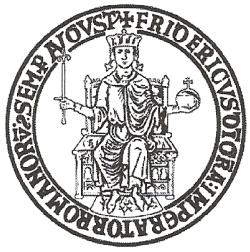
UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF AGRICULTURAL SCIENCE



Ph.D. IN FOOD SCIENCE

Development of enzymatic processes for the recovery of by-products from oilseeds

Coordinator
Professor Amalia Barone

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to my future

I. Abstract

In a circular economy, it is very important to try to recycle all the by-products of the enterprise. Even more interesting is the possibility of giving economic and nutritional value to the latter. For companies that process nuts and oilseeds, an important waste consists of pieces of different sizes that cannot be reused except as animal feed. One of the ways to recover this by-product is to extract the oil, since this type of seed contains a large amount of fatty acids as a source of energy for their germination.

Nowadays, the technology used to produce oil from oilseeds is based on the extraction in organic solvents or on the use of high pressures. Both technologies may have drawbacks. Safety and environmental issues have prompted the development of aqueous extraction for oil recovery from oilseeds. Enzymes are considered a useful biotechnological tool for the extraction of high-quality oil. Enzymatic treatments that improve oil extractability from oilseeds represent a follow up method to produce oil for companies. In addition, another degree of innovation derives from the development of enzymes immobilized on nanoparticles capable of being mixed with the solid to be liquified and improving its performance. It is known that immobilization not only allows for economic savings thanks to the enzyme reusing, but it has also the technological advantage of increasing the efficiency of enzymes. It is well known that oilseeds are a good source of ω-3 and ω-6 fatty acids and α-tocoferol. These have significant and valuable benefits on human health that classify them as a nutraceutical oil.

Given these assumptions, the proposed research would allow the introduction of an innovative technology based mainly on the biotechnological use of enzymes with low environmental impact and economically profitable. Furthermore, these studies have added value to the company waste, both economically and nutritionally. So, the main objective of this study was the development of an immobilized-enzyme system able to improve both extractability and yield of oil from oilseed. Papain-coated magnetic nanoparticles were developed and used as a biotechnological tool with low environmental impact and economically profitable, to extract oil from oilseed.

This study focuses on the development of enzymes processes that are able to increase the oil extractability. In order to obtain more information about oil extractability, we used the different enzymes, such as protease, cellulose and pectinase, which degrade the principal

components of the oilseed structures. Moreover, different time, concentration of enzymes and buffer ratio was studied. The oilseeds were ground, incubated with the enzymes properly, and then centrifuged. (At the end of each experiments, was taken a photo to analyse the amount of extracted oil).

The oilseeds have been grounded, properly incubated with the enzymes, and then centrifuged. Papain, a serine-proteases, bonded to magnetic nanoparticles (Fe_3O_4), was used for the oil extraction. Different raw materials, such as pumpkin seeds and walnut, work in different way, ending up with different results. Furthermore, the immobilization can drastically reduce the economic impact positively affecting the relative industries. Certainly, with the enzymatic treatment it is possible to extract oil from oilseeds and to improve the extraction of oil in term of yield and avoiding the use of solvent.

This study demonstrated that the enzymatic treatment allows to extract oil from oilseeds, avoiding the use of solvents and improving the process in terms of yield. Moreover, showed that aqueous enzyme assisted oil extraction gave higher yield than the simple aqueous oil extraction. Also, the oil quality does not decrease compared to the control extracted in hexane but is much better than the oil from local market. Furthermore, papain-coated magnetic nanoparticles work in the same ways of the free papain used for the oil extraction. So, with a view to a circular economy, the possibility to recovery the papain-coated magnetic nanoparticles through a magnet, allow to reuse the enzyme reducing the process cost.

II. Sommario

Nell'ambito di un'economica circolare è molto importante cercare di recuperare tutti i sottoprodotti aziendali. Ancor di più interessante è la possibilità di aggiungere valore economico e nutrizionale a quest'ultimi. Per quanto riguarda le aziende che lavorano frutta secca e oleaginose, uno scarto importante è formato da pezzi di varia dimensione che non è possibile riutilizzare se non come mangime per gli animali. Una delle vie che è possibile utilizzare per recuperare questo sottoprodotto è quello di estrarre l'olio, in quanto questa tipologia di semi ha una grande quantità di acidi grassi come fonte energetica per la propria germinazione.

Al giorno d'oggi, la tecnologia utilizzata per produrre olio da oleaginose si basa sull'estrazione in solventi organici o sull'utilizzo di alte pressioni. Entrambe le tecnologie possono avere degli svantaggi, che hanno portato alla ricerca di nuove tecnologie legate ad un'estrazione dell'olio che rispettino la sicurezza e l'ambiente. Questi motivi hanno spinto lo sviluppo dell'estrazione acquosa per il recupero dell'olio dai semi oleosi. Anche questa tecnologia porta degli svantaggi considerevoli, dovuti principalmente alle basse rese di estrazione, che rendono la tecnica poco applicabile a livello industriale. È noto che i trattamenti enzimatici migliorino l'estraibilità dell'olio da oleaginose e per questo rappresentano un follow-up per la produzione di olio per le aziende. Gli enzimi, infatti, sono considerati un utile strumento biotecnologico per l'estrazione di olio di alta qualità. Inoltre, un altro grado di innovazione deriva dallo sviluppo di enzimi immobilizzati su nanoparticelle in grado di essere miscelati nella soluzione per migliorarne l'estrazione. È noto che l'immobilizzazione non solo consente risparmi economici grazie alla possibilità di riutilizzare gli enzimi più volte, ma ha anche il vantaggio tecnologico di aumentare l'efficienza degli enzimi. Tra l'altro di notevole importanza è la natura salutistica dell'olio estratto da oleaginose. Infatti, è noto che i semi oleosi sono una buona fonte di acidi grassi ω-3 e ω-6 e di α-tocoferolo. Questi hanno benefici significativi e preziosi sulla salute umana che li classificano come un olio nutraceutico.

Alla luce di questi presupposti, la ricerca proposta consentirebbe l'introduzione di una tecnologia innovativa basata principalmente sull'utilizzo biotecnologico di enzimi a basso impatto ambientale ed economicamente redditizia. Quindi, l'obiettivo principale di questo studio è stato lo sviluppo di un sistema di enzimi immobilizzati su nanoparticelle in grado di migliorare sia l'estrazione che la resa di olio dai semi oleosi. Sono state sviluppate

nanoparticelle magnetiche rivestite di papaina e utilizzate come strumento biotecnologico a basso impatto ambientale ed economicamente redditizio, per estrarre olio da sottoprodotto di un'azienda che lavora oleaginose. Inoltre, questi studi hanno apportato un valore aggiunto allo scarto aziendale, sia a livello economico che a livello nutrizionale. Questo studio si concentra sullo sviluppo di processi enzimatici in grado di aumentare l'estrazione dell'olio. Per ottenere maggiori informazioni sull'estrazione dell'olio, abbiamo utilizzato i diversi enzimi, come proteasi, cellulosa e pectinasi, che degradano i principali componenti delle strutture dei semi oleosi. Inoltre, sono stati studiati tempi, concentrazione di enzimi e rapporto tampone diversi. I semi oleosi sono stati macinati, incubati adeguatamente con gli enzimi e quindi centrifugati. (Alla fine di ogni esperimento, è stata scattata una foto per analizzare la quantità di olio estratto).

I semi oleosi sono stati macinati, opportunamente incubati con gli enzimi e quindi centrifugati. La papaina, una serina-proteasi, legata a nanoparticelle magnetiche (Fe_3O_4), è stata utilizzata per l'estrazione dell'olio. Materie prime diverse, come i semi di zucca e la noce, lavorano in modo diverso, ottenendo risultati diversi. Inoltre, l'immobilizzazione può ridurre drasticamente l'impatto economico che influisce positivamente sulle relative industrie. Certamente con il trattamento enzimatico è possibile estrarre olio dai semi oleosi e migliorare l'estrazione dell'olio in termini di resa ed evitando l'uso di solventi.

Questo studio ha dimostrato che il trattamento enzimatico permette di estrarre olio dai semi oleosi, evitando l'uso di solventi e migliorando il processo in termini di resa. Inoltre, ha dimostrato che l'estrazione di olio assistita da enzimi acquosi ha dato una resa maggiore rispetto alla semplice estrazione di olio acquoso. Inoltre, la qualità dell'olio non diminuisce rispetto al controllo estratto in esano ma è molto migliore rispetto all'olio del mercato locale. Inoltre, le nanoparticelle magnetiche rivestite di papaina funzionano allo stesso modo della papaina libera utilizzata per l'estrazione dell'olio. Quindi, in un'ottica di economia circolare, la possibilità di recuperare le nanoparticelle magnetiche rivestite di papaina tramite un magnete, permette di riutilizzare l'enzima riducendo il costo del processo.

III. Resumen

En una economía circular, es muy importante intentar reciclar todos los subproductos de la empresa. Más interesante aún es la posibilidad de dar valor económico y nutricional a estos últimos. Para las empresas que procesan nueces y semillas oleaginosas, un desecho importante consiste en piezas de diferentes tamaños que no pueden reutilizarse excepto como alimento para animales. Una de las formas de recuperar este subproducto es extraer el aceite, ya que este tipo de semilla contiene una gran cantidad de ácidos grasos como fuente de energía para su propria germinación.

Hoy en día, la tecnología utilizada para producir aceite a partir de semillas oleaginosas se basa en la extracción en disolventes orgánicos o en el uso de altas presiones. Ambas tecnologías pueden tener inconvenientes. Las cuestiones de seguridad y medioambientales han impulsado el desarrollo de la extracción acuosa para la recuperación de aceite a partir de semillas oleaginosas. Las enzimas se consideran una herramienta biotecnológica útil para la extracción de aceite de alta calidad. Los tratamientos enzimáticos que mejoran la extractabilidad del aceite de las semillas oleaginosas representan un método de seguimiento para la producción de aceite para las empresas. Además, otro grado de innovación deriva del desarrollo de enzimas inmovilizadas sobre nanopartículas capaces de mezclarse con el sólido a licuar y mejorar su rendimiento. Se sabe que la inmovilización no solo permite un ahorro económico gracias a la reutilización de enzimas, sino que también tiene la ventaja tecnológica de incrementar la eficiencia de las enzimas. Es bien sabido que las semillas oleaginosas son una buena fuente de ácidos grasos ω-3 y ω-6 y α-tocoferol. Estos tienen importantes y valiosos beneficios para la salud humana que los clasifican como aceite nutracéutico.

Por supuestos, la investigación propuesta permitiría la introducción de una tecnología innovadora basada principalmente en el uso biotecnológico de enzimas de bajo impacto ambiental y económicamente rentable. Además, estos estudios han aportado un valor añadido a los residuos de la empresa, tanto a nivel económico como nutricional. Entonces, el objetivo principal de este estudio fue el desarrollo de un sistema de enzimas inmovilizadas capaz de mejorar tanto la extractabilidad como el rendimiento de aceite de semillas oleaginosas. Se desarrollaron nanopartículas magnéticas recubiertas de papaína y se utilizaron como herramienta biotecnológica de bajo impacto ambiental y económicamente rentable para extraer aceite de semillas oleaginosas.

Este estudio se centra en el desarrollo de procesos enzimáticos capaces de incrementar la extractabilidad del aceite. Para obtener más información sobre la extractabilidad del aceite, se utilizaron las diferentes enzimas, como la proteasa, celulosa y pectinasa, que degradan los principales componentes de las estructuras oleaginosas. Además, se estudiaron diferentes tiempos, concentraciones de enzimas y relación de tampones. Las semillas oleaginosas se trituraron, se incubaron adecuadamente con las enzimas y luego se centrifugaron. (Al final de cada experimento, se tomó una foto para analizar la cantidad de aceite extraído).

Las semillas oleaginosas se han molido, se han incubado adecuadamente con las enzimas y luego se han centrifugado. Para la extracción de aceite se utilizó papaína, una serina-proteasas, unida a nanopartículas magnéticas (Fe_3O_4). Diferentes materias primas, como pipas de calabaza y nueces, funcionan de forma diferente y terminan con resultados diferentes. Además, la inmovilización puede reducir drásticamente el impacto económico que afecta positivamente a las industrias relativas. Ciertamente, con el tratamiento enzimático es posible extraer aceite de semillas oleaginosas y mejorar la extracción de aceite en términos de rendimiento y evitando el uso de solventes.

Este estudio demostró que el tratamiento enzimático permite extraer aceite de oleaginosas, evitando el uso de disolventes y mejorando el proceso en términos de rendimiento. Además, mostró que la extracción acuosa de aceite asistida por enzimas dio mayor rendimiento que la extracción acuosa de aceite simple. Además, la calidad del aceite no disminuye en comparación con el control extraído en hexano pero es mucho mejor que el aceite del mercado local. Además, las nanopartículas magnéticas recubiertas de papaína funcionan de la misma manera que la papaína libre utilizada para la extracción de aceite. Entonces, con miras a una economía circular, la posibilidad de recuperar las nanopartículas magnéticas recubiertas de papaína a través de un imán permite reutilizar la enzima reduciendo el costo del proceso.

1. Introduction

1.1 Oilseeds

Oilseeds are those seeds or plants which are grown chiefly for their oil. These crops are capable of accumulating fatty substances in seeds or fruits, the extraction of which is the first and most beneficial use for consumption (oils, margarines, etc.) or industry (fuels, lubricants, solvents, soaps, etc.) (Gunstone 2002). For centuries, oilseeds have been cultivated and used as a source of food, oil, medicines, cosmetics, dyes, soaps, and other products. Their seeds contain energy for the germinating embryo mainly as oil, unlike cereals which contain energy in the form of starch (McKevith, 2005). While some of them, such as peanuts and sunflower, require little treatment before consumption, others, such as soybean, need to be soaked, cooked, ground, or fermented before consumption. Most oilseeds find their first and most beneficial use in the extraction of oil for consumption or industrial use; the residue of high protein content remaining after the oil extraction, which in the past was of little value, during the last hundred years has acquired increasing interest and has become an interesting source for the feeding of farm animals.

All oilseeds, together with legumes, are characterized by a high protein content, often more than double that of cereals, and there is no other food of natural origin with such high values. Lipid content, which within each species is generally inversely correlated to protein content, varies from 19% on a dry basis in soybean to about 50% in peanut and sunflower (Singh *et al.*, 1999); even higher values are found in walnut whose oil content can exceed 70% of dry weight (Amaral *et al.*, 2003). Proteins from oilseeds have the potential, in suitable and balanced mixtures with other vegetable proteins, to improve both the quality and quantity of dietary proteins for the purpose of direct human intake. This requirement makes them an interesting alternative to animal proteins. The dietary fiber content, in terms of cellulose plus lignin, is usually higher the smaller the seed, since the dietary fiber is essentially localized in the integuments, often in association with phenolic compounds (Nevara *et al.*, 2021). The mineral salt content of oilseeds is subject to high variability with varietal and crop-related factors; available data are sparse or incomplete. In addition, oilseeds are rich in compounds such as phytates, tannins, phenolic substances, and oxalates, which have the property of chelating metals by forming insoluble complexes with them, thus depriving these metals of biological

importance (Erdman 1979). In other words, they render some important microelements such as zinc and iron unabsorbable, and to a lesser extent macroelements, such as calcium and magnesium, by removing them from the body. Therefore, even with these plant sources and their derivatives, the gap between the presence of the micro- and macroelements and their bioavailability can be considerable and requires careful nutritional evaluation. As previously reported, whole oilseeds are a source of fiber, phosphorus, iron, and magnesium; many oilseeds are also a source of vitamin E (with antioxidant activity), niacin, and folate. Whole oilseeds also contain phytoestrogens, a group of substances including lignans and isoflavones. In addition, some phytoestrogens may have antioxidant properties (Goldberg 2008).

Industrial processes for obtaining edible oil from oilseeds generally involve a solvent extraction step, sometimes preceded by pressing. Safety concerns with the use of organic solvents, in the past, led to attempts to develop aqueous extraction but these were unsuccessful mainly due to the low oil yields (Eapen *et al.,* 1966; Hagenmaier *et al.,* 1975).

As well known, from an industrial point of view, the main product of oilseeds is oil, but the residue of extraction, initially considered insignificant, has gradually gained importance over the last 100 years because of its high protein content, until, in the case of soybean, it has become the main resource for feeding. The introduction of appropriate technologies has also made it possible to obtain protein preparations with properties suitable for human consumption and which, in perspective, can be proposed as a suitable means of improving the protein content and quality of the food ration of the population in large nutritional areas. Although animal products are preferred as a source of protein, and will continue to be so in the future, their availability to a large part of the world's population remains very low. It can be calculated that the annual world production of soy, which is about 260 million tons, would be sufficient for more than half of the earth's inhabitants if consumed directly by humans, assuming a daily requirement of 65 g of protein. On the other hand, it is also true that there is a positive evidence from biomedical research for partial replacement of traditional animal protein sources with plant protein sources such as oilseeds and legumes. Even though the various oilseeds, such as walnut, peanut, rapeseed, sesame, sunflower, pumpkin seeds can potentially be an interesting source of protein for human nutrition, it is only for soy that so much research has been done in the last decades, and thanks to that a transfer as food in various markets is possible.

The Besana Group is based in San Gennaro Vesuviano, near Naples, with the main production plant and other two industrial plant, one in UK, near London and another one in Ogliastro Cilento, in Campania region, where is located the Besana's chocolate factory, Vittoria Chocolatery. This plant is among the most innovative and technically advanced in Europe, for nuts, seeds, and dried fruit. The Besana Group is very committed to sustainability utilising ethical business practices, investments in the environment and reduction of emissions. Factory wastes are recycled by third parties as fuel, and industrial waste from all Besana sites is contractually processed by recycling companies. The Besana group is always looking for new sustainable and eco-friendly way to operate in the nuts sector.

The company carries out different kind of processes on their raw materials: toasted, caramelized, different kind of cut (chopped, diced, slice, sticks, fine-cut, meal), paste, smoked, roasted&salted, salted, truffled, just-infused, spiced, and flavoured. Therefore, in the company production process of semi-finished products all the deshelled nuts or seeds are recovered in the form of flours or pieces and sold to the feed industry. Given this assumption, a company that always put the attention on the circular economy, the recovery of most of its by-products became very important. Moreover, where it is possible, a parallel goal is even more adding qualitative and economic value to the by-product and the final product.

Based on Besana's industrial processes, walnut kernels and pumpkin seed by-products were found to be the best samples for the purpose of this work. In fact, in the analysis of the company's by-products, pumpkin seeds were considered as the seeds from which there was the largest amount of waste in the form of pieces of different sizes that could not be reused in another process already present in the company. On the other hand, walnut kernels, which did not have such a high amount of waste as pumpkin kernels, were considered in this study because they are very interesting from an economic point of view, due to the large amount of oil they contain.

1.1.2 Walnuts kenerls

Structures and properties

The fruit walnut or white walnut (*Juglans regia L., 1753*) has Persian origins and is the best known and economically most important member of the genus *Juglans*, dicotyledonous angiosperms belonging to the family *Juglandaceae* (Figure 1.1). The fruit (seed) is incorrectly

called nut but is a drupe with a woody endocarp. The name of the genus comes from the Latin *Iovis glans* (Jupiter's acorn).

Figure 1.1 - *Juglans regia* L., 1753. Scientific classification: Domain: *Eukaryota*; Kingdom: *Plantae*; Division: *Magnoliophyta*; Class: *Magnoliopsida*; Order: *Juglandales*; Family: *Juglandaceae*; Genus: *Juglans*; Species: *regia*. Common names: white walnut, common walnut, royal walnut.

Walnuts are consumed all over the world, both fresh and roasted. China is the largest producer in the world, followed by Iran, the United States, Turkey, Ukraine, Mexico, Romania, and India. Walnuts grow well in temperate and mild climates, and California is the main growing area for nuts in the United States. Among the species with nuts, walnut is ranked third in the world for distribution and cultivation. Recently, the Italian nut sector has experienced a significant reduction in acreage, which has led to a decline in the quantities produced. Italy has gone from being a self-sufficient country to an importer of nuts in shell, mainly from the United States, France, and South America, where genetic improvement programs and related technological innovations have been implemented. The current Italian market of walnuts for fresh consumption is characterized by the massive presence of varieties of foreign origin (Lara and Chandler), except for Sorrento and Malizia cultivars, which are native varieties and are

mainly used for local production and consumption. In Italy, *Juglans regia* is grown throughout the peninsula, at altitudes from sea level to heights of over 1000 meters in the south and 800 meters in the north. The region most traditionally associated with the cultivation of *Juglans regia* is Campania, with over 50% of the national production, followed at a great distance by Piedmont, Veneto, Lazio, Tuscany, Calabria, Sicily, and Basilicata. Plantations, mainly established for dual purpose (fruit and timber), often consist of groups of plants arranged on the edges of fields for other tree and perennial crops or in double rows on the borders of rural properties.

Walnuts are known to prevent cardiovascular disease and the development of other chronic diseases, including type 2 diabetes, hypertension, cancer, and neurodegenerative diseases. Nuts provide a near-perfect blend of protein, healthy fats, fiber, plant sterols, antioxidants, and many vitamins and minerals; in fact, they contain 62-68% lipids, 24% protein, 12-16% carbohydrates, 1.5-2.0% cellulose, and 1.7-2.0% minerals. They are an excellent source of high-energy foods, providing 630 kcal per 100 g of walnut (Duke 2018). Although rich in fat, epidemiological studies and clinical trials suggest that regular consumption of walnuts does not contribute to obesity and may even promote weight loss on a low-energy diet. The presence of bioactive compounds in walnuts is of great interest as they may be a cost-effective source of natural antioxidants for use in functional foods and nutraceuticals. Due to their numerous beneficial properties, a daily intake of 25 g of walnuts is recommended.

The seed or kernel of walnut accounts for 30-40% of the nut weight, which mainly depends on the cultivar. The seed of this tree nut has a high content of oil (52-70%), in which polyunsaturated and monounsaturated fatty acids predominate (Savage *et al.,* 1999; Greve *et al.,* 1992; Martínez *et al.,* 2006; Prasad 2003), as well as oleic acid and linoleic acid, which are sensitive to oxidation. In addition to oil, walnuts provide considerable amounts of protein (up to 24% of walnut kernel weight), carbohydrate (12-16%), fiber (1.5-2%), and minerals (1.7-2%) (Lavedrine *et al.,* 2000; Savage 2001; Sze-Tao *et al.,* 2000; Wardlaw & Insel 1996). Walnuts have a lower concentration of antioxidant tocopherol than other nuts, such as hazelnuts, almonds, peanuts, etc. (Fukuda *et al.,* 2003). Moreover, the lipid autoxidation suppression properties are associated with the antioxidants present in walnut kernel. Walnut kernels contain high amounts of various phenolic compounds, and the slightly astringent taste may be associated with the presence of phenolic compounds.

The main components of the lipid fraction are triacylglycerols; free fatty acids, diacylglycerols, monoacylglycerols, sterols, sterol esters, and phosphatides are all present only in minor quantities. The main fatty acids present in the walnut are oleic (18: 1), linoleic (18: 2), and linolenic (18: 3). The most abundant is a ω-6 fatty acid or linoleic acid.

They also contain a relatively high percentage of a beneficial ω-3 fat called alpha-linolenic acid (ALA). This constitutes approximately 8-14% of the total fat content. Walnuts are the only ones that contain significant amounts of ALA. The latter is considered particularly useful for heart health. In addition, it helps reduce inflammation and improve the composition of fats in the blood. ALA is also a precursor of the long-chain omega-3 fatty acids EPA and DHA, which have been linked to numerous beneficial health effects.

Walnut proteins can be categorized into four major categories, albumin, globulin, prolamin, and glutelin. Contain relatively low levels of lysine and high levels of arginine (Ruggeri *et al.,* 1998). The high content of arginine in walnuts represents a beneficial property as it is converted into nitric oxide, a potent vasodilator that can inhibit platelet adhesion and aggregation.

Walnuts are an excellent source of various vitamins and minerals, including:

- Vitamin E: Compared to other oil crops, walnuts contain high levels of a special form of vitamin E called ω-tocopherol. Vitamin E has a high antioxidant activity and plays an important role against the oxidation of fats in the lipid membranes.
- Vitamin B6: This vitamin can strengthen the immune system, a deficiency of vitamin B6 can cause anemia.
- Folic acid: also known as folic acid or vitamin B9, folic acid has important biological functions. A folic acid deficiency during pregnancy can cause birth defects.
- Copper: This mineral promotes heart health. It also helps maintain bone, nerve, and immune system function.
- Phosphorus: about 1% of our body is made up of phosphorus, a mineral found mainly in bones.
- Manganese: This mineral is found in modest quantities in nuts, whole grains, fruits, and vegetables.

Walnuts contain a complex blend of bioactive compounds. They are exceptionally rich in antioxidants, which are concentrated in the thin, brown cuticle. Some antioxidant molecules found in walnuts include:

- Ellagic acid: this phenolic antioxidant is found in high quantities in walnuts, together with other related compounds such as ellagitannins, which are hydrolyzable tannins, molecules consisting of sugar whose hydroxy group is esterified with hexahydroxydiphenic acid (HHDP). Ellagic acid can reduce the risk of heart disease and helps suppress cancer formation.
- Catechin: catechin is part of a group of antioxidant substances belonging to the category of flavonoids that can have various benefits for heart health.

1.1.3 Pumpkin seeds

Structures and properties

Pumpkin belongs to the genus *Cucurbita* and the family *Cucurbitaceae*. Pumpkin is a versatile fruit and is used in agriculture, commerce and as an ornamental plant. Pumpkins are grown around the world for a variety of reasons, with versatile uses in cooking for both the fleshy shell, the seeds, and even the flowers, most of which are edible (Devi *et al.,* 2018).
Like other members of the *Cucurbitaceae*, pumpkin fruits bear numerous seeds (Figure 1.2). Generally, pumpkin seeds are by-products in the food industry.

Figure 1.2 - Images of a mature pumpkin (A) and pumpkin seeds (B) (Patel 2013).

The pumpkin varies in size, shape, color, and weight. The genus *Cucurbita* comprises five species, *C. maxima*, *C. pepo*, *C. moschata*, *C. ficifolia*, and *C. turbaniformis. C. pepo* having the greatest range of variation, especially as regards the characteristics of the fruit. *C. pepo* is a native species of North America and has been cultivated there for several thousand years. It is claimed that *C. pepo* is more persistent and less susceptible to spoilage, which is certainly reflected in the quality of the oil obtained.

Pumpkin seed oil is used as edible oil in some countries in Africa and the Middle East and as salad oil in the south of Austria and adjacent regions in Slovenia and Hungary (Gohari *et al.,* 2011). Depending on the polyphenolic pigments present, the color of pumpkins varies from golden yellow to orange, with an initial weight of 4-6 kg, which can reach 25 kg in larger specimens. The seed content in pumpkin ranges from 3.52% to 4.27% of the total weight. Pumpkin seeds are a source of modest amounts of lipids, proteins and carbohydrates as shown in Table 1.1. It has high nutritional value, provides good quality oil, is an excellent source of protein and has pharmacological activities such as antidiabetic, antifungal, antibacterial, anti-inflammatory and antioxidant activities.

Table 1.1 - Chemical composition of whole pumpkin seed and hulled seed (Elinge *et al.,* 2012).

Composition	Mean value ± S.D.	
	Whole pumpkin seeds	Kernel
Moisture, %	5.53±0.26	4.43±0.44
Crude protein, %	28.90±1.36	31.98±1.18
Crude fat, %	31.75±0.45	38.29±1.51
Crude fiber, %	4.59±1.01	4.26±0.43
Ash, %	6.90±0.14	2.36±0.10
Total Carbohydrates, % (by difference)	27.86±1.50	33.11±2.94
Phytic acid, %	1.867±0.01	1.547±0.03

However, among the predominant fatty acids in pumpkin seeds we find linoleic and oleic acids, which belong to the unsaturated fraction and account for 73% of the lipid fraction. In contrast, among the saturated fatty acids, stearic and palmitic acids are the most abundant (Table 1.2).

Table 1.2 - Fatty acid composition of deshelled pumpkin seeds (Widy-Tyszkiewicz *et al.,* 2012).

Fatty acid	Mean value (%)
Myristic ($C_{14:0}$)	0.18±0.03
Palmitic ($C_{16:0}$)	16.41±0.95
Stearic ($C_{18:0}$)	11.14±1.03
Palmitoleic ($C_{16:1}$)	0.16±0.04
Oleic ($C_{18:1}$)	18.14±0.60
Erucic ($C_{22:1}$)	0.76±0.13
Linoleic ($C_{18:2}$)	52.69±0.92
Linolenic ($C_{18:3}$)	1.27±0.22
Total saturated	27.73±1.8
Total unsaturated	73.03±0.78
Monounsaturated	19.06±0.49
Polyunsaturated	53.97±1.15

Pumpkin seeds have many health benefits, they contain a variety of minerals such as magnesium, manganese, copper, and zinc (Table 1.3). Pumpkin seeds have an antiparasitic effect due to the presence of the amino acid cucurbitin.

Table 1.3 - Mineral composition of Cucurbita pepo L. seeds (mg / 100 g dry weight) (Elinge et al., 2012).

Element	concentration
Ca	9.78±0.03
Mg	67.41±0.05
Na	170.35±0.08
K	237.24±0.09
P	47.68±0.04
Fe	3.75±0.02
Zn	14.14±0.02
Mn	0.06±0.01
Co	2.17±0.02

1.1.4 Oil-bodies

Seeds store oils in subcellular particles called oil bodies (Figure 1,.3). The organelles offer ample area for fast mobilization of oil (TAG) throughout spermatophyte growth. Oil bodies are the best organelles in plant cells. every oil body incorporates a hydrophobic TAG matrix covered by a monolayer of phospholipids (PL) with embedded proteins. These proteins, called oleosins, are many and completely cover of the oil body.

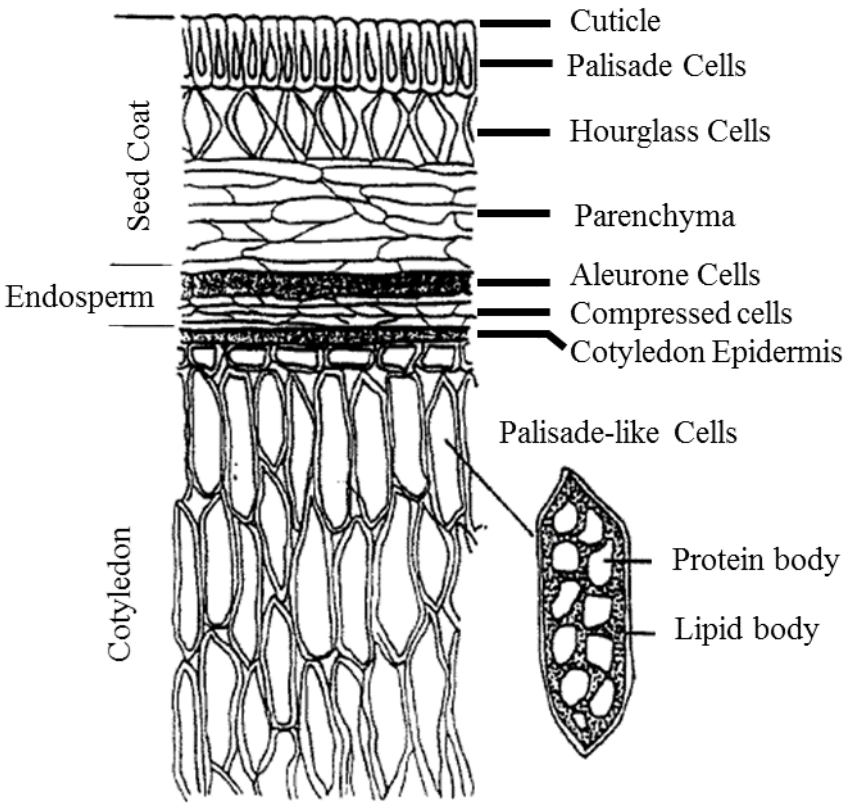


Figure 1.3 - Cotyledon and endosperm microscopic structure of soybean grain (Rosenthal et al., 1996).

The oil bodies are organelles contained in the seeds of all plants (Figure 1.4); they are more present in oil seeds, but they are also found, for example, in corn, and are evidently present in different tissues in varying concentrations and sizes.

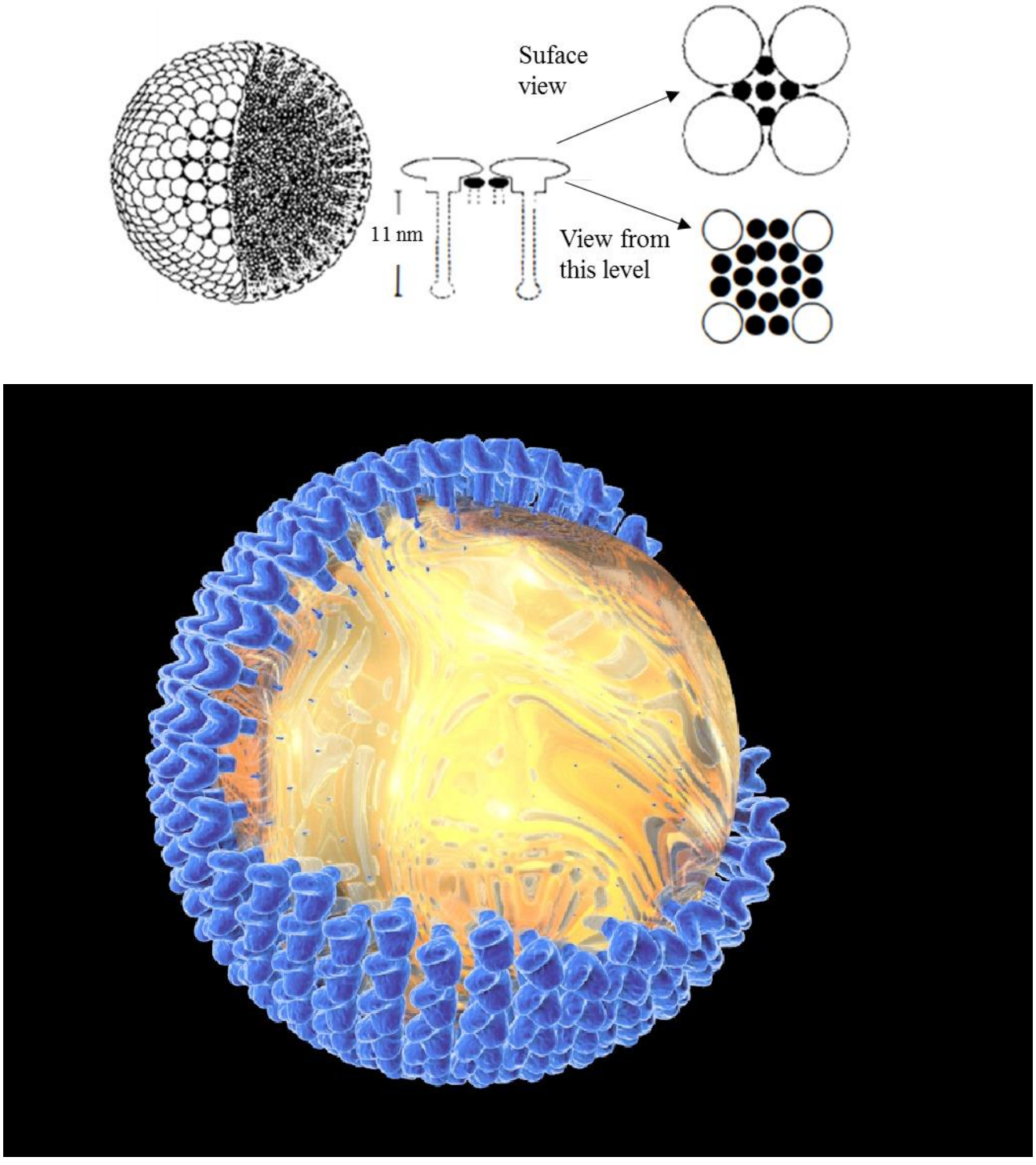


Figure 1.4 – Mais oil bodies reconstruction and 3D model.

They serve as a reserve when the seed needs to germinate, this energy source is obtained by the degradation of triglycerides (TAGs) present in the oil bodies. The oil bodies are in direct contact with the endoplasmic reticulum because these oil bodies originate from the endoplasmic reticulum. They are composed of triglycerides synthesized by enzymes of the endoplasmic reticulum and by a special protein kit consisting of oleosins. Oleosins are low

molecular weight proteins (13 kD to 16 kD). The surface of oil bodies is completely covered by oleosins, with N- and C-terminal ends in the cytosol and a long hydrophobic domain facing the interior of the oil body, where triglycerides are located (Huang 1992).

1.2 Extraction techniques for edible oil

In the food industry, the main use of oilseeds concerns the production of edible oils, for the extraction of which various processes can be used whose general operations can be outlined in Figure 1.5. Methods for extracting the oily component from plant matrices (fruits and seeds) involve the use of mechanical systems (physical methods) or solvents (chemical methods).

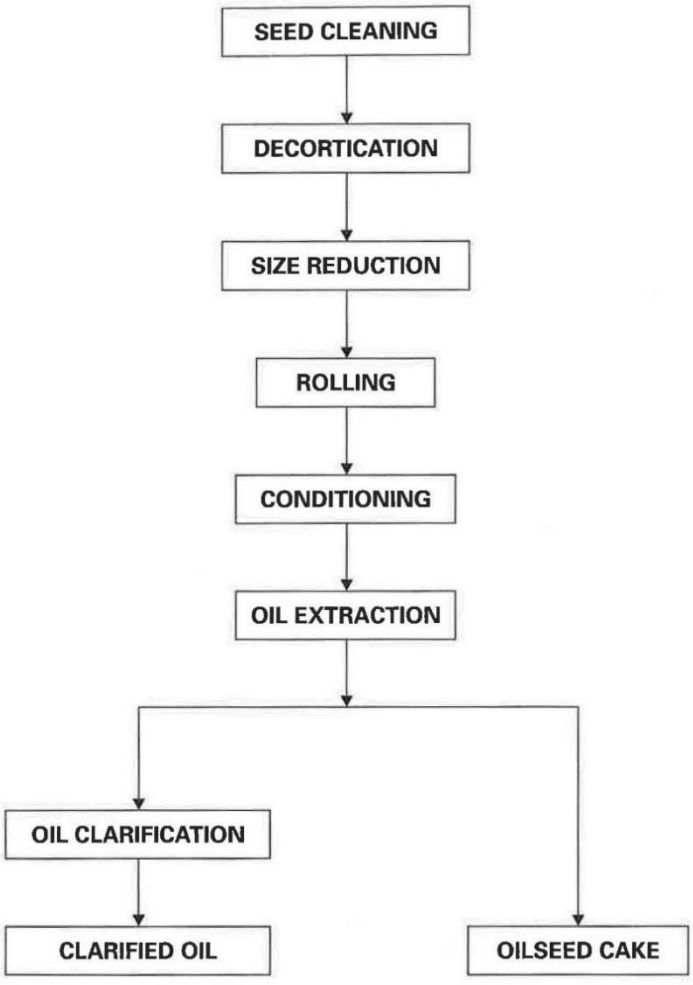


Figure 1.5 - Main stages of the oil extraction process from oleaginous plants (the actual process varies according to the types of seeds and the method chosen) (Head *et al.,* 1995).

Industrial processes for the extraction of edible oil from oilseeds generally use a two-step process (extraction and refining). Oil can be extracted from seeds either by pressing (using a screw press) or by the use of solvents (mainly by percolating the solvent through the prepared seeds). Hexane is the preferred solvent; hexane-based processes have been in commercial operation for a long time. For such processes, it is possible to achieve oil yields in excess of 95% with a solvent recovery of over 95%. The oil produced then goes through a series of refining pro-cesses to remove unwanted components which may affect taste, smell, appearance or storage stability. Examples include:

- Degumming, which removes a range of substances from the oil which would separate out on storage.
- Neutralisation, which reduces free fatty acids (FFA)and the oxidation products of FFA.
- Deodorisation, which reduces the level of FFA and removes odours, off-flavours and other volatile components from the oil to ensure the oil has an acceptable taste and shelf-life.

The extraction of oil from various seeds and fruits, unlike olives, aims solely at obtaining the highest possible yield, since the oil must still undergo rectification, however. This means that critical processing operations can be carried out in a drastic way, but always to the detriment of the quality of the oil obtained. Edible oils account for 2/3 of the total volume of oils produced and are used for direct use or in industry for margarine, mayonnaise, bakery products, while the non-edible oils, which account for the remaining 1/3, are used in the production of various products, including paints, detergents and in the cosmetics sector.

For centuries, various methods have been used to extract oil from oilseeds. The purpose of these extraction methods has been to optimize the process by harvesting the maximum amount of oil present in oilseeds at minimum cost. Currently, there are four basic methods of vegetable oil extraction in the world: chemical extraction, supercritical fluid extraction, steam distillation, and mechanical extraction. New methods are constantly being developed that eliminate the critical points of traditional extractions, among which aqueous extraction assisted by enzymes is a promising strategy.

1.2.1 Mechanical extraction

The most common method of extracting edible oil from oleaginous material, which has been practiced for thousands of years, is mechanical pressing of oilseeds. Mechanical extraction is based on compression of the oleaginous material. Through pressing, the oil is separated from the oleaginous material (solid-liquid mixture) by external compressive forces in machines called presses. This method guarantees the extraction of an uncontaminated cake, low in fat and high in protein, at a relatively low cost. The disadvantage of this method is the low efficiency.

1.2.2 Solvent extraction

Solvent extraction can be done by immersion or percolation. In the first case, the matrix is completely immersed in the solvent, either statically or dynamically, for the time necessary for extraction, in the second, however, the matrix is contained in perforated baskets and is continuously sprayed from above by the solvent which crosses and then comes out from the lower part of the basket.

In order to improve the efficiency of solvent extraction and reduce the processing cost, extrusion process has been used as a pre-treatment of oilseeds. The advantages of using this pre-treatment are:

- Increase in the speed of oil extraction,
- increasing the amount of oily material present in the extractor
- Increase of the extractor capacity,
- Reduction of steam requirement in the desolventizer.

Supercritical fluid extraction (SFE) is a similar technique to conventional solvent extraction, but the solvent is not a liquid but a gas above its critical point. The supercritical fluid most commonly used in oil extraction is CO_2, primarily because it does not remove molecular oxygen and is not a toxic liquid. The extraction efficiency depends on the temperature, pressure, contact time between the extraction fluid and the oil material, and the solubility of the oil in the extraction fluid (Ionescu et al., 2014). Moreover, organic solvents such as hexane can contribute to industrial emissions of volatile organic compounds (VOCs). The production of VOCs in the conventional process is of particular concern because they can react with other

pollutants in the atmosphere to produce ozone and other photochemical oxidants that can be dangerous to human health and cause damage to crops. In addition, VOCs are themselves "greenhouse gases"; some are carcinogenic and have toxic properties.

1.2.3 Aqueous extraction

Aqueous oil extraction is now an established technology in the fat and edible oil industry, as it offers many advantages over conventional extraction. Among them, the most important is certainly the low degree of contamination. Unfortunately, however, it is an underdeveloped technology due to its low extraction yield. For this reason, another aqueous extraction method is the one in which enzymes are added, capable of breaking the cell structures and extracting a larger amount of oil. Moreover, in this case, the aqueous part of the process simultaneously extracts the protein part of the oil seeds. This method is used by large companies as the process produces high quality products and increases the yield. The seeds are placed in water and enzymes are added to digest the solid material. Finally, the separation of the remaining enzymes and oil is carried out using a liquid-liquid centrifuge. Sometimes, after the centrifugation, a demulsification requirements is needed to recover oil when emulsions are formed.

1.3 Enzymes in food processes

Enzymes are proteins with powerful catalytic activity. They are synthesized by biological cells and in all organisms, they are involved in chemical reactions related to metabolism. Therefore, enzyme-catalyzed reactions also proceed in many foods and thus enhance or deteriorate food quality. Relevant to this phenomenon are the ripening of fruits and vegetables, the aging of meat and dairy products, and the processing steps involved in the making of dough from wheat or rye flours and the production of alcoholic beverages by fermentation technology. Enzyme inactivation or changes in the distribution patterns of enzymes in subcellular particles of a tissue can occur during storage or thermal treatment of food. Since such changes are readily detected by analytical means, enzymes often serve as suitable indicators for revealing such treatment of food. Examples are the detection of pasteurization of milk, beer or honey, and differentiation between fresh and deep-frozen meat or fish. Enzyme properties are of

interest to the food chemist since enzymes are available in increasing numbers for enzymatic food analysis or for utilization in industrial food processing. (Belitz *et al.,* 2009).

Food processing through the use of biological means is a consolidated approach. The trend of designing and implementing processes involving the use of enzymes has increased steadily over time. Enzymes used in large-scale applications, including food production, account for a portion of this market. These include enzymes used in bakery products, beverages and beer, dairy products, dietary supplements, fats and oils. A representative list of enzymes and their role in food and feed processing is presented in Table 1.4 (Fernandes, 2010).

Table 1.4 – Enzyme utilized in food and feed processing (Fernandes 2010).

Class	Enzyme	Role
Oxidoreductases	Glucose oxidase	Dough strengthening
	Laccases	Clarification of juices, flavor enhancer (beeer)
	Lipoxygenases	Dough strengthening, bread whitening
Transferases	Cyclodextrin Glycosyltransfeases	Cyclodextrin production
	Fructosyltransefases	Synthesis of fructose oligomers
	Transglutaminases	Modification of viscoelastic properties, dough processing, meat processing
Hydrolases	Amylases	Starch liquefication and saccharification; Increasing shelf-life and improving quality by retaining moist, elastic, and soft nature; Bread softness and volume, flour adjustment, ensuring uniform yeast fermentation; Juice treatment, low calorie beer
	Galactosidase	Viscosity reduction in lupin and grain legumes used for animal feed, enhanced digestibility
	Glucanase	Viscosity reduction in barley and oat used for animal feed, enhanced digestibility
	Glucoamylase	Saccharification
	Invertase	Sucrose hydrolysis, production of invert sugar syrup
	Lactase	Lactose hydrolysis, whey hydrolysis
	Lipase	Cheese flavor, in-situ emulsification for dough conditioning, support for lipid digestion in young animals, synthesis of aromatic molecules
	Proteases (namely, chymosin, papain)	Protein hydrolysis, milk clotting, low-allergenic infant-food formulation, enhanced digestibility, and utilization, flavor improvement in milk and cheese, meat tenderizer prevention of chill haze formation in brewing
	Pectinase	Mash treatment, juice clarification
	Peptidase	Hydrolysis of proteins (namely, soy, gluten) for savoury flavors, cheese ripening
	Phospholipases	In-situ emulsification for dough conditioning
	Phytases	Release of phosphate from phytate, enhanced digestibility
	Pullulanase	saccharification
	Xylanase	Viscosity reduction, enhanced digestibility, dough conditioning
Lyases	Acetolactate decarboxilase	Beer maturation
Isomerases	Xilose (Glucose) isomerase	Glucose isomerization to fructose

The enzymes use in food and feed processing is readily understandable given their specificity, their ability to operate under varying pH, temperature, and pressure conditions while exhibiting a high number of activities and high turnover, and their high biodegradability. Enzymes are also generally considered as a natural product. In legislation, enzymes used in food can be divided into food additives and processing aids. Most food enzymes are considered processing aids for food production, and only a few are used as additives, such as lysozyme and invertase.

Enzymes used in food processing are usually sold as enzyme preparations containing not only the desired enzyme but also metabolites of the production strain and many other substances added as stabilizers. All these substances should be safe under good manufacturing practice guidelines (Li e*t al.,* 2012).

In industrial practice, there are many substrates (carbohydrates, proteins, fats, fibers) on which different enzymes act according to their specificity.

Enzymes are classified according to the reaction they catalyze:

- oxidoreductase: electron transfer.
- transferases: Group transfer reactions.
- hydrolase: hydrolysis reaction.
- lyase: addition of double bond groups or formation of double bonds by removal of groups.
- isomerase: transfer of groups within molecules to form isomers.
- ligase: formation of C-C, C-S, C-O, C-N bonds, by means of condensation reactions coupled to the cleavage of ATP.

1.3.1 Hydrolases

Cellulase

Cellulases (EC 3.2.1.4) are a family of enzymes, mainly produced by fungi (Ahmed & Bibi 2018) and bacteria (Sadhu & Maiti 2013) that belong to the hydrolase family and which catalyze the hydrolysis of 1,4-β-D-glycosidic bonds in cellulose, lichenin, and β-D-glucans cereals. However, forms of cellulase are also present in animal and plant organisms, often different in structure and reaction mechanism. Other names used for cellulase are endoglucanase, Endo-1,4-beta-

glucanase, carboxymethyl cellulase, endo-1,4-beta-D-glucanase, beta-1,4-glucanase, beta-1,4-endoglucan hydrolase. In the nomenclature it defines the reaction catalyzed by this enzyme as an endohydrolysis of the beta 1,4 glycosidic bonds in the cellulose; hydrolysis can also occur in the glycosidic bonds of lichenin and other glucans. Three-dimensional structure of cellulase define by Selvam *et al.* (2017) is shown in figure above.

Cellulases are used in lots of biotechnological applications, such as fiber modification in the paper and textile industries, but they also have great ability in the rising industry of ethanol production from lignocellulose. (Selvam *et al*., 2017)

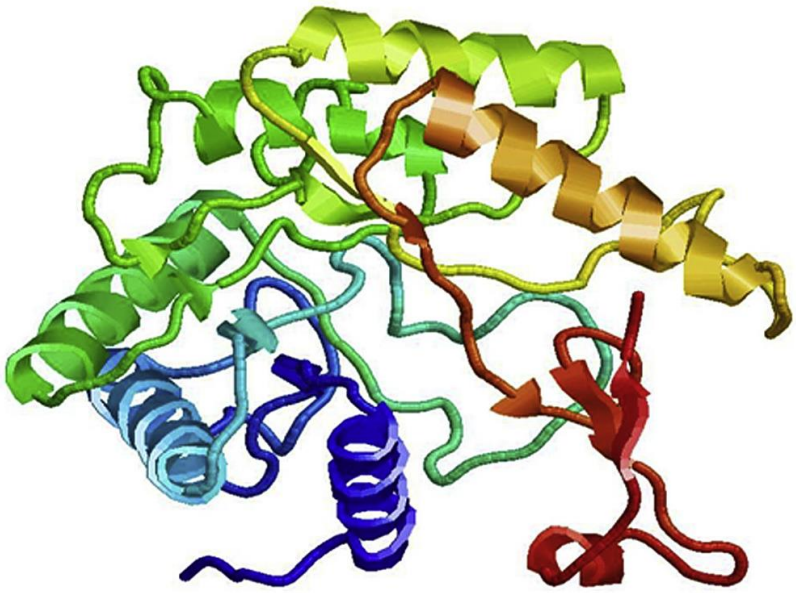


Figure 1.6 - 3D structure of cellulase from Acinetobacter sp (Selvam *et al.,* 2017).

Papain

Papain is a proteolytic enzyme belonging to the class of hydrolases and is extracted from the unripe fruit of papaya. Catalyzes the hydrolysis of proteins, with high specificity for peptide bonds.

Papain (EC 3.4. 22.2) is a cysteine protease isolated from papaya latex, but also contains other cysteine endopeptidases, such as glycyl endopeptidase (lysostafins) and chymopapain.

Papain is used in various biotechnological processes such as the production of soaps, bubble baths and in the food industry in the production of protein hydrolysate, confectionery, beer, dairy and finally in the textile and tanning industries (Omeje et al., 2014).

Proteolytic action occurs preferentially at peptide bonds with basic amino acids, especially arginine, lysine followed by phenylalanine residues (Mamboya, 2012).

Papain consists of a single polypeptide chain, has a molecular weight of 23,406 Da and is composed of 212 amino acids with four disulfide bridges; the residues at positions Gln19, Cys25, His158 and His159 are catalytically important. The graphical representation of the amino acid composition of papain is shown in Figure 1.5.

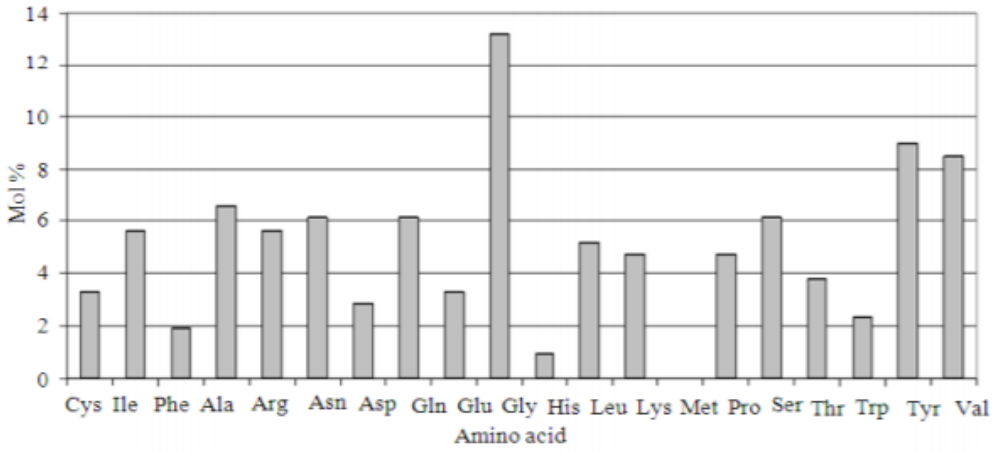


Figure 1.5 – Graphic representation of aminoacidic composition of Papain (Mamboya, 2012).

Papain is a stable and active cysteine hydrolase under a wide range of conditions. It is also very stable at high temperatures.

Papain is denatured with high concentrations of agents such as 8M urea or organic solvents such as 70% EtOH. The optimum pH for papain activity varies over a wide range from 3 to 9 and depends on the substrate; the optimum temperature is 65 ° C. Papain is stabilized by three disulfide bridges: the polypeptide chain is folded in length, creating a strong interaction between the side chains that contributes to the stability of the enzyme.

Its three-dimensional structure (Figure 1.6) consists of two distinct structural domains with a gap between them. This contains the active site, which contains a catalytic dyad that has been compared to the catalytic triad of chymotrypsin. The catalytic dyad consists of the aminoacids cysteine-25 and histidine-159.

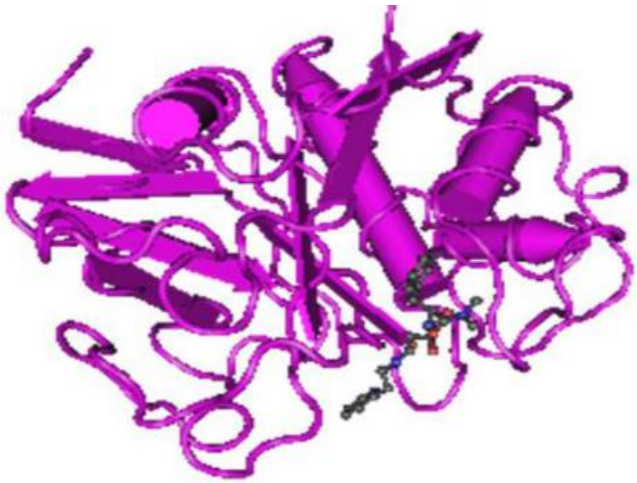


Figure 1.6 – Papain ternary structure (Mamboya, 2012).

Papain is used to tenderize meat; the major meat proteins responsible for tenderness are myofibrillar proteins and connective tissue proteins (Khanna & Panda, 2007). Proteolytic enzymes are used to hydrolyze proteins and papain has been widely used as a common ingredient in brewing and meat processing. The importance of papain in tenderizing meat in the food industry is similar to that of collagenases, which are also used in processing hides. Papain can also serve as a clarifying agent in many food industries processes.

Bromelain

Bromelain has been identified as an active component and as a major protease of *Ananas comosus* (Figure 1.7). Bromelain is a cysteine-proteases in pineapple tissue plant. Because of its anti-inflammatory and anti-cancer activities, as well as its ability to induce apoptotic cell death, bromelain has proved useful in several therapeutic areas (de Lencastre Novaes *et al.,* 2016). Moreover, bromelain has been used commercially in many applications in the food, beverage, tenderization, cosmetic, and textile industries.

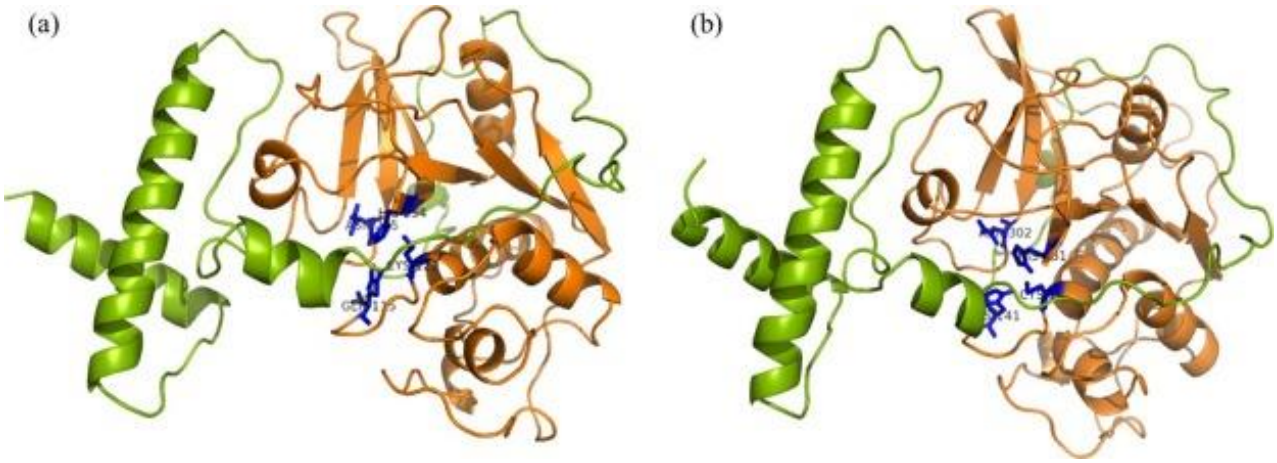


Figure 1.7 Model domain organisation of two different isoform of bromelain by Ramli *et al.* (2018).

Pectinase

For years, researchers from all over the world have been paying special attention to the biotechnological potential of pectinase or pectinolytic enzymes, as they are of great interest at the industrial level. Among these, alkaline pectinases have been the most interesting in the field of biotechnology for major applications in the textile sector, degumming of vegetable raffia fibers, treatment of pectic wastewater, production of paper and fermentation of coffee and tea (Hoondal *et al.,* 2002). Protopectinases, polygalacturonases, lyases and pectin esterases are among the extensively studied pectinolytic enzymes. Alkaline pectinase have significant importance in biotechnological field with their applications in fruit juice extraction and its clarification (Alkorta *et al.,* 1998), scouring of cotton (Calafell *et al.,* 2004), degumming of plant fibers (Brühlmann *et al.,* 1994), waste water treatment (Tanabe *et al.,* 1988), tea and coffee fermentations (Haile *et al.,* 2019), in poultry feed additives and in the alcoholic beverages (Jayani *et al.,* 2005) and food industries (Pasha *et al.,* 2013). Another important industrial process that uses pectinases is the aqueous extraction of oil from olives, to obtain virgin or extra-virgin oil as also reported by Najafian *et al.,* (2009). *Aspergillus niger* is the most used fungal species for industrial production of pectinolytic enzymes.

1.4 Nanotechnology

Nanotechnology is an interdisciplinary subject. Indeed, there has been a growing interest in nanotechnologies in the scientific world with contributions from the world of physics, chemistry, biology. This interest extends to the most widespread scientific fields, among which we find medicine, agriculture, food, textile and environment. The contributions from these different disciplines and their complementary nature in the synthesis, characterization, understanding and applications from daily to more complex and sophisticated technologies have implemented the foundations of nanotechnology.

There are many techniques available to synthesize different types of nanomaterials in the form of colloids, powders, wires, thin films etc. Some of the already existing conventional techniques to synthesize different types of materials are optimized to get novel nanomaterials and some new techniques are developed.

The methods for synthesizing nanomaterials can be through physical methods (i.e., based on temperatures, evaporation, mechanical methods, etc.), chemical methods (such as colloids, microemulsions, etc.), or even biological methods (which use biological sources such as bacteria or DNA to synthesize nanomaterials).

In general, the synthesis of nanomaterials can be done in two different and opposite ways: starting from large materials and breaking them down to obtain nanomaterials (top down) or starting from the individual elements that will form the nanomaterials and assembling them (bottom up). Moreover, nanomaterials can be both organic and inorganic and can concern different scientific fields.

As far as this work is concerned, the nanomaterials analyzed and studied are mainly inorganic in nature. Indeed, the focus has been on magnetite nanoparticles.

1.4.1 Fe_3O_4 Magnetic nanoparticles

As already discussed above, nanomaterials are widely used in different fields of applications and for different purposes. Magnetite nanoparticles are widely used and known in literature. Primarily, they are used for medical purposes, both diagnosis or therapy (Ghazanfari *et al.,* 2016; Mccarthy & Weissleder 2008) or as a drug carrier (Yuanbi *et al.,* 2008); for environmental issue (Liu *et al.,* 2008) and so on. All the purposes for which magnetite nanoparticles (MNP) are used dur their main cause to a very important function inherent in

their structure, namely the ability to bind molecules easily. Moreover, due to their reactivity to magnetism, magnetite nanoparticles can be transported, conveyed, or tracked by the use of magnets. Indeed, there are many scientific articles in the literature that show many types of molecules bind to MNP, all of which differ from each other to perform the desired function. The bond that is formed is very often a covalent bond, which allows for good stability of the particle created. In addition, once stabilized, these particles usually remain unchanged because they are inorganic.

2. Aim of the Work

The objective of this work was to develop a new extraction system able to improve the extractability and yield of oil from oilseed. The studio is at the heart of an industrial project to recycle the by-products of a company that processes oilseeds. In this context, the question arises of assessing the nature of the by-product produced by a leading company in the sector. Given these assumptions, the aim of this work was to develop a process that would provide an alternative to the use of solvents that are difficult to dispose of or of high pressures that produce an oil of poor quality. In addition, the process should be based on the principle of circular economy and environmentally friendly. Therefore, in this case, it was decided to optimize the process by using immobilized enzymes that would allow their recovery and reuse to reduce both costs and waste. Finally, some ideas for the use of the waste generated by the developed process were evaluated.

3. Materials and Methods

3.1 Materials

By-product and whole deshelled pumpkin seeds and walnut kernels were provided by Besana S.p.A. Papain (EC 3.4.22.2, 100000 U/g), Cellulase (EC 3.2.1.4, 20000 U/g), Bromelain (EC 3.4.22.33, 2400 GDU/g), Laccase (EC 1.10.3.2;10000 U/mL), Xylanase (EC 3.2.1.8; 20000 U/g); and Pectinase blend for oil (Pectinase ≥ 20000 U/g; Cellulase ≥ 10000 U/g; Hemicellulase ≥ 10000 U/g) were obtained from Creative enzyme (Shirley, NY 11967, USA). Viscozyme and the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Precision Plus Protein™ All blue Prestained Protein Standards and Bio-Rad Protein Assay Dye Reagent Concentrate were from Bio-Rad.

3.2 Methods

3.2.1 Walnut and Pumpkin Seeds processing

Pumpkin seeds and walnut kernel by-products were ground to a fine powder by using a coffee grinder at high speed. The obtained flours were used as starting material for the solvent or enzyme-assisted aqueous oil extractions.

3.2.2 Solvent Oil Extraction (SOE) process

For the solvent extraction, 5 g of seeds powder were incubated with 50 mL of n-hexane at 60°C and 150 rpm for 1 hour in orbital shaker (Forma Scientific, USA). The organic phase (n-hexane/oil mixture) was collected after centrifugation at 8000×g for 10 minutes and then the solvent was distilled at 40°C/335 mbar using a rotary evaporator (Heating Bath B-490, Büchi, Switzerland) in order to obtain the extracted oil which was quantified by weighing. Each sample was extracted in three replicates for statistical analysis. The mean value of the recovered oil for each kind of seed was taken as the maximum extractable oil from that seed sample and used to calculate the percentage of oil extraction in enzymatic process.

3.2.3 Enzyme-assisted Aqueous Oil Extraction (EAOE) process

The process steps for the Enzyme-assisted Aqueous Oil Extraction are reported in Figure 3.1 In all the experiments, the ground seeds were dispersed in aqueous phase, incubated in the presence of enzymes and then, the oil was recovered after centrifugation. The amount of extracted oil was quantified by imagine analysis. Several process parameters were evaluated to assess the best conditions in terms of oil extraction yield, such as the type of enzyme, the solid-to-buffer ratio, the incubation time. All the experiments were run in triplicate.

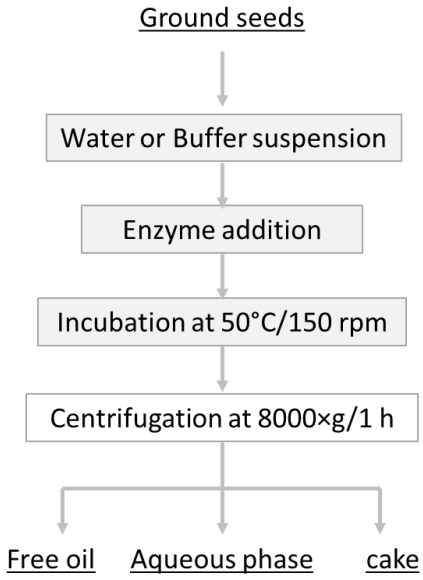


Figure 3.1 - Schematic representation of the process followed for the Enzyme-assisted Aqueous Oil Extraction method. The filled boxes indicate the points where different conditions have been tested for the determination of the best process parameters.

Enzyme choice

The EAOE process was performed with cellulase, papain, bromelain, and "pectinase blend for oil" enzymes. The ground seeds powder (2 g) was dispersed at 1:3 solid-to-buffer ratio in different kind of buffers (50 mM TRIS-HCl, pH 8, and 50 mM sodium acetate buffer, pH 5), according to the optimum pH range for the enzyme activity. Then, the appropriate enzyme was added to each sample at the final concentration of 100 mg/mL. After incubation overnight at 50°C and 150 rpm in orbital shaker, the extracted oil was recovered by centrifugation as described. For the samples subjected to a treatment with two enzymes in sequence, the incubation started with the first enzyme and the second one was added to the mixture 4 h

later. Control samples for Aqueous Oil Extraction (AOE), were also carried out in the same conditions except for the absence of enzymes.

Solid-to-buffer ratio

The EAOE process was also carried out with different ratios of solid-to-buffer (1:2; 1:3, 1:5, w/v), using papain enzyme 20 mg/g (with respect to the solid material) and 50 mM sodium acetate pH 5, as buffer. The samples were incubated for 4, 8, 16, 20, 24 and 30 hours at 50°C and 150 rpm in orbital shaker.

Kinetics of oil extraction

Oil extraction kinetics experiments were conducted as previously described at 1:3 solid-to-buffer ratio/pH 5/50°C/150 rpm, with increasing concentrations of papain (0, 60, 100 ,150, 200 and 300 U/mL). The yield of extracted oil was determined at set times (0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 20, 22 and 24 hours).

3.2.4 Nanotechnology

Synthesis of magnetic nanoparticles

A specific amounts of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) in a molar ratio of Fe^{3+}:Fe^{2+}= 2:1 were dissolved in distilled water and the pH was adjusted to 10.0 with NaOH under continuous stirring. Then, the reaction mixture was heated at 70℃, for 30minutes (Ma et al., 2003, Koneracka *et al. 1999*). The resulting magnetic nanoparticles were collected by centrifugation (10 minutes 8000 rpm) (HERMLE Labortechnik GmbH Z-326-K), washed with distilled water, and stored wetted at 4°C for future analysis.

Synthesis of dialdehyde starch

The dialdehyde starch (DAS) was synthesized starting from 4 different soluble starches (from potato, corn, rice and wheat) and sodium periodate ($NaIO_4$) as oxidant agent. 15.5 g sodium periodate were completely dissolved in 200 mL of distilled water at 20°C, then 60.0 g of the specific starch were added under vigorous mechanical stirring. The reaction was kept at 35°C and pH 7.0 during 4 or 18 h, after that the slurry was centrifuged per 10 min at 2000 rpm (HERMLE Labortechnik GmbH Z-326-K) and the pellet was washed with 200 mL of distilled

water at 35°C. The obtained DAS powders were dried for 24 h at room temperature (Zhang et al. 2007). The carbonyl content (CHO%) of the dialdehyde starch was determined according to Wing and Willett (1997). Briefly, 0.25 g of dry sample were completely dissolved in distilled water (75 mL) and the pH was adjusted to pH 3.2 with 0.15 M HCl, before adding 15 mL of hydroxylamine hydrochloride. The solution was heated to 40°C for 4 h and after that was rapidly titrated to pH 3.2 with 0.15 M HCl. A water sample and a starch sample were used as controls. The CHO% was calculated as:

$$CHO(\%) = \frac{N * 0{,}028 * (Vcontrol - Vsample) * 100}{m}$$

where *N* is the normality of titrant used for titration, *0,028* are the milliequivalent of the carbonyl groups, *V* is the volume (mL) of titrant used for the control or the sample, and *m* is the mass of the sample expressed in grams. The DAS showing the higher percentage of carbonyl group was chosen for the subsequent analysis (Figure 3.2).

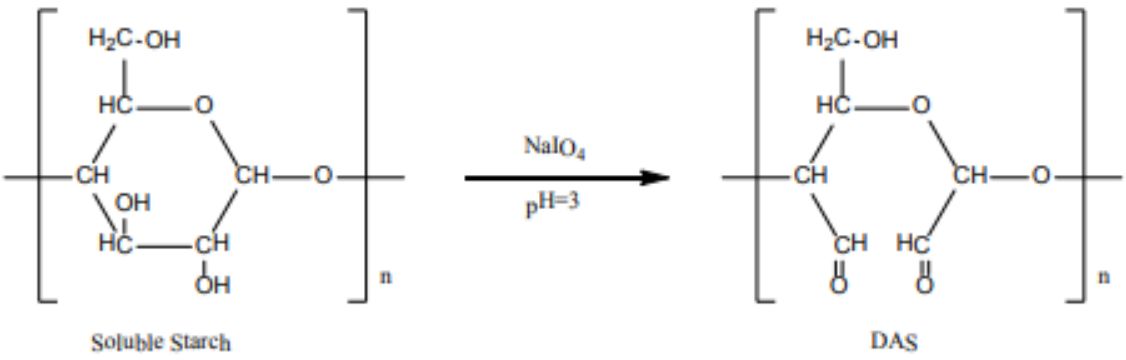


Figure 3.2 The soluble starch reaction with $NaIO_4$ as oxidant for DAS synthesis.

Nanoparticle coating with dialdehyde starch

50 mg of Fe_3O_4 magnetic particles were dispersed in 25mL of distilled water and then 7,5 mL of 1% DAS aqueous solution were added. The mixture was sonicated of 5 minutes (repeating duty cycle 0.5 sec-High; power level maximum; LabSonic U, B.Braun) before adding 1µL of epichlorohydrin and starting an incubation of 6h at 40°C and 150 rpm in orbital shaker (Forma Scientific, USA). Finally, the coated magnetic particles were collected by centrifugation (10 minutes 8000 rpm-HERMLE Labortechnik GmbH Z-326-K).

Enzyme binding

For the papain immobilization, 50 mg of DAS-coated particles were dispersed in 15 mL of 2% papain solution. The binding was carried out at 37°C for 6h with stirring in orbital shaker (Forma Scientific, USA) at 150 rpm. The supernatant was removed by centrifugation (5 min 13000 rpm-HERMLE Labortechnik GmbH Z-326-K) and stored at 4°C for further analysis together with the immobilized papain.

The amount of papain immobilized to MNP was determined by measuring the initial and final concentrations of papain in the supernatant, after MNP centrifugation using the Bradford method with the papain stock solution as a control.

3.2.5 Papain Enzymatic study

Enzymatic assays and specific activity determination

The proteolytic activity of papain was followed evaluating the release of tyrosine equivalents in assays mixture after incubation with the enzyme. The papain was tested in two different buffers (50 mM TRIS-HCl, pH 8 or 50 mM sodium acetate, pH 5) and in the presence or absence of 5 mM cysteine and 2 mM EDTA as cofactors. Distilled water in substitution of buffers was used for the control samples. The assays were performed with 1% of papain and 0.25% of sodium caseinate used as substrate, in a final volume of 0.5 mL. After 10 min of incubation at 50°C and 150 rpm, the reaction was stopped by adding 0.5 mL of cold TCA 10% and then the samples were incubated at 4°C for 10 minutes and centrifuged at 14000×*g* for 5 min. The absorbance at 280 nm of the supernatant was taken for the enzymatic activity evaluation. The assay conditions in which the maximum proteolytic activity of papain was measured were used for the determination of its specific activity. One unit of enzymatic activity was defined as the amount of enzyme that produce an absorbance change of 0,1 OD under the established assay conditions.

Immobilized papain was also tested in optimal conditions for the enzymatic activity.

Kinetic parameters determination

The kinetic parameters of both free or immobilized enzyme were determined through assays at increasing sodium caseinate concentrations, from 0.1 to 0.5% in the best fitting assay conditions (50 mM TRIS-HCl, pH8/ 5 mM cysteine/ 2 mM EDTA). The V_{max} and K_m values,

respectively the maximum reaction rate attained at infinite substrate concentration and the Michaelis-Menten constant were evaluated using the Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}\,[S]} + \frac{1}{V_{max}}$$

where [S] is the substrate concentration.

Re-usability of immobilized papain

The reusability of papain immobilized on MNP was analysed at optimal assay conditions with 0.5 % sodium caseinate substrate, for 10 min incubation intervals and 11 successive cycles. After each interval, the papain-MNPs were collected by centrifugation at 4000xg75 min, and then resuspended in a new assay mixture; while the supernatant was treated with TCA at final concentration of 5% and then used for the determination of tyrosine equivalent as above described. Free papain and simple DAS-MNP were used as controls. All the experiments were done in triplicate for the statistical analysis.

3.2.6 Oil quality analysis

Titrable acidity

The acidity of oil samples was calculated trough the titrable method (Reg. CEE 2568/91, allegato II). 1 g of oil was added to 10 mL of ethanol 96%- ethyl ether solution (1:2) containing 2-3 drop of phenolphthalein. This solution was titrated with NaOH 0.1 N to the toning of the solution.

The acidity was expressed as % of oleic acid obtained by the following formula:

$$FFA\ \%\ (g\ of\ oleic\ acid\ in\ 100\ g\ of\ oil) = \frac{\mathrm{V} * \mathrm{N} * 28.2}{\mathrm{m}}$$

Were V is the volume, in mL, of NaOH used for the titration, N is the normality of NaOH solution and “m” the mass in grams of the oil sample.

Peroxides number determination

Peroxides were determined with the Ferric Thiocyanate Method (Shantha & Decker 1994; Mihaljevic et al., 1996). 50 mg of oil sample were added to 450 µL of acetone and 1 mL of ferrous reagent (iron ammonium sulphate 0.1% /ammonium thiocyanate 0.6%). After 3 minutes the absorbance was read at 485 nm. The blank was done without oil. The peroxide

value was calculated as milliequivalents per kg of oil. All the samples were analysed in triplicate.

Preparation of phenolic extracts

Methanolic extracts from aqueous, and enzyme assisted extracted oils were prepared as follows: The samples (200 mg) were added to 400 µL of acetone and 1 mL of 1 mL of 7:3 (v/v) methanol-water solution, and mixed for 30 sec on vortex, after which they were centrifuged at 4000xg for 10 min. Then, the upper phase of n-hexane, containing the oil, was discarded and the methanolic extract, containing the antioxidant compounds, was stored at -18°C until the following analyses. Solvent extracted oil and commercial oil were used for the comparison.

Total phenolic compounds

Total phenolic compounds were analysed with the Folin-Chocalteu method (Matthaus 2002). 50 µL of methanolic extract were added to 880 µL of deionized water and 70 µL of Folin-Chocalteu reagent. The solution was shacked at vortex for 1 minute and incubated in dark at room temperature for 5 minutes. Then, 70 µL of Na_2CO_3 7,5% (w/v) and 530 µL of deionized water were added and the samples were incubated at 45°C for 15 minutes in dark condition; finally, the absorbance at 760 nm was measured. The total amount of phenolic compounds in the sample was expressed as mg of gallic acid equivalents (GAE) by extrapolation from a calibration curve obtained with known concentrations of gallic acid.

Antioxidant activity

The antioxidant capacity of methanolic extracts from aqueous and enzyme assisted extracted oils was evaluated by DPPH scavenging activity (Matthaus 2002). 50 µL of methanolic extract were mixed with a methanolic solution of DPPH at a final concentration of 1 mM. The mixture was vigorously shaken and incubated at room temperature for 1 h, then the absorbance at 517 nm was measured. The antioxidant capacity of samples was calculated according to the formula:

$$\%DPPH = \frac{A_0 - A_s}{A_0} * 100$$

were A_0 is the absorbance of DPPH solution and A_s the absorbance of the oil sample.

3.2.7 Protein analysis

Preparation of protein isolates

Solid cake and aqueous slurry residues obtained respectively after solvent (SOE) and aqueous (AOE) or enzyme-assisted aqueous (EAOE) oil extraction were used as starting material for alkaline extraction and isoelectric precipitation of proteins as described elsewhere (Shevkani & Singh, 2014). These defatted materials were dispersed in deionized water at a final ratio of 1:10 and settle down for 15 minutes to solubilize the albumin fraction. Then, the pH of the dispersions was raised up to 10.0 by using 1 N NaOH. The dispersions were stirred for 1 h at room temperature and then centrifuged at 8000×g for 20 min. Supernatants were collected and the pH was adjusted to 5 by adding drop to drop 1N HCl while stirring. The samples were incubated at 4°C overnight and finally centrifuged at 8000×g for 20 min. The pellets, containing the protein isolate, were resuspended in water, and neutralized at pH 7, then freeze-dried and stored in a dry place. Aliquots of all fractions (precipitates and the supernatants) were also collected and analysed by SDS-PAGE.

Bradford Assay

The protein content of solubilized samples was determined by the Bradford method using BSA as protein reference (Bradford, 1976).

SDS-PAGE

The SDS-PAGE was performed according to Laemmli (1970) using 15% resolving gel and 5% stacking gel at constant voltage (80 V). The marker used was the Precision Plus Protein^{tm} All blue Prestained Protein Standards Bio-Rad. The samples were prepared in reducing buffer containing β-mercaptoethanol and then denaturated at 100°C for 2 minutes. After the electrophoretic separation of proteins, the gel were stained with Comassie brilliant blue R-250 0,5% (Methanol:Acetic Acid :Water 5:1:4) and destained in Methanol:Acetic Acid:Water 1:1:8. A digital camera was used to acquire the image of the gels.

Total organic nitrogen determination

The total organic nitrogen determination in both seed flours and protein isolate samples was carried out through Kjeldahl method (Kjeldahl, J. Z. Anal. Chem. 1883, 22, 366). Briefly, the samples, were opportunely weighted and added to 7 g of potassium sulphate and 6 mg of selenium powder as a catalyst. Then 7 mL of sulfuric acid and 5 mL of hydrogen peroxide were added, and sample were subjected to mineralization in SpeedDigester K-425 and Scrubber K-415 (Büchi, Switzerland) following a thermal ramp of 180°C/10 min, 350°C/15 min and 480°C/ 2h. After mineralization, the samples were suspended in 50 mL of 32% NaOH (w/v) and distilled in 70 mL of 4% (w/v) boric acid with a Distillation Unit K-350 (Büchi, Switzerland).
The total obtained ammonia [W(N)] was determined by potentiometric titration to pH 4.6 with 0.1 N HCl and calculated as:

$$W(N) = \frac{\left[V_{(HCl\ sample)} - V_{(HCl\ blank)}\right] * F * c * M(N)}{m * 1000}$$

Where:

Formula		*notes*
$V_{HCl\ sample}$	Volume of HCl used for sample titration	mL
$V_{HCl\ blank}$	Volume of HCl used for blank titration	mL
F	molar reaction factor	**(HCl=1)**
c	molar concentration of HCl	mol/L
M(N)	molar weight of N	**14,007 g/mol**
m	weight of sample	g
1000	conversion factor	from mL to L

The organic nitrogen (%N) and protein (%P) contents in samples were respectively estimated as:

$$\%N = W(N) * 100\%$$

$$\%P = W(N) * 5.7 * 100\%$$

3.2.8 Technological properties

Protein solubility

The protein solubility at different pH, from 2 to 10, was determined through Bradford assay (Shevkani *et al.,* 2015). 200 mg of protein isolates were dissolved in 20 mL of deionized water and the pH adjusted in a range to 2, 4, 6, 8 and 10 with 0,1 N of HCl or NaOH or solution. The samples were shaked for 1 hours and then centrifuged at 8000×*g* for 10 minutes. The supernatant was collected, and the protein concentration was measured by Bradford assay.

Water absorption capacity (WAC)

The water absorption capacity of proteins was determined using the method described by Rodriguez et al. (2005); the protein samples (50 mg) were mixed with 750 µL of distilled water and vortex-shaked for 1 min. The protein suspensions were incubated at room temperature for 30 min and then centrifuged at 3000 rpm for 20 min at RT (Microfuge 18 Beckman Coulter^{tm}). The supernatant was decanted, and the tube was drained at 45° angle for 20 min. Water absorption capacity was calculated by dividing the volume of water absorbed by the weight of the protein sample. All analyses were performed in triplicate.

Oil absorption capacity (OAC)

The oil absorption capacity was determined using the method described by Lin and Zayas (1987); 50 mg of protein sample was vortex-mixed with 750 µL of sunflower oil for 1 min. The emulsion was incubated at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min at RT (Microfuge 18 Beckman Coulter^{tm}). The supernatant was decanted, and the tube was drained at a 45° angle for 20 min. The weight of oil absorbed was divided with the weight of the protein sample, to obtain the oil absorption capacity of the sample.

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined using the method described by Klompong *et al.* (2007). 100 mg of each protein isolate samples were mixed with 10 mL of deionised water, and the pH was adjusted to 2, 4, 6, 8, and 10. Each protein solution was mixed with 1:3 (v:v) of sunflower oil, the mixture was homogenised with Ultraturrax T25 (IKA, Germany) at a speed of 20,000 rpm for 1 min. 50 µL of the aliquot of the

emulsion were transferred (using pipette) from the bottom of the container at 0 and 10 min after homogenisation and mixed with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using UV-Visible spectrophotometer (JASCO V-730). This was used to calculate EAI and ESI using the method suggested by Pearce and Kinsella (1978):

$$\text{Emulsifying activity index (EAI) } (m^2/g): \frac{2*\mathrm{T}}{0{,}25*\mathrm{C}}$$

$$\text{Emulsion stability index (ESI) (min): } \frac{A_{10}*\Delta t}{\Delta A}$$

where *C* is the weight of protein in grams. *T* is the turbidity and 0,25 is a constant defined by the Mie theory for light scattering (Monk et al., 1969): $T = \frac{2{,}303*A_0}{l}$ where 2,303 is a conversion factor to change the base of the logarithms, A_0 is the absorbance at 0 min after homogenisation and *l* is the optical lenght; A_{10} is the absorbance at 10 min after homogenisation; $\Delta t = 10\ min$; and $\Delta A = A_0 - A_{10}$

Foaming properties

Foaming capacity and stability were determined according to the method described by Sze-Tao and Sathe (2000). In a 50 mL falcon-tube, 20 mg of each protein sample were mixed with 20 mL of distilled water, and the pH was adjusted to 2, 4, 6, 8, and 10. These protein solutions were vortex-mixed for 3 min. The shaked protein solutions were photographed with a digital camera to measure the volume of the foam using Adobe Photoshop software. The total sample volume was taken at 0 min for foam capacity and up to 60 min for foam stability. Foam capacity and foam stability were then calculated:

$$\text{Foam capacity (FC) (\%): } \frac{V_a - V_b}{V_b} * 100$$

$$\text{Foam stability (FS) (\%) = } \frac{V_{60} - V_b}{V_b} * 100$$

were V_a is the volume after shaking in mL; V_b is the volume before shaking in mL and V_{60} is the volume after 60 minutes.

Least gelation concentration (LGC)

LGC was determined using the method described by Abbey and Ibeh (1988); different protein samples were mixed with 1 mL of distilled water in a centrifuge tube to obtain 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% concentrations (w/v). The centrifuge tube was heated for 1 h in a

boiling water bath, cooled rapidly under running tap water and further cooled for 2 h in a refrigerator at 4°C. The least gelation concentration was regarded as the concentration at which the sample from the inverted tube did not drop or slide.

4. Results and Discussion

4.1 By-product choice

4.1.1 Besana S.p.A.

During the time spent in the company, different processes were analysed. The semi-finished sector of company was the one that produced the largest amount of waste. So, in this area different types of products have been examined. This area is dedicated to the production of seeds roasted, caramelized, salted, smoked etc. Relevant processes for the recovery of waste by-products are the various types of cuts, such as pieces, flours and slices, and least paste. Finding a use for these wastes is a way for the company to recover all the waste that is not intact. Therefore, the focus was placed on the market demand for the various types of products. Cashews, pistachios, hazelnuts, walnuts, pumpkin seeds etc. are the most popular oilseeds also required in the form of flour or paste. Among the seeds that produce the most waste, based on their fragility, the attention was on walnuts and pumpkin seeds. Given this assumption, walnut and pumpkin seeds were chosen as the subject of this thesis.

4.1.2 Walnut and Pumpkin Seeds characterization

Total oil was determined by solvent oil extraction using n-Hexane (Table 4.1). The solvent oil extraction of walnut samples gave a value about 52,6 g of oil per 100 g of seeds. According to *Amaral et al., (2003), Bada et al., (2010) and Oliveira et al., (2002)*, the total oil yield in walnut varies from 52 to 67 % of weight based on the variety, the cultivar, geographical location, genetics factor, etc. Pumpkin seeds samples gave a value of 36,6 g oil/100 g of product. Pumpkin seeds oil is reportedly present in the range of 27–49 g oil/100 g of pumpkin seeds (Younis *et al.*, 2000; Alfawaz 2004; Fruhwirth and Hermetter 2007; Meru *et al.*, 2018). A second round of SOE on the residual pumpkin seeds and walnut kenerls' cake, does not increases the amount of oil that can be extracted. Thus, a value of 53 g oil/100 g of walnut and 37 g oil/100 g of pumpkin seeds were taken as 100% of oil recovery.

Table 4.1. Chemical composition (%) of walnut kernels and pumpkin seeds. Ash, oil, and protein content were determined by AOAC, SOE and Kjeldahl method, respectively.

Samples	Ash	Oil	Proteins	Carbohydrates[a]
		%		
Walnut kernels	6,85±0,012	52,6±0,84	24,9±0,15	15,65
Pumpkin seeds	3,82±0,015	36,6±0,62	53,4±0,48	6,18

Values are reported as mean ± standard deviation of three replicates (n=3).
[a]Calculated as complement to 100%

Different method can be used to determinate the protein content in food samples. The Kjeldahl and Dumas methods measure nitrogen, Bradford and Lowry method measure the protein solubilized in a solution by spectroscopic analysis. These different methods show different results based on sensitivity and accuracy, the most used AOAC methods for determining total nitrogen is the Kjeldahl method. So, total protein content of walnut and pumpkin seeds was determinate trough total organic nitrogen analysis. The results are reported in Table 4.1.

Walnut protein content was roughly higher than 24% (wt), as reported by *Jahanban-Esfahlan et al., (2019)*. According to *Glew et al., (2006)*, pumpkin seeds protein content was approximately 54% of total weight.

4.2 Study of Enzymatic process

4.2.1 Enzyme-assisted Aqueous Oil extraction (EAOE) process

Conventional oil extraction processes are different depending on the plant. Enzyme-assisted Aqueous Oil extraction (AEOA) was proposed to obtain different edible oil, this method is normally used for fruit processing. Oil from oilseeds can be processed by cold pressing and/or solvent extraction with n-Hexane. AEOA can be an alternative method to extract oil from oilseeds. Generically, an Enzyme-assisted Aqueous Oil Extraction (EAOE) process consists in the dispersion of the ground seeds in aqueous phase containing specific enzymes which promote the disintegration of the tissue structure with consequent outcrop of the oil, which can then be recovered by centrifugation.

Oilseeds are composed mostly of oil from 25 to 75% (Kumar & Sharma, 2008; Sze-Tao *et al.,* 2000) and proteins from 20 to 60% (Rezig *et al.,* 2013; Moure *et al.,* 2006) depending on the seed, a lower part is composed of carbohydrates. Due to the chemical composition of oilseeds, commercial proteases and cellulase enzymes or a mix of them were selected for the AEOA extraction. Mechanical stirring for a defined time is applied to the samples to allow the enzyme to work and, at the end of the process, the centrifugation allows to separate the oil from the aqueous part. Figure 4.1 shows result of EAOE applied to walnut and pumpkin seeds at a laboratory scale: the distribution of different phases after centrifugation is well visible.

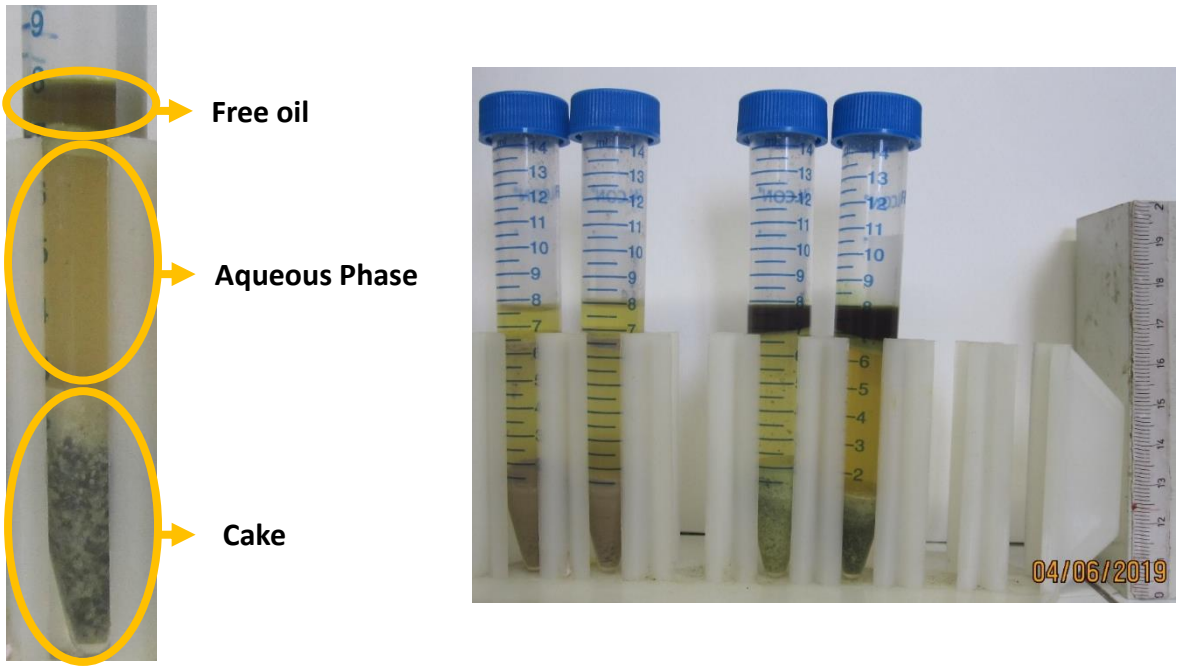


Figure 4.1 Walnut and pumpkin seeds oil extraction with AEOA method.

The use of enzymatic process for oil extraction is essential to reduce or avoid the use of organic solvents, and at the same time it allows to extract and recover the proteins from the samples. In most of the aqueous enzyme-assisted oil extraction procedures reported in the literature, commercial enzyme preparations containing varieties of enzyme activities, such as cellulase, pectinase and amylases, were used. For example, *Yusoff et al. (2017)* used a mixture of protease and cellulase to maximize the oil recovery from *Moringa oleifera*. Moreover, *Lanzani et al. (1975)* used a mixture of protease, cellulase, and α-1,4-galacturonideglucanohydrolase for maximal oil recovery. Furthermore, this method has an added value when the recovery of oil is obtained from by-products.

Enzyme choice

Commercial enzyme preparations, with different enzyme activities, such as cellulase, pectinase and protease, were used to study enzyme-assisted aqueous extraction. Normally the enzyme required for the best oil extraction in term of yield depends on the sample. In this case, preliminary studies were done on the pumpkin seeds by-product. Based on this by-product, papain, bromelain, cellulase, “pectinase blend for oil” and mixture of papain and cellulase were chosen to study the best enzyme for the oil extraction. The oil content in pumpkin seeds is reported from 30% to 50% based on the variety and grow conditions. As shown in Figure 4.2, the solvent extraction with n-hexane was took as a maximum yield of extractable oil from the matrix and was reported as a percentage. Different proteases and cellulases were chosen because of the content of pumpkin seeds, in addition to oil, also a substantial percentage of proteins and polysaccharides. All the commercial enzymes were used in optimal conditions as reported in commercial data sheet. A control was done without enzymes. The highest oil extraction with EAOE method was reached when papain, a protease, was added to the sample, showing a recovery of 80% of total extractable oil (Figure 4.2). Moreover, as evidenced from the Figure 4.2, the combination of papain and cellulase in the same EAOE process did not showed improvement in oil extraction. A parallel set of experiments was carried out on walnut kernel by-products subjected to EAOE process with the same enzymes, producing similar results. As for the pumpkin seeds, also for walnut kernel the papain resulted the most effective enzyme, showing an oil recovery of about 80% (data not shown).

Given these results, papain was then chosen as model enzyme and used for the following experiments.

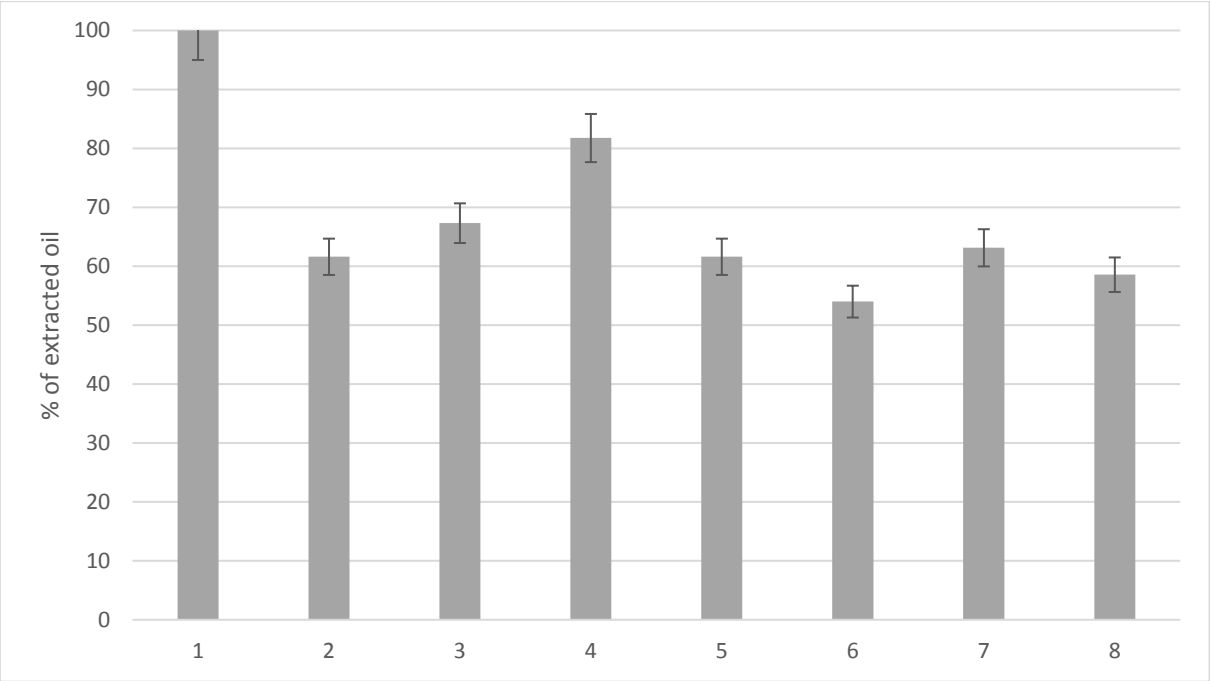


Figure 4.2 EAOE on pumpkin seeds by-products performed with different kind of enzymes. The % of extracted oil is referred to the maximum extractable oil recovered with the solvent extraction (SOE) in n-Hexane (sample 1). Sample 2, control process (without enzymes); samples from 3 to 6, EAOE treated with cellulase, papain, bromelain and "pectinase blend for oil" respectively; sample 7, EAOE treated with papain (4 hours) and then cellulase; sample 8, EAOE treated with cellulase (for 4 hours) and then papain. Values are reported as mean ± standard deviation of three replicates (n=3).

Solid-to-buffer ratio

As shown in Figure 4.3 before, papain was the best enzyme for the oil extraction. Commercial papain used in this process works at pH 5 and 50°C as optimal conditions reported in datasheet. As reported from *Sharma et al. (2002)*, that worked with peanut, the maximum recovery of oil was obtained when the pH used for the extraction is near to the isoelectric point of the protein, that for pumpkin seeds is pH 5,5. So, fixed these parameters, the ratio between solid and liquid was evaluated. As shows in the figure 3.3, after 8 h the recovery of oil is higher than 50% of extractable oil for the solid-to-buffer ratio of 1:2 and 1:3, instead of 1:5 that reach the 50% after 20 h. Moreover, after 20 h of enzyme incubation the recovery of oil gets to the plateau in any concentration of buffer used. Furthermore, its shows that the ratio 1:3 was found to give the maximum oil recovery, that is almost the 70% of oil that can be recovered with solvent extraction.

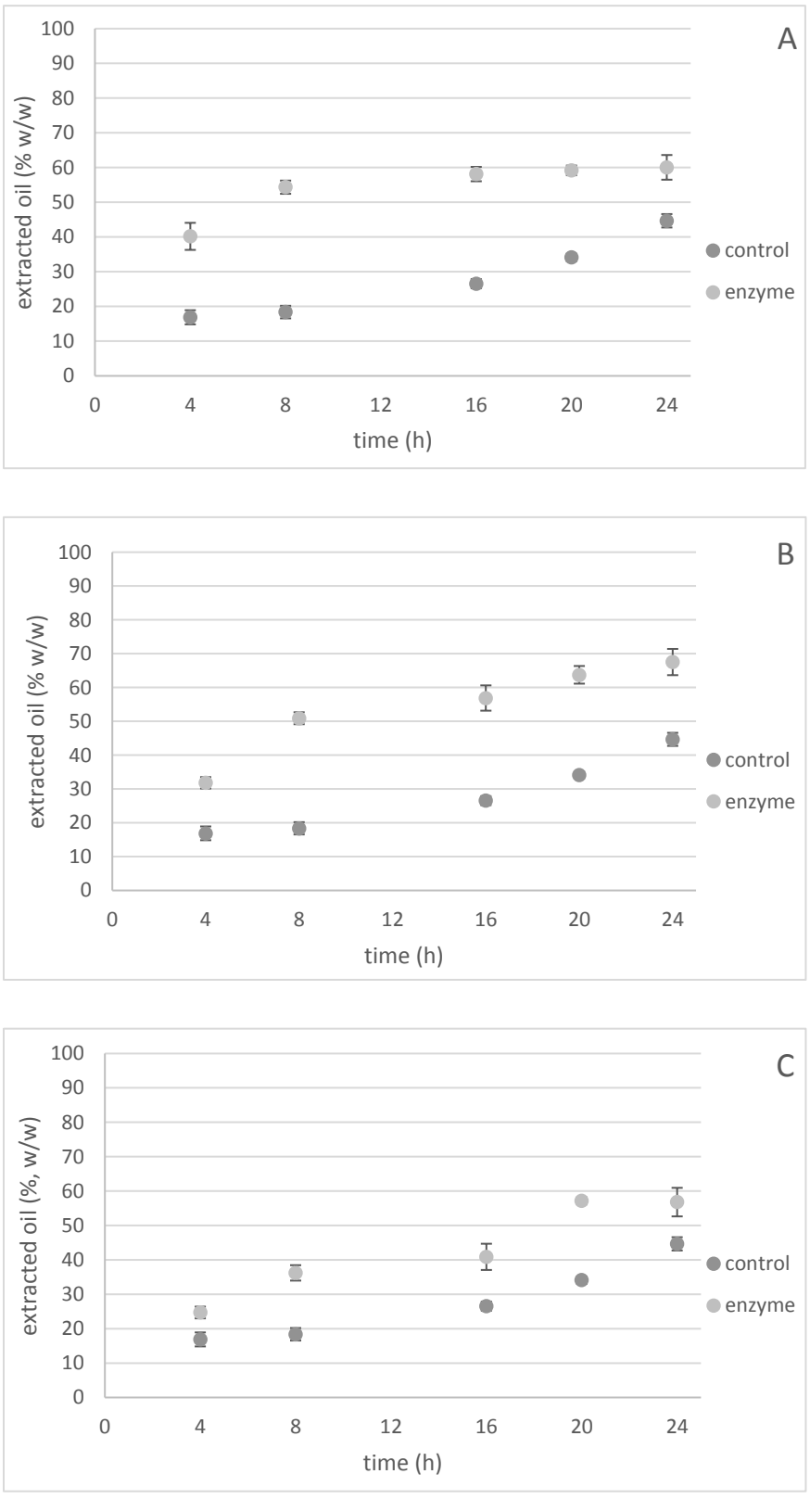


Figure 4.3 EAOE on pumpkin seeds by-products performed with different buffer ratios (solid-to-buffer) 1:2 (A); 1:3 (B); 1:5 (C). The percentage of recovered oil is referred to the maximum extractable oil recovered with the solvent extraction (SOE) in n-Hexane. Dark grey dot shows the control (aqueous extraction) and light grey dot shows the sample treated with papain. Values are reported as mean ± standard deviation of three replicates of three independent experiments (n=9).

Kinetics of oil extraction

Subsequently, the oil extraction kinetic was studied. As shown in figure 4.4, the oil yield increases with enzyme concentration increasing. To better understand the best process parameters, the U/mg of enzyme were chosen including the quantities previously used for the study of the solid: liquid ratio. Thus, in this case 60 U/mL, 100 U/mL and 150 U/mL, respectively, reflect the amounts of enzyme used for the 1:5, 1:3 and 1:2 ratios. Therefore, given the results shown in the figure, it is possible to confirm that the ratio 1:3 is the best, as it increases the extractability of the oil at 24 h of enzymatic incubation. Moreover, as regards the quantity of enzyme that allows the greatest extractability, from Figure 4.4 it is clear the difference between the control (without enzyme) and the enzyme's samples. The trend of oil extractability with an enzymatic process is mostly the same in all samples with a 10% difference from the lowest to the highest concentration of enzyme. This difference is more evident when the enzyme is not present, in fact the yield of recovered oil is lower and a decrease of 20% is observed. The highest oil yield is obtained when the enzyme concentration does not exceed 200 U/mL. Furthermore, the yield increases with longer time of incubation and reaches a maximum value after 24 h, where more than 70% of oil can be recovered, according to *Yusoff et al. (2017)* that recover from 70 to 80% of oil from peanut with best conditions. So, the optimal conditions were established as: 1:3 powder-to-liquid ratio, 200 U/mL of papain and 24 hours of incubation time for the maximum yield of extractable oil with enzymatic process; and 1:3 powder-to-liquid ratio, 100 U/mL of papain and 8 hours of incubation time to recover half part of oil extractable with solvent.

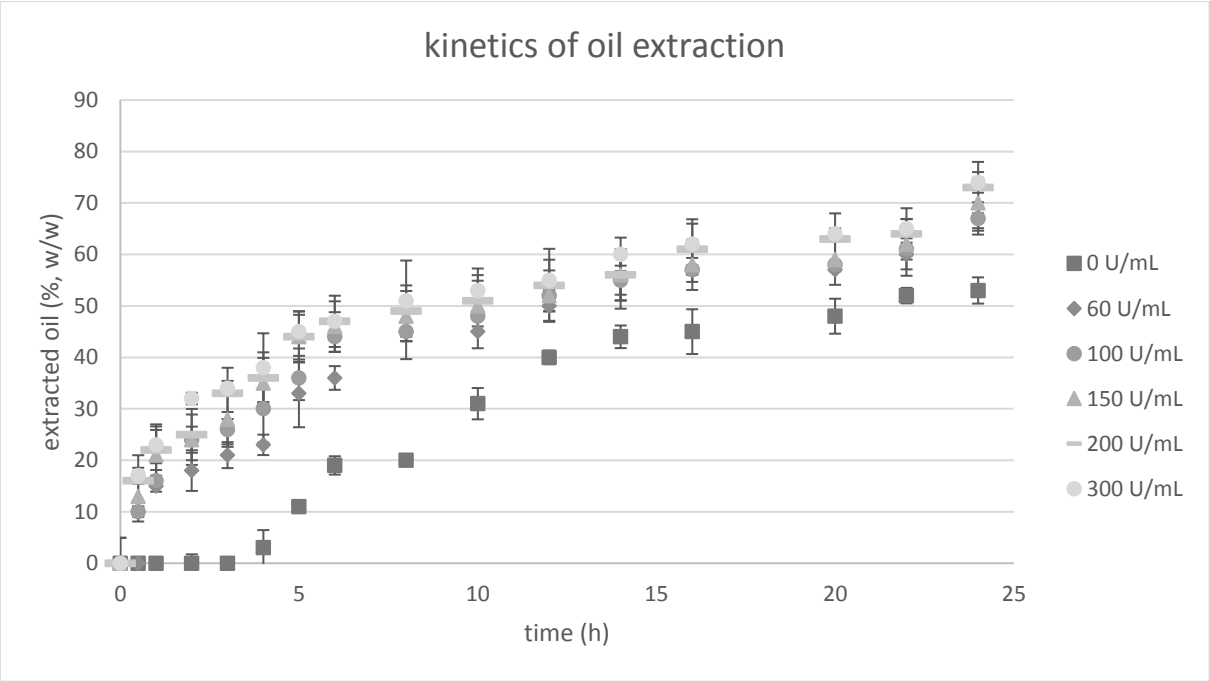


Figure 4.4 Kinetics curves of EAOE on pumpkin seeds by-products performed with different concentrations of papain. The percentage of recovered oil is referred to the maximum extractable oil recovered with SOE in n-Hexane. Square dot shows the control (without enzyme); rhombus dot the 60 U/mL; light green circle dots the 100 U/mL; triangle dot the 150 U/mL; line the 200 U/mL and dark green circle dot shows the 300 U/mL. Values are reported as mean ± standard deviation of three replicates of three independent experiments (n=9).

4.3 Nanotechnology

Synthesis of magnetic nanoparticles

Magnetic particles were synthesized in alkaline and hydrothermal conditions as shown by *Koneracka et al. (1999)*. The particles, black in colour, revealed a strong magnetic response as demonstrate in the figure 4.5.

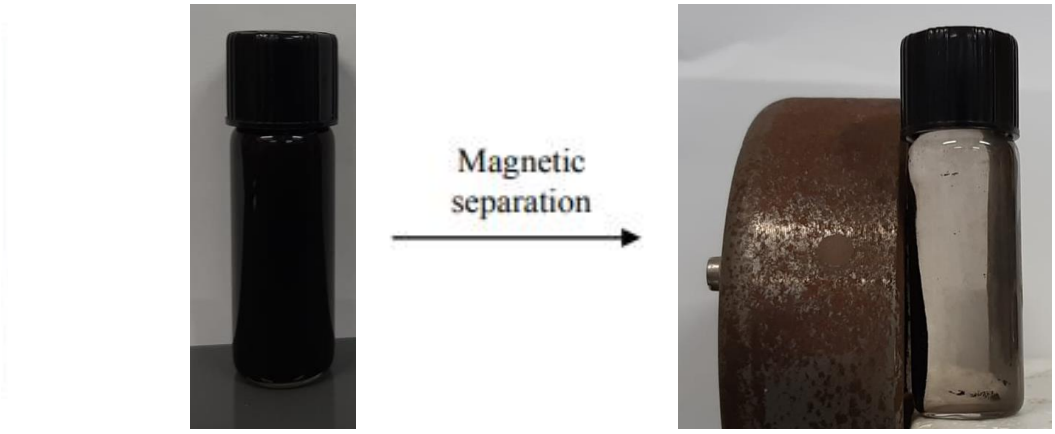


Figure 4.5 Magnetic response of MNP

Synthesis of dialdehyde starch

The dialdehyde starch (DAS) was synthesized with soluble starch from different origin (corn, rice, potato and wheat) and sodium periodate ($NaIO_4$) as oxidant agent, following the protocol of *Zhang et al. (2007)*. After oxidation of the glucose ring, two aldehydic groups appear on each opened ring of DAS molecules.

The carbonyl content (CHO%) of the dialdehyde starch was determined according to Wing and Willett (1997). Figure 4.6 shows the carbonyl groups content of the obtained 4 different types of (DAS). Corn DAS and rice DAS show the higher amount of carbonyl groups respect to potato and wheat DASs. So, these two types of DAS were studied to find the best DAS linker between magnetic particles and enzyme. The controls, normal starch of the different type, show no carbonyl groups as expected (data not shown).

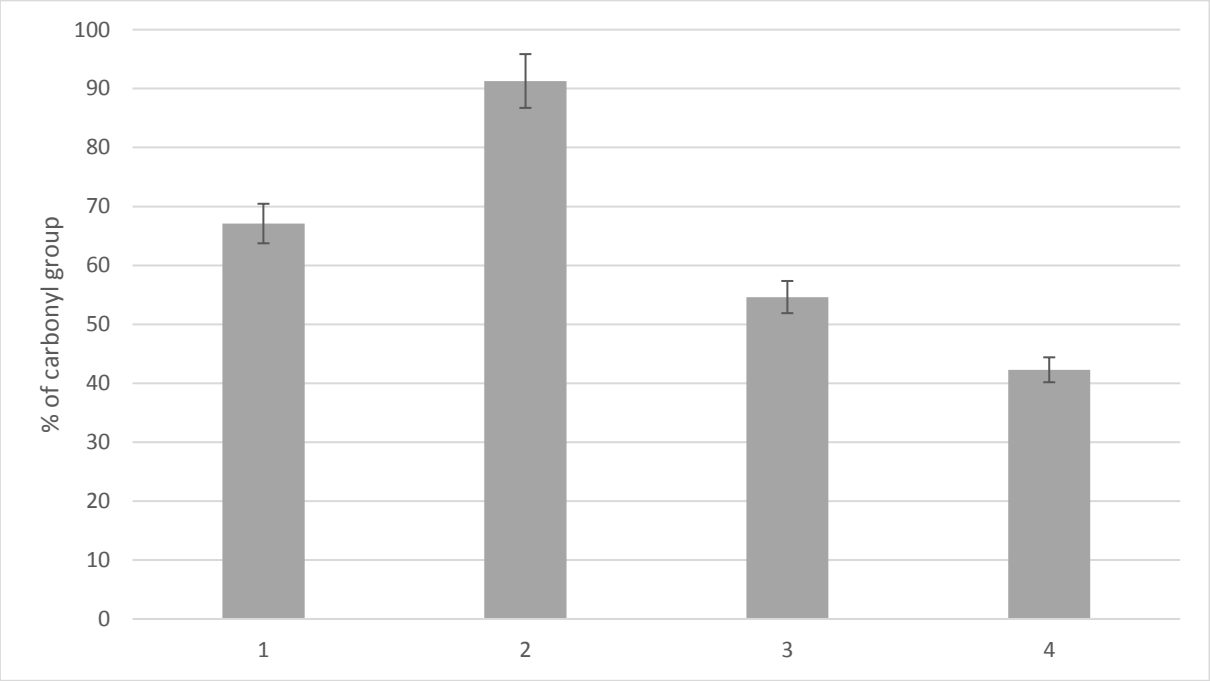


Figure 4.6 Percentage of carbonyl groups detected on DAS prepared with different starches. 1- corn starch; 2- rice starch; 3- potato starch, 4- wheat starch. Values are reported as mean ± standard deviation of three replicates (n=3).

Nanoparticles coating with dialdehyde starch

Activation of binding groups is often performed on linkers rather than on the enzyme protein, thus reducing the risk of decreasing the catalytic activity of the enzymes. Thus, the magnetic

particles were coated with different DAS linkers by epichlorohydrin reaction (Figure 4.7). Figure 4.7 shows the formation of the linkage helped by the epichlorohydrin.

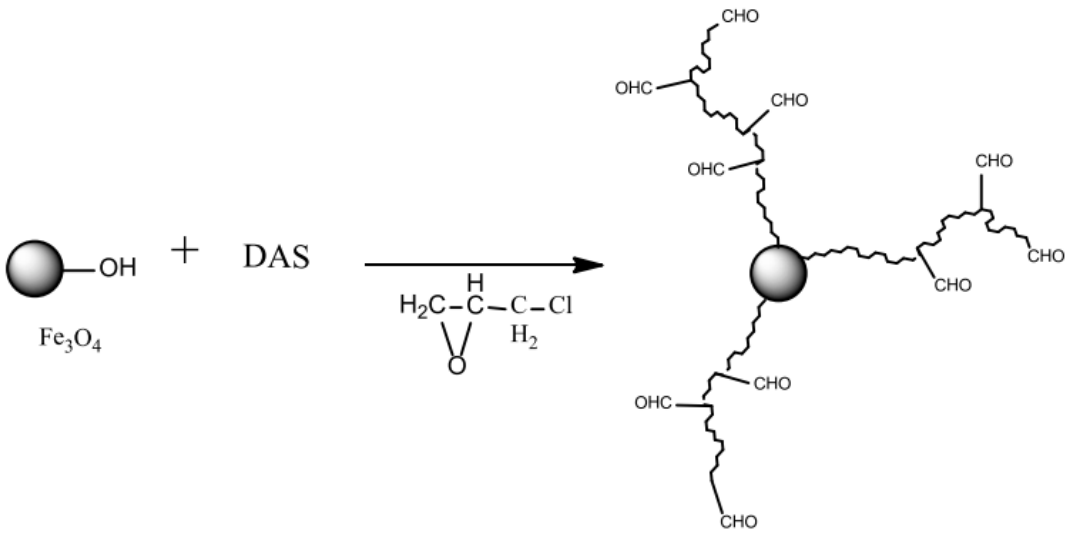


Figure 4.7 The coating of magnetic particles with DAS trough epichlorohydrin.

A coating reaction with native starches was also performed as controls. The yield of starch or DAS linker coated on magnetic particles was calculated as the difference between the concentration of the stock solution used for the linkage, and the concentration of DAS in the supernatant after the linkage. The glucose equivalent concentration was detected with phenol-sulfuric acid assay following the protocol of *Masuko et al. 2005*. Figure 4.8 shows the percentage of starch or DAS coated to magnetic nanoparticles (MNP). Results evidenced the highest efficiency for both types of starch to be coated on magnetic particles (Figure 4.8, samples 1 and 2). Moreover, a consistent amount of rice and corn DAS was found on coated MNP with a coating efficiency of 86 and 72 % respectively (Figure 4.8, samples 3 and 4).
This was a crucial step for the following ones for investigating the importance of using a linker between the particles and the enzymes, and the type of selected linker.

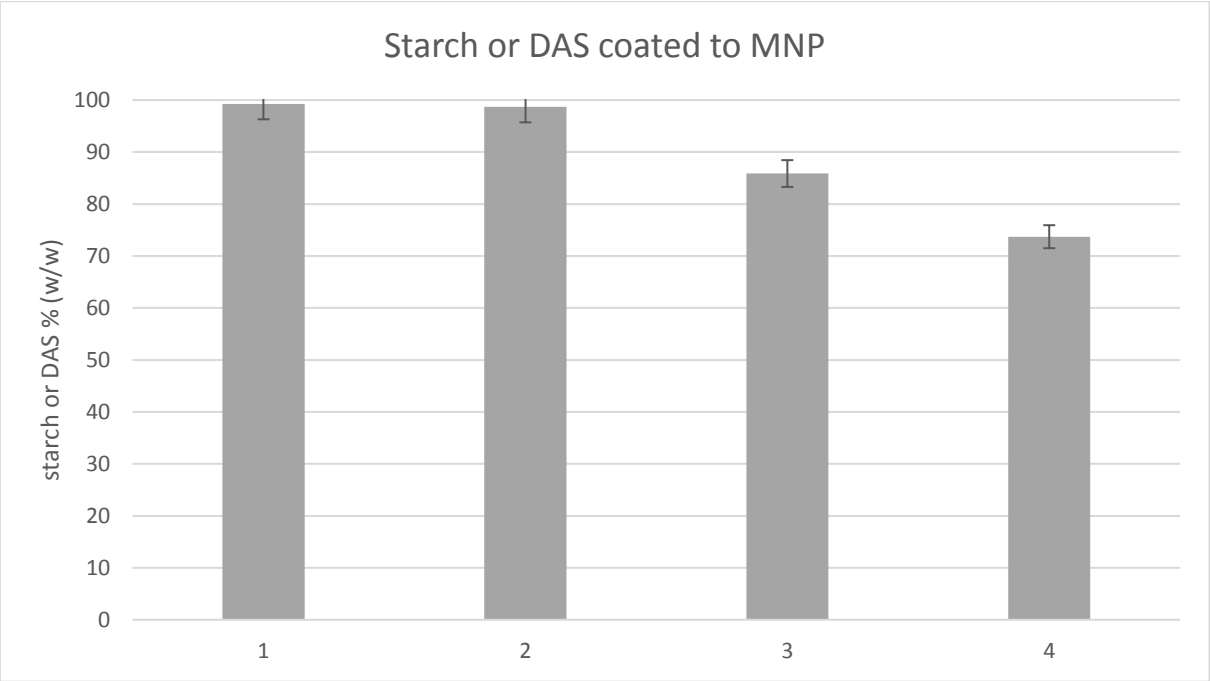


Figure 4.8 Percentage of coated starches or DAS on magnetic particles. 1, rice starch; 2, corn starch; 3, rice DAS; 4, corn DAS. Values are reported as mean ± standard deviation of three replicates (n=3).

Enzyme binding

Immobilization experiments were conducted with purified papain from Creative enzyme (Figure 4.9). As known from literature, papain is 23 kDa, the electrophoretic profile showed a major representative band between 20 and 25 kDa, and two very weak signals at lower and higher molecular weight.

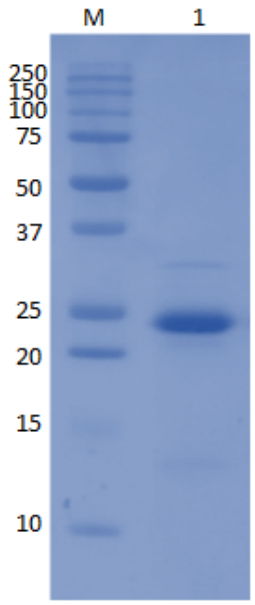


Figure 4.9 Electrophoretic profile in 15% SDS-PAGE under reducing conditions of papain. (1) Papain sample. M, Molecular weight marker (Precision Plus Protein, All blue Prestained Protein Standards -Biorad).

The immobilization of papain on magnetic nanoparticles was evaluated by Bradford assay and percentage of immobilized papain was calculated as a difference between the concentration of the papain solution used for the linkage and the remaining papain in the supernatant after the linkage. The performance of dialdehyde starch from rice and corn was investigated (Table 4.2). Because CHO groups were distributed over the surface of magnetic microspheres, papain was immobilized on the activated magnetic particles by covalent bond. In addition to covalent binding, non-specific sorption, ionic interactions, and hydrogen bonds probably occurred between papain and DAS. However, non-specific sorption could be decreased by extensive washing after binding; in fact, in table 4.2 is reported the detachment of papain after the first wash and after a total of 5 washes, when no more papain was observed in supernatant. The binding efficiency was slightly higher for corn DAS-coated MNPs compared to the rice ones (47 vs 46%). This result is apparently in contrast with the higher degree of CHO groups and the higher coating efficiency found in rice-DAS, which means a higher availability of binding sites for papain, respect to corn-DAS. However, the accessibility of CHO groups for the two considered DAS could be different because of the different amylopectin content of the two starches from which they come. Thus, the more branched corn DAS could offer a better distribution of CHO groups on the MNP shell, favouring the binding of more papain molecules and then, the amount of papain on corn-DAS MNPs and rice-DAS MNPs is nearly the same. Therefore, corn DAS demonstrated a better performance in papain binding.

Table 4.2 Percentage of immobilized and detached papain on corn-DAS and rice-DAS coted magnetic particles.

enzyme	Corn DAS	Rice DAS
attached	47±0,15	46±0,15
Detached (first wash)	51±0,15	51±0,15
Detached (end wash)	53±0,15	54±0,15

Finally, the importance of the functionalization of MNPs with corn DAS for papain binding was demonstrated by control experiments in which the enzyme was linked directly to MNPs, or to starch-coated MNPs. A very low percentage (<5%) of papain was able to directly bind on MNPs and this value became a little more than double when corn starch was coated on MNPs, as shown in Figure 4.10. Therefore, the increased amount of papain bound on DAS was the result

of the exposure of CHO groups on the surface of the flexible magnetic particles that allowed the covalent linkage of papain. About the nature of papain binding in the other contexts, it could be hypothesized that hydrogen bonds or electrostatic interactions occurred between the protein and the hydroxyl groups on the surface of MNPs and Starch-coated MNPs.

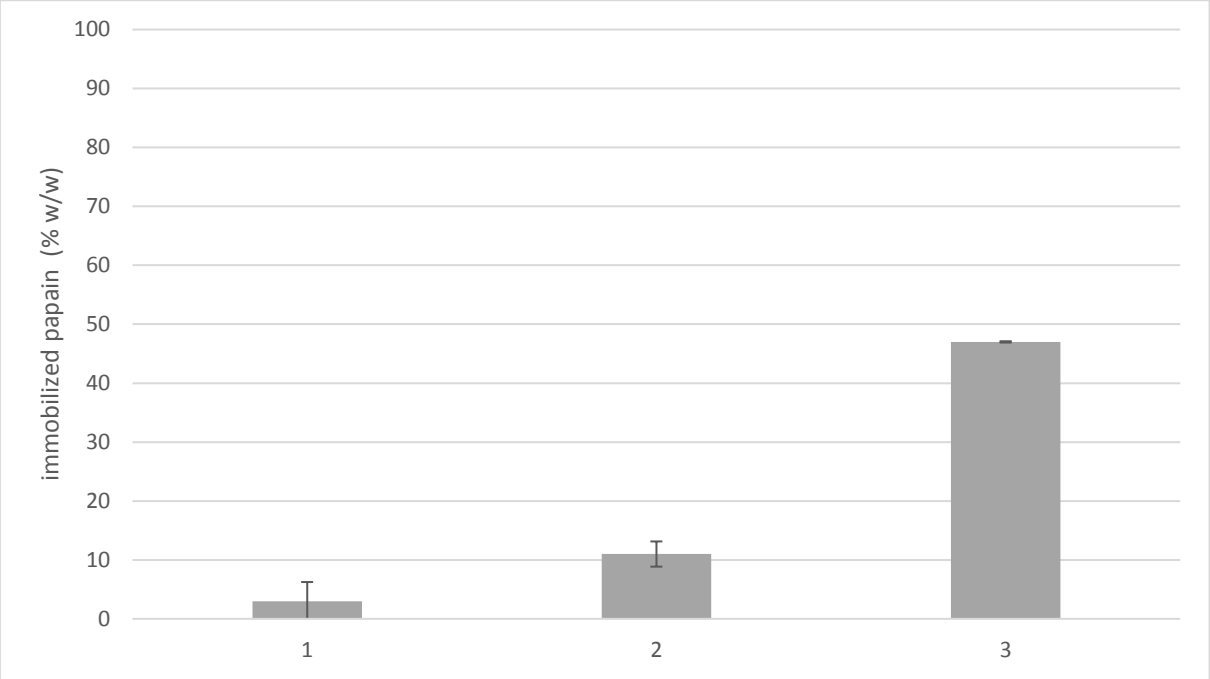


Figure 4.10 Percentage of immobilized papain on magnetic particles. 1, papain linked directly on the magnetic particles; 2, papain linked on corn starch-coated magnetic particles; 3, papain immobilized on corn DAS-coated magnetic particles. Values are reported as mean ± standard deviation of three replicates (n=3).

4.4 Papain Enzymatic study

Enzymatic assays and specific activity determination

In order to evaluate enzyme kinetics and compare data between free and immobilized papain, the best conditions for papain activity were sought. The enzyme activity is generally determined as substrate converted or product formed per time unit. The assays were conducted in different conditions with sodium caseinate as a substrate and the levels of tyrosine equivalents released after 10 min of incubation were taken as reference of enzyme activity. It is well known from literature that, like many enzymes, papain also needs cofactors to best perform its catalysis. Furthermore, buffers at different pH have been studied to better understand the activity of this enzyme. Moreover, water tests were carried out to know the efficiency of papain under these conditions and were taken as a control reference. The

experimental tests showed that the optimal conditions for the hydrolysis reaction of sodium caseinate catalysed by papain were 50 mM TRIS-HCl, pH 8 in the presence of 5 mM cysteine and 2 mM EDTA at 50°C (Figure 4.11).

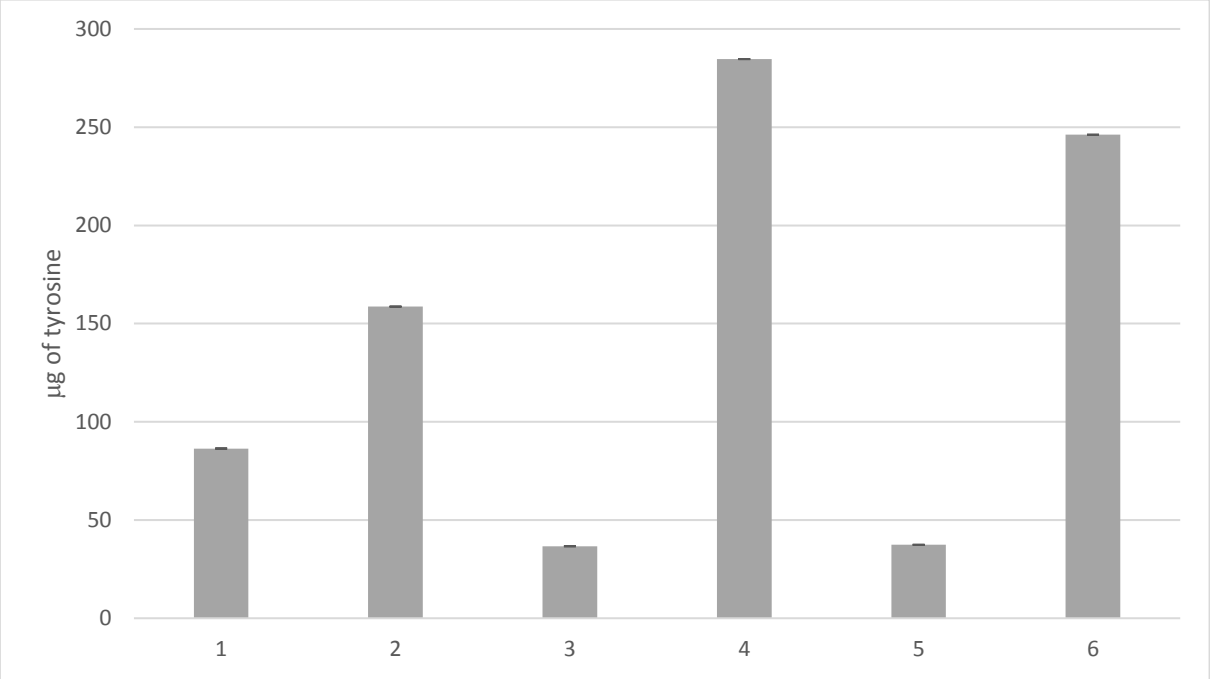


Figure 4.11 Papain enzymatic activity (ug of tyrosine equivalent) in different buffers at 50°/10 min. The hydrolysis reaction was conducted in water (samples 1-2), 50 mM TRIS-HCl, pH 8 (samples 3-4) and 50 mM sodium acetate, pH 5 (samples 5-6), in the absence (1,3 and 5) or in the presence (2, 4 and 6) of 5 mM cysteine and 2 mM EDTA. Values are reported as mean ± standard deviation of three replicates (n=3).

The specific activity of enzyme was determined considering 1 unit of enzyme activity as the amount of enzyme that produces an absorbance increase of 0,1 OD after incubation at 50°C for 10 min in optimal assay conditions. Therefore, assay tests at different papain concentrations were set up, keeping all other parameters constant. The enzymatic activity obtained was then shown in the graph as a function of the amount of papain used in the assay. The specific activity was derived from the equation of the interpolating using the angular coefficient as a parameter of proportionality. For the free papain, the equation was y= $1{,}49x$, and then, the specific activity was found as ~15 U/mg (1U: 0,1 OD = xU: 1,49). On the other hand, the immobilized papain showed an equation of y= $0{,}1x$, and then the specific activity was 1 U/mg (1U: 0,1 OD = xU: 0,1).

Kinetic parameters determination

The kinetic parameters K_m and V_{max} of free and immobilized papain were calculated using the Lineweaver–Burk linearization, and are shown in Table 4.3. High K_m values are related to a low affinity of enzymes for their substrate, and thus the immobilization decreases the substrate affinity. An increase in V_{max} indicates a faster rate of product formation in the catalytic reaction. As reported from Nadar & Rathod (2016), the immobilized enzyme can limit the flexibility of the enzyme and consequently the accessibility of the substrate. So, K_m increases, and the substrate concentration needed to reach the half of V_{max} for the immobilized enzyme is higher compared to the free papain, while the V_{max} value of the catalysis is higher for immobilized papain, which suggests that the immobilized enzyme catalyzes a faster hydrolysis of the sodium caseinate used as a substrate.

Table 4.3 Kinetic parameters of free and immobilized papain.

Papain	**k_m (mg/mL)**	**V_{max} (OD/min)**
Free	0,57±0,06	0,01±0,003
Immobilized	3,49±0,08	0,032±0,002

Re-usability of immobilized papain

To evaluate the reusability of the papain immobilized on corn-DAS coated MNPs, the activity was analysed for 11 successive cycles in the optimal conditions assay. The activity of fresh immobilized papain in first cycle was taken as 100%. Results are reported in Figure 4.12.

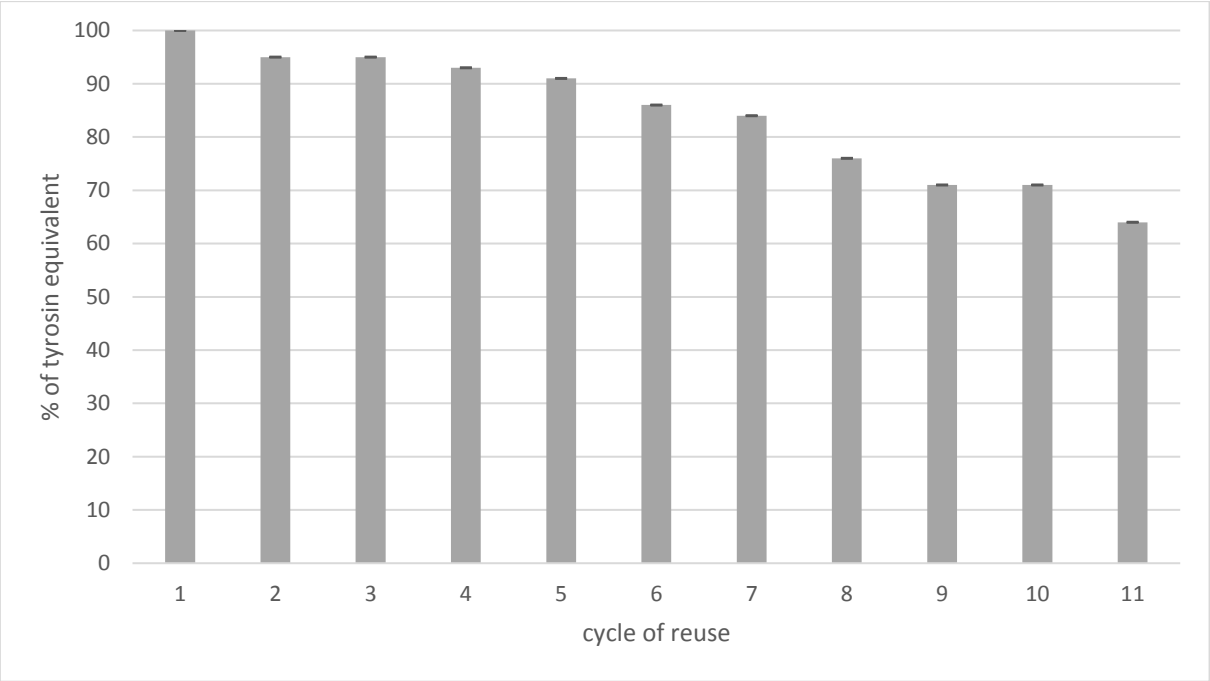
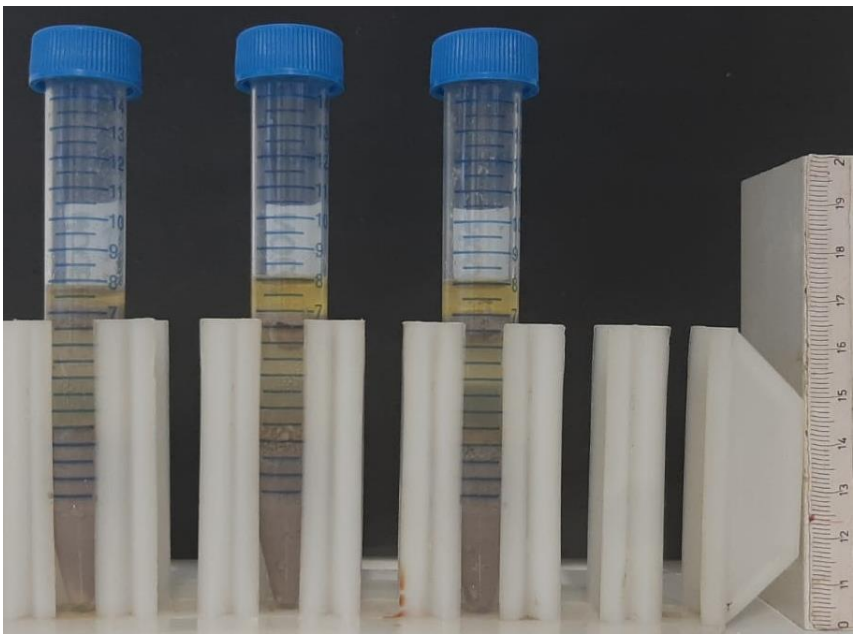


Figure 4.12 Reusability of papain immobilized on corn-DAS coated MNPs for 11 consecutive cycles. The % of tyrosine equivalent produced in each cycle is reported as mean ± standard deviation of three replicates (n=3).

Immobilized papain showed a good reusability, as it retained the 70% of residual activity after 10 cycles of using, according to what was reported by Lei et al. (2004). Moreover, the activity of papain decreased to 90% of its initial value after five cycles.

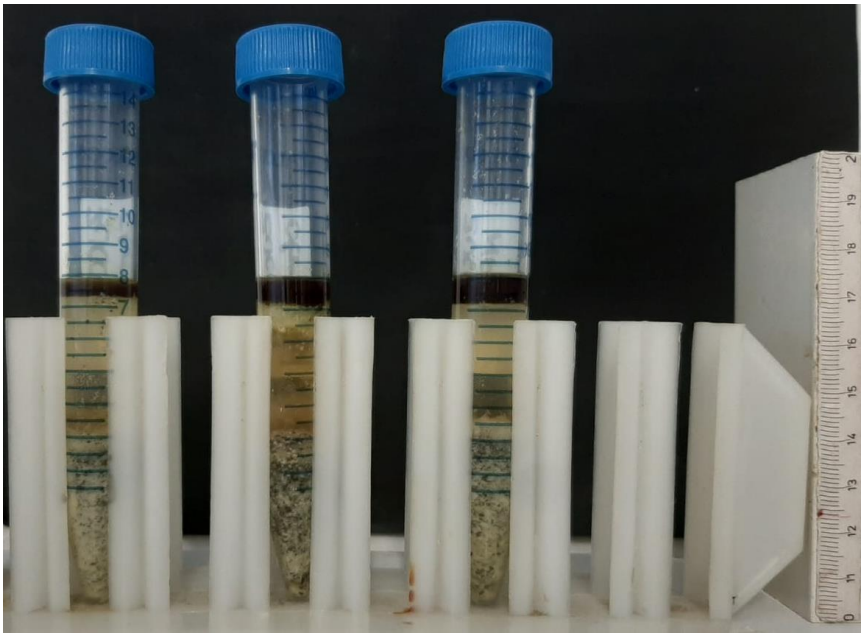
EAOE with immobilized papain

The efficacy of the immobilized papain was tested in EAOE process applied to walnut kernels and pumpkin seeds by-products, and the performance was compared to the free papain, as well as with the AOE process considered as a control (Figure 4.13). For the EAOE experiments the same Units of either free or immobilized papain were used. Results showed that, for both pumpkin seeds and walnuts by-product samples, the amount of extracted oil is equal (72 to 82%). As previously demonstrated, the control sample without enzyme (AOE process), showed a reduced oil extractability (Figure 4.13).



A

1 2 3



B

1 2 3

Figure 4.13 EAOE process on walnut (A) and pumpkin seeds (B) by-products performed with free papain (2) and immobilized papain (3). AOE process is used as control (1).

In conclusion, the immobilization of papain does not affect its oil extractability performance in EAOE processes. Moreover, the encouraging results obtained in the re-usability experiments highlight the possibility to reuse the immobilized papain for several times also in an EAOE process. Finally, the separation and recovery of the immobilized enzymes by an external magnetic field makes this process highly convenient, where the reuse for multiple cycles is easily achieved, reducing the total processing costs (Shahrestani *et al.,* 2016).

4.5 Oil quality analysis

Titrable acidity and Peroxide number

In evaluating the quality of vegetable oils, titratable acidity (TA) is a parameter that allows to determine the degree of hydrolysis of triglycerides, an undesirable characteristic, an index of low freshness for the oil. In this study, the TA values for walnut and pumpkin seeds oils obtained by SOE and EAOE process were compared (Figure 4.14). For pumpkin seed oils there were no differences between the products obtained with the two processes and, in fact, the determined TA values are the same. On the other hand, for walnut oil, the TA values obtained with the EAOE process were slightly higher (0,56 vs 0,42), suggesting a higher activity of lipases during the EAOE process compared to the SOE. In any case, both the TA values for the walnut oils were lower than those found for pumpkin seed oils.

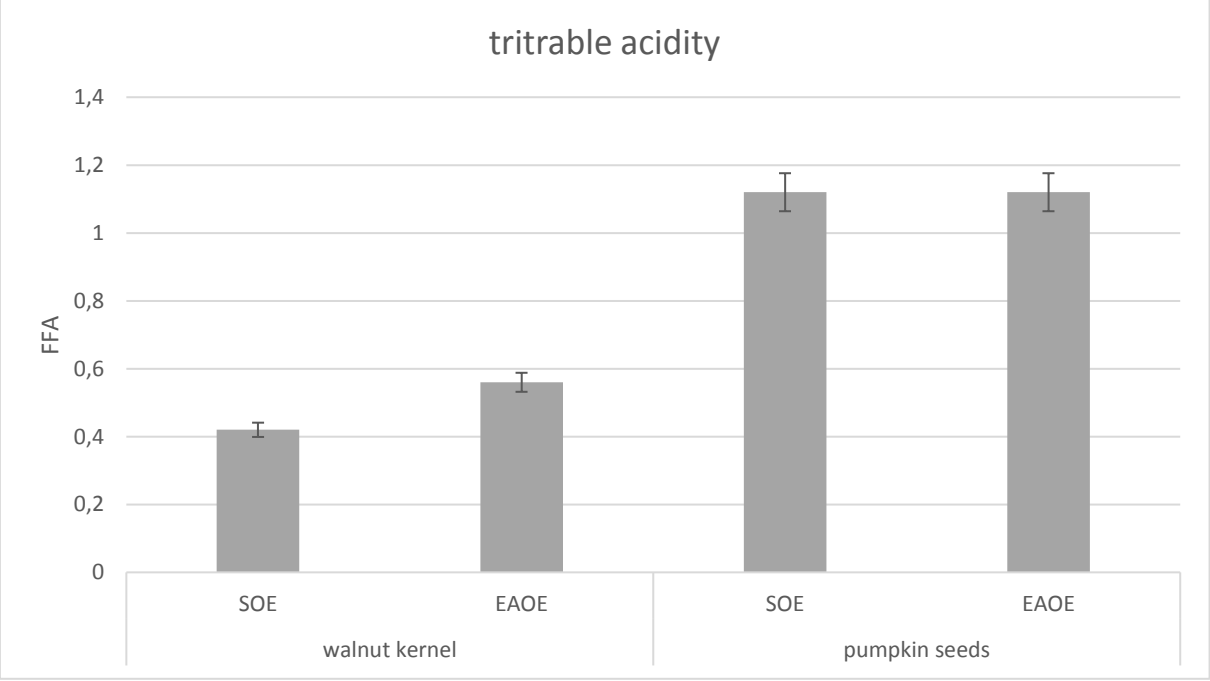


Figure 4.14 Titrable acidity of walnut and pumpkin seeds oils obtained by SOE and EAOE processes. Values are reported as mean ± standard deviation of three replicates (n=3).

Another important quality parameter for the vegetable oils is the number of peroxides, which are products of primary oxidation of the oil and therefore undesirable. The quality of the oils is inversely proportional to the number of peroxides. Analysing the peroxide values obtained for walnut and pumpkin seed oils, it can be observed that the products extracted with SOE

show a double peroxide value compared to those obtained with EAOE (Table 4.4). This result suggests that the EAOE process offers greater oxidative stability for the oil in the extraction stages.

Table 4.4 Peroxide number of walnut and pumpkin seeds oils obtained by SOE and EAOE processes.

Sample	process	Peroxide number (meq O_2/Kg oil)
walnut oil	SOE	0,61±0,16
	EAOE	0,36±0,07
pumpkin seeds oil	SOE	1,37±0,25
	EAOE	0,58±0,07

Values are reported as mean ± standard deviation of three replicates (n=3).

Total phenolic compounds and antioxidant activity

Plant foods are rich in phytochemicals, among that phenolic compounds play beneficial effects on human health. Walnut and pumpkin seeds are a good source of phenolic compounds Câmara & Schlegel (2016) and Fukuda *et al.* (2003), which exsert high antioxidant activity thanks to their hydroxyl groups and phenolic rings. Total Phenolic Compounds (TPC) were determined in walnut and pumpkin seeds oils (Table 4.5). The experimental results showed that the TPC content was not affected by the oil extraction method, although the pumpkin seed oils exhibited a higher value compared to the walnut oil, regardless of the oil extraction method.

Table 4.5 TPC content of walnut and pumpkin seeds oils obtained by SOE and EAOE processes.

Sample	process	TPC (µg of GAE/mL)
walnut oil	SOE	28,5±2,7
	EAOE	24,3±0,54
pumpkin seeds oil	SOE	53,8±10,12
	EAOE	54,6±1,28

Values are reported as mean ± standard deviation of three replicates (n=3).

The antioxidant is a molecule that protects other molecules, as biological molecules by the damage caused by free radicals. In this work, the DPPH assay has been used to study the antioxidant activity of walnut and pumpkin seeds oil obtained by SOE and EAOE processes. As shown in Figure 4.15, the percentage of inhibition of the DPPH radicals was much higher in both products subjected to EAOE process, thus suggesting that the latter allows to preserve the antioxidant activity of walnut and pumpkin oils compared to the SOE process. Moreover, in walnut oil the antioxidant activity was greater than that found in pumpkin seeds oil. Considering that the TPC values of the latter were higher, it can be hypothesized that the quality of the polyphenols of the walnut oil, compared to that of pumpkin seeds oil is better.

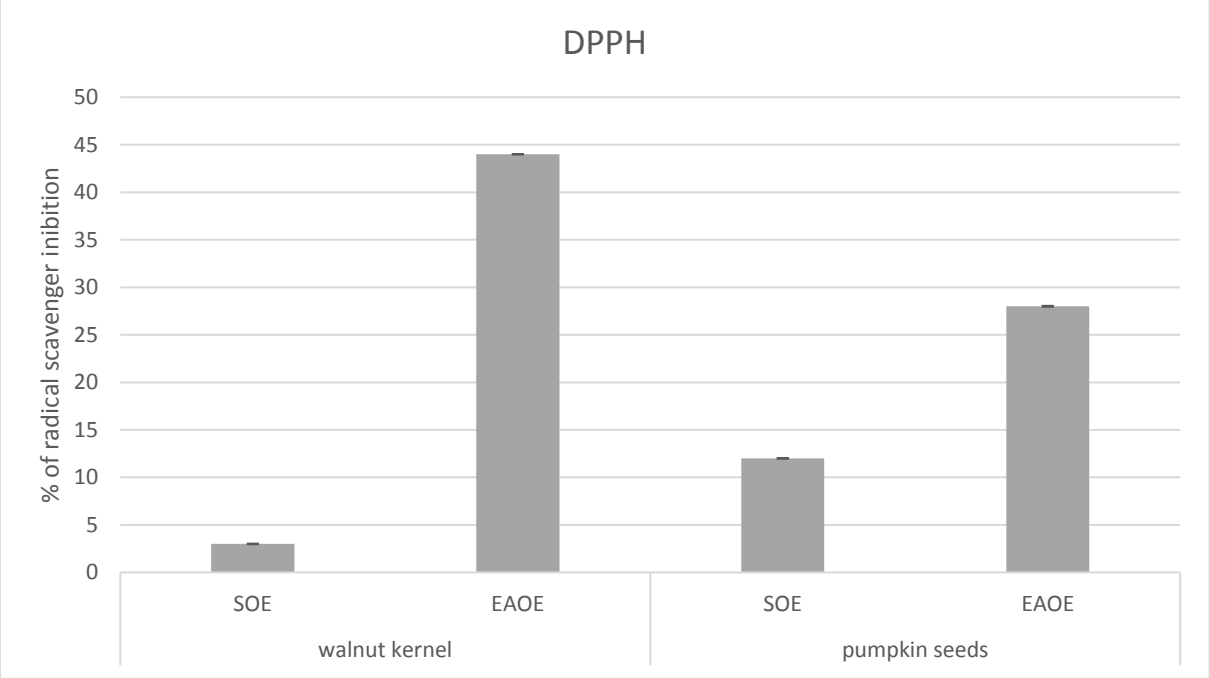


Figure 4.15 Antioxidant activity of walnut and pumpkin seeds oils obtained by SOE and EAOE processes. Values are reported as mean ± standard deviation of three replicates (n=3).

4.6 Protein analysis

Characterization of AOE and EAOE protein isolates

Solid cake and aqueous slurry residues obtained respectively after aqueous (AOE) or enzyme-assisted aqueous (EAOE) oil extractions were used as starting material for alkaline extraction and isoelectric precipitation of proteins as described in Method's section.

To evaluate from a qualitative point of view the walnut kernels and pumpkin seed proteins during the various fractionation steps to obtain the protein isolates, aliquots of the protein fractions were taken and subjected to electrophoretic analysis in 15% SDS-PAGE in reducing conditions. In detail, the protein component of the following preparations was analysed:

(1) soluble protein fraction at pH 10 (supernatant at pH 10);

(2) protein isolate obtained at pH 5 (pellet at pH 5).

The electrophoretic profiles of the walnut and pumpkin seed proteins recovered from AOE process are shown in Figures 4.16 A and B respectively.

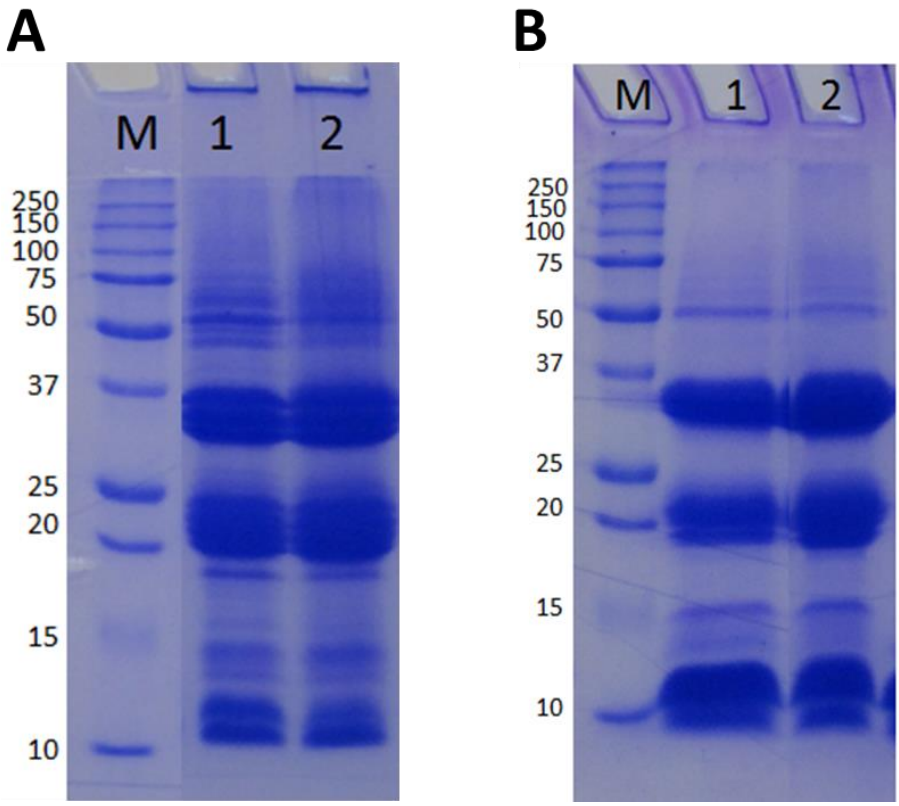


Figure 4.16 - Electrophoretic profile in 15% SDS-PAGE under reducing conditions of walnut (A) and pumpkin seeds (B) protein samples from AOE process during isoelectric precipitation. (1) soluble protein fraction at pH 10 (supernatant at pH 10); (2) protein isolate obtained at pH 5 (pellet at pH 5). M, Molecular weight marker (Precision Plus Protein^{tm} All blue Prestained Protein Standards -Biorad).

Walnut kernels and pumpkin seed samples show similar electrophoretic profiles in the two fractions. In fact, as shown in Figure 4.16 the lane 1 and 2 exhibit same protein species. Obviously, different seeds, as walnut and pumpkin seeds are, show different electrophoretic patterns. Therefore, a recovery of all protein species is obtained in qualitative and quantitative terms.

Similarly, the electrophoretic profiles of papain pre-treated walnut kernel and pumpkin seed proteins recovered from EAOE process are shown in Figures 4.17 A and B, respectively.

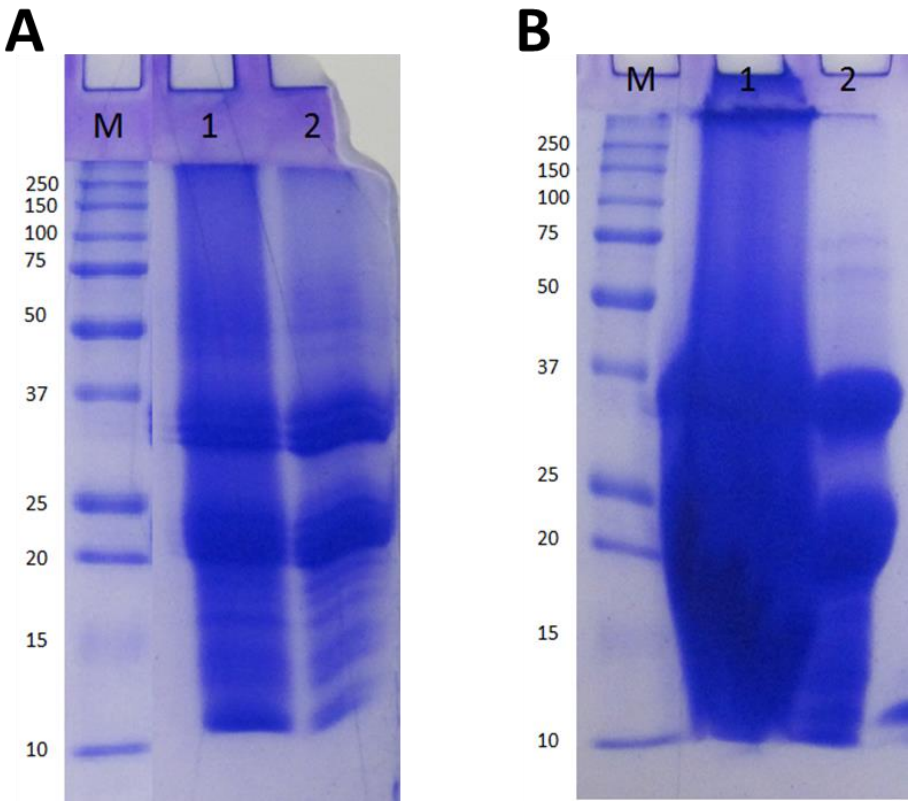


Figure 4.17 - Electrophoretic profile in 15% SDS-PAGE under reducing conditions of papain pretreated walnut (A) and pumpkin seed (B) protein samples from EAOE process during isoelectric precipitation. (1) – -soluble proteolyzed fraction at pH 10 (supernatant at pH 10); (2) - proteolyzed isolate obtained at pH 5 (pellet at pH 5). M, Molecular weight marker (Precision Plus Protein^{tm} All blue Prestained Protein Standards -Biorad).

As described before, also protolyzed sample of both seeds are well extracted by alkaline and isoelectric precipitation. Observing the electrophoretic profile of walnut samples after papain digestion (Figure 4.17-A), it seems that the enzyme does not had a very strong proteolytic activity on walnut proteins, compared to the pattern showed above (Figure 4.16-A) where the proteins were undigested. Although it is possible to notice a smear, which indicates the low proteolytic action. In the same way, the results for the pumpkin seed samples indicate that

the digestion with papain does not have strong activity. However, intense bands below 40 kDa are found in the protein isolate at pH 5 (Figure 4.17-B), compared to the protein profiles of Figure 4.16-B. Anyhow, as it possible to see from Figure 4.17 A and B, the intensity of the bands shown an enrichment recovery in term of quantity of protein species in both samples. Finally, the absence of visible bands in the high molecular weight regions (Figure 4.16), together with the appearance of lower molecular weight bands (Figure 4.17) demonstrate that papain had performed its catalytic action.

4.7 Technological properties

Protein solubility in water

The solubility of the isolated walnut and pumpkin seed proteins and their partially hydrolysed products was evaluated in a pH range from 2 to 10. The results are shown in Figures 4.18 A and B, where proteins are indicated with the acronym of the process from which they come, namely AOE (protein isolates after aqueous oil extraction) and EAOE (hydrolysed protein isolates after enzyme-assisted aqueous oil extraction). For all the analysed samples, both from walnut and pumpkin seeds proteins, the trend is the typical U-shaped trend of protein solubility, as reported in the literature for other protein species.

Both walnut protein isolate from AOE and EAOE process showed the maximum solubility at pH 2. Then, a dramatical drop of solubility was observed at pH 4 where the proteolyzed EAOE proteins exhibited a lower solubility. At pH 6-8, the solubility of both AOE and EAOE proteins start rising with a similar trend, till a good solubility was reached at pH 10 (Figure 4.18 A). Figure 4.18-B reports the results obtained for pumpkin seeds protein isolate derived from AOE and EAOE process. As described for walnut, also pumpkin seeds proteins showed the highest solubility at pH 2 with a progressive reduction at pH 4 until pH 6, where the lowest solubility for both AOE and EAOE samples is described. A significant differece is evident at pH 8, where AOE pumpkin seeds protein isolate maintains a very low solubility, while the EAOE hydrolysed proteins showed a solubility of 90% which was reached only at pH 10, from AOE proteins.

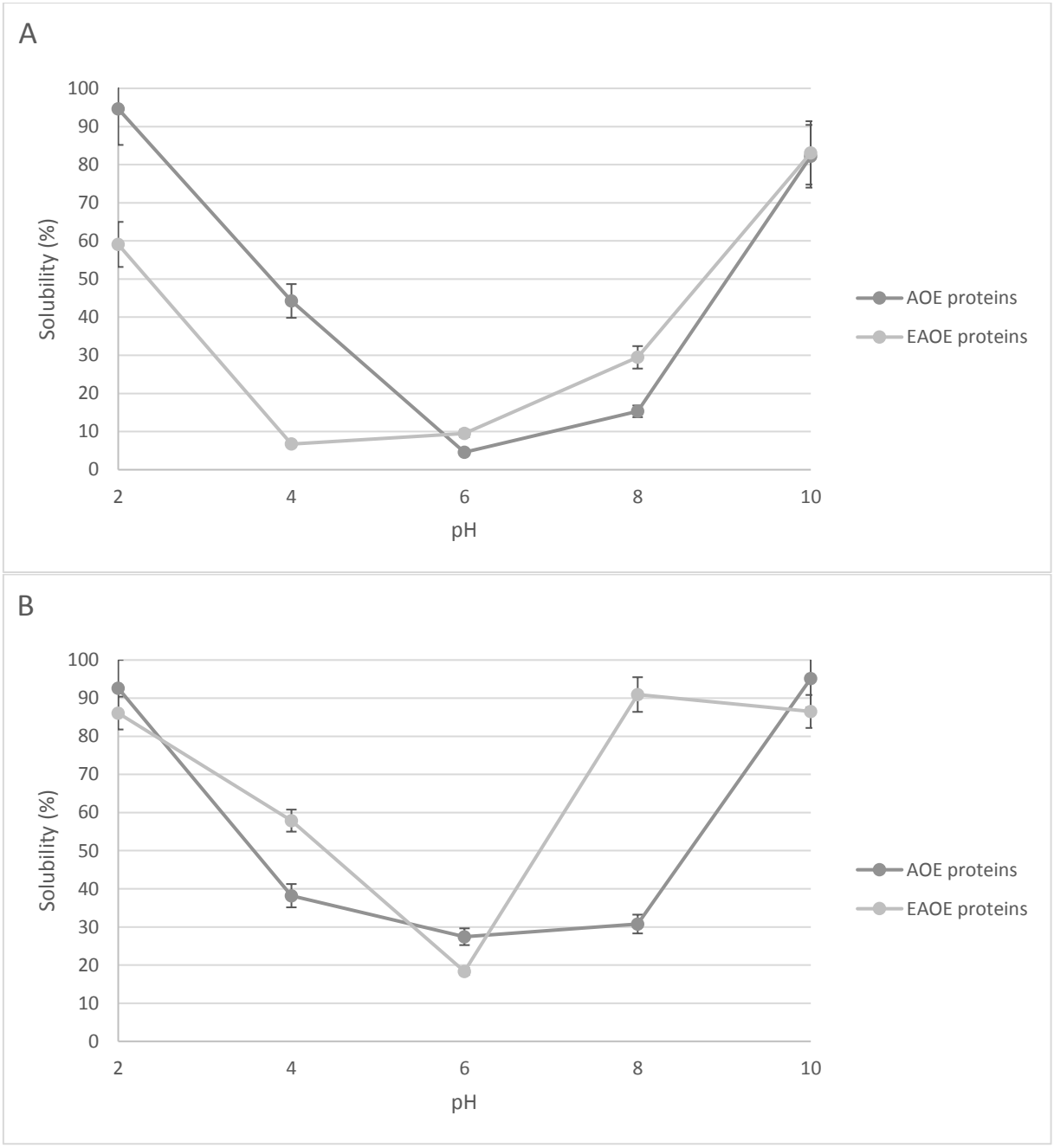


Figure 4.18 Solubility of walnut kernel (A) and pumpkin seeds (B) protein isolates from AOE and EAOE processes.

Water absorption capacity (WAC)

Water absorption capacity is expressed as the quantity in grams of water absorbed by the dry samples of protein based on initial sample weight (g). Walnut and pumpkin proteins showed opposite behaviour. On one side, walnut kernels AOE protein isolate showed a higher water absorption capacity then EAOE walnut proteins. In the other side, when EAOE pumpkin seeds proteins were proteolyzed the WAC value increased compared to the one of the corresponding AOE proteins (Figure 4.19). Overall, AOE walnut kernels proteins showed the

better WAC. These experimental results highlighted as the same enzyme treatment (applied in the EAOE process) leads to a different protein behaviour when applied on different protein sources such as walnut kernels and pumpkin seeds: in one case the partial proteolysis drastically worsens the WAC index of walnut kernel proteins, bringing it from about 3.5 to just over 1, while in the other case it improves it, albeit to a limited extent.

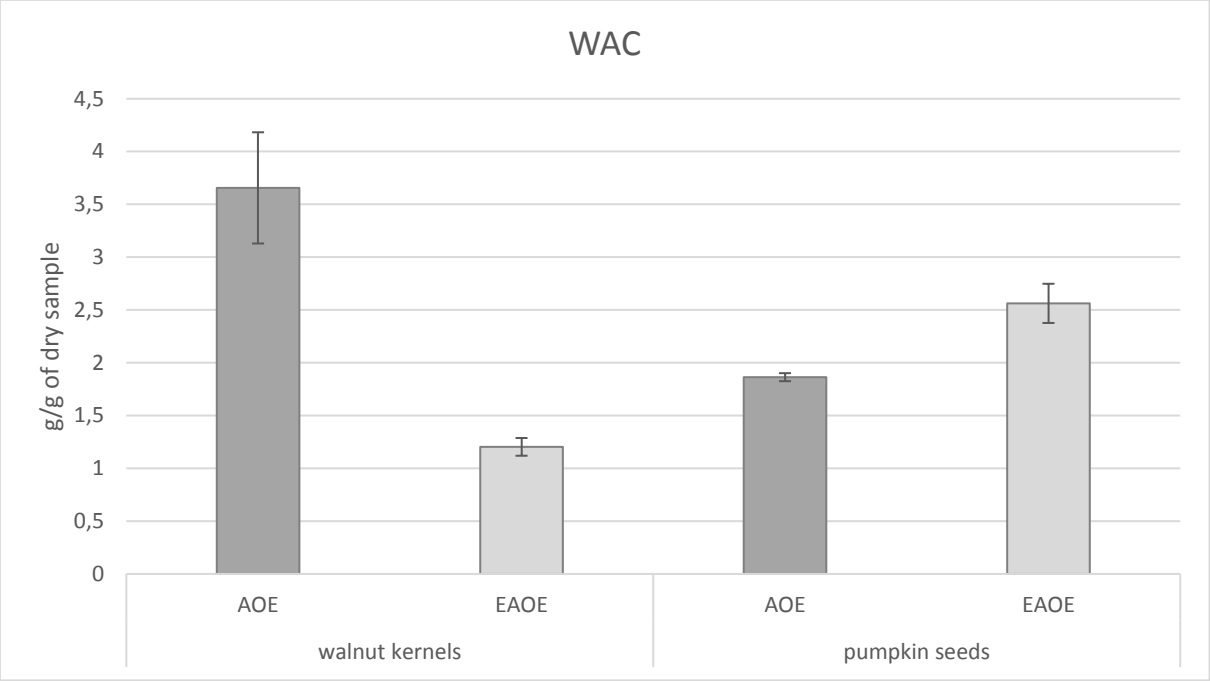


Figure 4.19 Water absorption capacity (WAC) of walnut kernel and pumpkin seeds protein isolates from AOE and EAOE processes.

Oil absorption capacity (OAC)

AOE walnut kernel protein isolates showed the best oil absorption capacity (Figure 4.20). This ability is less when the proteins are enzymatically pre-treated during the EAOE process. As already observed for the WAC, also for the OAC the walnut kernel proteins show good absorbing capacities compared to the corresponding EAOE protein isolate. In the same way, pumpkin seed samples show similar OAC trend to WAC: the proteolysis improved the oil absorption capacity, as demonstrated from results in Figure 4.20.

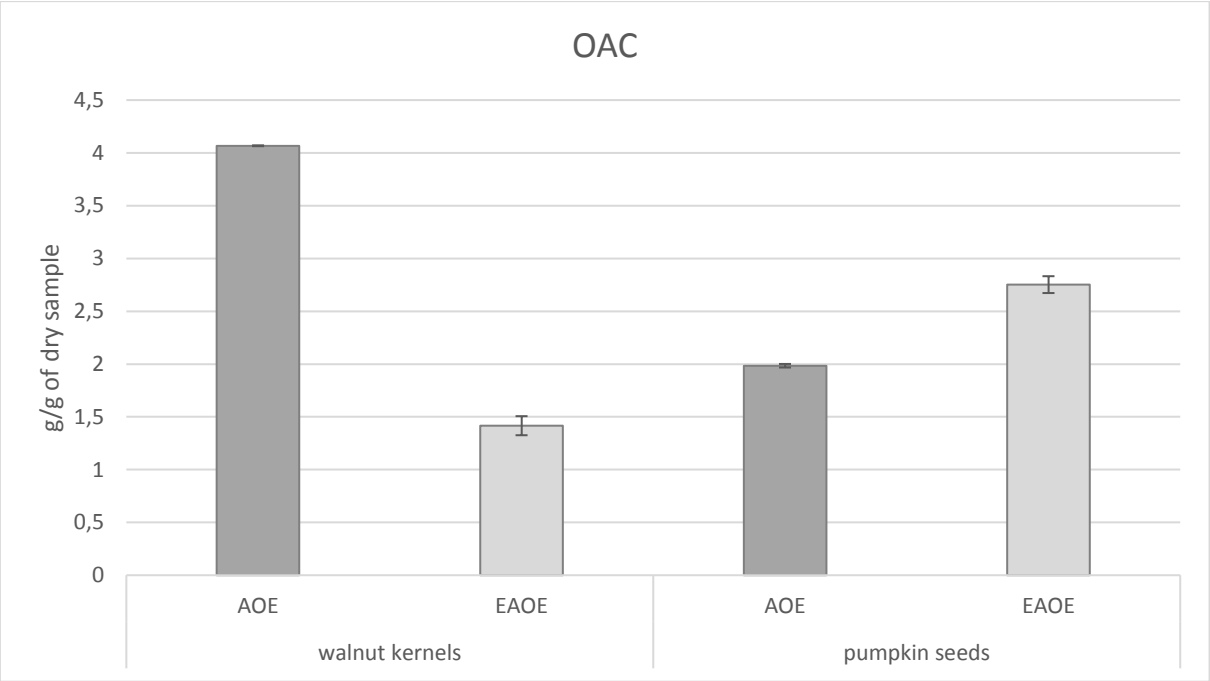


Figure 4.20 Oil absorption capacity (OAC) of walnut kernel and pumpkin seeds protein isolates from AOE and EAOE processes.

Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) as function of the pH of both walnut and pumpkin seeds protein isolates from AOE and EAOE processes are shown in Figure 4.21 A and B. At pH 2 all samples show the highest emulsifying activity that significantly decreases at pH 4 and remains constant at subsequent pH values (6, 8 and 10). In general, both pumpkin seed proteins from AOE and EAOE processes showed good emulsifying activity at all pHs. Protein samples from AOE both walnut and pumpkin seeds showed similar trends in EAI, with peaks at pH 2 and pH 8, while low emulsifying activity was observed at the other pH values (4, 6 and 10). The EAOE hydrolysed protein samples, on the other hand, exhibited good emulsifying properties at pH 2 and pH 10. Therefore, the enzymatic treatment improved the emulsifying activity at pH 10. *Mutilangi et al., (1996)* reported that more hydrophobic peptides contribute to stability of the emulsions. On the other hand, low molecular weight peptides may not be amphiphilic enough to exhibit good emulsifying properties (Chobert *et al.,* 1988). An emulsion system is generated by the absorption of peptides on oil droplets surface formed in the homogenization and the formation of a membrane that preserve the coalescence of the oil droplets (*Dickinson & Lorient, 1994*). According to literature, the obtained results showed that the stability of the proteolyzed samples was higher mostly for walnut sample. As for EAI, also the ESI parameter is pH dependent. In particular, the EAOE

proteolyzed pumpkin seeds proteins exhibited the greatest stability pH 2 and those of walnut at pH 8 (Figure 4.21 B).

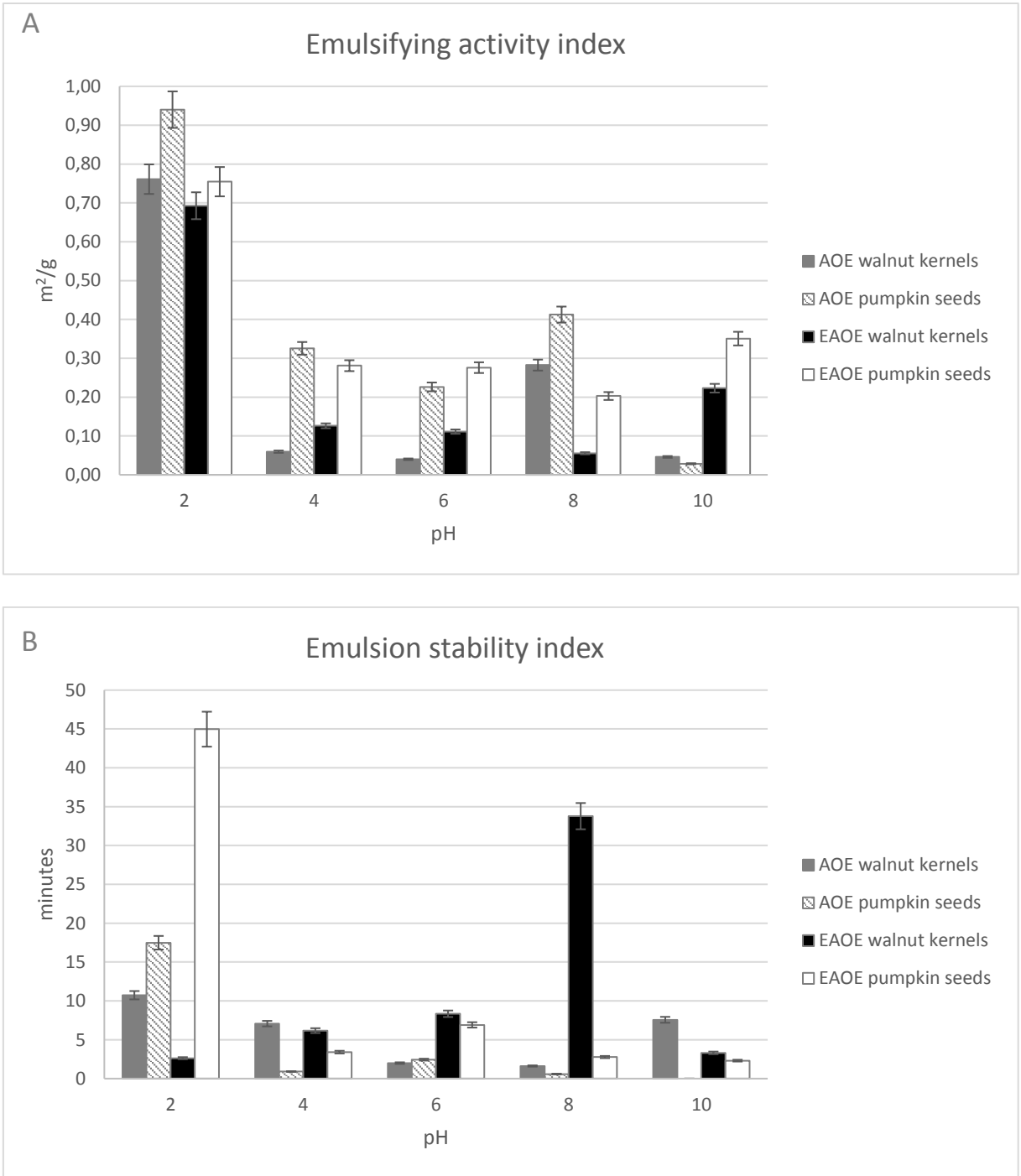


Figure 4.21 Emulsifying activity (A) and emulsion stability (B) of walnut kernels and pumpkin seeds proteins isolates from AOE and EAOE processes as function of pH.

Foaming properties

The foaming properties of the walnut and pumpkin seed protein isolates from AOE and EAOE processes were pH-dependent (Figure 4.22 A and B). As reported by Rodríguez-Ambriz (2005) with lupin samples, proteins and proteolyzed of both the seeds were found to have good foaming capacities at pH 2.

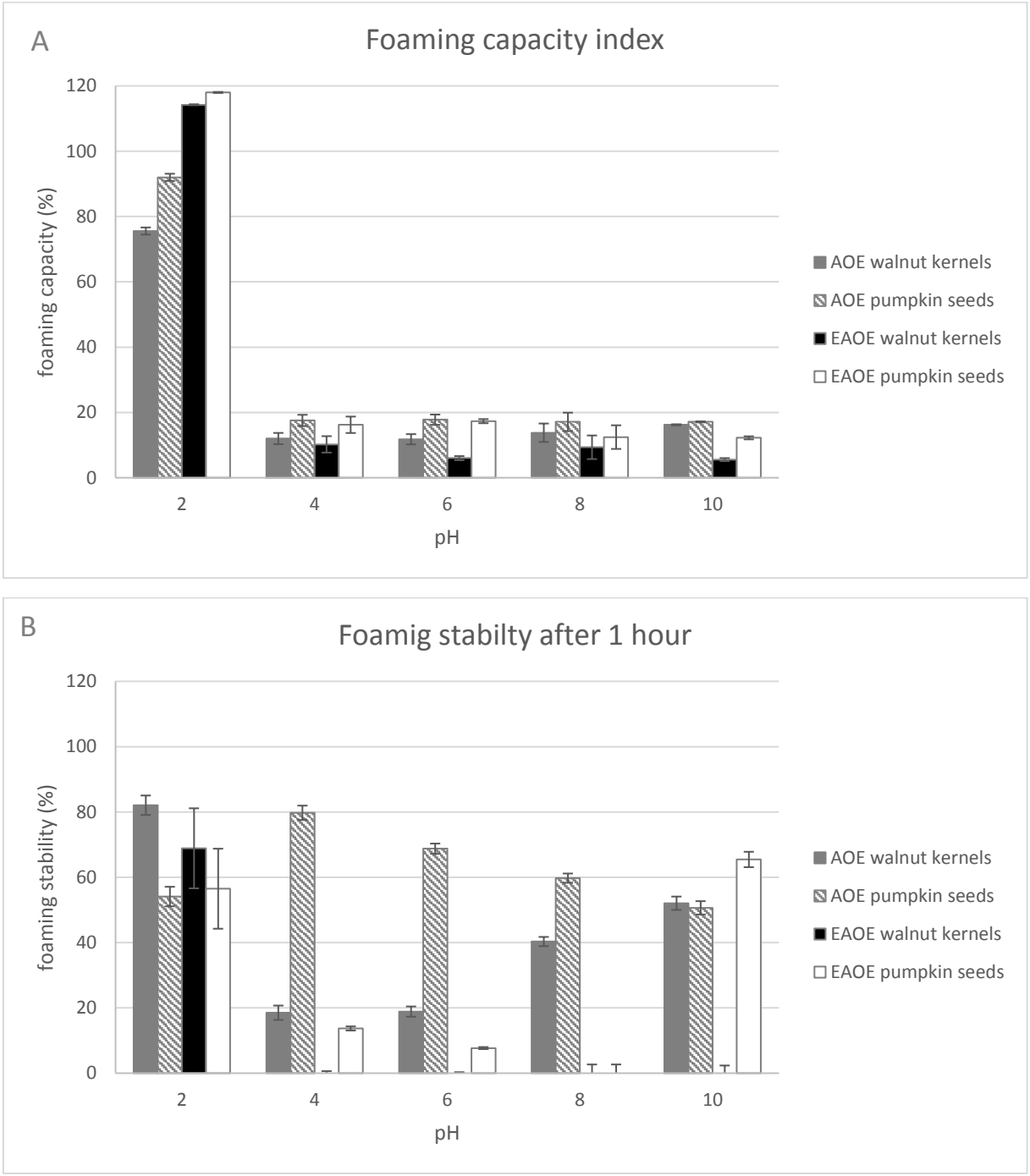


Figure 4.22 Foam capacity (A) and stability (B) of walnut kernels and pumpkin seeds proteins isolates from AOE and EAOE processes as function of pH.

At the other studied pHs, the foam formation trend was almost similar and never exceeds 20% of the initial volume, as shown in Figure 4.22-A. Moreover, EAOE proteolyzed walnut protein isolates resulted in a very high stability at pH 2 which totally disappears at the other pH values; while AOE walnut proteins foaming stability registered the maximum value (more than 80%) at pH 2 followed by a sensible drop to 20% at pH 4 and 6, and by a progressive increase at basic pHs (8 and 10) (Figure 4.22-B). On the contrary, AOE pumpkin seed protein isolates showed a good stability at all the pH values, thus indicating thata, despite the low foam formation, this is persistent over time. Finally, EAOE proteolyzed pumpkin seed proteins showed high foam stability at pH 2 and 10. These trends are significant for the functions that foam has in food technologies, as well as for preparations that require stable foams over time.

Least gelation concentration

AOE and EAOE of walnut and pumpkin seeds protein isolates were investigated for the gelling properties. For this purpose, solutions with increasing concentration (from 2 to 20%) were subjected to thermal treatment and sudden cooling to induce the formation of the gel (Abbey *et al.,* 1988). The analyses demonstrated that AOE and EAOE of walnut protein isolates were able to form gels at all the tested concentrations. Instead, aqueous solutions of AOE pumpkin seed protein isolate showed gelling properties only starting at a concentration of 14%. Unfortunately, this gelling capacity was lost when the proteins were treated with papain in EAOE process, indicating that the partial proteolysis of the protein components prevented subsequent aggregation to form a network with gel properties.

5. Conclusions and future perspectives

Nowadays, great attention is paid by to the recycling and refining of the waste produced. For this reason, many companies are increasingly looking for new processes that can be easily scaled up from a managerial point of view and that are both economically beneficial and sustainable. As extensively discussed, the partner company of my PhD was accumulating flours and pieces as an unused by-product. Since oil is the main use of oilseeds, the idea was to utilise the waste, which can only be resold in the form of animal feed, as starting material for oil extraction. Considering the need to operate in the waste utilisation with low environmental impact, it was decided to study an innovative and environment friendly process. It has been known for some time from the literature that it is possible to extract oil from oilseeds by aqueous processes with or without the aid of enzymes. In fact, Rosenthal et al (1996) described in detail the processes for obtaining oil by aqueous processes, and their potential advantages over solvent-based processes currently used. These advantages relate to environmental, safety, and possibly economic aspects; however, this kind of process depends on type of chosen seeds. In fact, in most of the enzyme-assisted aqueous oil extraction procedures reported in the literature, commercial enzyme preparations containing varieties of enzyme activities, such as lipase, cellulase, pectinase and amylases, were used. For example, Yusoff *et al.* (2017), while working with *Moringa oleifera*, used a mixture of protease and cellulase to maximize the oil recovery. Their results show that protease rise up the recovery of oil because of the high level of proteins in *Moringa oleifera*. Based on the results largely describe before, even pumpkin seeds and walnut kernels are rich in proteins. Therefore, the enzyme chosen in this study was a protease and in particular, a commercial papain. This work showed that enzyme-assisted aqueous oil extraction gave higher yield than the simple aqueous oil extraction. In that case, the proteases had an important role because of the high level of proteins of the matrix. Therefore, papain may help the recovery of oil wich was also affected by other parameters, such as time and solid-water ratio.

Moreover, aspects relating to water recycling and enzyme reutilization must also be considered, in order to make the process more attractive for companies. As a matter of the fact, the immobilization of the enzyme was carried out. About this, functional magnetic DAS (dialdehyde starch) nanoparticles have been prepared through covalently linking DAS and Fe_3O_4 by epichlorohydrin. Being synthesized by common industrialized raw materials, and due

to the convenient magnetic operation, as well as the extraordinary reusability, the MP-DAS-papain particles have a great potential to be used as environmentally friendly and economical method for the enzyme-assisted aqueous oil extraction from oilseeds. Moreover, the quick and operative immobilization and reuse methods of immobilized enzymatic systems offer the possibility of cost reduction in biotechnological applications. This binding method can be used to bind other enzyme with different functions. Hence, it represents a very economical way to use them in industrial projects. For process optimization, one must not only consider the yield obtained in the extraction step, but also the stability of the resulting oil-in-water emulsion and its implications on downstream processing.

Considering these conclusions and the results shown in this dissertation, the future perspectives are many, but the most important one is certainly the development of a pilot plant involving the use of immobilized enzymes on magnetite nanoparticles. In parallel, other features as long-term storage stability and, in general, storage stability conditions should be necessarily developed. In a context of industries circular economy, a possible strategy could be to integrate into the process the already existing systems of oil extraction by centrifugation, widely used in the olive oil extraction sector. Through these methods, applying the innovation of enzyme recovery through a magnet, a possible company scale-up is certainly not a gamble.

6. Supplementary data

6.1 Microscopy

6.1.1 Methods

Light microscopy

The whole walnut kernels were paraffine-embedded and frozen under liquid nitrogen. Then 0.2 mm thick slices were cut by microtome (rotative cryostat microtome Leica CM 1860) and placed on a specimen slide for optical microscope. The samples were dewaxed through washing with a short alcohol series (100%, 95%, 85% and 70%), and then the specimen slides were transferred in 100 mM sodium acetate buffer (pH 5,4). For the enzymatic treatment, papain or cellulase were added at a final concentration of 0.5% (w/v). The samples were incubated overnight with single enzymes under mild rocking shaking. The sample treated with both enzymes was first subjected to the cellulase action for 4 hours, and then, after a gentle washing in the same buffer, incubated overnight with papain. Untreated controls were also done in same buffer without enzymes. All the specimen slides were washed with buffer and with increasing alcohol series (70%, 85%, 95% and 100%), before colouring for 2 minutes with 1% SUDAN BLACK solubilized in 100% ethanol. Excess of dye was removed by exhaustive washing with 100% ethanol. Finally, the slides were close with cover slip and analysed at optical microscope (Leica AmScope 40X-2000X LED). Representative micrographs from all the samples were selected.

Scanning electron microscopy (SEM)

Walnut kernels were cut in transverse and longitudinal slices and de-fatted by incubation with n-Hexane for 1 hour at 40°C/200 rpm in orbital shaking. After the solvent removing, the slices were dried 18 hours at room temperature, and then placed in 100 mM sodium acetate buffer pH 5,4 containing 1,5% (w/v) of either papain or cellulase and incubated overnight under mild rocking shaking. For the double enzyme treatments, the samples were sequentially immersed for 4 hours in the first and for 18 hours in the second enzyme solutions, with an exhaustive washing in between. After treatments, all slices were frozen and freeze-dried to obtain the completely dehydrated samples. Buffer incubated slices as well as simply de-fatted samples

were taken as controls. Dehydrated seed tissues were mounted on aluminium stubs and coated with gold by means of DC sputtering (Sputter and Carbon Coater Agar Scientific B7340) in order to make the sample conductive. The microstructure of samples was observed at a magnification of 500 and 1000× with LEO EVO 40 SEM (Zeiss, Germany) with a 20 kV acceleration voltage. Representative micrographs from all the samples were selected.

6.1.2 Results and Discussions

Light microscopy

Pre-fixed sections of walnut were treated with different enzymes, stained with Sudan Black and observed by light microscopy. The oil bodies appeared as distinct bodies and coloured of black because of the dyeing. Moreover, it is possible also to observe the cell wall that surround the oil bodies. Figure 6.1 shows the images of samples subjected to treatments with different enzymes compared with the untreated controls. In section A the untreated sample shows the intracellular space full of oil bodies and the tissue surface seems to be covered by something like a film. This film does not cover the whole surface in section B, when the sample was re-hydrated with buffer, but the intracellular space is still full of oil bodies. Sections C and E show samples treated with cellulase (C) and cellulase and papain (E) and in both the film disappears from the surface, the tissue structure appears like in section B: it is possible to distinguish both the cell wall and the oil bodies. Finally, section D shows the image of the walnut slice treated with papain. In that section, the cell wall appears intact, but the intracellular space is completely empty, demonstrating that the papain can damage the oil bodies provoking the release of oil from the cell. According to *Ellis et al., (2004)*, the LM can show only a surface image (the first layer of cells), so, what it appears is that the sample treated with papain releases only the superficial oil.

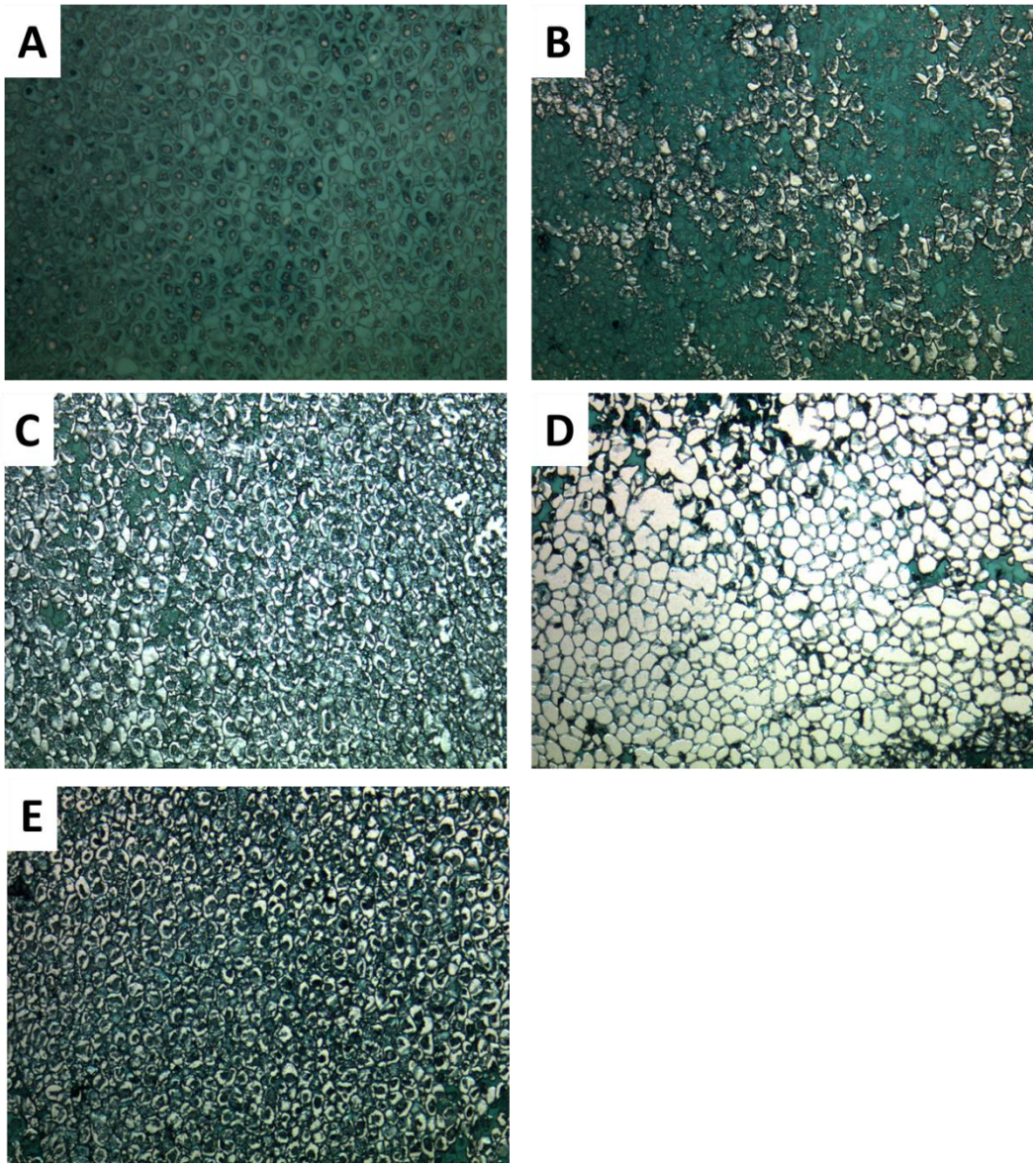


Figure 6.1 Optical microscopy of walnut samples treated with enzymes. A- untreated dried walnut slice; B- untreated hydrated walnut slice used as control (no enzyme); C- sample treated with cellulase; D- sample treated with papain and E- sample treated with cellulase for 4 h followed by papain incubation overnight; all samples were stained with Sudan Black.

Scanning electron microscopy (SEM)

Figure 6.2 shows scanning electron microscopy of walnut samples treated with enzymes, papain and cellulase, or mixture of them and the controls. As reported by *Dave et al., (2019)* with the SEM analysis the spherical droplet can be associate to oil bodies in oilseeds. As in the LM, also in this case it is possible identify a film that surround the whole surface of the untreated dry sample (section A) compared to the hydrated control (section B). In sections A, B, C and E it is possible to observe droplets, so oil bodies are viewed in all these samples. In section D, the droplets are fewer due to the papain hydrolysis. Moreover, in samples C and E

the organized structure that appear in section D was destroyed because of the cellulase hydrolysis that hollowed out the cell wall and the walnut structure. So, in section C and E the three-dimensional vision is more evident due to the enzyme's action.

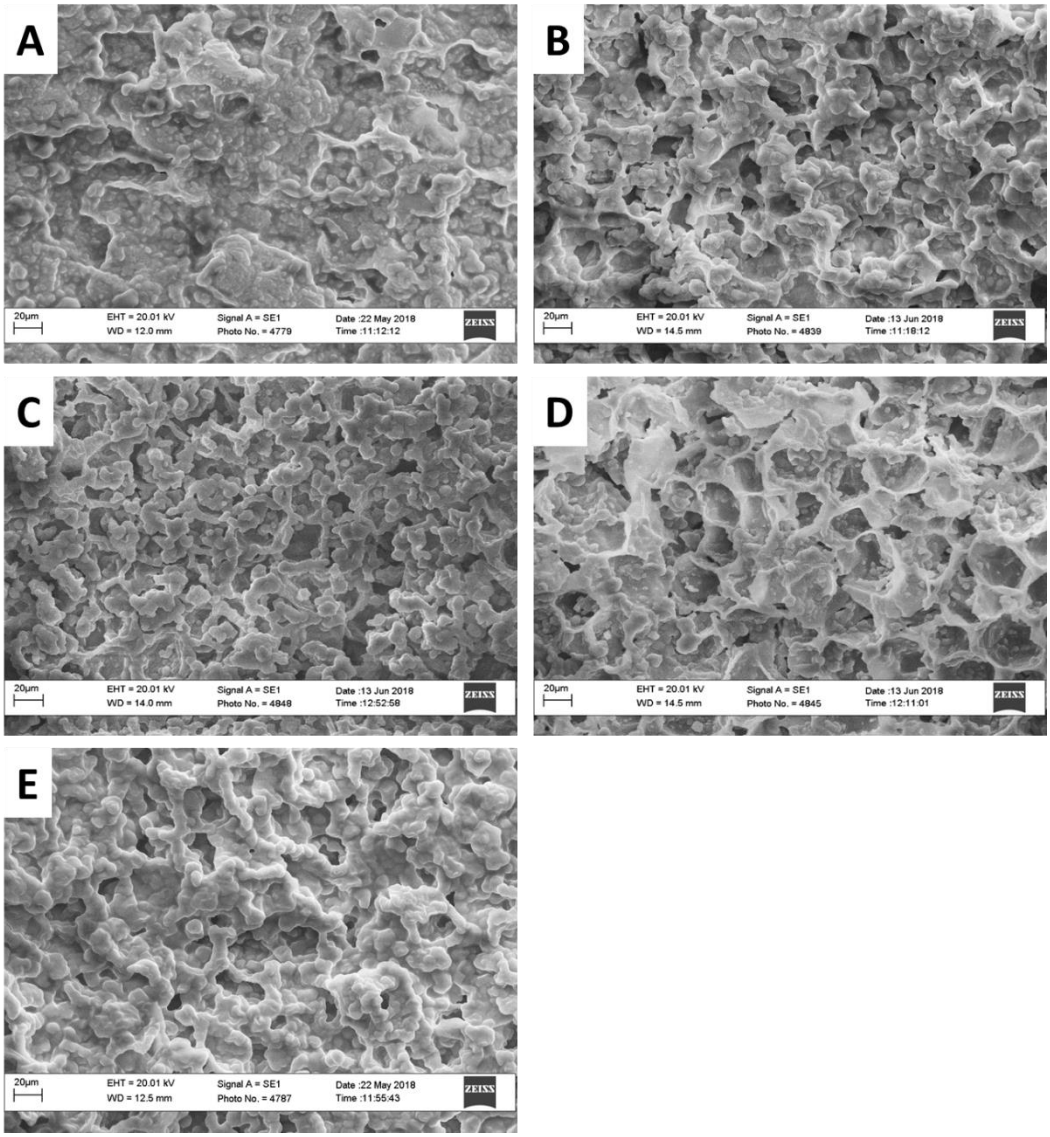


Figure 6.2 Scanning electron microscopy of walnut samples treated with enzymes. A- untreated dried walnut slice; B- untreated hydrated walnut slice used as control (no enzyme); C- sample treated with cellulase; D- sample treated with papain and E- sample treated with cellulase for 4 h followed by papain incubation overnight.

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Conference Proceedings

Conference:	XXIII workshop on the developments in the Italians PhD research on food science, technology and biotechnology. Oristano 19th-20th-21st Sept 2018
Poster title:	Strategies for enzymatic extraction of oil from oilseeds
Authors:	Serena Marulo, Angela Sorrentino, Paolo Masi

Conference:	SLIM 2019 – 9° Shelf Life International Meeting, Naples, Italy, June 17-20th 2019
Poster title:	Recovery of proteins and peptides from oilseeds by-products
Authors:	Serena Marulo, Angela Sorrentino, Alessia Ramondo, Aniello Falciano, Pierfrancesco Motti, Paolo Masi
Award:	Best Poster Award

Conference:	XXIV workshop on the developments in the Italians PhD research on food science, technology and biotechnology. Florence 11-13 September 2019.
Poster title:	Enzyme-assisted aqueous extraction of oil from oilseeds by-products
Authors:	Serena Marulo, Angela Sorrentino, Paolo Masi

Conference:	EFF2021 the 3rd International Conference on ENGINEERING FUTURE FOOD, Naples, Italy 23-26 May 2021
Poster title:	Aqueous Enzyme Assisted Oil Extraction from Oilseeds by using papain-coated Magnetic Microparticles
Authors:	Marulo Serena, Ramondo Alessia, Aliberti Giuliana, Di Pierro Prospero, Regalado-Gonzáles Carlos, Masi Paolo

Conference:	35th EFFoST International Conference 2021 Lausanne, Switzerland 1-4 November 2021
Poster title:	Aqueous enzyme-assisted oil extraction from pumpkin seeds (*Cucurbita maxima*)
Authors:	Serena Marulo, Angela Sorrentino, Prospero Di Pierro, Alessia Ramondo, Paolo Masi
Notes	Submited Abstract

Awards

- **Best poster Award**

Conference	SLIM 2019 – 9° Shelf Life International Meeting, Naples, Italy, June 17-20th 2019
Poster title	Recovery of proteins and peptides from oilseeds by-products
Authors	Serena Marulo, Angela Sorrentino, Alessia Ramondo, Aniello Falciano, Pierfrancesco Motti, Paolo Masi

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