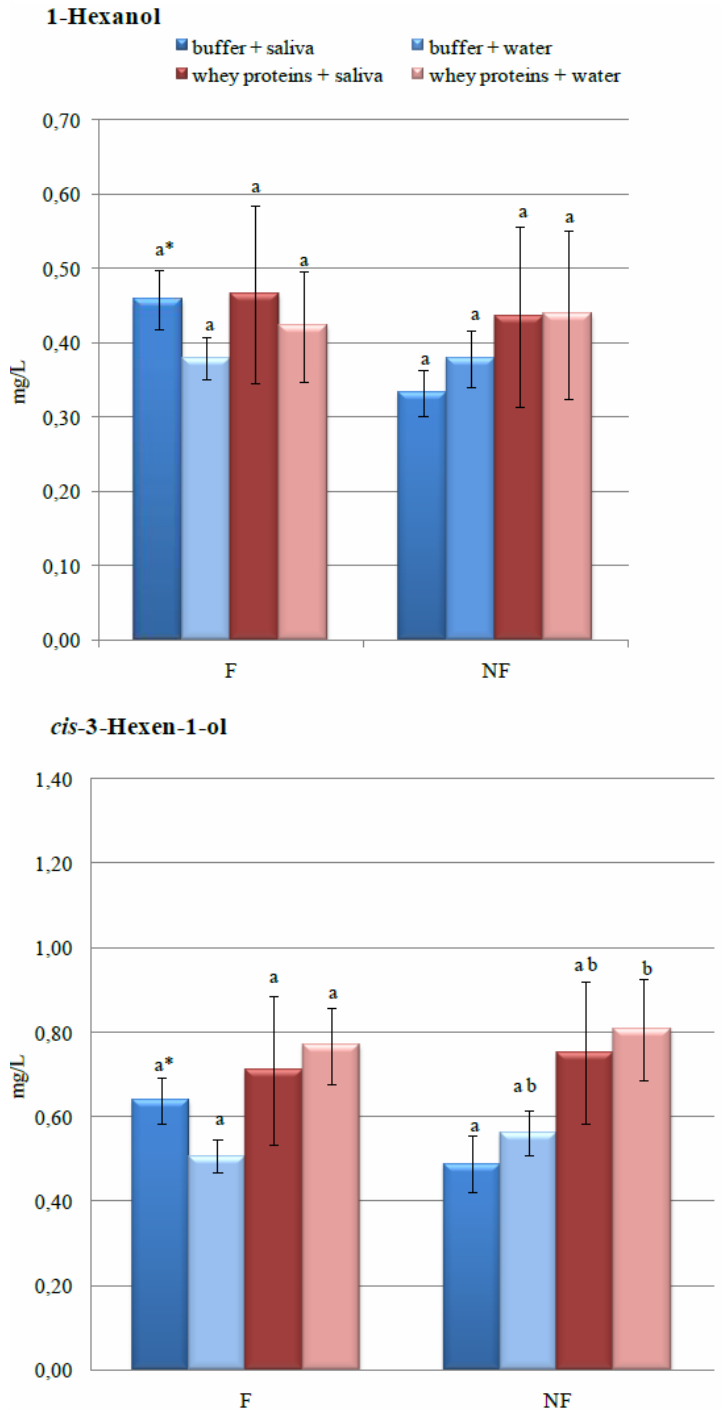

Fig. 5.12. Headspace concentration of alcohols in the systems with filtered (F) and non-filtered (NF) EVOO, with or without the addition of whey protein isolate. Different letters indicate statistically significant values ($p<0.05$).

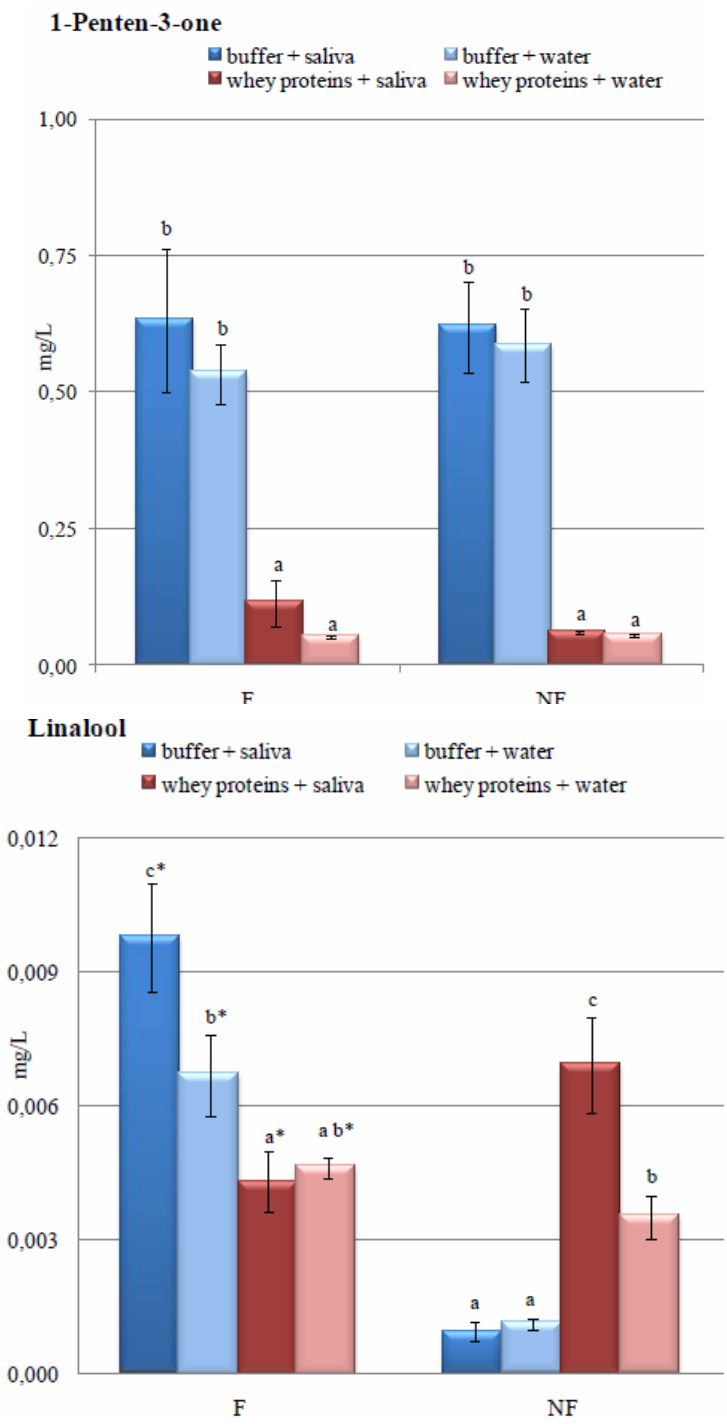

Fig. 5.13. Headspace concentration of 1-penten-3-one and linalool in the systems with filtered (F) and non-filtered (NF) EVOO, with or without the addition of whey protein isolate. Different letters indicate statistically significant values ($p<0.05$).

In conclusion, the use of a real matrix showed a different behaviour compared to a "simulated" virgin olive oil. Probably this effect is linked to the fact that the oil is a matrix consisting of more than one hundreds of compounds, which involves a greater competition in the interaction with the saliva and/or whey proteins, but also in the distribution of the different phases. A demonstration for this is also the fact that the two extra virgin olive oils, showing differences the quantity and the volatile fraction, behave in a different way. However, the filtered EVOO was generally more similar to the refined oil.

Conclusion for topic 3

In the present chapter of the dissertation, the analysis of volatile compounds of an oil-in-water emulsion was performed to verify the effect of pairing extra virgin olive oil with a milk product. A dynamic headspace technique was used to simulate the conditions of the mouth (RAS, Retronasal Aroma Simulator, i.e. a simulator of the aroma perceived by the nose).

Referring to the specific objectives of this work, the following conclusions can be drown:
1. The presence of whey proteins significantly change the overall aroma when pairing extra virgin olive oil and milk products.

2. The retro-nasal aroma release was more influenced by the presence of polyphenols. This effect could be of practical interest for its applications, since that would lead to an oil richer in polyphenols which corresponds to a more intense retronasal aroma in food preparations made with EVOO and dairy products. Further confirmations are needed in simulated systems by increasing concentrations of polyphenols, and supported by sensory analysis.

3. The use of a real matrix, i.e. EVOO, showed a different behaviour for some volatile compounds compared to a simulated system. This effect is probably related to the fact that the oil is a matrix consisting of more than one hundred volatile compounds that involves a greater competition in the interaction with the saliva and/or whey proteins, but especially in the distribution of the different phases. However, the extra virgin olive oil filtered was more similar to the refined oil.

These findings open new possibilities of interpretation on the principles governing the release of the flavour and perception during the consumption of extra virgin olive oil, therefore, may be useful for the formulation of foods with improved flavour characteristics. Indeed, whey proteins together with the polyphenols, which are mostly by- products arising from the respective processing industries, could be used for the preparation of functional foods, and help to solve, as well, the problem of disposal of such by-products for their valorisation.

General conclusions and future research needed

The present dissertation has reported on olive oil emulsions and dispersion to investigate on their stability and aroma release. In the first part, O/W emulsions were produced by using natural hydrocolloids and the phenolic extracts from olive mill wastewater, and the consequent effect on the physical and chemical emulsion stability was assessed.

In the second part of the work, the attention was put on the interaction of olive oil, phenolics and other compounds on the release of volatile compounds which are key aroma molecules from olive oil. Lastly, an in vitro device simulating the human mouth was used to investigate the retronasal aroma release in such dispersions, and using human saliva to also assess the possible interaction with salivary proteins.

The study of the physical and chemical stability of O/W emulsions in a not easy topic, especially when complex mixtures are considered. Moreover, the addition of compounds which are expected to be antioxidant does not always lead to an antioxidant system, as establishing when the surface potential of an emulsion droplet interface would be antioxidative or prooxidative is difficult to predict In addition, it is currently not clear which has more impact on emulsion oxidative stability, the presence of positive charge or the absence of negative charge and therefore more research is needed to better understand this relationship and the effect of ingredient interactions on the antioxidant or pro-oxidant effect of olive minor constituents.

The presence of phenolic compounds in virgin olive oil is one of the most important parameter affecting the sensory impact and health properties of olive oil, both with the volatile compounds and high oleic acid content. The great amount of phenolics differentiates virgin and EVOO from refined olive oil and any other vegetable oil. Moreover, the relevant of study the phenolic fraction of olive and olive oils is also related to the important aspect of olive mill by-products. There is therefore a huge stress both from the industry, institutions and scientific bodies to investigate this latter topic to find valuable solutions for the recovery and use of phenolic extracts. This might help in solving the problem of environmental pollution due to the negative effect of polyphenols on the microbial community, and phenolic extracts could be valuable ingredients for the formulation of functional foods.

Further developments are expected in the next future for this specific research topic. Indeed, there is a wide range of food products which might be created starting from emulsions or dispersions, and the effect of different hydrocolloids added to the system might dramatically change both the stability of the final product and the aroma release and perception.

Moreover, as virgin olive oil is regarded as the most precious vegetable fat, it was particularly important to study its behaviour in emulsions and the possible interactions due to the presence of other ingredients, present both as natural constituents of added for specific purposes.

The ability of “food-grade” particles to control lipid oxidation was confirmed in this study. It would be interesting to use different types of “food-grade” particles (particle size and morphology) to extend the present findings. In the present study, food-grade ingredients were used for the preparation of the emulsion systems, whereas no sensory analysis has been carried out. It would be also interesting to study different particle size in the future, by changing the homogenization during the emulsion production.

In terms of future work expected from the development of the present research topics, there are several aspects that need further investigation. For example, further research is need to assess the properties studies in the present work at different pH, more close to those of some commercial products, i.e. acidic pH. This information might be greatly useful at industrial level for practical reasons, and might also greatly affect the interactions taking place in such system due to the different charges of the molecules.

The model here presented is a starting model which can be applied on a wide variety of food products, and other stabilizers might be tested depending on the characteristics of the final products needed. Also, other approaches might be tested to retard lipid oxidation in the presence of these extracts, in particular by testing other coating materials of using a new approach based on double emulsions, in which the biophenols from olive mill wastewater have greater freedom to act as antioxidant in the product during the storage.

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Annex I

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Food & Function

PAPER

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Physical and oxidative stability of functional olive oil-in-water emulsions formulated using olive mill wastewater biophenols and whey proteins

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The present paper reports on the use of phenolic extracts from olive mill wastewater (OMW) in model olive oil-in-water (O/W) emulsions to study their effect on their physical and chemical stability. Spray-dried OMW polyphenols were added to a model 20% olive O/W emulsion stabilized with whey protein isolate (WPI) and xanthan gum, in phosphate buffer solution at pH 7. The emulsions were characterised under accelerated storage conditions (40 °C) up to 30 days. Physical stability was evaluated by analysing the creaming rate, mean particle size distribution and mean droplet size, viscosity and rheological properties, while chemical stability was assessed through the measurement of primary and secondary oxidation products. The rheological behaviour and creaming stability of the emulsions were dramatically improved by using xanthan gum, whereas the concentration of WPI and the addition of encapsulated OMW phenolics did not result in a significant improvement of physical stability. The formation of oxidation products was higher when higher concentrations of encapsulated polyphenols were used, indicating a possible binding with the WPI added in the system as a natural emulsifier. This paper might help in solving the issue of using the olive mill wastewater from olive processing in formulating functional food products with high antioxidant activity and improved health properties.

Introduction

Olive oil production technology causes major environmental problems in countries where its production is mainly localized, *i.e.* the Mediterranean area, as the industry produces a high output of liquid by-products represented by the olive mill wastewater (OMW). Due to the high concentration of phenolic compounds,[1] this waste could be conveniently converted into a valuable source of antioxidant compounds, which can be added to a variety of foods to develop a functional product with better nutritional properties.[2]

In the last few years, new technologies have been tested and applied for the extraction of phenolic compounds from OMW, particularly membrane processes which involve ultrafiltration in combination with nano-filtration and reverse osmosis.[3] The concepts behind its production by membrane separation techniques were reported by other researchers.[4,5] For their convenient storage and use, water phenolic extracts must be dried, and spray-drying has been applied to the OMW obtained from membrane filtration. The use of these phenolic extracts in emulsions is of interest at the industrial level for the production of a wide range of functional food products, such as mayonnaise, creams, sauces and other spreads.

Emulsions are kinetically unstable systems, and their instability is due to many mechanisms, including creaming, coalescence and flocculation.[6,7] Therefore, stabilizers and emulsifiers are needed to provide physical stability to avoid emulsion phase separation. Food emulsions are often multiphase systems containing more than one biopolymer, *e.g.* mixtures of proteins and polysaccharides.[8] Thickening agents are mainly polysaccharides, *e.g.* xanthan gum, maltodextrin, galactomannans, starches, pectin, carboxymethylcellulose, *etc.*, used to increase the viscosity of the continuous phase.[7,9]

Milk proteins (caseinate and whey proteins) are hydrocolloids used in many food systems, owing to their good solubility and behaviour.[10] Being surface active, whey protein isolate (WPI) is adsorbed on the oil–water interface in the form of a protective film.[11–13] Proteins are usually less effective emulsifiers than synthetic surfactants, but their use in the food industry has been increasing due to the trend to use "clean label" ingredients or "natural" products.

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Influence of Olive Oil Phenolic Compounds on Headspace Aroma Release by Interaction with Whey Proteins

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Supporting Information

ABSTRACT: The release of volatile compounds in an oil-in-water model system obtained from olive oil−whey protein (WP) pairing was investigated by considering the effect of phenolic compounds. Human saliva was used to simulate mouth conditions by retronasal aroma simulator (RAS) analysis. Twelve aroma compounds were quantified in the dynamic headspace by SPME-GC/MS. The results showed significant influences of saliva on the aroma release of virgin olive oil (VOO) volatiles also in the presence of WP. The interaction between WP and saliva leads to lower headspace release of ethyl esters and hexanal. Salivary components caused lower decrease of the release of acetates and alcohols. A lower release of volatile compounds was found in the RAS essay in comparison to that in orthonasal simulation of only refined olive oil (without addition of saliva or WP), with the exception of hexanal and 1-penten-3-one, where a significantly higher release was found. Our results suggest that the extent of retronasal odor (green, pungent) of these two volatile compounds is higher than orthonasal odor. An extra VOO was used to verify the release in model systems, indicating that WP affected aroma release more than model systems, while saliva seems to exert an opposite trend. A significant increase in aroma release was found when phenolic compounds were added to the system, probably due to the contrasting effects of binding of volatile compounds caused by WP, for the polyphenol−protein interaction phenomenon. Our study could be applied to the formulation of new functional foods to enhance flavor release and modulate the presence and concentrations of phenolics and whey proteins in food emulsions/dispersions.

KEYWORDS: *phenolic−aroma interaction, volatile compounds, SPME-GC/MS, aroma release, human saliva, RAS*

INTRODUCTION

Olive oil is a staple in the Mediterranean diet, considered as one of the most health-promoting nutritional habits worldwide. It is characterized by a high intake of monounsaturated fatty acids, which have showed to help in lowering cholesterol and heart disease.[1] In comparison to seed oils, virgin olive oil (VOO) has a peculiar flavor and biophenol-rich polar fraction which gives rise to other benefits to human health, due to the important nutritional role played by the high percentage of oleic acid.[2,3] Therefore, an increase of VOO biophenol consumption, without an increase in fat intake, should be recommended by health authorities in order to encourage the general population to use it. The delicate flavor of VOO is first perceived during inhalation, when the odorants are released into the headspace; they pass through the external nostrils and stimulate the olfactory receptors in the nasal cavity (nasal route). Then the aroma is perceived while tasting VOO, when the odorants interact with the receptors by migrating from the mouth to the nasal cavity via the nasopharynx (retronasal route). Simultaneously other sensations take place: e.g., bitterness, sweetness, astringency, and pungency. VOO phenolic compounds are responsible for the astringency and pungency perceptions; the former sensation acts by stimulating taste receptors, while the latter is perceived by the trigeminal nerve. On the other hand, volatile compounds are responsible for the odor and aroma by stimulating the olfactory receptors.[4,5]

VOO is mainly employed in Mediterranean countries in many food preparations or as an ingredient in salad dressings. Generally, there are two distinct ways to pair VOO with food: complementary and contrasting approaches. A complementary flavor is obtained when two similar ingredients are blended. This results in the enhancement of primary flavors, while a contrasting approach consists in tasting each ingredient separately. Pairings of VOO with salads, vegetables, pesto, tomato sauces, etc. are examples of the first case. The latter example could be the case of pairing VOO and fresh mozzarella cheese in the famous and delicious "*insalata Caprese*." In fact, the unique flavor of a "strong" VOO (bitter, pungent, and fruity) is in contrast with the delicate texture and taste of fresh mozzarella (sweet, acid, and milk note). Frequently, ricotta cheese is also used as an ingredient in many recipes with VOO to obtain a sweetening effect of bitter-pungent notes in strong VOOs. It is not surprising that renowned chefs pair VOO with many different foods, in order to obtain new sensations and create new culinary experiences. When VOO is combined with dairy products, an oil-in-water emulsion or dispersion is produced. The presence of different phases and the level of volatile compounds affect their partitioning. In fact, lipophilic aroma compounds tend to move in the oil phase and their concentration considerably decreases in the continuous phase. In contrast, hydrophilic compounds tend to move in the aqueous phase.[6] In addition, nonvolatile matrix components, such as whey proteins, affect aroma release by interacting with aroma compounds.[7−9] Furthermore, it must be considered that

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Annex II

Preliminary experiments for the definition of emulsions conditions

Preliminary results for emulsion ingredient definition and operative conditions

The use of model systems has been applied to understand the characteristics and interactions of many food systems, including emulsions. The most common approach is to study one variable per time, however this does not allow to monitor the influence of the interactive effects of many variables.

The selection of emulsion ingredients and their preliminary experimental ranges were based on literature research for vegetable oils (Mirhosseini et al., 2008b; Comas et al., 2006) and xanthan gum (Mirhosseini et al., 2008a: Sun et al., 2007; Hemar et al., 2001). Furthermore, the concentrations of olive biophenols extract was based on the calculation of medium-high levels of phenolics on EVOOs, also considering the actual concentration of phenolic compounds in the spray-dried powder. Therefore, the final concentration of biophenols in the model emulsions was similar to the levels found in EVOO.

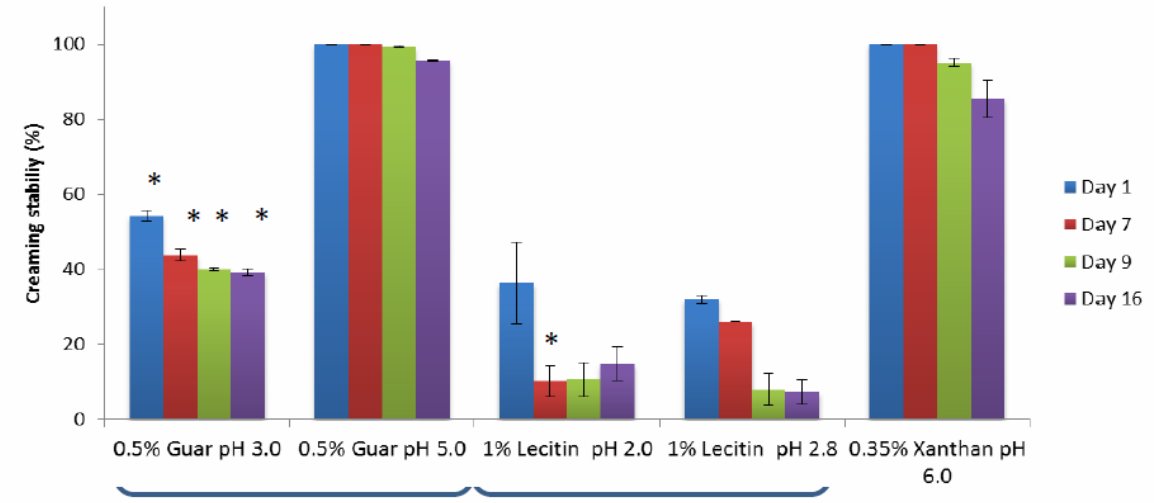


Figure II.1. Creaming stability of 20% O/W emulsions by changing the type of stabilising agent and pH of the system, over storage at 20 °C. Several hydrocolloids and stabilizers were assessed in preliminary experiments, and xanthan gum was eventually used for further experiments. Asterisks indicate statistically significant differences between the same hydrocolloid group ($p<0.05$).

As shown in **Fig. II.1**, the stability toward emulsion physical separation (creaming stability) in O/W emulsions was affected by the different hydrocolloids used as emulsion stabilisers, as expected. Depending on the type of stabiliser used, i.e. guar gum, lecithin and xanthan gum. These emulsions were formulated only with the lipid and water phase and the hydrocolloid tested, and stored at room temperature for the evaluation. Two different pH conditions were also tested to check their effect on creaming stability, and this parameter resulted to be significant for some hydrocolloids only, specifically for guar gum. In this case, the lowering in pH caused a reduction in creaming stability of almost half of the compared stability. On the contrary, a statistically significant difference was only observed at 1 week storage for lecithin-stabilised emulsions.

Sixteen days of storage at room temperature were able to significantly affect the creaming rate in all the studied systems, whereas it should be considered that the concentrations used were in the upper portion of the concentrations commonly reported in literature.

Overall, the highest stability was observed for guar gum at pH 5.0 and for xanthan gum. Being xanthan gum able to retard cream separation at a lower concentration than guar gum, and also considering its positive properties over guar gum (for which gelation is the main mode for controlling emulsion stability), this hydrocolloid was used for the formulation of the final emulsions, as described in **Chapter 3**.

Rheological measurements made on O/W model emulsions

20% O/W emulsions stabilized by WPI

The effect of using increasing concentrations of WPI on O/W emulsions on the physical properties was assessed by rheological measurements. Rheological tests were performed using a cone-plate geometry (CP 40/4ºC) over a shear rate of 0.1-100 s^{-1} at 25±0.1ºC.

As shown in **Fig. II.2**, no change in shear rate was obtained changing the WPI concentration in the range 0.5%-2%.

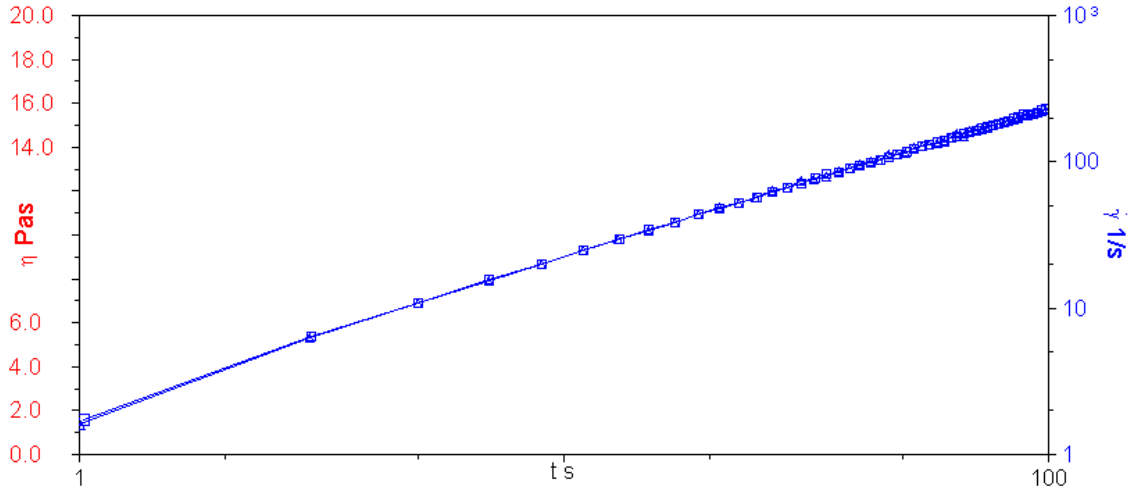


Figure II.2 Shear rate of WPI-stabilized 20% olive oil-in-water emulsions (0.5%, 1% and 2%), as function of time of analysis (1-100 s).

From the analysis of these emulsions, a significant higher viscosity was recorder for 0.5% WPI respect to the other samples, as shown in **Fig. II.3**.

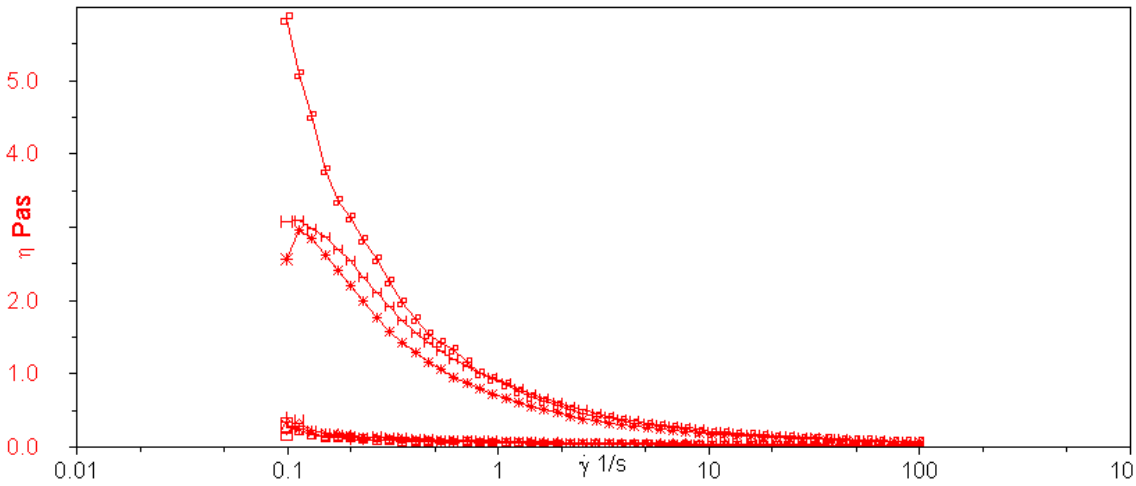


Figure II.3. Viscosity as function of share rate (0.1-100 1/s) of WPI-stabilized 20% olive O/W emulsions. Each curve for the 3 higher values represent replicates for emulsions added with 0.5% WPI.

20% O/W emulsions stabilized by WPI and 0.1% of Xanthan gum

Emulsions at different levels of WPI (5%, 10% and 5% thermally treated at 60 ºC for 30 min) were characterized under oscillatory test (**Fig. II.4**). The addition of 0.1% Xanthan gum is justified by the need of a stabilizer agent together with the WPI that were been described as emulsifiers agents only, being their sole presence inappropriate for a stabile O/W emulsions. Xanthan gum and Arabic gum were also widely applied in emulsions and in particular in salad dressing emulsions with low pH value (Paraskevupoulou et al., 2005).

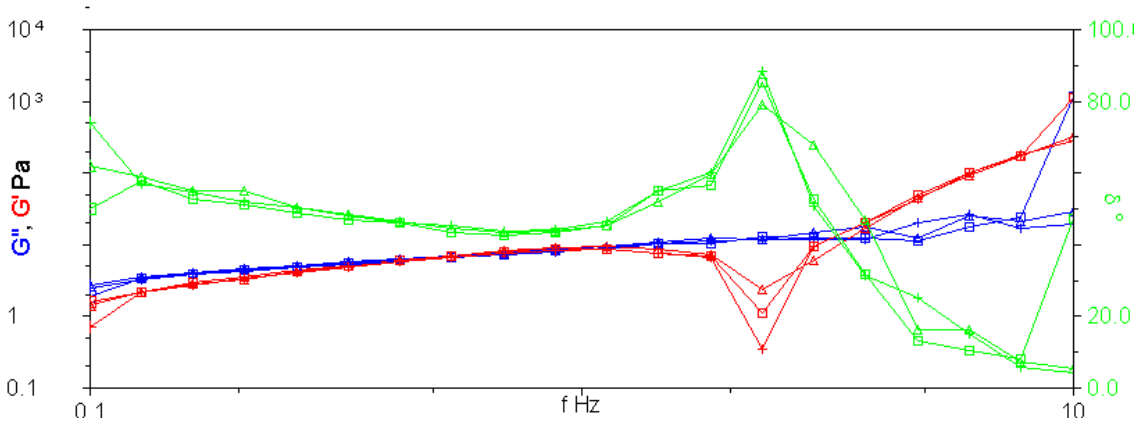


Figure II.4. Viscous moduli (G''), elastic moduli (G') and phase angle (right axis) of 5% WPI-stabilized 20% O/W emulsion with the addition of 0.1% of xanthan gum.

In **Fig. II.5** the oscillatory measurements were reported for different concentrations of WPI O/W emulsions. Thermal treatment of whey protein was briefly tested by heating the solutions to check its effects on emulsion behavior. The thermal treatment caused a significant lower elastic modulus (G') around 2.3 Hz, and a higher phase angle in the same frequency. Higher protein concentration caused both slightly higher elastic and viscous moduli. However, the thermal treatment was not considered as a variable for the formulation of the final O/W emulsions due to the other issues related to heating and consequent gelation which involves further mechanisms.

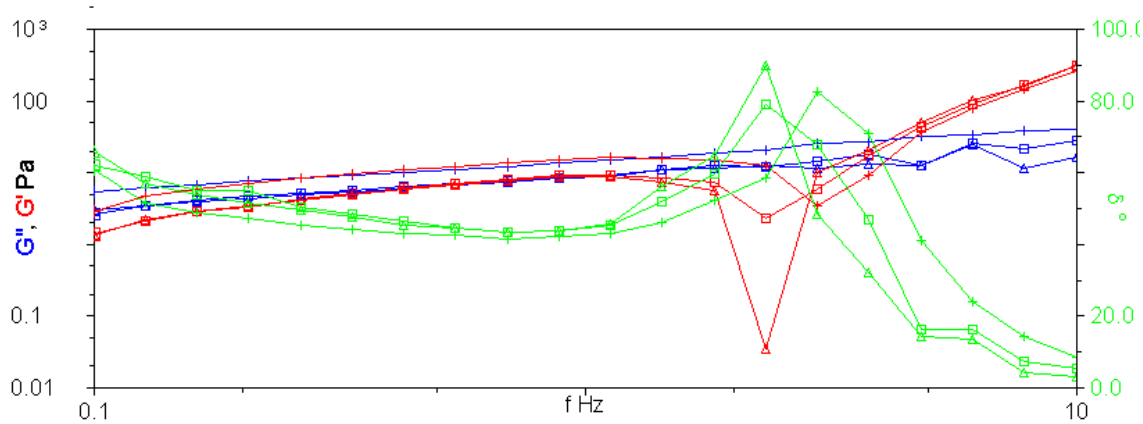


Figure II.5. Oscillatory test for 20% O/W emulsions stabilized by addition of 5% WPI (square), 5% thermally treated WPI (triangle) and 10% WPI (+).

A plane 40 mm plate was used for the viscosity analysis of a mayonnaise-like 20% olive oil-in-water emulsion. The emulsion was composed by the addition of 1.5% xanthan gum and 1% Guar gum (Silva et al., 2010). The pH (2.55 and 4) was changed to verify its effect on physical stability (creaming value) and rheological parameters. These pH values represent the

range of pH for most food emulsions and salad dressing, e.g. made from the addition of lemon juice or wine vinegar, these two presenting a pH of circa 2.5 and 4.5 respectively.

Yield stress test performed from 0 to 60 s was reported in **Fig.II.6**. In **Fig.II.7** the viscosity was reported in function of the shear rate. No significant differences were found as effect of the different pH. The shear rate values were reported in **Table II.1** for the samples used in the preliminary experiments.

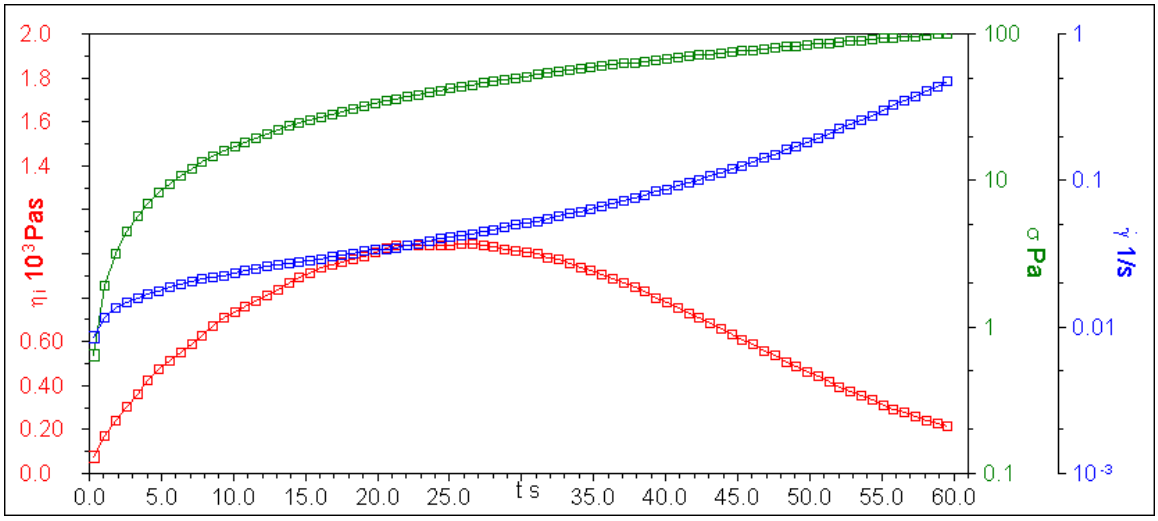


Figure II.6. Yield stress test of 20% O/W emulsion stabilized by 1.5% xanthan gum and 1.0% guar gum.

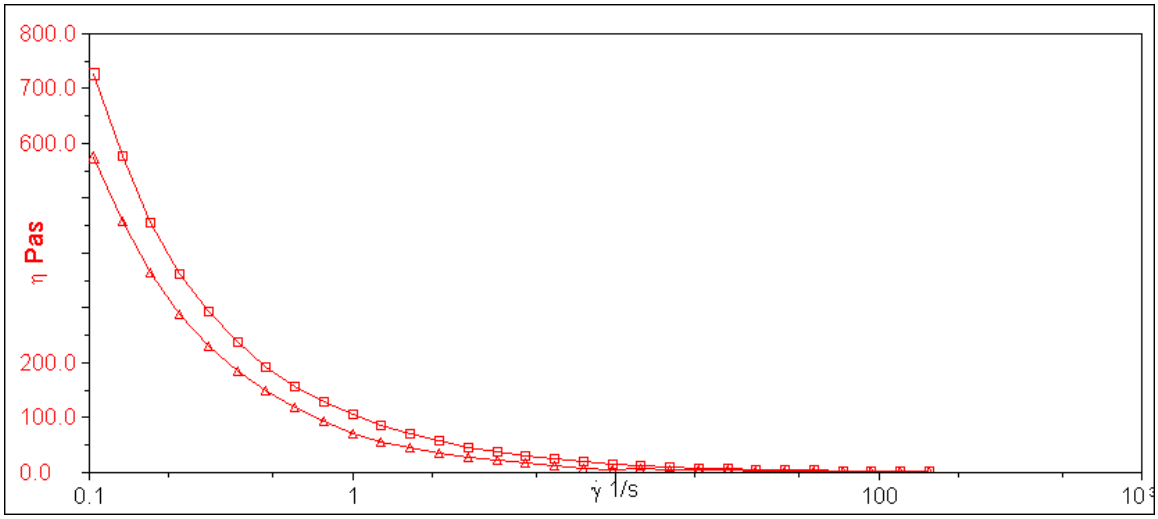


Figure II.7. Shear rate of model 20% O/W emulsions stabilized by 1.5% xanthan gum and 1.0% guar gum at different pH values (square: 2.5, triangle: 4.0).

Table II.1. Rheological measurements of 20% O/W emulsions stabilized by using 1.5% xanthan gum and 1.0% guar gum. Mean values of replicate measurements.

	Time s	**Temperature** °C	**Shear Rate** 1 s^{-1}	**Shear Stress** Pa	**Viscosity** Pa s^{-1}	**Strain**
1, 1	60.01	25	0.1463	40.23	275.1	23.36
1, 2	180	25	22.63	60.22	2.661	3716
1, 3	300	25	156.4	83.11	0.5314	2.258E4
1, 4	420.1	25	156.4	91.03	0.582	4.144E4
1, 5	540.1	25	21.54	54.42	2.526	4.515E4
1, 6	660.1	25	0.139	22.68	163.2	4.517E4

Assessing the effect of polyphenols and proteins on the stability and rheological parameters of 20% O/W emulsions

20% Olive oil-in-water emulsions were prepared by adding to the aqueous phase 1% WPI as emulsifier and 0.2% Xanthan gum as stabilizer. The effect of polyphenol addition was evaluated by adding 1 and 2% of olive mill wastewater polyphenol previously extracted by a membrane process and spray-dried using Maltodextrin as coating agent. The polyphenols concentration in the powder was about 10%.

The emulsions were produced as described in the previous sections for other emulsions.

1.5 mL of emulsion were placed on the steel plate and a thin layer of silicone oil was added to avoid evaporation. A 40/4 cone-plate was used as the viscosity was > 1 Pas and < 100 Pas. An isotherm of 25 ºC was used.

A C-VOR 150 rheometer was used for the rheological analysis, as described in the following sections. A controlled-shear rate was applied from 0.1 to 10 Pa to assess the effect on viscosity.

Evaluating the interaction between polyphenol and proteins in a O/W emulsion stabilized by Xanthan gum

Spray-dried olive mill wastewater extract coated by maltodextrin was added to a 20% and 50% O/W emulsion stabilized by 0.2 and 0.5 % of whey protein isolate (WPI), respectively. 0.2 % of Xanthan gum was added as stabilizer in all the formulations.

The emulsions were prepared as following.

Polyphenols were added to the aqueous phase in order to obtain a polyphenol concentration similar to that found in high quality extra virgin olive oil (range 200-400 ppm). WPI was added as 10% respect to the oil phase, e.g. in 20% O/W emulsion, for a total volume of 100 mL, the amount of added WPI was 0.2 g.

Proteins and polyphenols were allowed to hydrate using a magnetic stirrer for about one hour. Then, 0.2% Xanthan gum was added to all the studied systems and the solution was gently stirred for 2 hours. The aqueous phase was used the subsequent day to allow fully hydration of xanthan gum.

One trial was done by using 40% O/W emulsions, 0.2% WPI, 0.2% polyphenols and 0.2% xanthan gum. Oscillatory test was performed according to Ercelebi and Ibanoglu, 2009. In particular, a stress sweep test (1 Hz at 25 C) was adopted, using a stress value of 1 Pa for all tests. The frequency range was 0.001-10 Hz.

In **Fig. II.8** an example is reported, for the 40% and 20% O/W emulsions previously described.

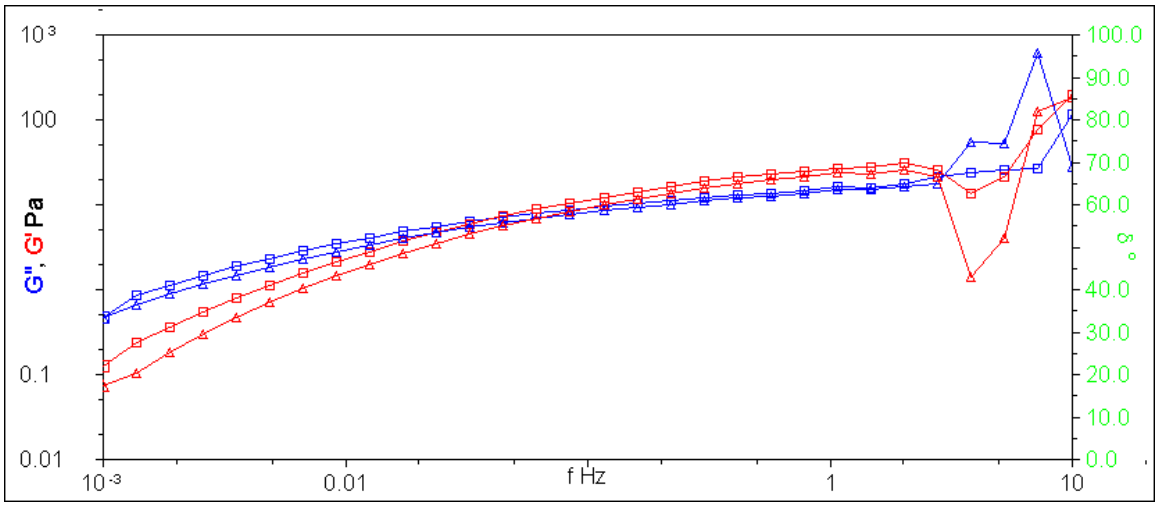


Figure II.8. Oscillatory test for 40% (triangle) and 20% (square) O/W emulsions prepared with the addition of 0.2% of olive mill wastewater polyphenol extract and stabilized by 0.2% WPI and 0.2% xanthan gum.

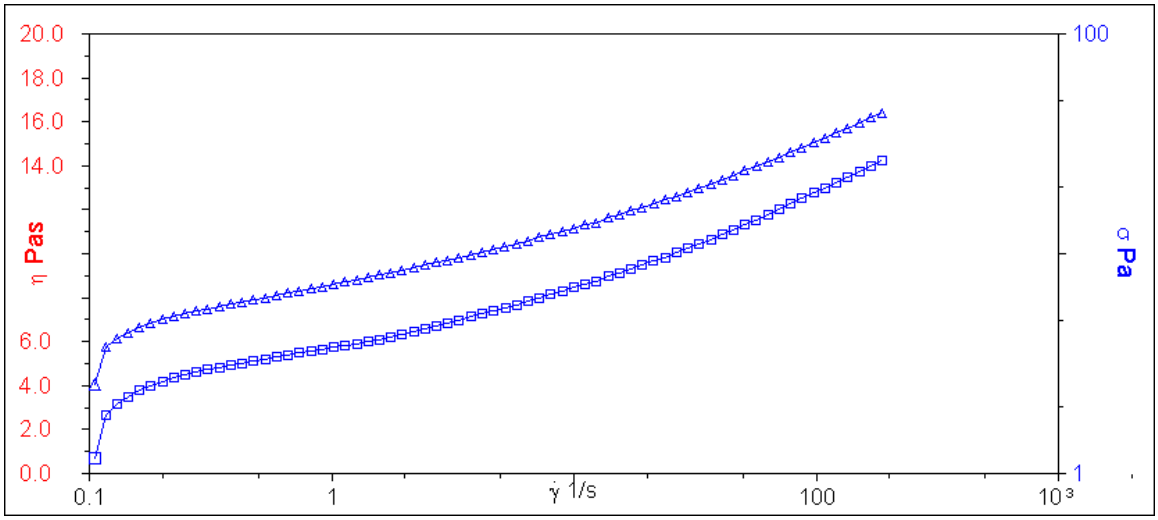


Figure II.9. Apparent viscosity of O/W emulsions made with only added OMW polyphenols, at 0.2 and 0.4% polyphenol concentration (square and triangle, respectively).

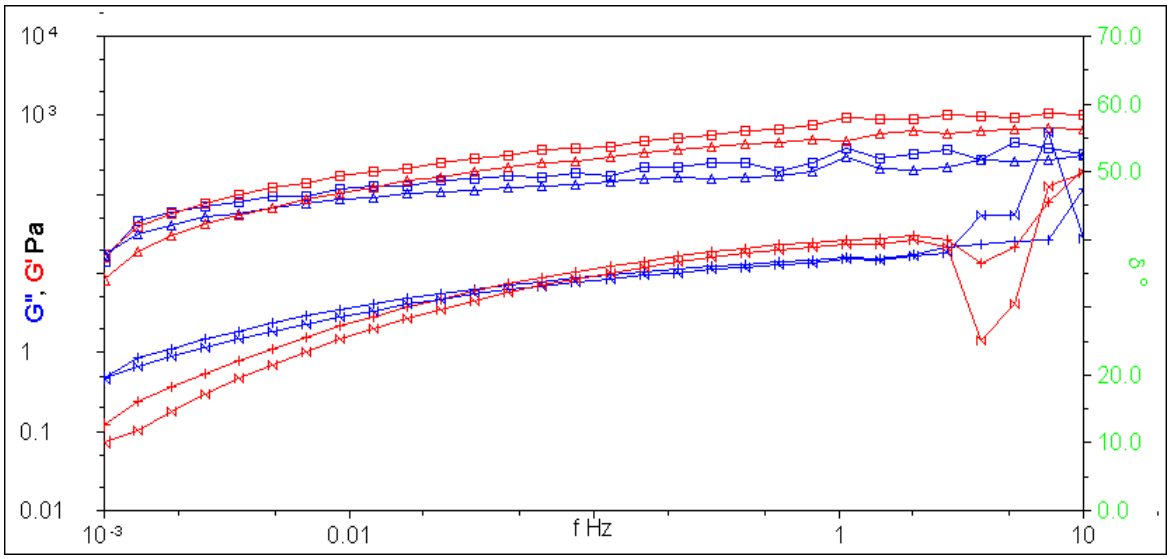


Figure II.10. Thixotropic measurements of O/W emulsions. **Square**: 50%O/W, 0.5WPI, 0.4 polyphenols. **Triangle**: 50%O/W, 0.5WPI, 0.2 polyphenols. +: 20%O/W, 0.2WPI, 0.2 polyphenols. **Z**: 40%O/W, 0.2WPI, 0.2 polyphenols

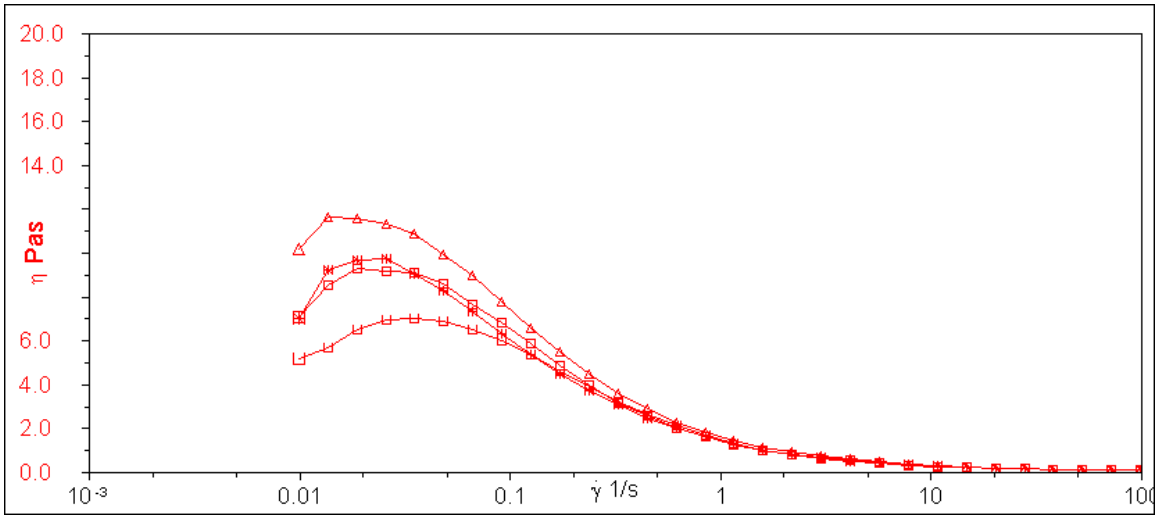


Figure II.11. Measurement of Viscosity at 25 ºC. Replicates of the same sample of 20% O/W emulsion stabilized by 0.2% WPI, 0.2% polyphenols and 0.2% xanthan gum. (Date of emulsification: 26 June. Date of analysis: 28 June).

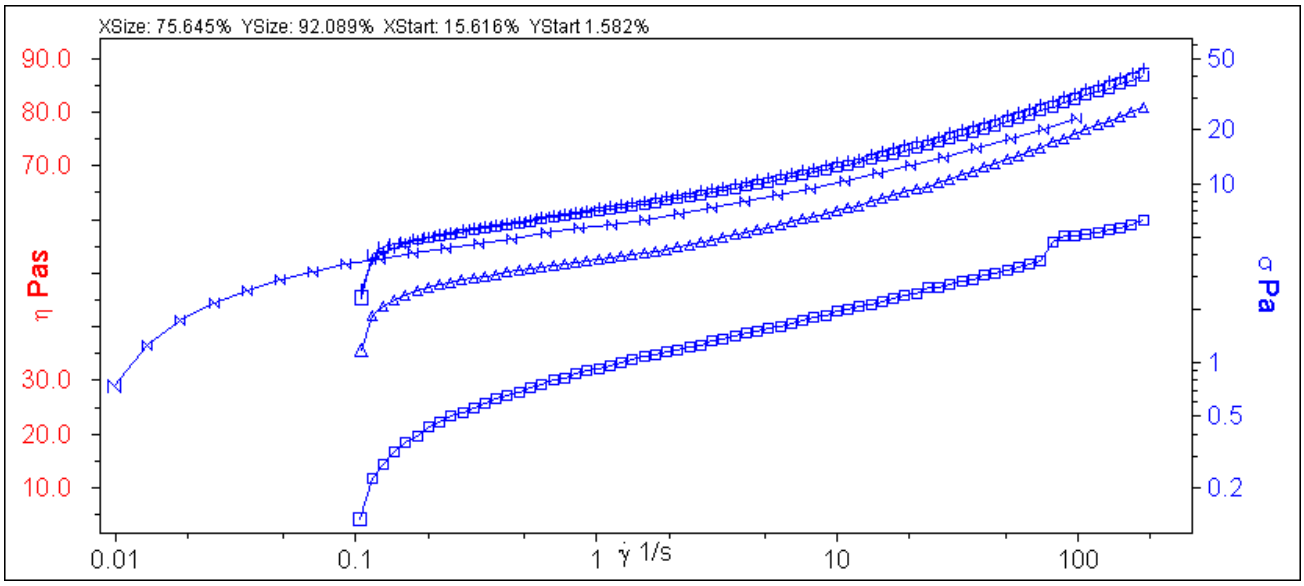
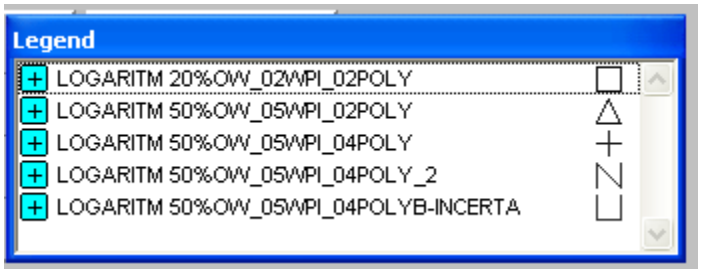


Figure II.12. Apparent viscosity, measured on a shear ramp, at controlled rate (0.01-100 s^{-1}).



Characterisation of OMW phenolic extract used in the experiments

Different fractions can be obtained using membrane purification systems from olive mill wastewater (OMW), as shown in **Fig. II.13**. The phenolic extracts used in the present research were obtained from the ultrafiltration retentate, whereas also those obtained from nanofiltration retentate to check its performances. The water extract was spray-dried by the LABS group (Department of Agriculture, University of Naples Federico II). Similar extracts were used for other research experiments, e.g. by Troise et al. (2014) who investigated their effects on the Maillard reaction in heated milk.

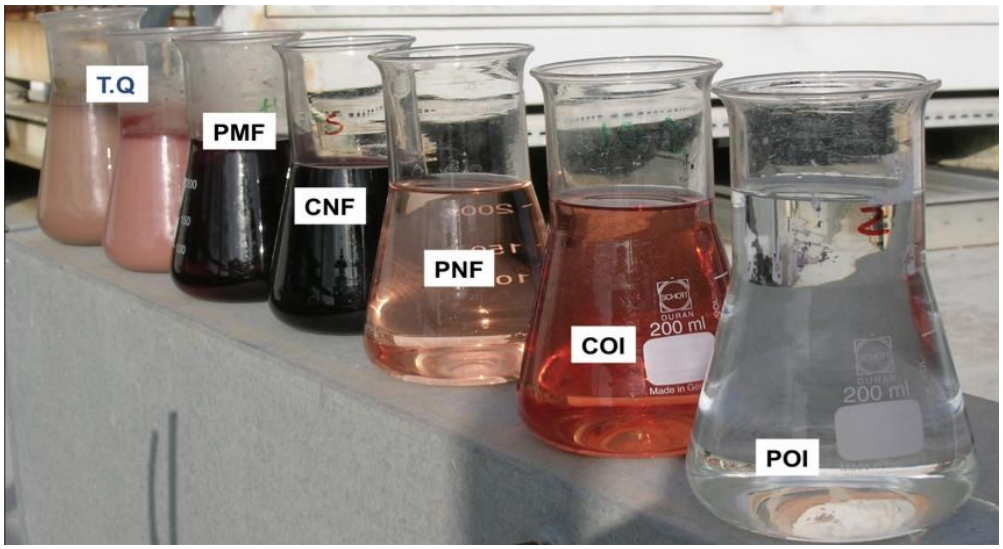


Figure II.13. Different liquid fractions separated from olive mill wastewater using membrane processing technologies to obtain spray-dried polyphenols used in the present study (from Vitagliano, 2013).

The spray-drying was done by using several coating materials, which are necessary for the encapsulation of the polyphenols. These included α-cyclodextrins, β-cyclodextrins and maltodextrins, and different concentration of phenolic extract to coating materials were tested to obtain the final powders.

The chromatograms obtained by HPLC-DAD to characterise the phenolic extracts are reported in **Fig. II.14**.

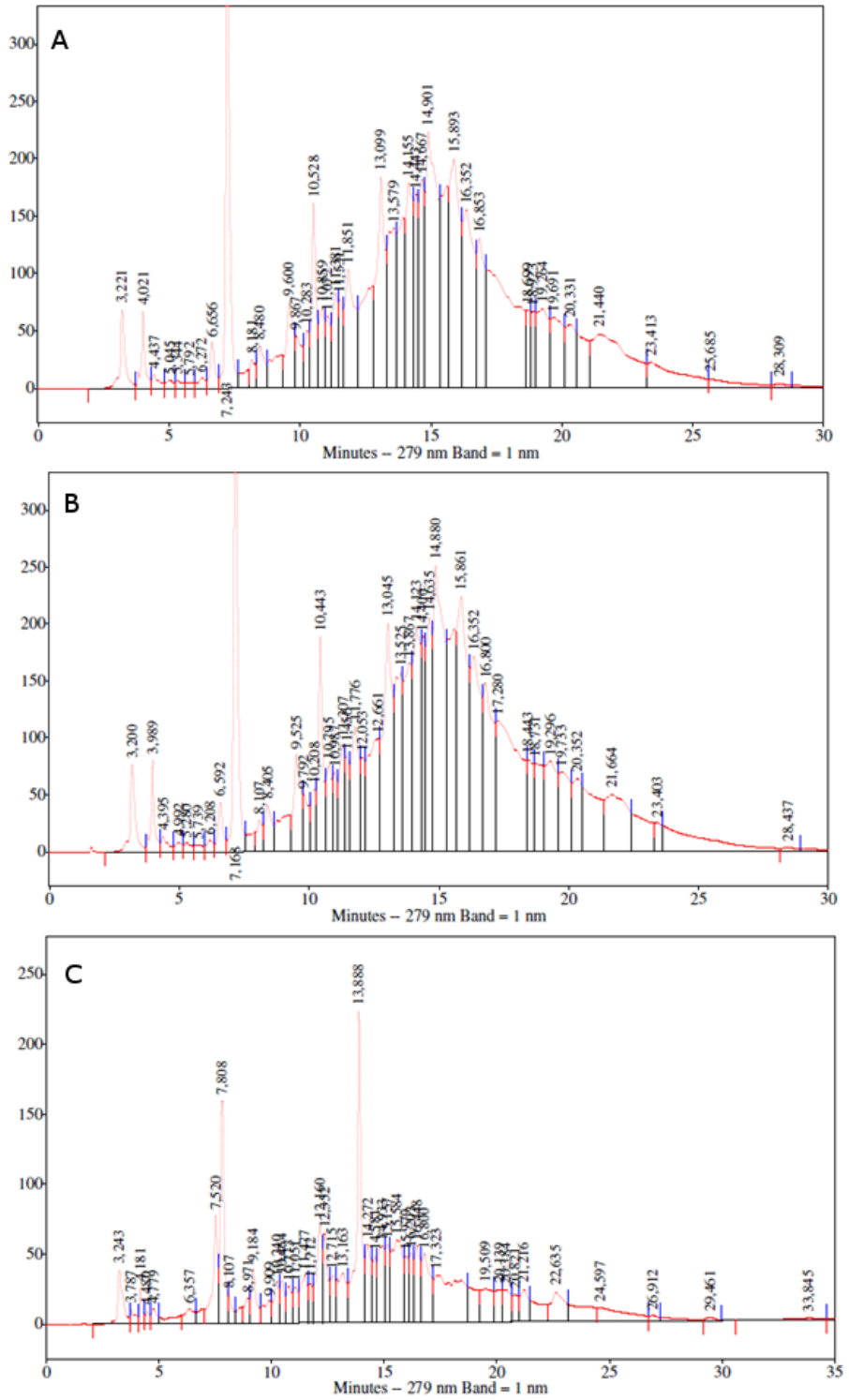


Figure II.14. Chromatograms by HPLC-DAD of phenolic compounds from OMW obtained from ultrafiltration retentate (A and B) or nanofiltration retentate (C), coated using β-cyclodextrins (A), maltodextrins (B) and α-cyclodextrins (C).

The concentration of total phenolic compounds analysed by HPLC was 22 and 23% of the total weight for the first two phenolic powders, respectively, assuming the sum of the total

chromatographic peaks as phenolics. On the contrary, the concentration of total phenolics in the third powder was about 6%.

These results were obtained by dissolving the powder extracts extracts (40 mg) in a 2 mL mixture of distilled water and methanol (1:1 v/v), and then filtered on filtering paper.

High molecular weight compounds (>500 Da) are retained by NF while short molecules (< 200 Da) can pass in the permeate stream (orthodiphenols and derivatives) the RO has to be considered as a concentrated form of NF permeate cause only water is able to permeate in RO filtration (Pizzichini and Russo, 2005).

HPLC analysis and chromatographic profiling of NF and RO retentate confirmed the performance of phenolic molecules separation: high molecular weight compounds (>500 Da) are retained by NF while short molecules (< 200 Da) can pass in the permeate stream (orthodiphenols and derivatives) the RO has to be considered as a concentrated form of NF permeate cause only water is able to permeate in RO filtration.

Effect of storage conditions on O/W emulsions chemical stability

Fig. II.15 shows the overall picture of the 17 runs (+1 blank without phenolic compounds) of 20% olive O/W emulsions formulated with different concentrations of OMW biophenol extracts, WPI and xanthan gum. The emulsions were stored under accelerated oxidizing conditions, i.e. 40 °C over 3 weeks, and analysed at longer exposure times as better reported in **Chapter 3**.

The samples shown result from the Response Surface Methodology model, and for each run a precise concentration of the factors was applied. The general plot of TBARS, i.e. secondary oxidation products, shows a dramatic increase up to the first week of storage, with obvious differences between the samples (more than three-fold differences), followed by a slight decrease up to the end of the storage period, with the exception of some samples for which a slight further increase was observed, probably due to further decomposition of primary oxidation products.

In terms of primary oxidation products, i.e. lipid hydroperoxides formation, the trend was similar and a plateau was reached after the second week of storage, with no further dramatic increase or decrease. It was also noted that the difference between samples (runs) was less dramatic than those observed for TBARS, in fact the maximum peak (at 1 week storage) shown a maximum variation in hydroperoxide concentration of circa two-folds.

The results are in agreement with previous research papers (Di Mattia et al., 2009; Di Mattia et al., 2010) who investigated the lipid oxidation of emulsions in presence of added polyphenols. However, from the samples a clear difference is not shown depending only from the presence of polyphenols as due to the effect of the hydrocolloids used in the formulation. Further discussion and data on this aspects were added in the **Chapter 3**.

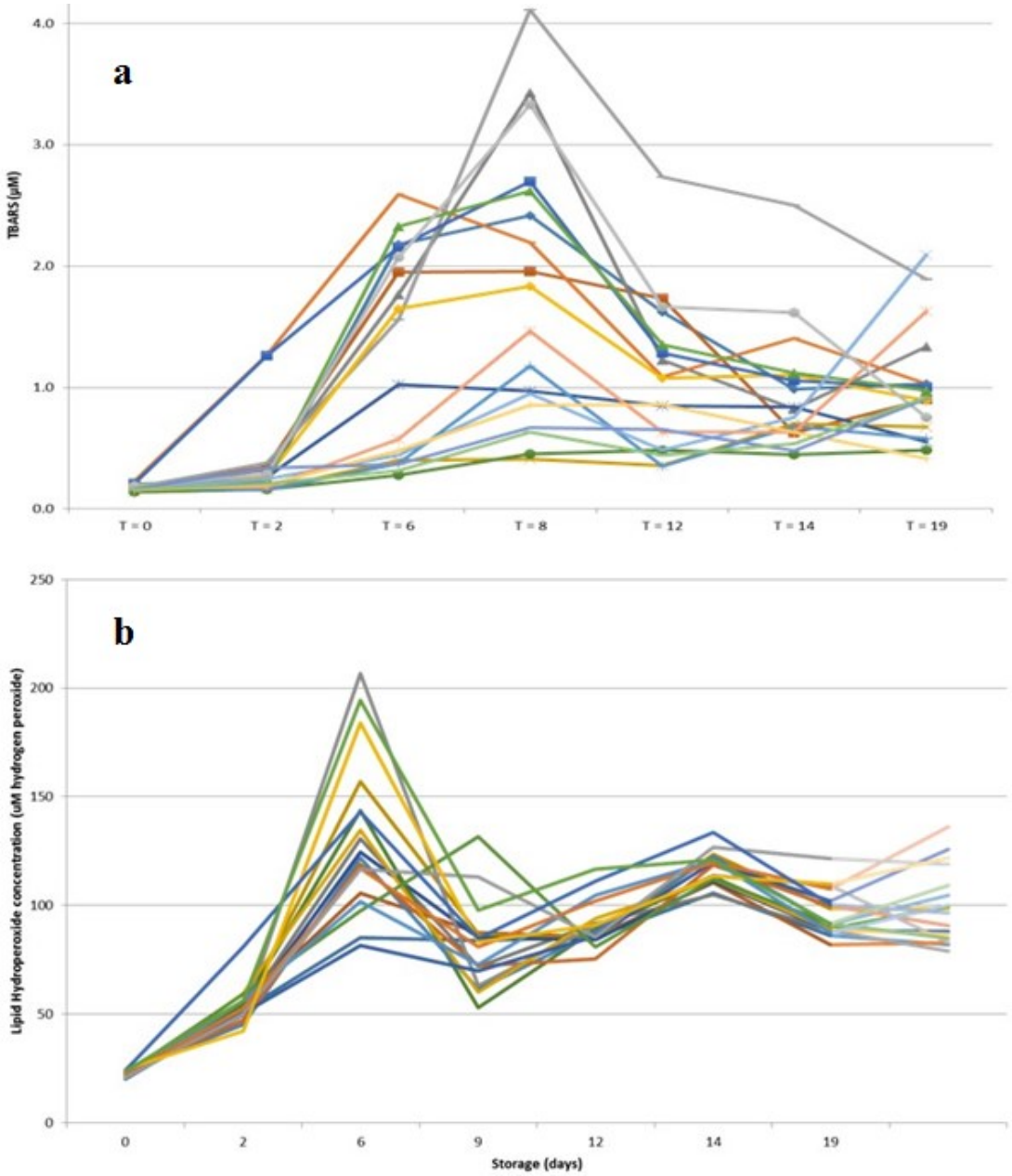


Figure II.15. Overall trends of primary and secondary oxidation products from olive O/W emulsions under accelerated storage conditions (40 °C). The emulsions were formulated using different concentrations of WPI, OMW biophenols and xanthan gum, as reported in the detail in the following chapter. **a**) TBARS; **b**) lipid hydroperoxides.

Characterisation of the emulsion droplet size by optical microscopy and light scattering techniques

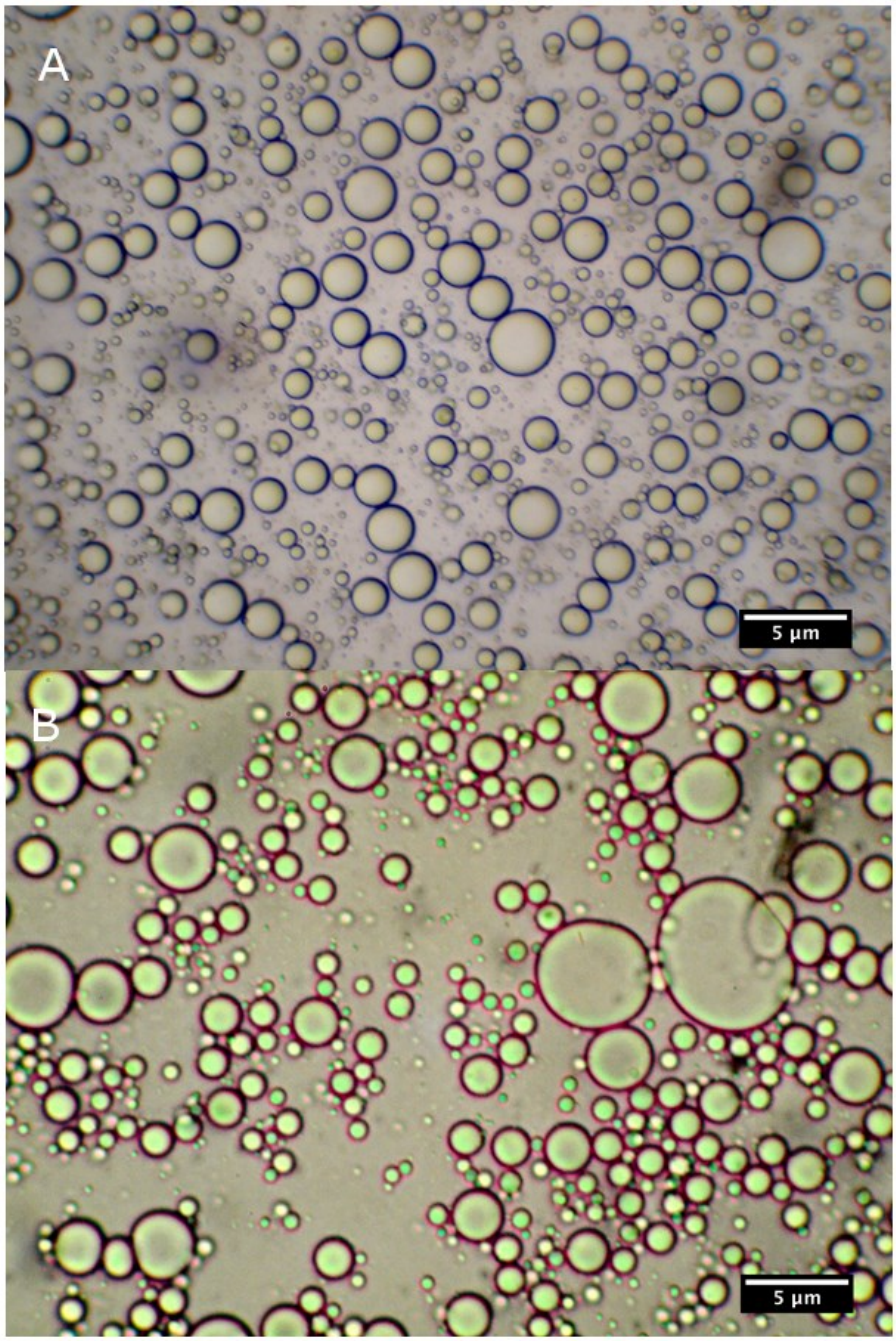


Figure II.16. Example of microscopic pictures obtained from olive O/W emulsions: A) more uniform particle size distribution obtained on fresh emulsions; B) effect of emulsion storage, with increase in particle size. These digital pictures were used to assess the mean particle size by image analysis over storage.

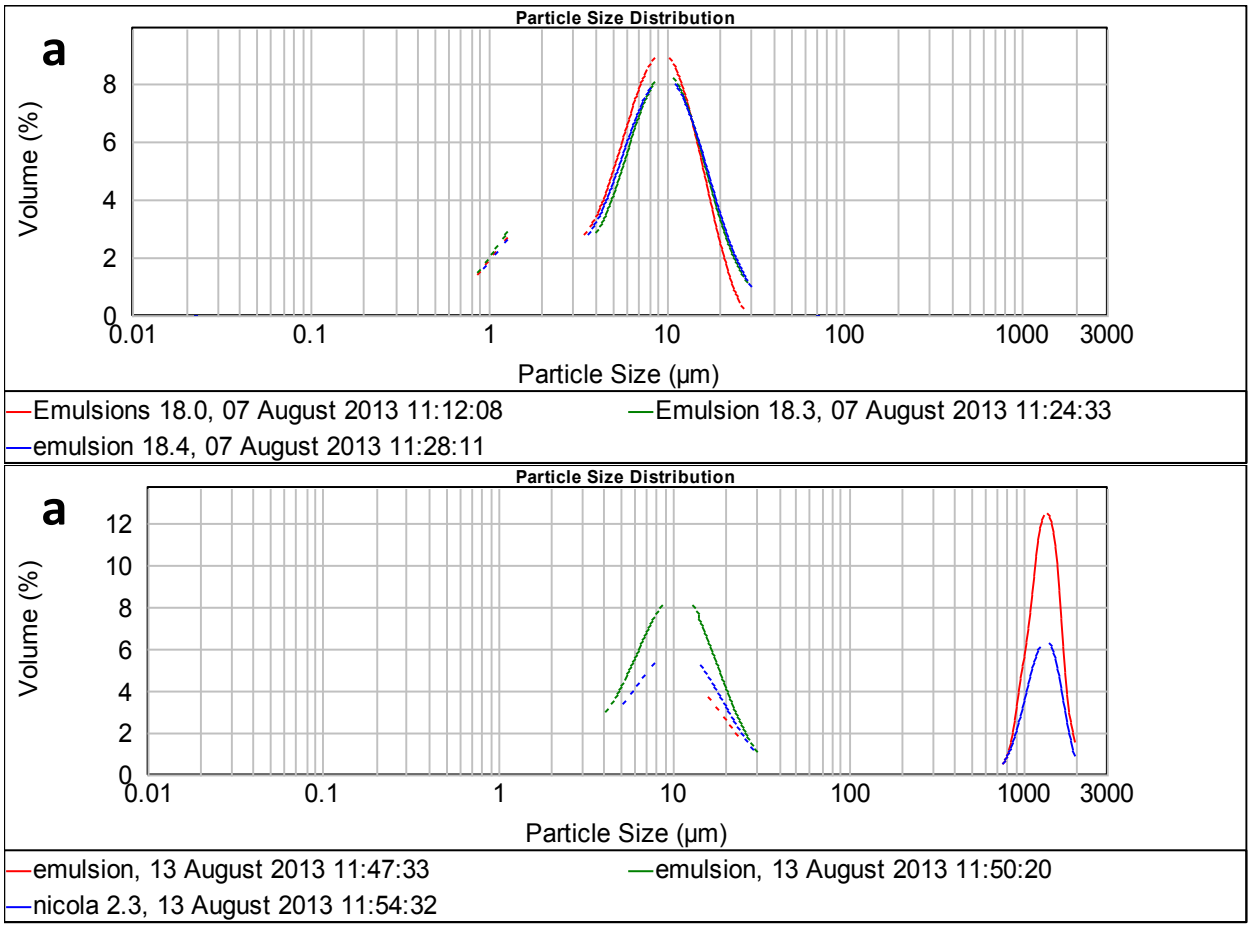


Figure II.17. Particle size distribution of 20% olive O/W emulsions obtained by light scattering techniques (Mastersizer), on freshly prepared samples (a) and after 1 week storage (b) under accelerated storage conditions. It is obvious the bimodal distribution of the emulsion particles. The data are from the same sample**.**

Response Surface Models obtained for O/W emulsions

The Response Surface models for the O/W emulsions reported and discussed in **Chapter 2** are fully reported in the present section to show all the plots. Whereas the plots which were not shown in the previous chapter did not result in a high coefficient of correlation and sufficient significance of the model, they are reported here to show the whole images which can help in have a better description of the emulsion behaviour.

As resulting from the graphs, the main interaction arises from the plot plolyphenols-PWI, while more reduced effects are caused by the interaction WPI-xanthan gum and xanthan gum-polyphenols. These latter two plots are very similar for all the three responses studied **(Fig. II.18, II.19, II.20)**. Better discussion and the whole statistical analysis and model performance evaluation is reported in Chapter 2.

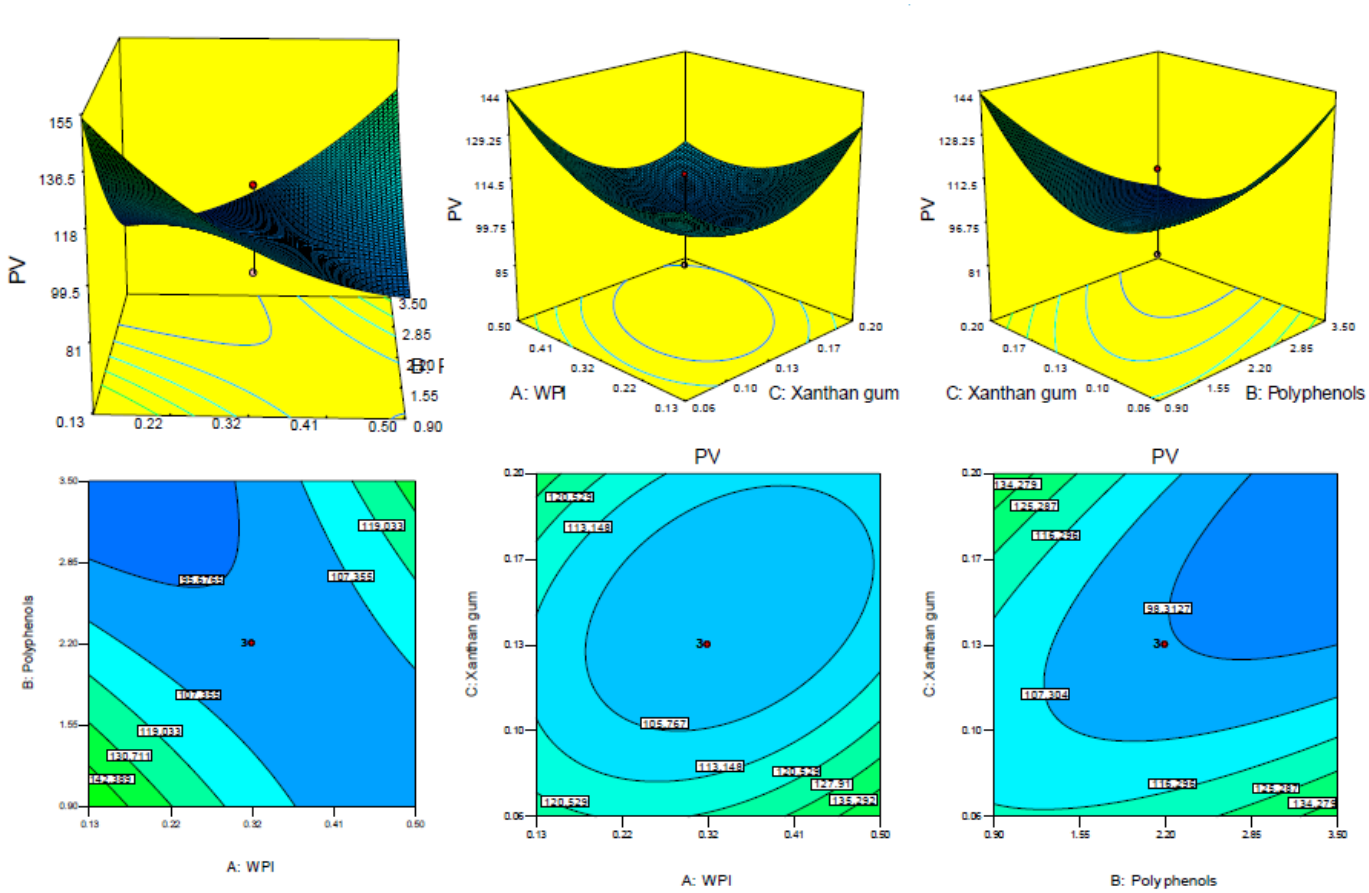


Figure II.18. Response Surface Methodology for Peroxide Value (PV) in olive O/W emulsions with added OMW polyphenols, whey protein isolate (WPI) and xanthan gum, over accelerated storage at 40 °C.

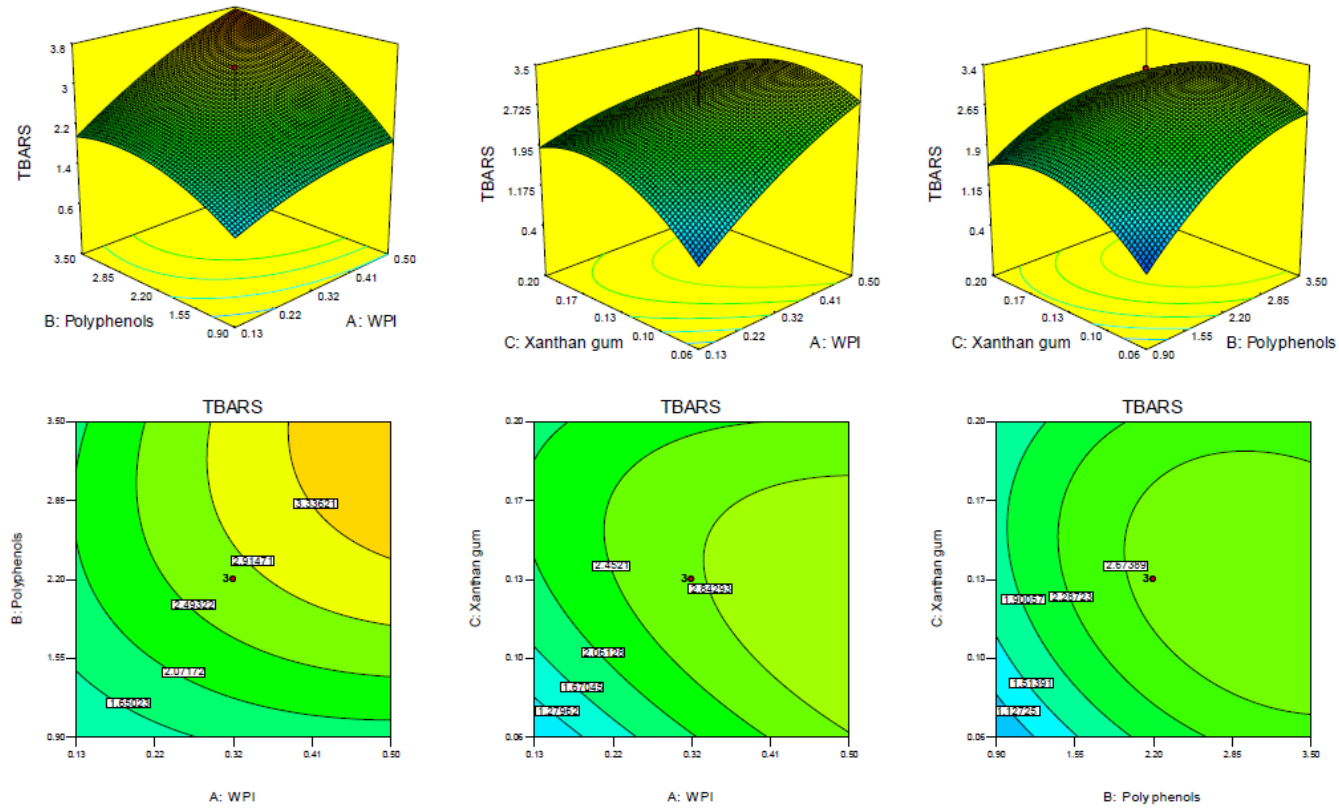


Figure II.19. Response Surface Methodology for secondary oxidation index (TBARS) in olive O/W emulsions with added OMW polyphenols, whey protein isolate (WPI) and xanthan gum, over accelerated storage at 40 °C.

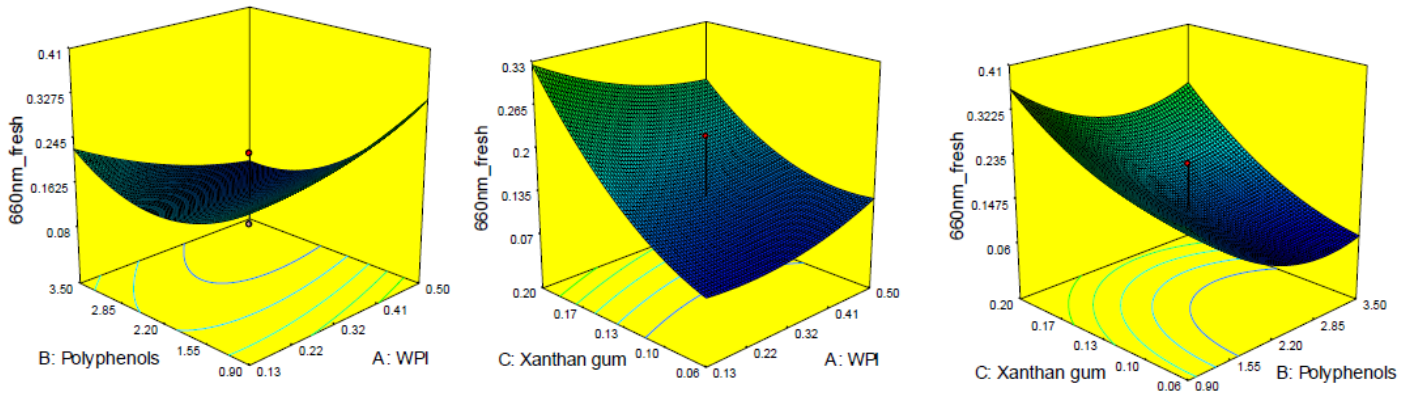


Figure II.20. Response Surface Methodology for cloudiness on fresh emulsions (measured as absorbance at 660 nm) in olive O/W emulsions with added OMW polyphenols, whey protein isolate (WPI) and xanthan gum, over accelerated storage at 40 °C.

Resolution - an indication of the confounding pattern of the design. Designs are classified as having one of the following resolutions: iii) designs which confound the estimates of the main effects with two-factor interactions. Such designs can be safely interpreted only if all two-factor interactions are small or non- existent. iv) designs which are capable of obtaining clear estimates of all main effects. However, some or all of the two-factor interactions are confounded with other two-factor interactions or block effects. The *Alias Structure* table described below indicates where the confounding occurs. v) designs which are capable of obtaining clear estimates of all main effects and all two-factor interactions. Higher order interactions, however, are confounded with these effects. In most cases, this is not a problem since third-order and higher effects are usually assumed to be small or non-existent. Resolution V designs are typically excellent selections.

Error degree of freedom - the number of degrees of freedom from which the experimental error may be estimated remaining after estimating all main effects, second-order interactions, and quadratic effects (if relevant). This is prior to any replication or addition of centerpoints. In general, at least 3 d.f. must be available if the statistical tests to be performed during the analysis are to have reasonable statistical power.

Centerpoints - the number of centerpoints to be added to the base design, which are additional experimental runs located at a point midway between the low and high level of all the factors. Each additional CenterPoint adds one degree of freedom from which to estimate experimental error. If the design involves a single categorical factor, the centerpoints will be placed at a middle level of the quantitative factors and divided equally between the two levels of the categorical factor.

Sum of Squares - the Type III sums of squares attributable to each term in the model. This measures the increase in the variance of the experimental error that would occur if each term was separately removed from the model. The sum of squares for total error is also included, where

$$S_{error} = \sum_{i=1}^{n} e_i^2 = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2$$

where *ei* is the *i-th* residual, measuring the difference between the observed response for run *i* and the value predicted by the fitted model.

Mean Square - the mean square associated with each term, obtained by dividing the associated sum of squares by its degrees of freedom. The mean squared error (MSE) estimates the variance of the experimental error

$$\hat{\sigma}^2 = MSE = \frac{S_{error}}{df_{error}}$$

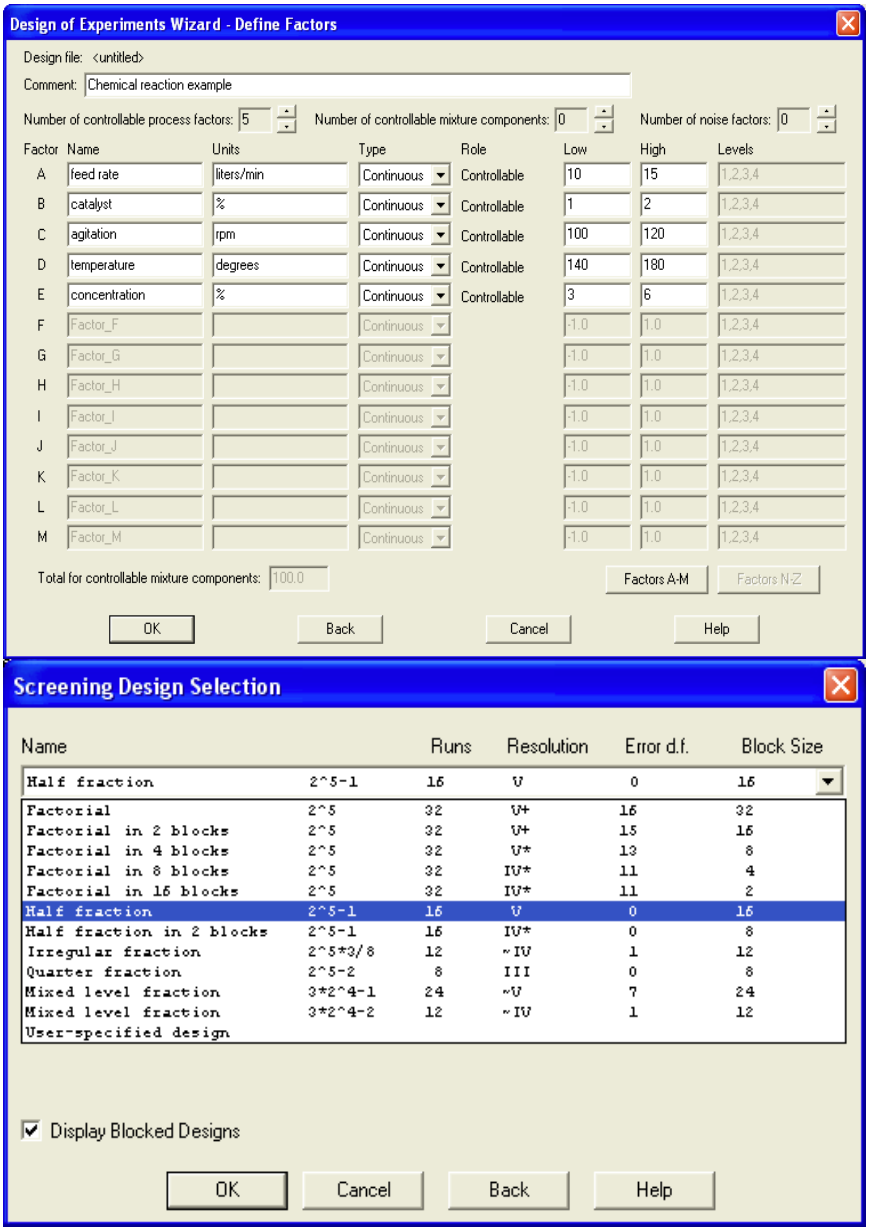


Figure II.21. Example of setting for the Response Surface Methodology design, for the number and types of controllable process factors (upper window), and the type of Design available (lower window) under the software package Statgraphics Centurion XVI.

F-Ratio - an F ratio which divides the mean square of an effect by the mean squared error, and may be used to determine the statistical significance of each effect:

$$F = \frac{MS_{effect}}{MSE}$$

P-Value - the P-Value associated with testing the null hypothesis that the coefficient for a selected effect equals 0, implying that the effect is not present. P-Values below a critical level (such as 0.05 if operating at the 5% significance level) indicate that the corresponding effect is statistically significant at that significance level.

R-squared - the percentage of the variability in the response variable that has been accounted for by the fitted model, calculated from the following equation:

$$R^2 = 100\left(1 - \frac{S_{error}}{S_{total}}\right)\%$$

R-squared ranges from 0% to 100% and measures how well the model fits the observed response data.

R-squared (adjusted for d.f.) - the adjusted R-squared accounts for the number of degrees of freedom in the fitted model. In situations such as the current one where the number of coefficients in the fitted model is large relative to the total number of runs, the ordinary R-squared statistic may overstate the ability of the fitted model to predict the response. The adjusted R-squared compensates for this effect by:

$$R^2_{adj} = 100\left[1 - \left(\frac{n-1}{n-p}\right)\frac{S_{error}}{S_{total}}\right]\%$$

Standard error of estimation - the estimated standard deviation of the experimental error, given by

$$\hat{\sigma} = \sqrt{MSE}$$

This value is used when constructing prediction intervals for the response.

Mean absolute error - the average of the absolute values of the residuals, given b

$$MAE = \frac{\sum_{i=1}^{n} |e_i|}{n}$$

This value indicates the average error in predicting the observed response using the fitted model.

Durbin-Watson statistic - a statistic calculated from the residuals according to the following equation:

$$DW = \frac{\sum_{i=1}^{n-1} (e_{i+1} - e_i)^2}{\sum_{i=1}^{n} e_i^2}$$

The Durbin-Watson statistic measures serial correlation in the residuals to determine whether there is any dependence between successive observations. In this case, it could detect drifts over the course of the experiment. A small P-value would indicate that the analyst should take a close look at the residuals to look for any trends, which may be done using the *Diagnostic Plots* graph option.

Pure error: an estimate calculated by pooling the variance within sets of observations at identical levels of *X*. It is "pure" in the sense that it estimates the experimental error ε whether or not the proper model has been selected.

Lack-of-fit: an estimate calculated from the deviation between the average response for each group of replicate values and the values predicted by the fitted model. If the model is not correct, this estimates value plus a positive quantity that measures the lack-of-fit of the selected model. The P-Value in the lack-of-fit line may be used to test the hypothesis that the current model is adequate. A small P-Value would indicate an inadequate model. In the current example, the P-Value is well above 0.05, so the selected model appears to be adequate.

Annex III

Example of application of Molecular Gastronomy in relation to virgin olive oil interaction and aroma release: the case of Neapolitan pizza

INTRODUCTION

Pizza is one of the most known and appreciated products of the Italian gastronomy, and it is undoubtedly known worldwide, whereas it has been also associated to fast-food restaurants and unhealthy nutritional habits. The first document where the word “pizza” appeared dated before 1000 AD, in southern Italy, and in 16th century many types of toppings were known, whereas the pizza Margherita, the most known Neapolitan pizza, was created by the Neapolitan pizzamaker Raffaele Esposito in 1889 (Danford, 1994). From its origin, virgin olive oil was commonly used for pizza, except for pizza "Mastunicola", where lard was used as a source of fat. Nowadays, it is difficult to find Neapolitan pizzerias that use extra virgin olive oil (EVOO), because other vegetable oils or "olive oils" (mixture of refined olive oil and virgin olive oil) are preferred, mainly due to their lower cost with respect to EVOO.

Pizza was a poor food product for poor people when it originated: the basic ingredients are water, flour, edible oil, salt and yeast. The pizza as it is known today started to be produced when tomato was imported from America and began to be diffused in the 16th century in Europe, but pizza was already known more than one thousand years ago. Except for the sole "bread basis", the most ancient pizzas were "mastunicola" (composed by lard, cheese and basil) in 1660, "marinara" (tomato, garlic, oregano, oil) in 1750 and "margherita" (tomato, mozzarella, oil) in 1850. In Margherita pizza, basil was added later, in 1889 to add the green color and reproduce the three colors of the Italian flag (De Bourcard, 1965).

Neapolitan pizza was recently awarded by the European Union as traditional speciality guaranteed product. The designation 'Pizza Napoletana' comprises well defined steps and only some ingredients could be used. *e.g.* the preparation of the dough includes a double fermentation, the first for about 2 hours, and the second leavening phase is made by using little “balls” of dough for 4-6 hours at room temperature for the fermentation. The dough is then carefully expanded by hand, ensuring to leave a thicker edge on the external part. Different types of pizza exist, depending on the type of topping, but the most traditional and known ones are “marinara” and “margherita”. The first one is made by using tomato sauce, olive oil and oregano, while the latter is made by using tomato sauce, mozzarella, olive oil and fresh basil. The cooking of the Neapolitan pizza takes place exclusively in wood-fired ovens, where it reaches a temperature of typically 485° C and cooking times should not exceed 60-90 seconds. The temperature usually reached by pizza is about 204-288 °C. The consistency of pizza has been described as soft, elastic, easily foldable, with a distinctive savory taste, derived from the rim which has the typical taste of well cooked bread, both with the sour taste of tomato, and the flavor of oregano, garlic, basil or baked mozzarella (EU Reg. 97/2010).

Lipids are an important part of the human diet, and beyond their nutritional value, they also possess important technological and sensory properties (Drewnlowski, 2009; Sandrou and Arvanitoyannis, 2000). Vegetable oils generally contain high amount of unsaturated fatty acids, which undergo rapid and dramatic changes in their compositions. *e.g.*, the oxidation rate of sunflower oil with respect to olive oil has been reported to be as double, because of its fatty acid composition. Virgin olive oil, instead, shows higher resistance to lipid oxidation, for the presence of the so-called minor compounds, particularly the

polyphenols, or “biophenols” (Obied, 2013). The effect of certain type of cooking by using fat has been widely studied, *e.g.* the chemical alterations of vegetable oils during deep-frying (Zhang et al., 2012).

Also, another important ingredient in pizza is tomato sauce. The thermal treatment of tomato has been reported to greatly modify its chemical (Gahler et al., 2003) and volatile composition (Mathias et al., 2002).

The flavor of pizza is strongly influenced by its volatile compounds. Many techniques have been used for sampling volatile compounds in food (Pillonel et al., 2002), and one of the most useful, rapid and accurate techniques is the solid phase micro-extraction (SPME) technique, followed by gas-chromatography and mass spectrometric analysis (GC-MS). This type of analysis has been applied in a wide variety of food (Roberts et al., 2000; Kataoka et al., 2000).

Little information was found in literature about the lipid composition and volatile profile of pizza, particularly for the “original” (traditional) preparation. Traditional pizza, which has been historically produced only in the city of Naples, has been scarcely studied, as few papers were found in literature. Some of these reported about the microbiological characteristics (Pepe et al., 2003; Coppola et al., 1996), and their main aim was to assess the effect the characteristics of the microorganism on the dough fermentation.

Thus, the aim of our work was to evaluate the contribution of extra virgin olive oil and other vegetable oils on the chemical characteristics, oxidation status as affected by pizza cooking and volatile profile of Neapolitan pizza.

MATERIALS AND METHODS

Standards and reagents

The following pure chemical compounds were used for the identification of volatile compounds in olive oil and pizza: octane > 99%, octene 98%, ethyl acetate 99.8%, 3-methyl-butanal 97%, 3-pentanone 99%, 1-penten-3-one ≥ 97%, 2-methyl-1-propanol ≥ 99.9%, 2-heptanone ≥ 99.9%, 1-heptanal ≥ 95%, 2-octanone ≥ 99.5%, 1-octanal 99%, (Z)-3-exenyl acetate 98%, (E)-2-heptenal 98%, nonanal 95%, (Z)-2-hexenol 95%, (E)-2-octenal ≥ 99.9%, 1-octanol 99%, (E)-2-decenal 97%, 1-nonanol ≥ 99.5%, (E)-2-pentanal 95%, 3-methyl-1-butanol ≥ 99%, (E)-2-hexenal 98%, 1-pentanolo 99%, hexyl acetate 99%, hexanol 99%, (E)-3-hexen-1-ol 98%, (E)-2-hexen-1-ol 96%, (Z)-3-hexen-1-ol 95%, (Z)-2-pentenol 98%, benzaldehyde ≥ 99.5 %, 1-penten-3-ol 98%, hexanal 95%, pentanal 97%, toluene 99.9% (Sigma-Aldrich, Steinheim, Germany). All other chemicals used for the chemical analysis were of laboratory grade and were bought all from Sigma-Aldrich (Steinheim, Germany).

Samples and pizza preparation

Extra virgin olive oil cultivar Ravece was provided by San Comaio (Villanova del Battista, Avellino, Italy). Refined olive oil was provided by IOBM s.r.l. (Benevento, Italy). The sunflower oil and the commercial olive oil were bought from the local market. The samples of pizzas were prepared in the pizzeria "Bocconcì" by chefs (“pizzaioli”) and the production parameters used were those of the “*Disciplinare di produzione Pizza Napoletana*” (Neapolitan Pizza guidelines). Vegetable oils and tomato sauce were submitted to an intense thermal treatment (300-400 °C) in a traditional wood-fired oven, in pizzeria Bocconcì (Portici, Naples, Italy). The time chosen for the treatment was 90 seconds, which corresponds to that traditionally used for pizza cooking.

With the exception of few pizza typologies (e.g. "*bufalina*", in which freshly cut tomato is used), the tomato commonly added to Neapolitan pizza is an industrially processed tomato sauce. After washing the fruits, they are boiled or heated by using other techniques, and finally crushed after separating peel and seeds, and adding the adequate doses of salt. Citric acid or other GRAS could be added to the product, which can undergo another thermal treatment for the pasteurization in the package. In our experiment, we used an

industrially produced tomato sauce. When pizza was prepared, dough was kneaded by hand. After the rising process, the dough is formed by hand and is about 3 mm thick. Then, toppings were placed on it, and olive oil or other vegetable oils were added as a final ingredient, immediately before pizza was placed into the oven. Oil was added by using a special and traditional copper or aluminum cruet that has a long nozzle, and the *pizzaiolo* started placing the oil from the center of the pizza toward its border, by creating an imaginary spiral. Depending on the type of pizza, or the preferences of *pizzaiolo* or consumer, sometimes oil may be added after pizza is cooked, when it is removed from the oven, before serving it. When both tomato sauce and olive oil are used, the first ingredient spread on pizza is tomato sauce, and then the olive oil as previously described (EU Reg. 97/2010).

PV, UV and fatty acid composition of vegetable oils

Determination of free acidity, peroxide value (PV) and spectrophotometric indices (K_{232}, K_{270}, ΔK) for vegetable oils were carried out according to the EC Regulation 2568/91. Fatty acid composition was determined by gas-chromatographic analysis of fatty acid methyl esters, as reported by Christie (1982), by using a gas chromatographer Shimadzu mod. GC-17A equipped with Flame Ionization Detector (Shimadzu Italia, Milano, Italy). The acquisition software was Class-VP Chromatography data system vers. 4.6 (Shimadzu Italia, Milano, Italy). The column was a capillary column FAME 60 m, i.d. 0.25 mm using a 50% cyanopropyl-methyl phenyl silicone stationary phase 0.25 mm thickening (Quadrex corporation, New Heaven, USA). The conditions used were as following: chamber hold at 170 °C for 20 min, then a thermal rate of 10 °C min^{-1} until 220 °C, hold for 5 min. Injector temperature and FID temperature were both set on 250 °C. Helium flow was 2 mL min^{-1}, split ratio was 1/60 and injected amount of sample was 1 µL. Compounds were identified by comparing fatty acids retention times with those obtained by analyzing a mixture of pure methyl ester fatty acids (Larodan, Malmoe, Sweden) used as standard compounds.

HPLC analysis of phenolic compounds

The analysis of phenolic compounds in EVOO was performed according to Vasquez-Roncero (1978), slightly modified. Ten grams vegetable oil were dissolved in 10 mL hexane and then extracted in a separating funnel using a water/methanol mixture (40:60 v/v) (3x7 mL); the hydro-alcoholic extract was washed using hexane and centrifuged for 10 minutes at 3500 rpm. The methanol phase was collected in a flask and evaporated in vacuum rotary evaporator (40 °C). A Shimadzu mod. LC-10ADVP HPLC with a UV-Vis Diode Array detector Shimadzu, mod. SPD-M10AVP (Shimadzu Italy, Milan, Italy) was used for analysis. The chromatographic conditions were chosen according to Tsimidou et al. (1992). Acquisition software was Class-VP Chromatography data system vers. 4.6 (Shimadzu Italy, Milan, Italy). The column was a reversed phase column (Spherisorb S5 ODS3 250 x 4.6 mm id) and the flow was 1 mL min^{-1}, injection volume 20 µL. Quantification was carried out by using an external standard (tyrosol) as reference compound for the calibration curve. The compounds identification was carried out by comparing retention time and UV spectra with pure reference compounds.

Analysis of volatile compounds by SPME-GC-MS

Analysis of volatile compounds was performed by sampling 3 mL vegetable oil or pizza homogenate, which were put in a 15 mL vial for the equilibration phase. In the case of pizza, 3 g sample was added in 5mL distilled water, and homogenized for 2 min by using an Ultraturrax blender (Ika-Werke, Staufen, Germany). Volatile compounds were analyzed by SPME-GC-MS, using a DVB-CAR-PDMS 50/30 µm 1 cm length fiber (Supelco, Bellofonte, USA). DVB/CAR/PDMS fiber was reported for the analysis of a wide variety of food matrices and it was reported as the most efficient fiber for the sampling of volatile compounds in some baked products (Rega et al., 2009), and in virgin olive oil (Vichi et al., 2003). Samples were mixed by using a magnetic stirrer for 10 minutes at 40 °C for the equilibration phase. Fiber was exposed to the sample for 30 minutes and the GC-MS analysis was performed according to Vichi et al. (2003). A GC-MS Shimadzu mod.

QP5050A (Shimadzu, Milan, Italy) equipped with a fused silica capillary column Supelcowax 10, 60 m length, 0.32 mm internal diameter and 0.50 µm film thickness of propylene glycol (Supelco, Bellefonte, USA) was used for the analysis. An electron impact source of 70 eV was used for the MS. Source temperature was 200 °C and the interface temperature was 250 °C. Scanning program ranged from 30 to 250 amu and scanning time was 0.4 sec. GC conditions were the following: column temperature was set at 40 °C for 4 min and increased 2.5 °C min^{-1} until 240 °C, hold for 3 min; injector temperature was 230 °C; carrier gas: Helium; column flow: 1.4 mL min^{-1}; split ratio: 1/20. The acquisition software used for the analysis was GC-MS Solution vers. 1.2 (Shimadzu, Milan, Italy). The identification was performed by comparing retention times and mass spectra of each compound with those of pure standards in the same operative conditions, when available. For the other compounds, a tentative identification was given on the basis of the mass spectra available in libraries of the acquisition software (NIST 27, NIST 147, SZTERP).

Statistical analysis

Three pizza replicates were cooked, and all the chemical analyses were carried out in triplicate. Data were statistically treated by two-way ANOVA to assess the effect of different types of vegetable oils and the effect of cooking on pizza composition by using XLStat vers. 6.1 software (Addinsoft, Paris, France). Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Quality indices for vegetable oils

Table III.1. Quality indices for vegetable oils used on the pizza, before and after cooking.

Vegetable oil	Peroxide value		K_{232}		K_{270}		ΔK	
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
EVOO	6.05aA*	12.5bA	1.81aA	2.54bA	0.16aA	0.36bA	-0.08aA	0.03bA
Refined olive oil	2.3aB	8.55bB	2.44aB	2.68aA	0.57aB	0.55aB	0.06aA	0.08aA
Olive oil	3.05aB	8.75bB	2.85aB	2.24aA	0.93aB	0.68bB	0.65aB	0.16bB
Sunflower oil	2.41aB	13.93bA	1.26aA	2.38bA	1.10aC	1.40bC	0.25aC	0.33aB

*Values are the average for three replicates of analysis. Different lower case letters indicate significant differences ($p<0.05$) as affected by cooking treatment, and different capital letters indicate significant differences ($p<0.05$) among different types of vegetable oils. EVOO: extra virgin olive oil. "Olive oil" is the commercial mixture of refined olive oil and virgin olive oil (EC Reg. 2568/1991).

Peroxide Values (PV) is a chemical index that has been used to evaluate the oxidative status of lipid foods. It is a measure of the extent to which the oil has undergone primary oxidation of unsaturated fatty acids. As reported in **Table III:1**, PV and spectrophotometric indices in vegetable oils showed significant differences as affected by oven cooking. In particular, a significant increase ($p<0.05$) of PV was measured in all samples, but sunflower oil showed a more drastic increase in PV, that is explained by its chemical composition rich in polyunsaturated fatty acid and to the absence of natural antioxidant compounds such as polyphenols that were found in EVOO. On the contrary, the thermal treatment did not cause significant changes in fatty acid profile, except for linoleic and linolenic acids ($p<0.05$), probably due to their oxidation. Beside the drastic change in PV due to the thermal treatment in oven, the vegetable oils analyzed remained below the law limits

for extra virgin olive oil (20 meq O_2/kg). The PV value is an index commonly used to measure the oxidation level of unsaturated fatty acids, and for a better analysis of primary and secondary oxidation of oils, UV indices are also used. The thermal treatment of pizza in traditional oven did not cause significant alteration in olive oil fatty acid composition, except for some fatty acids. As shown in **Table III.2**, linoleic acid (18:2) decreased slightly in extra virgin olive oil as effected by thermal treatment. This could be easily explained by the high oxidation rate reported for this fatty acid, which is also the most common source of volatile compounds produced in heated vegetable oils (Frankel, 2005). A significant decrease was also observed for linolenic acid (C18:3) in extra virgin olive oil. In the case of refined olive oil, a different pattern was observed. Palmitoleic acid (C16:0) increased more than double after pizza cooking. In this case, also a significant decrease of 11-octadecenoic acid (C18:1 ω7) and icosanoic acid (C20:0) was observed. Oleic acid (C18:1) decreased significantly, but the most remarkable difference in fatty acid profile was observed for eicosenoic acid (C20:1), which increased more than ten times the initial amount.

Table III.2. Effect of pizza cooking on fatty acid composition in extra virgin olive oil and refined olive oil used in the preparation of the traditional Neapolitan Pizza, before and after cooking in wood fired oven.

Fatty acid (%)	**Extra virgin olive oil**		**Commercial olive oil**		**Sunflower oil**	
	Crude	*Cooked*	*Crude*	*Cooked*	*Crude*	*Cooked*
C 16:0	13.19aA*	13.19aA	13.72aA	13.69aA	7.38aB	7.41aB
C 16:1 ω9	0.12aA	0.07bA	0.10A	nd	nd	nd
C 16:1 ω7	0.75aA	0.61aA	1.38aB	1.18aB	0.20aA,B	0.11bC
C 17:0	0.07aA	0.05aA	0.07A	nd	0.04aA	0.01bA
C 17:1	0.10aA	0.08aA	0.10A	nd	nd	nd
C 18:0	3.23aA	3.61aA	2.73aB	2.58aB	3.43aC	3.45aA,B
C 18:1	69.39aA	69.55aA	64.78aB	66.47bB	33.07aC	32.89aC
C 18:1 ω7	1.89aA	1.89aA	2.40aA	2.45aB	0.85aB	0.78aC
C 18:2	9.26aA	8.77bA	13.29aB	12.24bB	53.73aC	54.11aC
C 20:0	0.37aA	0.46aA	0.40aA	0.37aA	0.08aB	0.15aB
C 18:3	0.70aA	0.61aA	0.51aA	0.44aB	nd	nd
C 20:1	0.22aA	0.22aA	0.25aA	nd	0.08aB	0.09aB
C 22:0	0.09aA	0.11aA	0.11A	nd	0.50aB	0.56aB
Squalene	0.53aA	0.77bA	0.11aB	0.58bA	nd	nd
O/L	7.49aA	7.90bA	10.71aB	5.40bA	0.61aC	0.61aB

* Values are the average of three replicates of analysis. Different lower case letters indicate significant differences (p<0.05) for the same type of vegetable oil as affected by thermal treatment, and capital letters indicate significant differences (p<0.05) for the same treatment but in different type of vegetable oils. nd: not detected. O/L: oleic to linoleic acid ratio.

The major secoiridoids in virgin olive oil have been described to be the isomer of oleuropein algucone and the dialdehydic form of elenolic acid linked to p-hydroxyphenolylethanol (Montedoro et al., 1993). Oven cooking of traditional Neapolitan pizza caused a drastic decrease of about 30% of EVOO polyphenols. Both simple phenols and complex fractions of polyphenols were affected, as reported in **Table III.3.** According to Carrasco-Pancorbo et al. (2007), the level of lignans decreased in a lesser extent with respect to other classes of phenolic compounds. In fact, the authors reported that acetoxypinoresinol was stable during the first hour of EVOO heating. The concentration of some phenolic compounds as Ty-EA, OH-Ty, OH-Ty-EA and OH-Ty-EDA decreased about 50% or more with respect to their initial concentration. Tyrosol did not decrease significantly due to the thermal process. This result was explained by the contemporary degradation of

tyrosol and the formation of degradation products from complex form of polyphenols, *i.e*. aldehydic and dialdehydic forms of tyrosol and hydroxytyrosol, which contributed to increase the concentration in tyrosol in the final product. The same result was previously reported in literature for EVOO cooking (Brenes et al., 2002; Pernice et al., 2007). This hypothesis was supported by previously published papers, where the hydrolysis of secoiridoids aglycones and the diffusion of free phenolics hydroxytyrosol and tyrosol from the oil phase to the water phase was reported (Pernice et al., 2007). The hydrolysis and partitioning of phenolic compounds is undoubtedly influenced by the pH of the medium, thus the presence of tomato sauce could be a very important factor implied in these phenomena. It has been reported that when olive oil and other vegetable oils are subjected to thermal treatments, hydroxytyrosol derivatives are the first antioxidants that are lost during thermal oxidation and tyrosol derivatives seem to be the most stable compounds (Brenes et al., 2002; Carrasco-Pancorbo et al., 2007). Our result confirmed that hydroxytyrosol derivatives are the first compounds to be oxidized, in fact OHTy-EDA decreased of about two third from its initial concentration.

Phenolic compounds in EVOO

Table III.3. Phenolic compounds (expressed as mg/kg tyrosol) in extra virgin olive oil added to Neapolitan Pizza, before and after pizza cooking in traditional ovens.

Phenolic compounds	**Extra virgin olive oil**	
	Crude	*Cooked*
OHTy*	22.20a	11.14b
Ty	36.15a	31.30a
n.i.	10.22a	5.63b
OHTy-EDA	93.38a	31.20b
Ty-EDA	7.50a	3.95b
PR	31.25a	21.62b
OHTy-EA	14.30a	5.39b
Ty-EA	23.90a	10.38b
Total	**254.66a**	**172.02b**

* OHTy: hydroxytyrosol; Ty: tyrosol; n.i.: not identified compound; OHTy-EDA: dialdehydic form of decarboxymethyl aglycone; PR: pinoresinol and acetoxypinoresinol; OHTy-EA: aldehydic form of decarboxymethyl aglycone. Values are the mean or triplicate analysis. Different letters on the same line indicate significantly different values (p<0.05).

Volatile compounds and interactions

Sixty-seven volatile compounds were identified in vegetable oils or in pizza by SPME-GC-MS analysis. As reported in **Table III.4**, 22 volatile compounds were identified both in cooked and uncooked EVOO, while only 8 compounds were identified in refined olive oil. Sunflower oil volatile compound found both before and after cooking were 10, and only 3 were found in sunflower oil, being mainly described as oxidation product. In cooked vegetable oils, 30 volatile compounds were identified in EVOO, 22 in refined olive oil, 18 in commercial olive oil and 20 in sunflower oil. In the case of pizza topped with tomato sauce, 22 and 21 volatile compounds were identified before and after sample cooking, respectively. When also EVOO was added to pizza topped with tomato sauce, 31 volatile compounds were identified in the headspace of cooked sample. Whereas our SPME-GC-MS analysis was not quantitative, the peak area for hexanal resulted to be strongly influenced by the type of vegetable oil and the thermal treatment. This result was expected, as the development of hexanal in foods has been related to lipid oxidation, particularly in fats with high amount of unsaturated fatty acids.

The main volatile compounds found in pizza were the followings: (E)-2-pentenal, 3-methylbutanal, (E)-2-hexenal, (Z)-2-hexenol, n-hexyl acetate and (Z)-3-hexenol. Some compounds such as hexanal, heptanal, 2-octanone, octanal, 3-methylbutanol and (Z)-2-decenal were described to derive from thermal degradation of EVOO and in general from heated vegetable oils (Kalua et al., 2007).

In refined olive oil, the main volatile compounds were the following: (Z)-3-hexenol, (E)-2-hexanal, hexanal, (Z)-3-hexenyl acetate. After pizza cooking, the volatile compounds arising from refined olive oil were octanal, 3-pentanone and (Z)-3-hexenol. Only this latter compound was previously found on the raw sample (not cooked pizza), and the other two compounds are probably oxidation products of unsaturated fatty acids. The abundance of nonanal with respect to the hexanal could indicate a significant higher oxidation. Hexanal has been report to derive from L-OOH hydroperoxides decomposition in position 13 of linoleic acid, which is 40 times more susceptible to auto-oxidation process (Frankel, 1998). (E)-2-Hexenal and (Z)-3-hexenol were the most abundant volatile compounds originated from EVOO, analyzed from cooked pizza.

As shown in **Fig. III.1**, the use of refined olive oil instead of EVOO gave rise to a different aromatic profile. The main volatile compounds in refined olive oil were (Z)-3-hexenol, (E)-2-hexanal, hexanal, (Z)-3-hexenyl acetate. In this vegetable oil, cooking caused the production of octanol, 3-pentanone, and (Z)-3-hexenol. Some of the cited compounds were reported to be characteristics of heated vegetable oils, with typical sensory notes (Kalua et al., 2007).

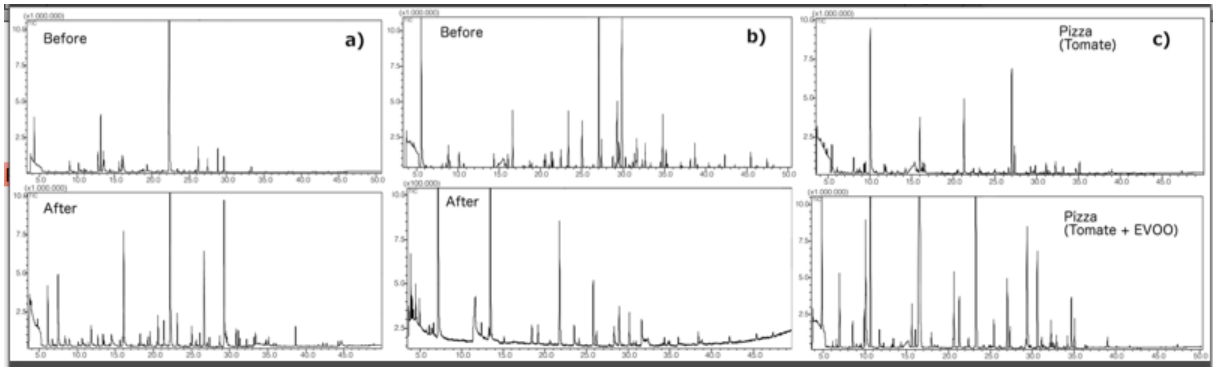


Figure III.1. Total ion chromatograms (TIC) obtained by SPME-GC-MS analysis of volatile compounds in a) extra virgin olive oil before and after cooking, b) tomato sauce before and after cooking, and c) traditional Neapolitan pizza cooked with tomato sauce or tomato sauce and extra virgin olive oil as topping.

Butanal, hexanal, heptanal, 2-octanone, octanal, nonanal, 3-methylbutanol, (E)-2-decenal were found in pizza cooked when only commercial olive oil was added (without tomato sauce), whereas only (E)-2-hexenal, (E)-2-pentenal and (Z)-3-hexenol were found in olive oil before cooking. Beside the intense thermal treatment, in cooked samples, (E)-2-hexanal and (Z)-3-hexenol were still identified. These compounds are characteristics of good EVOOs and they have been described with pleasant sensory notes. This result could be explained by the very rapid process, which is about 90 seconds. The relative abundance of nonanal with respect to the amount of hexanal found in some samples indicates a thermal oxidation of oleic acid, from the decomposition of hydroperoxides in position 13 of oleic acid.

The analysis of commercial tomato sauce was performed before and after cooking. Our result from the volatile compounds found in crude tomato sauce are in accordance with previously published papers (Servili, 2000), being the most abundant compounds as following: methanol, 3-methylbutanal, ethanol, 3-pentanone, hexanal, 2-pentylfuran, 3-methylfuran, α-fellandrene, heptanal, octanone, 6-methyl-5-hepten-3-one, (Z)-3-hexenol, 2-isobutylazol, nonanal, (E)-2-octenal and (E,E)-2,4-heptadienal. In tomato sauce after cooking, some compounds drastically decreased, in particular ethanol and 6-methyl-5-hepten-2-one, and new volatile compounds were detected, as malonic acid, (Z)-2-pentenol and octanal.

Table III.4. Volatile compounds in different vegetable oils used for pizza topping as affected by thermal treatment in a traditional wood fired oven, and the effect of tomato sauce and extra virgin olive oil (EVOO) on pizza aroma.

Volatile compound	I.M.*	Extra virgin olive oil		Refined olive oil		Commercial olive oil		Sunflower oil		Pizza + Tomato		Pizza + Tomato + EVOO	
		Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
Acids													
Butanoic acid	RT									P			
Malonic acid	TI										P		
Alcohols													
Ethanol	RT			P	P	P	P		P	P		P	P
3-Pentenol	RT	P	P	P	P						P	P	
3-Methylbutanol	RT	P	P	P	P					P		P	P
Hexanol	RT	P	P		P	P				P		P	
(E)-2-Pentenol	RT										P		
(Z)-3-hexenol	RT	P	P	P	P	P	P	P	P	P		P	P
Heptanol	RT					P	P			P	P	P	P
(Z)-3-Octenol	RT		P		P								P
1-Octen-3-ol	RT	P	P		P								P
Octanol	RT	P	P		P	P	P		P		P	P	P
Nonanol	RT			P		P			P	P			
2-Phenyl ethanol	RT					P	P					P	P
Benzyl alcohol	RT									P			
Aldehydes													
2-Methylbutanal	TI	P									P		P
3-Methylbutanal	TI	P							P		P	P	P
Pentanal	RT		P			P		P				P	
Hexanal	RT			P	P	P	P	P	P	P	P	P	P
(E)-2-Pentenal	RT	P					P						
(Z)-2-Pentenal	RT	P	P				P						
Heptanal	RT		P			P		P		P	P	P	
Octanal	RT				P	P	P		P		P		
(E)-2-Hexenal	RT	P	P	P									P
(E)-3-Heptenal	RT	P	P										
Nonanal	RT		P	P			P			P	P		
(Z)-2-Octenal	RT	P	P		P					P	P		
(Z)-2-Nonenal	RT								P				
(E,E)-2,4-Heptadienal	TI	P	P		P		P		P				P
(Z)-2-Decenal	RT	P	P										
(E,E)-2,4-Decadienal	TI	P	P		P		P						
2-Furan carboxyaldehyde	TI										P	P	P
2-Methylpropanal	TI										P		P
Alkanes													
Pentane	RT			P	P		P			P	P	P	P
Hexane	RT	P		P		P			P		P	P	P
Heptane	RT		P	P		P	P		P			P	
Octane	RT	P		P		P			P	P			P

1-Methoxy hexane	TI	P		P	P				P			P		
2,5,5-Trimethylheptane	TI							P	P					
2-Methyl-5-ethyl heptane	TI						P		P					P
Alkenes														
Octene	RT			P		P		P						P
2,4-Dimethyl heptene	RT			P					P			P		P
2,4-Dietyl-1,5-hexadiene	TI										P			
1,5-Hexadiene	RT		P											
Ethyl benzene	RT									P				
1,4-Pentadiene	TI	P												
1,5-Octadiene	RT	P												
3,7-Decadiene	RT	P	P		P							P		P
(1,2,4)-Trimethylbenzene	TI													P
Esters														
Methyl acetate	RT						P				P			P
(Z)-3-Hexenyl acetate	RT	P	P		P	P		P			P	P		P
n-Hexyl acetate	RT		P			P						P		
Furan														
3-Methylfuran	RT									P				
Ketones														
3-Pentanone	RT	P		P	P	P								P
1-Penten-3-one	RT	P												
2-Heptanone	RT				P	P								P
2-Octanone	RT	P	P							P				
6-Methyl-5-hepten-2-one	TI	P	P			P				P		P		P
(Z)-3-Octen-2-one	RT	P	P	P	P	P								
Terpenes														
Linalool	TI									P		P		
α-Fellandrene	RT									P				
(Z)-β-Ocimene/Limonene	TI	P	P			P	P		P					
(E)-β-Ocimene	TI		P			P	P							P
α-Copaene	TI	P	P						P			P		P
α-Murolene	TI	P	P						P					
α/β-Farnesene	TI	P	P		P	P			P	P	P			P
Thiazole														
2-Isobutyl thiazole	TI									P	P			

The differences found in the volatile composition of pizzas as affected by cooking process indicated by many changes caused by thermal treatment, but strong differences were observed when different types of vegetable oils were used as toppings, or when tomato sauce was added. In fact, thermal process is widely known to cause intense transformation due to heat and mass transfer, and leads to the production of many newly formed volatile compounds (Rega et al., 2009). Maillard reaction, caramerization and lipid oxidation have been described as the main phenomena responsible of the development of baked products flavor. 2-Isobutylthiazole, such as other thiazoles, is a common product of the Maillard reaction found in different processed foods, where amino acid reacts with reducing sugars. In particular, these compounds have been reported to be responsible for tomato-leaf aroma.

To differentiate the specific effect of EVOO in the final volatile profile of pizza, a sample of pizza was prepared by the sole addition of tomato sauce, while the second one was prepared by adding tomato sauce and EVOO. In the first one, the following compounds were identified: malonic acid, nonanal, 6-mehtyl-5-hepten-2-one, 3-pentanone, 3-methylbutanol, (E)-2-hexenol, (Z)-3-hexenol, ethanol, (E)-2-hexenol, (E,E)-2,4-heptadienal, heptane. In the second case, the compounds derived from EVOO were as following: 3-methylbutanol, octanol, 2-heptanone, 2-methylpropanol, (Z)-3-hexenylacetate and (Z)-3-hexenol. Some compounds from tomato sauce were not identified when EVOO was added to pizza, particularly malonic acid, nonanal, 3-methylbutanol, (E,E)-2,4-heptadienal and heptane.

This effect could be explained by the interactions of some EVOO volatile compounds with those arising from tomato sauce, but also for the possible “protective effect” of EVOO on tomato oxidation.

The presence of ethanol was attributed to the fermentation of pizza dough, as it has been reported as one of the most abundant fermentation products, together with 1-propanol, 2-methyl-1-propanol and 3-methyl-1-butanol (Gobbetti et al., 1995).

In the case of 2-phenyletanol, this compound was reported both in tomato and in olive oil. In the first one, it has been described to derive from amino acid decarboxylase synthesis (Tieman et al., 2006). In the case of virgin olive oil, this molecule was previously reported as a relatively abundant component of its aroma, being also described as a potent odorant (Kiritsakis, 1998). Its absence in refined olive oil was expected, due to the refining process of olive oil that causes the loss of almost all the volatile compounds. For EVOO, the absence of 2-phenylethanol could be probably due to typicality of the EVOO cultivar used in our experiment. Benzyl alcohol was reported as an aroma compound in fresh tomato and tomato products, but in the case of thermal processing, a drastic decrease of its amount was reported (Markovic et al., 2007). The same behavior was described for other compounds such as (Z)-3-hexenal, 6-methyl-5-hepten-2-one, geranial and neral (Markovic et al., 2007).

(Z)-3-Hexenal possesses fresh green, fruity and sweet sensory notes, characteristics of fresh tomato, but this compound was also frequently found in good EVOO. This compound is responsible for the positive attribute "tomato" or "tomato leaf" reported for some EVOOs.

2-Methylpropanal, 2-methylbutanal, and 3-methylbutanal are Strecker’s aldehydes derived from valine, isoleucine, and leucine, respectively. The latter two compounds were reported both in the headspace of baked products (Rega et al., 2009), and in virgin olive oil headspace (Kalua et al., 2007). The first compound, 2-methylpropanal, is a newly formed volatile compound produced from the dough baking, and it was only detected in cooked pizzas.

Nonanal, (Z,Z)-2,4-decadienal, 1-octen-3-ol and 2-penthylfuran were found as degradation products of linoleic acid, as already discussed above. Octanol was found both in vegetable oils and in uncooked pizzas: this result was in accordance with previous papers, this compound being found at the beginning of baking and up to the first 5 minutes of baking (Rega et al., 2009). 3-Methylbutanal is a Strecker's aldehyde and was reported to gradually increase in a linear trend during dough baking. In our experiment, this compound was also found in some vegetable oil, particularly in EVOO before cooking. In fact, 3-methylbutanol was reported as a component in virgin olive oils headspace, and also a key aroma compound described as "malty" (Kalua et al., 2007). The same behavior was reported in literature for nonanal, whereas in our case we did not found the same result. Probably, this is due the different baking time, 90 seconds for traditional pizza with respect to very longer times up to 25 minutes reported in literature (Rega et al., 2009), and it also seems dependent upon the storage of vegetable oils. Another hypothesis could be the further thermal degradation of this compound, which caused the production of other volatile compounds with lower molecular weight.

Whereas this work reports for the first time an investigation upon the effect of vegetable oil and cooking on the traditional Neapolitan pizza, it has several limitations mainly due to the natural high variability in composition in virgin olive oils. In fact, for the phenolic composition, the range from 100 up to 600 mg/kg polyphenols could be found in EVOO, thus a drastic effect on the stability of vegetable oil during cooking is

expected. Also, the volatile composition of such a complex media, *i.e.* virgin olive oil and tomato sauce, can dramatically change according to different origins, variety and processing practices. Finally, pizza was processed without considering mozzarella or other types of cheeses, a fundamental component in many types of pizza, which is likely to strongly modify both the aroma of the final product and the amount and composition of fats. In this case, a higher intake of saturated fatty acid would be possible.

Food pairing is a new concept in the international food research. It could be defined as the study of the combination of different ingredients to determine their sensory effect, which depend upon the interactions of flavor and aroma of each ingredient. Whereas the scientific studies are quite new (Traynor et al., 2013; Ahn et al., 2011), the principle of using different ingredients and assess their effect on the final product were undoubtedly used from ancient times, for example in the Neapolitan pizza. In our opinion, pizza could be considered as one of the brightest example of the "Mediterranean molecular gastronomy". In fact, Molecular Gastronomy is a scientific discipline that investigates the transformations of ingredients that occur while cooking, and finally seeks to explain the “deliciousness” of a dish. We added the term “Mediterranean” to refer to some ingredients or dished typical of the culinary traditions of the Mediterranean people, such as virgin olive oil. Among the aims of the "Mediterranean Molecular Gastronomy" one could cite the study of the molecular characteristics that could explain the "magic" of some traditional dishes or some food ingredients peculiars of the Mediterranean gastronomy. Thus, the research about the maximization of the nutritional properties and health benefits of these ingredients and/or foods, both with their sensory properties and behavior during cooking is a key concept in this field of investigation (Sacchi et al., 2014)

Whereas the ingredients analyzed in our study, *i.e.* vegetable oils and tomato sauce, share many volatile compounds, the highly appreciated aroma of traditional pizza is unlikely to be only attributed to the addition effect of the volatile compounds. In fact, according to Traynor et al. (2012), the success of the food pairing is much more complicated than simply pairing foods that share common key compounds, because positive and negative synergistic or antagonist effects could take place. Further study about this topic would also justify some of the effects obtained in our study as affected by the volatile-volatile or volatile-matrix interactions among vegetable oils, tomato sauce and pizza dough.

CONCLUSIONS

The effect of the type of vegetable oil used in the preparation of traditional Neapolitan pizza was studied, with particular emphasis on the chemical composition and the effect of cooking on pizza quality. Virgin olive oil seems to strongly characterize Neapolitan pizza, for its desirable fatty acid composition, its polyphenols and its aroma compounds. The fatty acid composition is particularly important to avoid excessive oxidation as occurred for sunflower oils. In the case of virgin olive oil, a positive effect of phenolic compounds was observed, whereas a strong decrease was obtained as affected by cooking. For the first time, a qualitative characterization of the volatile compound in traditional Neapolitan pizza with tomato sauce and EVOO was reported. It seems that the final volatile pattern strongly depends not only upon the single ingredients used, particularly the type of fat, but also on their interaction during cooking, that cause the formation or retention of volatile compounds.

In conclusion, virgin olive oil should be preferred both to refined olive oil and sunflower oil, for its higher resistance to the thermic stress caused by high temperature of the oven and for the complex volatile compounds to obtain the best final product. If the target of the chef or food industry technologist is the maximization of the nutritional properties of pizza, it is recommended to add extra virgin olive oil after cooking, which avoids the decrease of EVOO phenolic compounds and probably provides a better sensory experience due to the bitter and pungent notes. Further studies are needed from a Molecular Gastronomy point of view to characterize the main variables responsible of the “deliciousness” of pizza, which would include chemical studies, engineering knowledge about the cooking phase and the application of sensory science and food pairing.

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