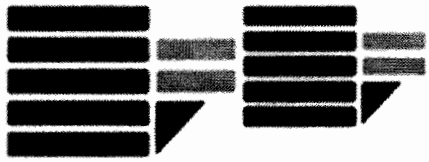

UNIVERSITA' DELLA CALABRIA

Department of Informatics, Modeling, Electronics and System Engineering

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Doctor of Philosophy

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XXVII Cycle

EXTRACTION OF NUTRACEUTICALS FROM NATURAL MATRICES

S. S. D.

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Coordinator/Supervisor: Prof. Bruno de Cindio

Tutor: Prof. M.S. Sinicropi

Ph-D: Dr Valentina Perri

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UNIVERSITA' DELLA CALABRIA

Dipartimento di Ingegneria Informatica, Modellistica, Elettronica e Sistemistica

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XXVII Ciclo

EXTRACTION OF NUTRACEUTICALS FROM NATURAL MATRICES

Il presente lavoro di tesi è finalizzato all'estrazione, sia con tecniche convenzionali che con tecnologie innovative, di nutraceutici da matrici vegetali.

Un nutraceutico è una sostanza (o principio attivo), con proprietà medicamentose, presente in un alimento. Esempi di nutraceutici sono i probiotici, gli antiossidanti, gli acidi grassi polinsaturi (ω-3, ω -6), le vitamine ed i complessi enzimatici. Tipicamente vengono utilizzati sotto forma di alimenti funzionali per prevenire le malattie croniche, migliorare lo stato di salute, ritardare il processo di invecchiamento ed aumentare l'aspettativa di vita.

In questo lavoro è stata investigata la possibilità di estrarre composti bioattivi mediante fluidi supercritici utilizzando la CO_2 come solvente, il quale presenta numerosi vantaggi sia operativi che d'impatto ambientale. Non è tossico, è disponibile ad un elevato grado di purezza e non lascia residui nell'estratto in quanto diffonde a temperatura ambiente; le sue proprietà solventi possono essere manipolate cambiando pressione, temperatura e tempo di estrazione ed inoltre, variando il pretrattamento della matrice vegetale ed aggiungendo particolari terre con differenti caratteristiche, si può raggiungere un'elevata selettività di estrazione.

Tra le piante che contengono nutraceutici la nostra attenzione è stata focalizzata sull' *Opuntia ficus-indica*, una pianta endemica del Sud America, molto invasiva e ben adattata in zone aride o semiaride come l'aria Mediterranea. La materia prima risulta quindi facilmente disponibile ed offre la possibilità di interagire sia a livello industriale (produzione di massa) che a livello delle piccole e medie imprese sul mercato alimentare, farmaceutico, nutraceutico e cosmetico.

Numerose ricerche affermano che la pianta è ricca di vitamine, minerali ed aminoacidi e rivelano inoltre la presenza di pigmenti colorati solubili in acqua ad alta attività antiossidante, chiamati betalaine, nei frutti di *Opuntia ficus-indica*. I cladoni,

contengono sia composti ad attività antiossidante, polifenoli quali rutina, isoquercitina, nicotiflorina e narcissina che una serie di polisaccaridi con importanti proprietà funzionali, reologiche e nutrizionali.

E' stata inizialmente investigata la possibilità di ottimizzare, mediante SFE-CO_2 la selettività di estrazione tra i polifenoli e le altre sostanze presenti in cladoni appartenenti a due differenti ecotipi di O*puntia ficus-indica* (L.) Mill. presenti in Calabria.

Dati bibliografici riportano che gli estratti dei frutti possiedono numerose attività biologiche quali antilipidemica, antiulcera, analgesica ed anticancro,

Obiettivi della seconda parte del lavoro sono da un lato quello di valutare la possibilità di estrarre le betalaine mediante SFE-CO_2 e dall'altro di valutare l'effettiva attività antiossidante dell'estratto da frutti di *Opuntia ficus-indica* in emulsioni sia di tipo O/A che A/O sottoposte ad ossidazione forzata e di valutarne le proprietà reologiche e la stabilità, effettuando sia misure di viscosità, per una valutazione delle proprietà di scorrimento, che misure in oscillatorio, per una valutazione delle proprietà di deformabilità della struttura del materiale.

Nell'ultimo anno è stato svolto un periodo di studi di sei mesi presso il Rowett Institute of Nutrition & Health, University of Aberdeen (United Kingdom): Il lavoro ha previsto l'identificazione e quantificazione, mediante l'utilizzo di tecnologie estrattive specifiche e mirate, di composti bioattivi presenti in nuove colture di cereali e pseudocereali,

Le classi di principi attivi sui quali è stata effettuata l'indagine sono: composti fenolici e flavonoidi, antocianine, catechine e saponine. Il lavoro contribuisce ad un ampio progetto di ricerca in corso chiamato "*Novel food crops*" sotto la guida dal governo scozzese.

CHAPTER I

VALORISATION OF THE AGRO-FOOD PRODUCTS

Introduction

Consuming a high level of dietary diversity is one of the most longstanding and universally accepted recommendations for human health at national, regional and international levels (WHO, Europe. 2011). Nowadays in the world there is a high interest in the issue of sustainable diets;.

Agriculture is the bedrock of the food system and biodiversity is critically important to food and agriculture systems because it provides the variety of life. Biodiversity includes the variety of plants, terrestrial animals and marine and other aquatic resources (species diversity), along with the variety of genes contained in all individual organisms (genetic diversity), and the variety of habitats and biological communities (ecosystem diversity). Biodiversity is essential for humanity, providing food, fibre, fodder, fuel and medicine in addition to other ecosystem services (SCBD, 2006).

It has been clearly shown that low-cost foods are those energy-dense (fat- and sugar-rich) and nutrient-poor, inducing both deficiencies and overweight consequences of inappropriate food choices, often driven by household income and education level.

The high energy content of most food consumed (meat, egs, butter, snacks etc.) can fit the important needs of people with a high energy expenditure, but is in excess for most urbanized sedentary people (Etiévant *et al.*, 2010); that are progressively less influenced by the local cultural heritage and a not suited integration in the local environment.

Just in the last two human generations ago are been generate large greenhouse gas emissions and are been promote marked alterations of ecosystems such as

biodiversity loss, deforestation, soil erosion, chemical contaminations, water shortage (Gallagher *et al.*, 2005).

The use and exploitation of plants widely disseminated or invasive in nature, which contain bioactive compounds, it would be the best way to have beneficial effects on health; both would reduce all those problems that we have in the long-distance transportation of fruits and vegetables from one country to another, resulting in a reduction of taste, texture and nutritional qualities.

1.1 CACTUS INVASIVES

The Mediterranean basin is one of the bio geographical regions with greater plant diversity in the world (Myers *et al.*, 2000) with approximately 10% (25,000 species) of the vascular flora present on 1.6% of the Earth's surface (Heywood, 1999). Calabria, located almost at the geometric centre of the Mediterranean, has ancient and rich traditions and a documented ethno botanical use for more than 600 food species. For all these reasons it is important to study, research and monitoring of species belonging or massively present to the territory.

1.1.1 Plants of genus *Opuntia* spp.

Among the plants that contain nutraceuticals our attention was focused on cactus pear, a plant tropical or subtropical belonging to the genus *Opuntia*, invasive, widespread and well adapted to arid and semi-arid zones such as the Mediterranean area. This genus is endemic of South America and up to now 377 species has been recognized, 104 have been wild in Mexico, and 60 of which are endemic to this country (Zhong *et al.*, 2010). It was an important part of the agricultural economy and diet of the ancient Mexicans of the Aztec empire; now cacti are distributed in different countries and all continents. Cacti may be used for both, as a crop for subsistence and for market-oriented agriculture, contributing to the food security of

the population in agriculturally marginalized areas (Andrade-Montemayor *et al.*, 2011).

In some countries as Mexico, Italy and South Africa, the variety *Opuntia ficus-indica* (L.) Mill. (OFI, Fig.1), the most commercial, is cultivated on considerable surface for industrial ends; in Mexico its culture stretches on a surface of 300.000 ha. (Khatabi *et al.* 2011).

Fig. 1.1: Plant *Opuntia ficus indica* (L.) Mill.

In North Africa, the cultivation of OFI cactus is used on the one hand against the soil erosion in arid areas, and on the other hand as a forage substitute during drought (Malainine *et al.*, 2003).

Due to agricultural problems, linked to increasing arid zones and declining water resources, *Opuntia* spp. is gaining importance as an effective source (Guevara-Figueroa *et al.*, 2010). The starting-material results available easily and it gives the possibility to interact both with the industrial mass production on different levels of markets such as: food, pharmaceutical and nutraceutical, but also with the local companies, because it infests the lands of the farmers. *Opuntia* was also traditionally

used to differentiate the properties of paesantry and to curb fires, the cladones have high water content, 95% in mass (Lopez *et al.*, 2009).

The stems are formed by groups of opaque-green cladones, with areolas that contain numerous thorns; they produce large yellow-orange flowers, and an edible fruit. The fruit can change in colour from green - yellow (less sweet) to reddish - purple (very sweet) and orange shades between two both. Several searches show that the Prickly pear is a plant very rich in vitamins, minerals, aminoacids and in sugars (Khatabi *et al.* 2011). The young cladones of OFI, also known as nopalitos, are consumed as vegetables, contain functional polyphenolics, like ruthin, iso-quercitrin, narcissin and nicotiflorin (Guevara-Figueroa *et al.*, 2010) and a series of polysaccharides, like pectins, having high molecular weight and important functional properties such as rheological, medicinal and nutritional (Medina-Torres *et al.*, 2000). The cladones are very perishable and they have a very short storage life, from 5 days at room temperature to 10 days in refrigerated environments (Lopez *et al.*, 2009). In the fruits are present pigments named betalains, widely distributed in the *Cactaceae* family. Several studies, carried out with different species of *Opuntia* fruits, have found a high betalains content. Betalains are water-soluble nitrogenous pigments, they can be divided into two major structural groups, the red and red-violet betacyanins (Latin *Beta*, beet and Greek *kyanos*, blue color) and the yellow betaxanthins (Latin *Beta* and Greek *xanthos*, yellow). Betacyanins can be further classified by their chemical structures into four kinds: betanin-type, amaranthin-type, gomphrenin-type and bougainvillein-type (Strack *et al.*, 1993). Both cladones that the fruits have a high percentage of pectins and fibers, which can increase fecal mass and intestinal motility, affecting cholesterol and glucose plasma levels; as reported in literature, fruits and stems of *Opuntia* have been traditionally used in oriental folk medicines to treat diabetes, hypertension, burns, edema and indigestion (Kim *et.al.*, 2010). It was also been reported that the extracts of fruits or stems exhibit hypoglycemic, anti-ulcer (Lahsasni *et al.*, 2003), anti-allergic, analgesic and antioxidant activity (Galati *et al.*,

2003; Tesoriere *et al.*, 2004; Stintzing *et al.*, 2005; Coria-Cayupan *et al.*, 2011). From the fruits and the stem extracts was isolated β-sitosterol, an active anti-inflammatory principle (Dok-Go *et al.*, 2003; Saleem *et al.*, 2006); fruit and flower infusions significantly enhance diuresis (Galati *et al.*, 2002). The work of Hfaiedh *et al.*, 2008 was to evaluate the protective effects of regular ingestion of juice from the prickly pear cactus (OFI) cladones against nickel chloride toxicity. In the liver, nickel chloride was found to induce an oxidative stress evidenced by an increase in lipid peroxidation and changes in antioxidant enzymes activities. Superoxide-dismutase (SOD) activity was found to be increased whereas glutathione peroxidase and catalase activities were decreased. These changes did not occur in animals previously given cactus juice, demonstrating a protective effect of this vegetal extract. The betacyanin pigments are also related to the preventive action against several diseases like inflammatory (including cardiovascular disease, asthma and rheumatoid arthritis), leukemia and cancer (Sreekanth *et al.*, 2007; Chang *et al.*, 2008).

1.2 CEREAL AND PSEUDOCEREALS

The low nutrient/fibre density of generally consumed food (raw and processed) is a widely acknowledged concern in all countries. As an example, the fibre, mineral, vitamin and anti-oxidant content of whole-wheat bread compared with refined-wheat bread is about three to fourfold higher, for the same amount of energy (Maillot *et al.*, 2007).

In the last five decades there is a trend to find new sources of dietary fiber, such as agronomic by-products that have traditionally been undervalued. Dietary fiber refers to parts of fruits, vegetables, crops, nuts and legumes that cannot be digested by humans. It is a well-established fact that the consumption of adequate amounts of dietary fiber reduces significantly the risk of degenerative diseases, including

diabetes, obesity, coronary heart disease, bowel cancer and gallstones. Dietary fibers also have technological properties that can be used in the formulation of foods, resulting in texture modification and enhancement of the stability of the food during production and storage (Ayadi *et al.*, 2009).

It must be emphasized that although the texture and sensory qualities of the products made from refined flours have been improved, the nutritive values of these products have become lower because most of the nutritional compounds such as minerals, vitamins, dietary fiber, resistant starch and micro constituents have been removed during refining of flours.

It has been argued that changes in agricultural production systems from diversified cropping systems towards ecologically more simple cereal based systems have contributed to poor diet diversity, a potential source of nutrients for the animal feed industries, micronutrient deficiencies and resulting malnutrition in the developed as well as developing world (Graham *et al.*, 2007).

Recent advances in cereal and pseudo-cereal based food researches have been reviewed; pseudo-cereals are broadleaf plants (non-grasses) that are used in much the same way as cereals (true cereals are grasses). Their seed can be ground into flour and otherwise used as cereals. New milling techniques have been applied to produce graded flours which contain large amounts of dietary fiber, vitamins and minerals and could be applied for substitution with the conventionally milled flour in bread-making to produce functional food products with high safety, palatability and nutrition. In addition, germination of cereal and pseudo-cereal grains helps to improve the chemical compositions, nutritive values and acceptability characteristics of the products (Morita *et al.*, 2013).

1.2.1 CHIA (*Salvia hispanica* L.)

Salvia hispanica L. (Fig ure 1.2), whose common name is chia, is an annual herbaceous plant that belongs to the *Lamiaceae* family and it is native from southern Mexico and northern Guatemala.

Fig. 1.2: Plant of *Salvia hispanica* L.

It has been cultivated from tropical to subtropical regions. Although plants are little frost-tolerant, they can be grown in greenhouses in some parts of Europe. Today, chia is grown commercially in Mexico, Bolivia, Argentina, Ecuador, and Guatemala. Its seeds have long been used by the Aztec tribes, they are important not only as food, but also for medicines and paints.

It is traditionally consumed in Mexico and the south western United States, in a minor extent in South America, though is not widely known in Europe. They have been investigated and recommended due to their content of oil, protein, antioxidant and dietary fiber content. Nowadays, chia seed are a potential source of nutrients for

the food and animal feed industries. Although chia is not a well-known food, its global production is increasing due to its healthy properties. Chia seeds are also used as nutritional supplements as well as in the manufacturer of bars, breakfast cereals and cookies in the USA, Latin America and Australia (Muñoz *et al.*, 2012).

The plant is day light sensitive and produces small white and dark seeds (Fig. 13); that one that produce white seeds are encoded by a single recessive gene.

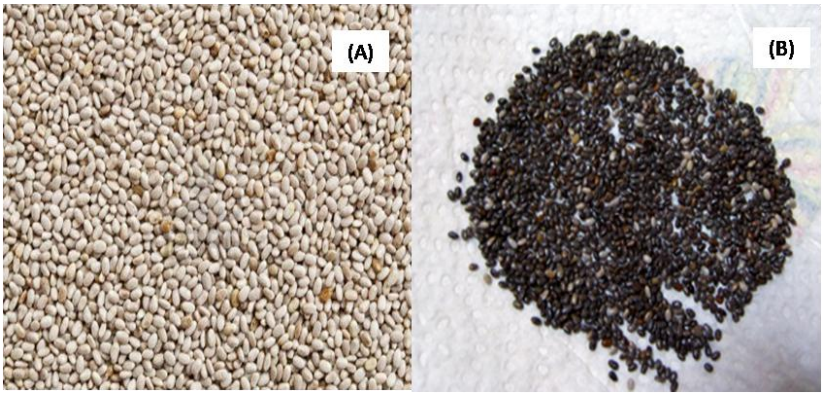

Fig. 1.3: White (A) and dark (B) chia seeds.

Their shape is oval, measuring about 2.0mm×1.5mm. Most of chia populations that grown today contain a low percentage of white seeds. In general, there is a little size difference between these seeds, white seeds are somewhat larger than the black ones; also, there are some differences in protein content and fatty acid composition (Ixtaina *et al*., 2008). Chia crop has been studied principally because of its oil quality; the seed contains between 0.25 and 0.38 g of oil/g seed, where the major constituents are triglycerides, in which polyunsaturated fatty acids (PUFAs, α-linolenic and linoleic acids) are present in high amounts. Ω-3 fatty acids play a very essential role in physiology, especially during fetal and infant growth and in the prevention of cardiovascular diseases, being antithrombotic, antiinflammatory, antiarrythmic and favoring plaque stabilization. The main compounds of the chia oil are volatiles and chemically unstable in the presence of oxygen, light, moisture and heat (Rodea-

González *et al.*, 2012). It also has high level of proteins (0.19 - 0.23 g/g seed), antioxidants, tocopherols (238 - 427 mg/kg) and polyphenols, being the major phenolic compounds chlorogenic and caffeic acids, followed by myricetin, quercetin and kaempferol (Martínez *et al*., 2012).

1.2.2 QUINOA *(Chenopodium quinoa Willd.)*

Quinoa is a species of goosefoot *Chenopodium quinoa* Willd. (Fig.1.4), a grain crop grown primarily for its edible seeds. It is a pseudo-cereal rather than a true cereal, as it is not a member of the true grass family.

Fig.1.4: Plant of *Chenopodium quinoa* Willd.

As a chenopod, quinoa is closely related to species such as beetroots, spinach and tumbleweeds; it is originated in the Andean region of Ecuador, Bolivia, Colombia and Peru, where it was successfully domesticated 3,000 to 4,000 years ago for human consumption. Similar *Chenopodium* species, such as pit seed goosefoot (*Chenopodium berlandieri*) and fat-hen (*Chenopodium album*), were grown and

domesticated in North America as part of the Eastern Agricultural Complex before maize agriculture became popular (Smith, 1999). Fat-hen, which has a widespread distribution in the Northern hemisphere, produces edible seeds and greens much like quinoa, but in smaller quantities. The nutrient composition is favourable compared with common cereals. Quinoa is also cultivated in England, Sweden, Denmark, Netherlands, Italy and France. Recently France has reported an area of 200 ha with yields of 1080 kg/ha and Kenya has shown high seed yields (4 t/ha) (Nascimento *et al.*, 2014).

Quinoa seeds are an excellent raw material for healthy and tasty foods. They are considered easy to digest, because gluten free, and are an unusually complete food, because they possess a well-balanced set of essential amino acids for humans {high methionine (4–10 g/kg DM) and lysine (51–64 g/kg DM) (Peiretti, 2013)}and are a good source of protein (12–18 g/100 g dry weight), fibre, phosphorus, magnesium, and iron. Its fatty acid (FA) composition has a high proportion of unsaturated FA, particularly oleic (OA, C18:1 n-9) and linoleic acid (LA, C18:2 n-6). Among main FA of the plant during growth, α-linolenic acid (ALA, C18:3 n-3) was the most abundant (from 385 to 473 g/kg of total FA), while LA content, which ranged from 146 to 176 g/kg of total FA, decreased with increasing growth until the shoot stage and then increased, while OA and stearidonic acid (C18:4 n-3) did not show differences in their content during growth (Peiretti *et al.*, 2013).

The whole seeds contains a large variety of antioxidant compounds, such as carotenoids, vitamin C and flavonoids. The opportunity to supplement or completely replace common cereal grains (corn, rice and wheat) with a pseudo-cereal of higher nutritional value (such as quinoa) is inherently beneficial to the public interests (Dini *et al.*, 2010).

Quinoa was important to the diet of pre-Columbian Andean civilizations (Keenet Haynes, 2008) and has been called a super food (Keppel, 2012). In fact, protein content is very high for a cereal/pseudo-cereal (14% by mass), yet not as high as

most beans and legumes. Quinoa's protein content per 100 calories is higher than brown rice, potatoes, barley and millet, but is less than wild rice and oats.

Due to its rheological properties, sensory characteristics, nutrient profile and stability, the gluten-free formulations based on quinoa confers a texture similar to corn based formulations. In parallel, the taste, smell and flavor influence and reinforce consumer preferences.

Quinoa is a good source of flavonoids and other bioactive compounds with putative health effects. Studies performed on animals have recently reported a gastro protective activity of quinoa seeds. These effects are mainly attributed to arabinose and arabinose-rich pectic polysaccharides that compose the dietary fibre of quinoa. Recognising the importance of quinoa ‘‘*in providing food security and nutrition and in the eradication of poverty*’’ the General Assembly of United Nations has designated, in its resolution A/RES/66/221, the year 2013 as being the International Year of Quinoa (Nascimento *et al.*, 2014).

The chemical composition of quinoa is closely connected to development of the plant with the quality of crop decreased with increasing morphological stages (Peiretti *et al.*, 2013).

Unfortunately, quinoa seeds contain bitter-tasting constituents located in the outer layers of the seed coat, making it essentially unpalatable. Therefore, most commercial quinoa seeds have been processed to remove their coating by washing or milling so to eliminate bitter compounds before consumption (Dini *et al.*, 2010).

After harvest, the seeds must be processed to remove the coating, they are in general cooked the same way as rice and can be used in a wide range of dishes. Theirs leaves are also eaten as a leaf vegetable, much like amaranth, but the commercial availability of quinoa greens is limited.

It was shown that the drying operation led to reductions of 10% in proteins, 12% in fat and 27% in both fibres and ashes(Miranda *et al.*, 2010).

Quinoa grains range in color from ivory to pinks, brown to reds, or almost black depending on the variety. There are over 120 species of *Chenopodium,* but only three main varieties are cultivated; one producing very pale seeds, called the white or sweet variety; a dark red fruited variety called red quinoa; and a black quinoa (Fig. 1.5).

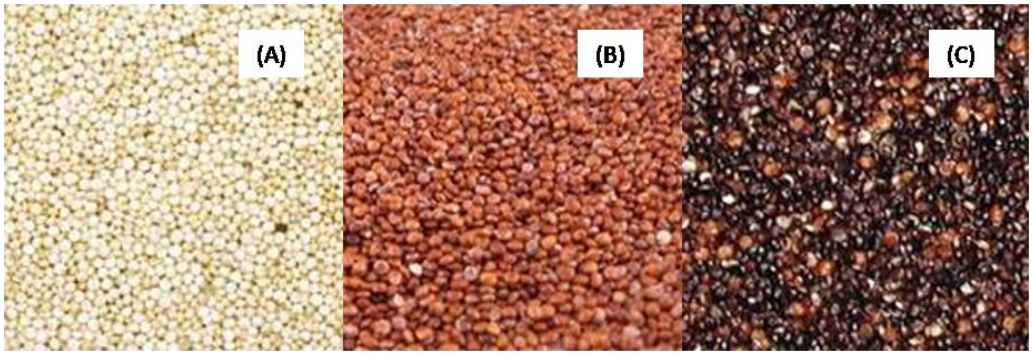

Fig. 1.5 : White or Ivory (A); red (B) and black (C) quinoa seeds.

The seeds are similar in size to millet, but are flat with a pointed oval shape and look like a cross between a sesame seed and millet.

The most common kind of quinoa available in stores produces white seeds, so it is often see it just called quinoa; sometimes it's also called ivory quinoa.

Cooks report that red quinoa holds its shape after cooking a bit better than white one, making it more suitable for cold salads or other recipes where a distinct grain is especially desirable. A bit earthier and sweeter than white quinoa, black quinoa keeps its striking dark color when cooked.

1.2.3 LINSEED *(Linum usitatissimum L.)*

Linseed or flax, the *Linum usitatissimum* L. (Fig. 6) is a versatile crop grown since prehistoric times for its seeds and fiber. More recently, linseed oil has come into focus due to its fatty acid (FA) composition. It is the richest agricultural source of α-linolenic acid (ALA), an essential dietary polyunsaturated fatty acid of Ω-3 class.

Fig. 1.6: Plant of *Linum usitatissimum* L.

Its use in industrial as well as food and feed products including a wide variety of nutraceuticals and health foods has been reported.

Many studies have established the health benefits of ALA in prevention of cardiovascular diseases, cancer, neurodegenerative and inflammatory diseases, and also in lowering blood glucose and cholesterol levels. Indian linseed germplasm consists of more than 3000 varieties with varying oil (30–40%) and ALA (40–50%) content. Varieties with less than 3% of ALA were also developed and used in linseed breeding, targeting production of oil with improved tolerance to rancidity. (Mueller *et al.*, 2010).

Desaturation is an important biochemical process in the FA biosynthesis pathway. Fatty acid desaturases (FADs) are the key enzymes that convert saturated FAs with single bond between two carbon atoms (C–C) to unsaturated FA with double bond (C═C) at a specific location in the fatty acyl chain. Desaturases are classified into two phylogenetically unrelated groups; the membrane bound fatty acid desaturases

and the soluble desaturases, although both are reported to be diiron-oxo enzymes. The distribution of fatty acids and fatty acid desaturases is ubiquitous, observed in all aerobic organisms including algae, fungi, mosses, higher plants and mammals. They play key roles in maintaining proper structure and function of biological membranes (Rajwade *et al.*, 2014).

The main objective of a study on twenty-four pregnant ewes was to evaluate the effects of supplementing lactating diets with extruded linseed on the fatty acid composition of intramuscular and subcutaneous fat depots of suckling lambs. Muscle fat and adipose tissue from the linseed treatment showed higher proportions of polyunsaturated fatty acids (PUFA). The results from this study have shown that dietary extruded linseed supplementation of lactating ewes enhances the nutritional quality of suckling lamb fat depots such as intramuscular and subcutaneous fats (Gómez-Cortés *et al.*, 2014).

Linseed is one of the most important cultivated plants concerning its linen and oil (FAO, 2008). A protein-rich linseed cake results as a by-product of linseed oil production, which is currently mainly used as animal feed or fertilizer. Linseed meal is reported to have a high nutritional potential, not only based on its high protein content, but also because of its water-soluble fiber fraction and lignan content. Lignans especially secoisolariciresinoldiglucoside (SDG), are claimed to be effective in reducing the risk of cardiovascular diseases and might be efficient in inhibiting the development of diabetes. The health benefits of these compounds are suggested to be due to their antioxidant activity, estrogenic and antiestrogenic properties (Mueller *et al*., 2010). There are two kind of linseeds, the Yellow or Golden that is usually treated by hot pressing to recover the oil and the Brown one (Fig. 1.7) that has a predominant market share in comparison to the Golden. Brown linseed is normally processed by a combination of cold and hot pressing.

Fig. 1.7: Yellow or Golden (A) and Brown (B) linseeds.

1.2.4 PUMPKIN (*Cucurbita pepo* L.)

Pumpkin is refers to certain cultivars of squash, most commonly those of *Cucurbita pepo* L. (Fig. 1.8), with smooth, slightly ribbed skin and deep yellow to orange coloration; it is a very common member of *Cucurbitaceae* family that grows widely in temperate and subtropical regions over the world.

Fig. 1.8: Plant of *Cucurbita pepo* L.

Some exceptionally large cultivars of squash with similar appearance have also been derived from *Cucurbita maxima* L. This plant has been employed in the food industry for the production of purees, juices, jams and alcoholic beverages. Pumpkin seeds (Fig. 1.9) are a rich source of bioactive compounds and they have been used frequently as functional foods or medicines. Pumpkin seed oil has been implicated in providing a broad spectrum of health benefits such as prevention of prostate disease, retardation of the progression of hypertension, mitigation of hypercholesterolemia and arthritis, reduction of bladder and urethral pressure and improving bladder compliance; alleviation of diabetes by promoting hypoglycaemic activity, lowering the levels of gastric, breast, colorectal and lung cancers and possessing also good antioxidant potential (Jiao *et al*., 2014). Owing to their composition and, more importantly, to a specific spectrum of fats present, pumpkin seed kernels are regarded as a valuable dietary component. In some countries they are served as a snack, mostly after being salted and roasted.

Fig. 9: Pumpkin seeds.

Pumpkin seed kernels are also used as additives to confectionery and bakery products instead pumpkin seed oil (especially that obtained from hull-less seeds) is utilised by both food and pharmaceutical industries (in food industry generally as salad oil). For many years pumpkin seed kernels have been used in complementary medicine – primarily as vermifuge. They are part of a minor group of plants and herbs containing fatty acids and phytosterols that are administered at the early stage of prostatic hyperplasia therapy (Nawirska-Olszanska *et al*., 2013).

It is evident that both the production of cold pressed pumpkin seeds oil (PSO) and its consumption have been on a constant increase in the last few year. Cold pressed PSO is a relatively new product on the Serbian market; the establishment of a chain of small oil mill plants for producing cold pressed oils was initiated in the late 1990s. Bioactive components, such as vitamins, provitamins, phytosterols, phospholipids and squalene, which are, together with some fatty acids, key PSO nutritional value factors. In addition, it has been proven that these components have a positive effect on human health in many different ways: they have anti-inflammatory and diuretic properties, alleviate negative symptoms of benign prostatic hyperplasia, help lower cholesterol levels, bind free radicals, and others (Rabrenovic *et al*., 2014)

With oil as their main component (which in many instances accounts for 50% of the total content), pumpkin seeds are regarded as a valuable source of protein and fat.

PSO includes fatty acids: palmitic (C 16:0), stearic (C 18:0), oleic (C 18:1) and linoleic (C 18:2). Their content, however, differs among the pumpkin varieties, and was found to dependent on the climate and cultivation conditions (Nawirska-Olszanska *et al*., 2013).

1.2.5 TRITICALE *(Triticosecale* Wittm.*)*

Triticale, *Triticosecale* Wittm. (Fig. 1.10) is a hybrid of wheat (*Triticum*) and rye (*Secale*) first bred in laboratories during the late 19th century. The grain was originally bred in Scotland and Sweden**.**

Fig.1.10 : Plant of *Triticosecale* Wittm.

Commercially available triticale is almost always a second generation hybrid, i.e., a cross between two kinds of primary (first cross) triticales. As a rule, triticale combines the yield potential and grain quality of wheat with the disease and environmental tolerance (including soil conditions) of rye. Only recently has it been

developed into a commercially viable crop. Depending on the cultivar, triticale can more or less resemble either of its parents. It is grown mostly for forage or fodder, although some triticale-based foods can be purchased at health food stores or to be found in some breakfast cereals. The *International Maize and Wheat Improvement Center Triticale Improvement* program wanted to improve food production and nutrition in developing countries. Triticale has potential in the production of bread and other food products, such as cookies, pasta, pizza dough and breakfast cereals (Mohamed *et* Gómez-Macpherson, 2004).

The protein content is higher than that one of wheat, although the glutenin fraction is lesser. The grain has also been stated to have higher levels of lysine; later research found its starch was particularly readily digested (Bird *et al.*, 1999).

1.2.6 POTATO BEAN *(Apios americana medikus)*

The genus *Apios,* belonged from family of *Fabaceae,* is a legume with species distributed worldwide. *Apios Americana* Medikus (Fig. 1.11) is sometimes called the potato bean, hopniss, Indian potato, hodoimo or American groundnut.

Fig 1.11. Plant of *Apios Americana* Medikus

It is a perennial vine that produces both edible tubers (Fig 1.12) and fleshy roots, which were consumed by native Americans and early settlers. The quality of *Apios* tuber protein has been investigated in three different species, and a high degree of variability is evident.

Fig 1.12 Tubers of *Apios americana* Medikus

The crude protein content, on a dry-mass basis, was highest in *A. americana* (16 %) followed by *A. fortunei* (13.3 %) and *A. priceana* (6.8 %) (Krishnan *et al*., 1996).

Its vine can grow to 1–6 m long, with pinnate leaves 8–15 cm long with 5–7 leaflets. The flowers are usually pink, purple, or red-brown, and are produced in dense racemes 7.5–13 cm in length; the fruit is a legume 5–13 cm long. Botanically speaking, the tubers are rhizomatous stems, not roots. *A. Americana* is native from America in which there is a renewed interest as a potential food crops (Ameny *et al*., 1994). Its natural range is from Southern Canada (including Ontario, Quebec, and New Brunswick) down through Florida and West as far as the border of Colorado (Reynolds *et al.,* 1990).

Due to the fact that the tubers contain a high content of proteins and are used as functional foods, the current research was focused on the functional element in the tuber; it has high nutritional values that attract consumers' attention and has been cultivated widely in northern Japan (Iway & Matsue, 2007).

It was showed that the AATI (a trypsin inhibitor purified from *A. americana* tubers) has the inhibitory effects on the proliferation of cancer cell lines, and has similar or stronger inhibitory activity that of SBBI (Soybean Bowman-Birk inhibitor). Agarose

gel electrophoresis of the genomic DNA showed that the DNA was broken into the fragment state by treatment with AATI. These results suggested that inhibitory effects on the proliferation of cells by AATI were caused by induction of apoptosis. Moreover, chemical modification of arginine or lysine residues in AATI resulted not only in the partial loses of protease-inhibitor activity, but also in the inhibitory effect on cancer cell lines. This result supported the rationality of *Apios* tubers as functional foods, and AATI may serve as an alternative resource for SBBI for medical applications and it can be used as a substitute for potato to meet the Western countries demands, which will be helpful to improve the cancer incidence by food intake methods (Zhang *et al.*, 2011). Epidemiological research results have shown that diets containing high amounts of soybean products contribute to low cancer incidence and mortality rates, particularly for breast, colon, and prostate cancers, which are more serious in Western countries. Since *Apios* tubers share similar characteristics of potato, perhaps it may be easier to substitute for potato without drastically altering the eating patterns of the Western population.

Potato bean contains a novel isoflavone (isoflavones are flavonoids widely found in plants), genistein-7-O-gentiobioside. Additionally, isoflavones have been proven to exert various pharmacological actions; such as carcinostatic (anticarcinogenic and anti-proliferative), antihypertensive, antioxidative, and antiallergic activities (Liu *et al*., 2005), gaining increasing interest as functional components of soybeans.

Recently were reported the protective effect of groundnuts against blood pressure elevation (Iwai *et al*., 2008).

Takashima *et al.* 2013 examined the biological activities of an alcohol extract of groundnut containing genistein-7-O-gentiobioside as the main component; the extract by itself did not show antioxidative activity instead genistein-7-O-genitiobioside, isolated, showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity in a concentration-dependent manner (Nara *et al.*, 2011).

Pretreatment of human breast carcinoma with the groundnut extract and soybean isoflavone increased gene expression of heme oxygenase-1 (HO-1), a major antioxidative stress enzyme. Although the groundnut extract hardly showed radical-scavenging capacity, it completely inhibited oxidative stress induced by radical initiator AAPH; the intracellular ROS level in the groundnut extract-treated cells was lower than that in untreated cells, and the GSH level was higher than in the untreated cells. These results suggest that the groundnut extract had isoflavone-like activity.

The isoflavone glycoside consumed as part of the diet is converted to its aglycone by the action of β-glycosidase enzymes of the intestinal bacteria; genistein-7-O-gentiobioside may be converted to genistein in the intestine (Takashima *et al*., 2013).

CONCLUSIONS

Traditional diets, containing a high proportion of lesser known and underutilized agro food products, are an ideal basis for sustainable diets, for chronic disease prevention and to obtain environmental benefits. In order to facilitate this, would be an appropriate enhancement of both plants that are found easily in nature, because highly invasive, such as O*puntia*, and cereals and pseudocereals, more or less cultivated, but not always well known because they are rich in bioactive compounds such as polyphenols and vitamins and dietary fiber as well.

Cultural heritage, food quality and culinary skills are key aspects determining sustainable dietary patterns and food security.

The current food production, food supply and food consumption system does not generally fit present and future human needs, because it is unable to satisfactorily feed everybody and relies on high fossil energy use, chemicals, and energy inputs, long-distance transport, low-cost human work and cultural loss.

This generate and contribute to have both micronutrient and fibre deficiencies as well as excess intakes of fat and sugar promoting overweight and obesity, in a general trend of reduced physical activity and body energy expenditure.

Therefore, the consumption of cereal and pseudoceral has been considered to have many physiological benefits related to “western diseases” such as coronary heart disease, colon cancer and diabetes. These vegetables are also easily to be used as feed or for the production of biofuels. Recently, researchers tried to find a good method to prepare cereal and pseudocereal foodstuffs containing sufficient amounts of nutritional compounds.

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CHAPTER 2

EXTRACTION OF METABOLITE BIOACTIVES FROM NATURAL MATRICES

Introduction

The history of plant's used for mankind is as old as the start of humankind. Initially, people used plants for their nutritional purposes but after the discovery of medicinal properties, this natural flora became a useful source of disease cure and health improvement across various human communities. Egyptian papyruses showed that coriander and castor oil were useful for medicinal applications, cosmetics and preservatives through thousands of recipes. During Greek and Roman period, a thousand of therapeutic uses of herbal plants were described by several scholars namely Hippocrates, Theophrastus, Celsus, Dioscorides and many others.

Romanians are known for their use of medicinal herbs since very long. For example, Herodotus (5^{th} century B.C) mentioned *Leonurus cardiac* was used by the people living north of the Danube river in his writings.

In 19^{th} century Romanian pharmacopoeia introduced herbal products and in 1904 the first institute of medicinal herbs was established in Cluj city. The use of herbal plants in the ancient time actually illustrates the history of bioactive molecules (Vinatoru *et al.*, 1997).

In the past, people had no idea about bioactive molecules but the use of these compounds was sufficiently diverse in different prospect. Typically, bioactive compounds of plants are produced as secondary metabolites. Every living body, from one cell bacterium to million cell plants, processes diverse chemical compounds for their survival and subsistence. All compounds of biological system can be divided into two broad areas. One is primary metabolites, which are the chemical substances

aimed at growth and development, such as carbohydrates, amino acids, proteins and lipids. Anothers are secondary metabolites, which are a group of compounds other than primary metabolites believed to help plant to increase their overall ability to survive and overcome local challenges by allowing them to interact with their surroundings. In different words, secondary metabolites are those molecules which are often produced in a phase of subsequent to growth, have no function in growth (although they may have survival function), are produced by certain restricted taxonomic groups of microorganisms, have unusual chemical structures, and are often formed as mixtures of closely related members of a chemical family. The production of secondary metabolites in different species is mainly selected through the course of evaluation and the particular need of that species. For example, synthesis of aroma by floral species to attract insect for their pollination and fertilization, and synthesis toxic chemicals has evolved toward pathogens and herbivores for suppressing the growth of neighboring plants.

Among secondary metabolites some of these have effects on biological systems which are considered as bioactive. Thus a simple definition of bioactive compounds in plants is: secondary plant metabolites eliciting pharmacological or toxicological effects in human and animals (Kittredge, 2012).

Extraction is the first step of any medicinal plant study, these methods are sometimes referred as ‘‘sample preparation techniques’’. It is true that development of modern chromatographic and spectrometric techniques make bioactive compound analysis easier than before but the success still depends on the extraction methods, input parameters and exact nature of plant parts.

Normally, a high extraction yield is required for an efficient process, although it will not necessarily ensure a high concentration of bioactive components.

Since some bioactive components are very sensitive to oxygen and, should be taken to prevent their oxidation and thermal degradation. Therefore, the extraction yield

and the bioactive components characteristics should also be considered when an extraction method is selected (Aspé *et* Fernández, 2011).

2.1 Classification and biosynthesis of bioactive compounds

Classification of bioactive compounds in different categories is still inconsistent rather it depends upon the intention of the particular classification. For example, biosynthetic classifications, which serve for simplify of the description of biosynthetic pathways, will not match the scope of pharmacological classification. According to Croteau *et al.* (2000), bioactive compounds of plants are divided into three main categories: (a) terpenes and terpenoids (approximately 25,000 types), (b) alkaloids (approximately 12,000 types) and (c) phenolic compounds (approximately 8000 types).

The majority of bioactive compounds belong to one of a number of families, each of which has particular structural characteristics arising from the way in which they are built up in nature (biosynthesis). There are four major pathways for synthesis of secondary metabolites or bioactive compounds:

1. Shikimic acid pathway;
2. Malonic acid pathway;
3. Mevalonic acid pathway;
4. Non-mevalonate (MEP) pathway.

Alkaloids are produced by aromatic amino acids (come from shikimic acid pathway) and by aliphatic amino acids (come from tricarboxylic acid cycle). Phenolic compounds are synthesized through shikimic acid and malonic acid pathways. Through mevalonic acid pathway and MEP pathway terpenes are produced (Ares *et al.*, 2013).

These secondary metabolites can have therapeutic actions in humans and they are often refined to produce drugs (Springbob *et* Kutchan, 2009).

2.2 Extraction of bioactive compounds

Considering the great variations among bioactive compounds and huge number of plant species, it is necessary to build up a standard and integrated approach to screen out these compounds carrying human health benefits. Farnsworth *et al.* (1985) reported an integrated approach showing sequence of medicinal plant study, which started from name collection of frequently used plants and ended at industrialization. It is only possible to conduct further separation, identification, and characterization of bioactive compounds followed by an appropriate extraction process. Different extraction techniques should be used in diverse conditions for understanding the extraction selectivity from various natural sources. All these techniques have some common objectives:

- to extract targeted bioactive compounds from complex plant samples;
- to increase selectivity of analytical methods;
- to increase sensitivity of bioassay by increasing the concentration of targeted compounds;
- to convert the bioactive compounds into a more suitable form for detection and separation;
- to provide a strong and reproducible method that is independent of variations in the sample matrix (Smith, 2003).

2.2.1 Conventional extraction techniques

Bioactive compounds from plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. In order to obtain

bioactive compounds from plants, the existing classical techniques are: (1) Soxhlet extraction, (2) Maceration and (3) Hydrodistillation.

Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet (1879). It was designed mainly for extraction of lipids but now it is not limited for this only. The Soxhlet extraction has widely been used for extracting valuable bioactive compounds from various natural sources and it is also used as a model for the comparison of new extraction alternatives. Generally, a small amount of dry sample is placed in a thimble. The thimble is then placed in distillation flask which contains the solvent of particular interest. After reaching to an overflow level, the solution of the thimble-holder is aspirated by a siphon. The siphon unloads the solution back into the distillation flask. This solution carries extracted solutes into the bulk liquid. Solute is remained in the distillation flask and solvent passes back to the solid bed of plant. The process runs repeatedly until the extraction is completed.

Maceration was used in homemade preparation of tonic from a long time. It became a popular and inexpensive way to get essential oils and bioactive compounds. For small scale extraction, maceration generally consists of several steps. Firstly, grinding of plant materials into small particle is used to increase the surface area for proper mixing with solvent. Secondly, in maceration process, appropriate solvent named as menstruum is added in a closed vessel. Thirdly, the liquid is strained off but the marc which is the solid residue of this extraction process is pressed to recover large amount of occluded solutions. The obtained strained and the press out liquid are mixed and separated from impurities by filtration. Occasional shaking in maceration facilitate extraction by two ways: (a) increase diffusion, (b) remove concentrated solution from the sample surface for bringing new solvent to the menstruum for more extraction yield.

Hydrodistillation is a traditional method for extraction of bioactive compounds and essential oils from plants. Organic solvents are not involved and it can be performed before dehydration of plant materials. There are three types of hydrodistillation:

water distillation, water and steam distillation and direct steam distillation (Vankar, 2004). In hydrodistillation, first, the plant materials are packed in a still compartment; second, water is added in sufficient amount and then brought to boil. Alternatively, direct steam is injected into the plant sample. Hot water and steam act as the main influential factors to free bioactive compounds of plant tissue. Indirect cooling by water condenses the vapor mixture of water and oil. Condensed mixture flows from condenser to a separator, where oil and bioactive compounds are separate automatically from the water (Silva *et al.*, 2005). Hydrodistillation involves three main physicochemical processes; Hydrodiffusion, hydrolysis and decomposition by heat. At a high extraction temperature some volatile components may be lost. This drawback limits its use for thermo labile compounds extraction. Extraction efficiency of any conventional method mainly depends on the choice of solvents (Cowan, 1999). The polarity of the targeted compounds is the most important factor for solvent choice. Molecular affinity between solvent and solute, mass transfer, use of co-solvent, environmental safety, human toxicity and financial feasibility should also consider in selection of solvent for bioactive compound extraction.

2.3 Non-conventional or innovative extraction techniques

The major challenges of conventional extraction require longer extraction time, requirement of costly and high purity solvent, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds (Luque de Castro *et* Garcia-Ayuso, 1998). To overcome these limitations of conventional extraction methods, new and promising extraction techniques are introduced. These techniques are referred as nonconventional extraction techniques. Some of the most promising techniques are ultrasound assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, pulsed electric field assisted extraction, supercritical fluid extraction and pressurized liquid extraction.

Some of these techniques are considered as ‘‘green techniques’’ as they comply with standards set by *Environmental Protection Agency*.

These include less hazardous chemical synthesis; designing safer chemicals, safe solvents auxiliaries, design for energy efficiency, use of renewable feedstock, reduce derivatives, catalysis, design to prevent degradation, atom economy, and time analysis for pollution prevention and inherently safer chemistry for the prevention of accident.

2.3.1 Ultrasound-assisted extraction (UAE)

Ultrasound is a special type of sound wave beyond human hearing. Usually, in chemistry it is 20 kHz to 100 MHz. Like other waves, it passes through a medium by creating compression and expansion. This process produces a phenomenon called cavitation, which means production, growth and collapse of bubbles. A large amount of energy can produce from the conversion of kinetic energy of motion into heating the contents of the bubble. The bubbles have temperature about 5000 K, pressure 1000 atm and, heating and cooling rate above 10^{10} K/s. Only liquid and liquid containing solid materials have cavitation effect. The main benefit of UAE can be observed in solid plant sample because ultrasound energy facilitates organic and inorganic compounds leaching from plant matrix (Herrera *et* Luque de Castro, 2005). Probable mechanism is ultrasound intensification of mass transfer and accelerated access of solvent to cell materials of plant parts. The extraction mechanism by ultrasound involves two main types of physical phenomena: (a) the diffusion across the cell wall and (b) rinsing the contents of cell after breaking the walls (Mason *et al.*, 1996). Moisture content of sample, milling degree, particle size and solvent are very important factors for obtaining efficient and effective extractions. Furthermore, temperature, pressure, frequency and time of sonication are the governing factors for the action of ultrasound. UAE have also been incorporated along with various classical techniques as they are reported to enhance the efficiency of a conventional

system. In a solvent extraction unit, an ultrasound device is placed in an appropriate position to enhance the extraction efficiency.

The advantages of UAE include reduction in extraction time, energy and use of solvent. Ultrasound energy for extraction also facilitates more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, selective extraction, reduced equipment size, faster response to process extraction control, quick start-up, increased production and eliminates process steps (Chemat *et al.*, 2008).

2.3.2 Pulsed-electric field extraction (PEF)

The pulsed electric field (PEF) treatment was recognized as useful for improving the pressing, drying, extraction, and diffusion processes during the last decade (Vorobiev *et al*., 2005). The principle of PEF is to destroy cell membrane structure for increasing extraction. PEF has been applied to improve release of intracellular compounds from plant tissue with the help of increasing cell membrane permeability (Toepfl *et al*., 2006).

During suspension of a living cell in electric field, an electric potential passes through the membrane of that cell. Based on the dipolare nature of membrane molecules, electric potential separates molecules according to their charge in the cell membrane. After exceeding a critical value of approximately 1V of transmembrane potential, repulsion occurs between the charge carrying molecules that form pores in weak areas of the membrane and causes drastic increase of permeability. Usually, a simple circuit with exponential decay pulses is used for PEF treatment of plant materials. It has a treatment chamber consisting of two electrodes where plant materials are placed. PEF treatment at a moderate electric field (500 and 1000 V/cm; for 10^{-4}- 10^{-2} s) is found to damage cell membrane of plant tissue with little temperature increase. Due to this reason, PEF can minimize the degradation of heat sensitive compounds. PEF is also applicable on plant materials as a pre-treatment

process prior to conventional extraction to lower extraction effort (Lopez *et al.*, 2009).

2.3.3 Enzyme-assisted extraction (EAE)

Some phytochemicals in the plant matrices are dispersed in cell cytoplasm and some compounds are retained in the polysaccharide- lignin network by hydrogen or hydrophobic bonding, which are not accessible with a solvent, in a routine extraction process. Enzymatic pre-treatment has been considered as a novel and an effective way to release bounded compounds and increase overall yield. The addition of specific enzymes like cellulase, α-amylase, and pectinase during extraction enhances recovery by breaking the cell wall and hydrolizing the structural polysaccharides and lipid bodies (Singh *et al.*, 1999). There are two approaches for enzyme-assisted extraction: (1) enzyme-assisted aqueous extraction (EAAE) and (2) enzyme- assisted cold pressing (EACP). Usually, EAAE methods have been developed mainly for the extraction of oils from various seeds. In EACP technique, enzymes are used to hydrolyze the seed cell wall, because in this system polysaccharide-protein colloid is not available, which is obvious in EAAE. Various factors including enzyme composition and concentration, particle size of plant materials, solid to water ratio, and hydrolysis time are recognized as key factors for extraction. The EAE is recognized as eco-friendly technology for extraction of bioactive compounds and oil because it uses water as solvent instead of organic chemicals (Puri *et al.*, 2012).

2.3.4 Microwave assisted extraction (MAE)

The microwave-assisted extraction is also considered as a novel method for extracting soluble products into a fluid from a wide range of materials, using microwave energy. Microwaves are electromagnetic fields in the frequency range from 300 MHz to 300 GHz. They are made up of two oscillating fields that are perpendicular such as electric field and magnetic field. The principle of heating using

microwave is based upon its direct impacts on polar materials. Electromagnetic energy is converted to heat following ionic conduction and dipole rotation mechanisms. During ionic conduction mechanism heat is generated because of the resistance of medium to flow ion. On the other hand, ions keep their direction along field signs which change frequently. This frequent change of directions results in collision between molecules and consequently generates heat. The extraction mechanism of microwave assisted extraction is supposed to involve three sequential steps: first, separation of solutes from active sites of sample matrix under increased temperature and pressure; second, diffusion of solvent across sample matrix; third, release of solutes from sample matrix to solvent. Several advantages of MAE have been described by Cravotto *et al*. (2008), such as quicker heating for the extraction of bioactive substances from plant materials; reduced thermal gradients; reduced equipment size and increased extract yield. MAE can extract bioactive compounds more rapidly and a better recovery is possible than conventional extraction processes. It is a selective technique to extract organic and organometallic compounds that are more intact. MAE is also recognized as a green technology because it reduces the use of organic solvent (Alupului, 2012).

2.3.5 Pressurized liquid extraction (PLE)

In 1996, Richter *et al.* first described PLE. This method is now known by several names; pressurized fluid extraction (PFE), accelerated fluid extraction (ASE), enhanced solvent extraction (ESE), and high pressure solvent extraction (HSPE) (Nieto *et al.*, 2010).

The concept of PLE is the application of high pressure to remain solvent liquid beyond their normal boiling point. High pressure facilitates the extraction process. Automation techniques are the main reason for the greater development of PLE-based techniques along with the decreased extraction time and solvents requirement.

PLE technique requires small amounts of solvents because of the combination of high pressure and temperatures which provides faster extraction. The higher extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate and, also decrease the viscosity and surface tension of solvents, thus improving extraction rate.

In comparison to the traditional Soxhlet extraction, PLE was found to dramatically decrease time consumption and solvent use (Richter et al., 1996).

2.3.6 Supercritical fluid extraction (SFE)

The application of supercritical fluid for extraction purposes started with its discovery by Hannay and Hogarth (1879) but the credit should also be given to Zosel who presented a patent for decaffeination of coffee using SFE (Zosel, 1964). Since this beginning, supercritical fluid technique has attracted wide scientific interest and it was successfully used in environmental, pharmaceutical and polymer applications and food analysis. Several industries have been using this technique for many years, especially, decaffeinated coffee preparation industries.

Every earthly substance has three basic states namely: solid, liquid and gas. Supercritical state is a distinctive state and can only be attained if a substance is subjected to temperature and pressure beyond its critical point. Critical point is defined as the characteristic temperature (Tc) and pressure (Pc) above which distinctive gas and liquid phases do not exist. In supercritical state, the specific properties of gas and/or liquid become vanish, which means supercritical fluid cannot be liquefied by modifying temperature and pressure. A supercritical fluid possesses gas-like properties of diffusion, viscosity, and surface tension, and liquid-like density and solvation power. These properties make it suitable for extracting compounds in a short time with higher yields. A basic SFE system consists of the following parts: a tank of mobile phase, usually CO_2, a pump to pressurize the gas, co-solvent vessel and pump, an oven that contains the extraction vessel, a controller

to maintain the high pressure inside the system and a trapping vessel. Usually different type of meters like flow meter, dry/wet gas meter could be attached to the system. Carbon dioxide is considered as an ideal solvent for SFE. The critical temperature of CO_2 (31°C) is close to room temperature, and the low critical pressure (74 bars) offers the possibility to operate at moderate pressures, generally between 100 and 450 bar. The only drawback of carbon dioxide is its low polarity which makes it ideal for lipid, fat and non-polar substance, but unsuitable for most pharmaceuticals and drug samples. The limitation of low polarity of carbon dioxide has been successfully overcome by the use of chemical modifier. Usually a small amount of modifier is considered as useful to significantly enhance the polarity of carbon dioxide. The properties of sample and targeted compounds and the previous experimental results are main basis for selection of the best modifier. The successful extraction of bioactive compounds from plant materials rely upon several parameter of SFE and most importantly these parameters need to be precisely controlled for maximizing benefits from this technique. The major variables influencing the extraction efficiency are temperature, pressure, particle size and moisture content of feed material, time of extraction, flow rate of CO_2, and solvent-to-feed-ratio.

The advantages of using supercritical fluids for the extraction of bioactive compounds can be understood considering following points: (1) The supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more penetration to sample matrix and favorable mass transfer. Extraction time can be reduced substantially by SFE in compared with conventional methods; (2) The repeated reflux of supercritical fluid to the sample provides complete extraction; (3) The selectivity of supercritical fluid is higher than liquid solvent as its solvation power can be tuned either by changing temperature and/or pressure; (4) Separation of solute from solvent in conventional extraction process can easily be bypassed by depressurization of supercritical fluid, which will save time; (5) SFE is operated at room temperature, so an ideal method for thermo

labile compound extraction; (6) In SFE, small amount of sample can be extracted compared with solvent extraction methods which will save time for overall experiment; (7) SFE uses little amount of organic solvent and considered as environment friendly; (8) On-line coupling of SFE with chromatographic process is possible which is useful for highly volatile compounds; (9) The recycling and reuse of supercritical fluid is possible and thus minimizing waste generation; (10) SFE scale can be arranged on specific purpose from few milligram samples in laboratory to tons of sample in industries; (11) SFE process provides information regarding extraction process and mechanism which can be manipulated to optimize extraction process. Supercritical fluid technology is one of the most innovative method for preparing bioactive products from plant materials used as supplements for functional foods (Liza *et al*., 2010) and it results as a promising technology both in food farming and pharmaceutical industry.

The extracts obtained by SFE-CO_2 are also regarded as *Generally Recognised As Safe* (GRAS) for the *American Food and Drug Administration*, being possible to add them to any food without undesirable effects for health. Numerous works have also been done on the application of SFE- CO_2 from vegetable by-products (Romo-Hualte *et al.*, 2012).

CONCLUSIONS

The ever growing demand to extract plant bioactive compounds encourages continuous searchs for convenient extraction methods.

Extractions of plant materials can be done by various extraction procedures. Non-conventional methods, which are more environmental friendly due to decreased use of synthetic and organic chemicals, reduced operational time, and better yield and quality of extract, have been developed during the last 50 years. To enhance overall yield and selectivity of bioactive components from plant materials, ultrasound,

pulsed electric field, enzyme digestion, extrusion, microwave heating, supercritical fluids have been studied, as non-conventional methods.

At the same time conventional extraction methods, such as Soxhlet is still considered as one of the reference method to compare success of newly developed methodology. Substantial number of scientific reports, book chapters and monographies exist where non-conventional methods were extensively reviewed.

The chromatography advancement and awareness about environment are two important factors for the development of most non-conventional extraction processes. However, understanding of every aspect of non-conventional extraction process is vital, as most of these methods are based on different mechanism and extraction enhancement is resulted from different process.

The efficiencies of conventional and non-conventional extraction methods mostly depend on the critical input parameters; understanding the nature of plant matrix; chemistry of bioactive compounds and scientific expertise.

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CHAPTER III

NUTRACEUTICALS OBTAINED BY SFE-CO_2 FROM CLADONES OF TWO DIFFERENT ECOTYPES OF *OPUNTIA FICUS-INDICA*

Introduction

Flavonoids are the most widely distributed group of polyphenols, a class of phytochemicals characterized by their phenolic structures, with >5,000 unique compounds found in various fruits, vegetables, grains, nuts, teas, wines and medicinal plants.

There is now growing interest in these compounds because they are reported to play an important role in the control and prevention of a series of neurodegenerative diseases such as Alzheimer and Parkinson, cancer and tumor genesis, cardiovascular injury, dysmetabolism, inflammation etc., possibly as the result of their *free-radical scavenging* activity (Pradeep & Guha, 2011; Li *et al.*, 2013, Ravishankar *et al.*, 2013).

The antioxidant potential of polyphenols has been correlated to the capacity of donating hydrogen radicals. The number and the configuration of H-donating hydroxyl groups are both important structural features influencing the antioxidant capacity of phenolic compounds (Silva *et al.*, 2009).

The health benefits of natural phytochemicals present in fruits, vegetables, oilseeds and herbs as antioxidants or functional foods promoted their extraction and use.

The conventional techniques to obtain plant extracts, such as steam distillation and organic solvent extraction, usually require several hours or days, spending a large volume of solvents, frequently toxics and with problematic garbage disposal.

Apart from difficulties in the extraction steps, the solute/solvent separation may result in degradation of the thermo-labile components and traces of the solvent used

could be present in the product, which may reduce quality assessment of the extraction yield.

Supercritical fluid technology is the most innovative method for preparing bioactive products from plant materials used as supplements for functional foods (Liza *et al*., 2010) and it results as a promising technology both in food farming and pharmaceutical industry.

The combined liquid-like solvating capabilities and gas-like transport properties, of supercritical fluids, make them particularly suitable for the extraction of diffusion-controlled matrices such as plant tissues (Liu *et al.*, 2009).

Among several gases and liquids researched, carbon dioxide remains the most commonly used fluid because of its low critical point (T_c = 31.1 C°, P_c = 7.38 MPa). Moreover, it's no toxic, availability in high purity at low cost and easily to separate from the extract. The solvent strength of supercritical fluid can be manipulated by changing pressure, temperature, and time of extraction, and also by varying the preventive treatment of the natural matrices, all of these make possible to obtain a remarkably high selectivity (Liza *et al.*, 2010).

The extracts obtained by SFE-CO_2 are also regarded as *Generally Recognised As Safe* (GRAS) for the *American Food and Drug Administration*, being possible to add them to all food without undesirable effects for health. Numerous works have also been done on the application of SFE-CO_2 from vegetable by-products (Romo-Hualte *et al.*, 2012).

In this work it has been investigated the possibility of phytochemicals extraction by using the supercritical fluids extraction (SFE) with CO_2 from cladones of two different ecotypes of O*puntia ficus-indica* (L.) Mill., optimizing the selectivity between phenolics and other substances.

3.1 PHYTOCHEMICALS PRESENT IN THE CLADONES

The young cladones of *Opuntia ficus-indica* contain functional polyphenols (Figure 3.1), like rutin, iso-quercitrin, nicotiflorin and narcissin (Guevara-Figueroa *et al.*, 2010), and a series of polysaccharides, having a high molecular weight and important functional properties such as rheological, medicinal and nutritional (Medina-Torres *et al.*, 2000).

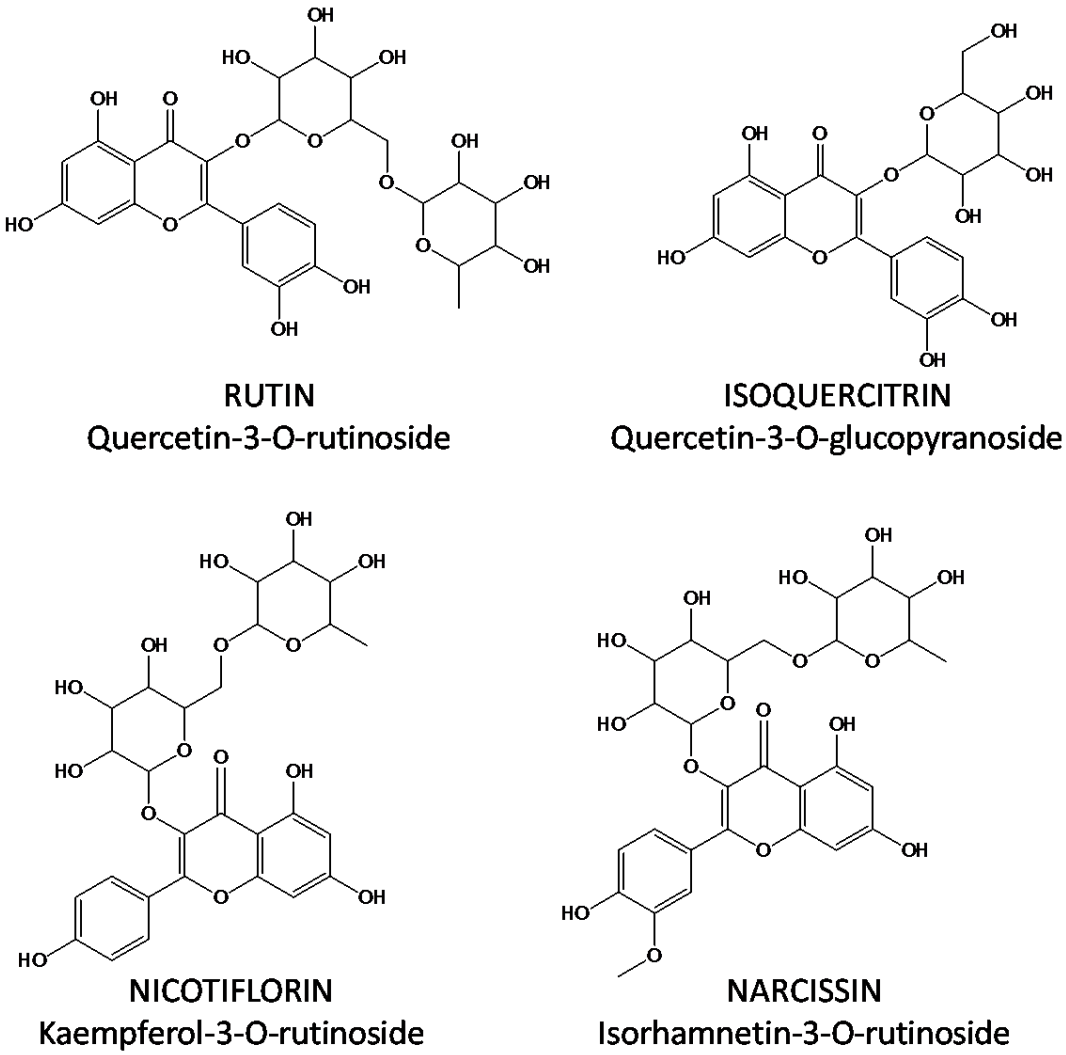

Figure 3.1: Polyphenols present in cladones of *Opuntia ficus-indica*

Rutin is found in many fruits and vegetables and over 130 therapeutic medicinal preparations, that have been registered as drugs worldwide, contain rutin in their formulations (Chua, 2013).

It was showed that rutin exerts *in vitro* cytotoxic effects on SW480 colon cancer cells and on HL-60 leukemic cells; it induces *in vivo* antitumor effects and it is lacks toxic effects on mice bearing SW480 colon tumor. Moreover, it exerts antiangiogenic properties (Alonso-Castro et al., 2013; Araujo et al., 2013) such as anti-inflammatory effects in UVB-irradiated hairless mouse skin by inhibiting expression of COX-2 and iNOS (Choi et al., 2014). In addition, rutin improves spatial memory in Alzheimer's disease transgenic mice by reducing Aβ-oligomer level and attenuating oxidative stress and neuroinflammation (Xu *et al.*, 2014) and it is a potential protective agent for acute lung injury via suppressing the blood gas exchange and neutrophil infiltration. The mechanism is down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation (Chen *et al.*, 2014); it shows a protective effect on cholestatic liver injury induced by biliary obstruction in rats (Pan *et al.*, 2014).

Isoquercitin, as glycosylated flavonoid, is rapidly absorbed and transformed, from gastro-intestinal tract, into glucuronidated quercetin, that was found to be the major form in plasma after oral administration of isoquercitrin in rats; such absorption might be related to the hydrolysis of the sugar moieties attached to its aglycone molecule (Chang *et al.*, 2005). Isoquercitin shows diuretic and potassium-sparing effects in spontaneously hypertensive rats (Gasparotto Junior *et al.*, 2011) and tumor suppression effects in co-treatment with bilberry extracts. It was determinate *in vitro* and *in vivo* antioxidant activity of isoquercitin (Silva *et al.*, 2009).

Also, nicotiflorin shows protective effects on reducing memory dysfunction, energy metabolism failure and oxidative stress in multi-infarct dementia model rats (Huang *et al.*, 2007) and reduces cerebral ischemic damage and up-regulates endothelial

nitric oxide synthase in primarily cultured rat cerebral blood vessel endothelial cells (Li *et al.*, 2006).

Cladones from Opuntia ficus indica are also an important source of fiber and can be used in the diet. In fact, in the last five decades there is a big trend in finding new sources of dietary fiber. Dietary fiber refers to parts of fruits, vegetables, crops, nuts and legumes that cannot be digested by humans. It is a well-established fact that the consumption of adequate amounts of dietary fiber reduces significantly the risk of degenerative diseases, including diabetes, obesity, coronary heart disease, bowel cancer and gallstones. Dietary fibers also have technological properties that can be used in the formulation of foods, resulting in texture modification and enhancement of the stability of the food during production and storage (Ayadi *et al.* 2009).

3.2. MATERIALS AND METHODS

3.2.1 Plant material

Two cladones ecotypes of *Opuntia ficus-indica* (L.) Mill. (Figure 3.2) were analysed in this study: one supposed to be a cultivar with hybrid origin, which often escapes, from cultivation and behave as an invasive species is almost spineless, and it was accepted with the name *Opuntia ficus-indica cult.* (*OFI cult*.); and the second one with white and hard spines, long about 3-4 cm, sharing some characters with *Opuntia amyclaea* Ten., *O. maxima* Miller and *O. ficus-barbarica* A. Berger, Hereafter we will use for the spiny ecotype the name *Opuntia ficus-indica* (L.) Mill. *s.l.* (*OFI s.l.),* because the studied local area lacks of taxonomic and nomenclatural study of the genus *Opuntia*.

Fig.3.2. Two ecotypes of *Opuntia ficus-indica* (L.) Mill under analysis: A) *Opuntia ficus-indica cult.* and B) *Opuntia ficus-indica* (L.) Mill *s.l.*

A part of cladones from *O ficus-indica cult.* were collected during June 2012 in Calabria (South of Italy); another part during August 2012 in order to check the differences due to the stage of maturation. During August were also collected cladones from *O. ficus-indica s.l.*

All the cladones, covered by spines and multicellular hairs or trichomes, were manually cleaned, cutted into small pieces and then homogenized (Figure 3.3) with a TYPE HR 2064 PHILIPS -600 W and stored at -18°C until the analysis.

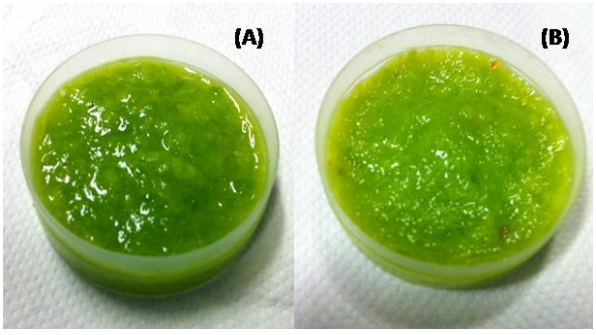

Fig.3.3. Homogenized from *OFI cult.* (A) and *OFI s.l.* (B)

3.2.2 Chemicals

Solvents as methanol (both analytical grade and for HPLC), trifluoroacetic acid, water (both analytical grade and for HPLC) and reagents such as *Folin-Ciocalteau* and Chlorogenic acid were purchased from Sigma-Aldrich (USA).

Ottawa Sand and an Spe-ed^{TM} PSE Matrix - Hydroscopic Samples Dispersing Agent (Diatomee Sand), used like SFE-CO_2 accessories, were purchased from Labservice Analytica S.R.L. (Italy).

Standards, for HPLC analysis and quantification, including Rutin, Isoquercitrin, Nicotiflorin and Narcissin were purchased from Extrasynthese (Lyon, France).

3.2.3 Preliminary analysis

The pH value of the homogenized system (pH 700, Eutech Instruments, Germany), the activity level (Novasina AW Sprint – TH 500, Switzerland), the Brix degrees (ATAGO, Hand Refractometer N Type Series, Japan) and the humidity (Mettler Toledo Moisture Analyzer HB43-S, Switzerland) were measured.

3.2.4 Exhaustive extraction and total phenolics content

The fresh cladones of *Opuntia ficus-indica* (L.) Mill. both ecotypes were extracted with methanol (48 h x 3 times) at 4°C. The same procedures was followed for a sample of *OFI cult.* and *OFI s.l.* Moreover, the exhaustive analysis was performed on samples after drying in oven for 360 min at 32±1°C; removing the 30% of weight.

The extraction solutions were filtered, concentrated and dried under *vacuum* at 35 ±1°C for the thermolability of the poliphenolic compounds.

The total phenol content of the cladones extracts was quantified using *Folin-Ciocalteau* reagent and chlorogenic acid, used as standards. The absorbance was measured at 726 nm (Perkin Elmer Lambda 40 UV/VIS spectrophotometer) and the total content was expressed (mean±S.D. of three determinations) as mg of chlorogenic acid equivalents for g of dry vegetal material (Singleton *et* Rossi, 1965).

3.2.5 Extraction of polyphenols with supercritical fluids

The supercritical extractions by CO_2, on the cladones collected in August from both ecotypes, were performed on a Spe-ed SFE extractor (Applied Separation, Allentown, PA, USA), following the necessary steps: loading in the steel vessel, pressurizing with CO_2 and waiting a transitory state of temperature. When a balanced state was obtained, the system was kept closed for 20 min to promote the diffusion of CO_2 inside the matrix, then the micrometric valve was opened until a constant flow of 1.5 L/min was reach.

The extract leaves the vessel through a valve, which is thermostated to avoid the possible solidification of CO_2. Experiments were carried out at 40°C, 110 bar and 250 bar by varying the preventive sample preparation with PSE accessories, as Ottawa Sand and an Spe-ed^{TM} PSE Matrix (Hydroscopic Samples Dispersing Agent). The different methods to prepare the samples, temperature and pressure of the process are listed in the following Table 3.1.

All samples after collection have been previously cleaned, homogenized and frozen; at the time of the analysis, some were centrifuged (3500 rpm for 10 minutes) and was used the precipitated to remove an aliquot of water present in the matrix. Other partially dried (32°C in oven) in order to reduce the amount of water but not totally so as to use a part of water as a natural co-solvent.

Sample		**Preventive preparation**	**T(°C)**	**P(bar)**
OFI cult.	CULT_20D	20 % Diatomee Sand	40	110
OFI cult.	CULT_20P	20 % Diatomee Sand	40	250
OFI cult.	CULT_90E_20O	Dried until 90% + 20 % Ottawa Sand	40	110
OFI cult.	CULT_30E_20O	Dried until 30% + 20 % Ottawa Sand	40	110
OFI cult.	CULT_C_20D	Centrifuged + 20 % Diatomee	40	110
OFI s.l.	SL_30E_20D	Dried until 30% + 20 % Diatomee Sand	40	110

OFI s.l.	SL_90E_20O	Dried until 90% + 20 % Ottawa Sand	40	110
OFI s.l.	SL_90E_20D	Dried until 90% + 20 % Diatomee Sand	40	110

Table 3.1: Different preparation of the samples before SFE separation.

The extraction time was 1 h. The final extracts were collected in glass tubes and frozen until analysis. Each extractions were carried out in duplicate.

3.2.6 HPLC analysis

The analysis of all the extracts both from solvent extraction and to SFE was carried out by means of HPLC using a Smartline HPLC system (Knauer, Germany). Chromatographic separation was carried out using a 2,0 mm ID ×150 mm L, with precolumn, C-18 TSKgel ODS-100 V, 21810 (TOSOH BIOSCIENCE), both thermostated at 40°C. The mobile phase, flow rate and gradient of elution utilized are reported below in the Table 3.1.

Time (min)	**Methanol [%]**	**Water + 0,1 % TFA [%]**	**Flow (mL/min)**
0,00	0	100	0,2
2,00	20	80	0,2
55,00	100	0	0,2
65,00	0	100	0,2

Table 3.1 Mobile phase, flow rate and gradient of elution utilized.

Absorbance spectra were recorded every 2s, between 200 and 450 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 254 and 280 nm. HPLC analysis was performed in duplicate. The wavelength used for quantification was 280 nm (Guevara-Figueroa *et al.*, 2010). The calibration curve was produced by the integration of absorption peaks generated from the analysis of a dilution serial of Rutin, Isoquercitin, Nicotiflorin and Narcissin.

3.3 RESULTS AND DISCUSSION

3.3.1 Preliminary analysis

Cladones by *Opuntia* genus showed a weakly acid pH, so allow an easier conservation of the homogenized system. The results are showed in Table 3.2.

Higher amount of saccharides was found in the sample of *Opuntia ficus-indica* (L.) Mill. *s.l.*, than that one of *Opuntia ficus indica cult*., whereas the water activity and humidity are almost equal.

PLANT	pH	a_W	°Brix	Humidity
OFI cult.	4,45±0.03	0,935± 0.03	6,5±0.1	96.7 ± 1,5
OFI s.l.	4,50± 0.02	0,933±0.02	7,8±0,2	96.8± 1,3

Table 3.2: Level of acidity, water activity, measure of Brix degrees and humidity of *Opuntia ficus indica cult.* and *Opuntia ficus indica s.l.*

3.3.2 Exhaustive extraction, total phenolics content and HPLC analysis

High content of total phenolics, with the *Folin-Ciocalteau* assay, (Table 3.4) has been observed in the samples dried in oven, Cult_DA and Sl_DA; these two show as well the better quantitative yield of extraction, calculated using the following equation:

The data for the yield of extraction at different sample preparation are shown in the following Table 3.3:

Plant and time of collection	Ref.	Sample Preparation	Yield of Extraction (%)
OFI cult. (June)	Cult_J	Fresh macerated	2,79 % ± 0,29
OFI cult. (August)	Cult_AD	Dried macerated	5,01 % ± 0,63

OFI cult. (August)	Cult_AF	Fresh frozen macerated	2,14 % ± 0,22
OFI s.l. (August)	Sl_AF	Fresh frozen macerated	2,19 % ± 0,25
OFI s.l. (August)	Sl_AD	Dried macerated	4,39 % ± 0,48

Table 3.3: Yield % of extraction (mean±S.D. of two determinations) of samples extracted with methanol

In the Table 3.4 are shown the total phenolic content expressed as mg phenols on 100 g of fresh raw material.

Plant and time of collection	**Ref.**	**Total phenolics content (mg/100 g raw material)**
OFI cult. (June)	Cult_J	170.01± 2.98
OFI cult. (August)	Cult_AD	320,13± 6.54
OFI cult. (August)	Cult_AF	170.01± 2.65
OFI s.l. (August)	Sl_AF	221,29± 5.93
OFI s.l. (August)	Sl_AD	414,90± 9.58

Table 3.4 Total phenolics content (mean±S.D. of three determinations) expressed as mg of total phenols /100 g of macerated raw material.

Despite the quantitative extraction yield was slightly higher in the sample Cult_J, the total phenolic content is almost unchanged between the fresh samples of Cult_J and Cult_AF. This indicates that the different maturation period don't influence the content of secondary metabolites, such as phenols.

Moreover, the total phenolic content is higher in the dried samples Cult_AD and Sl_AD, probably because drying process is designed to dehydrate the matrix in order to stop the common enzymatic processes; the aqueous environment of the cytoplasm of plant cells, could damage the active compounds (Capasso *et al.*, 2006).

The highest content of total phenols was found in the sample of Sl_AD, perhaps because being an wild ecotype is less affected by climate change, more adaptable than a cultivated plant.

In Figure 3.3 are reported the data, obtained by the HPLC analysis, of extractions with solvent.

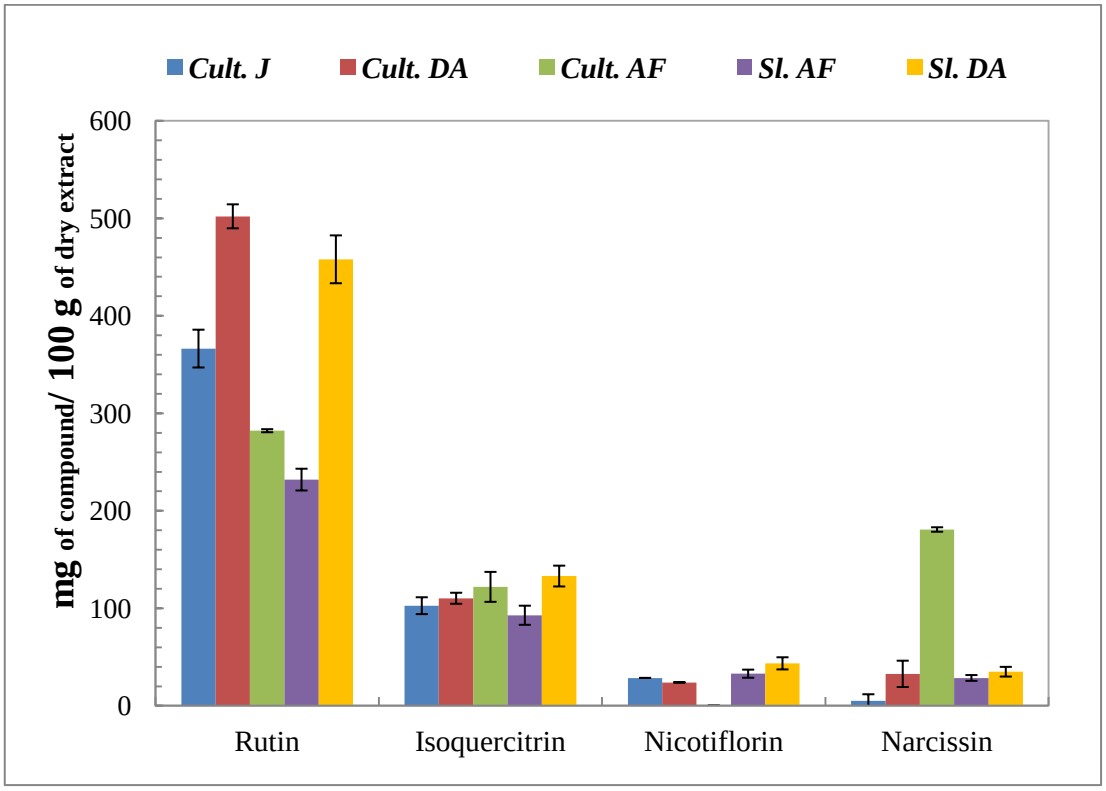

Figure 3.3: Quantification in mg of standards in 100 g of dry extract.

The polyphenol present in greater amount in all species is Rutin; the dried samples (Cult_AD and Sl_AD) contain greater amount than the fresh. It follows Isoquercitin, present in a similar quantity in all the samples. Then they follow, in lesser amount, Nicotiflorin and Narcissina respectively, both slightly variables except for sample Cult_AF. The variability in the amount can be probably due to changes in the secondary metabolism of the plant, for example a different exposure to the sun or to climate change that increased the production of Narcissin instead of Nicotiflorin, not identified in this sample.

3.3.3 Extraction with supercritical fluids and HPLC analysis

In this paragraph the results of the supercritical extraction are reported. The yield of extractions is calculated by using the following equation:

$$\frac{\qquad\qquad}{\qquad\qquad}$$

The results are reported in Table 3.5.

Plant	Ref.	Yield of extraction
OFI cult.	CULT_20D	0,48 % ± 0,07
OFI cult.	CULT_20P	1,00 % ± 0,13
OFI cult.	CULT_90E_20O	0,88 % ±0,11
OFI cult.	CULT_30E_20O	2,14 % ± 0,35
OFI cult.	CULT_C_20D	1,81 % ±0,15
OFI s.l.	SL_30E_20D	0,1 % ±0,03
OFI s.l.	SL_90E_20O	0,25 % ±0,05
OFI s.l.	SL_90E_20D	0,02 % ± 0,01

Table 3.5: Yield % of extraction (mean±S.D. of two determinations) of different samples by SFE-CO_2.

The results of HPLC quantification on mg of compound for 100g of material loaded (mean±S.D. of two determinations) for *OFI cult.* are showed on Figure 3.4, instead for *OFI Sl.* on Figure 3.5.

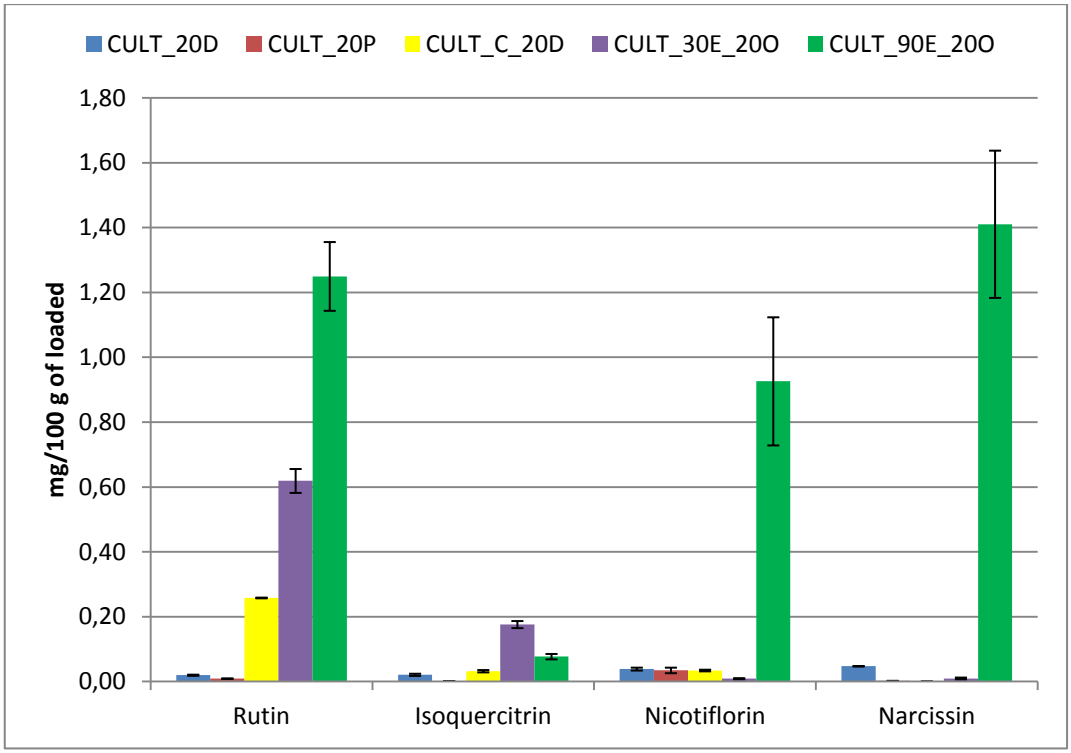

Figure 3.4: Quantification on mg of compound for 100g of loaded material for OFI cult.

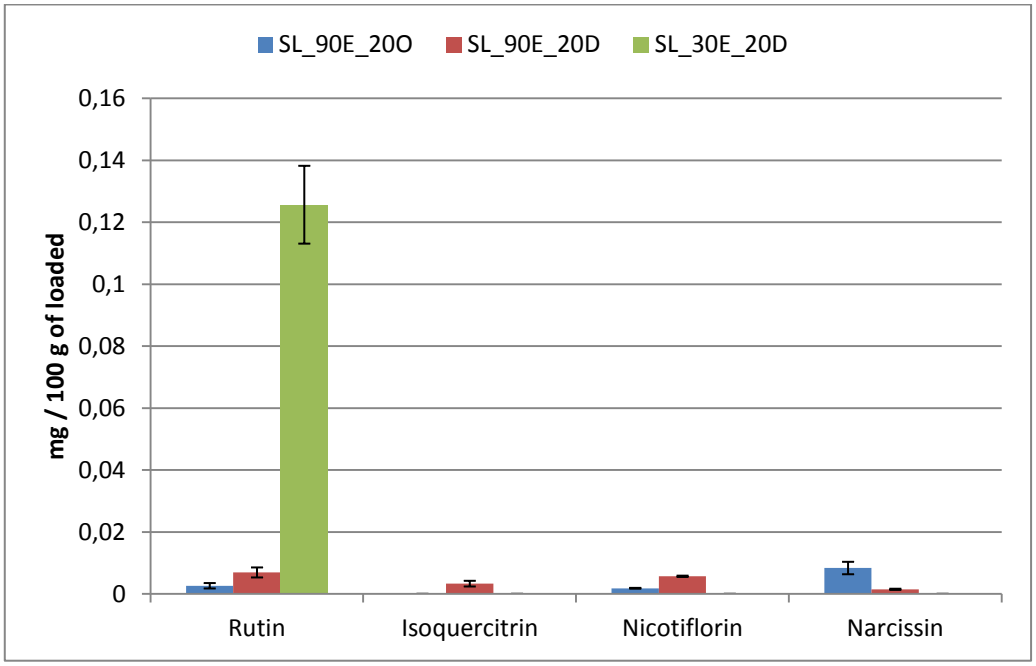

Figure 3.5: Quantification on mg of compound for 100g of loaded material for OFI cult

The yields of extraction with SFE-CO_2 are lower than that one with solvent as well as the amount of polyphenols, but resulting extracts don't need to be separated from solvent, they are purer and cleaner.

The better results were obtained with the samples of *OFI cult*.. spite to the *OFI s.l.* The best yield of extraction was 2,14%± 0,35, obtained with the sample CULT_30E_20O of *OFI cult* dehydratated before at 30% and after added with 20% (of the total weight loaded for the extraction) of Ottawa Sand at 110 bar. Probably the Ottawa Sand prevents preferential pathways within the extraction chamber and allows a better diffusion of CO_2 in the plant matrix and it doesn't adsorb water, which acts as co-solvent.

At the increase of the dehydratation to 90% for the sample CULT_90E_20O, it follows a decrease of the yields % quantitative, but an improvement is obtained with regard to the selective extraction of polyphenols. In this sample we have the highest quantities of Rutin, Narcissin and Nicotiflorin

The treatment of *OFI cult.* with 20% of Diatomee Sand (sample CULT_20D) implies a reduction of both quantity and selectivity of extraction of polyphenols, perhaps due to the fact that the Diatomee Sand is hygroscopic. This sand absorbs water from the surrounding environment reducing the function of co-solvent of water naturally present in the matrix. Being polyphenols polar compounds, the pressure was then raised to 250 bar for the sample CULT_20P, maintaining the same sand, to try to increase the polarity of CO_2, but the effect of the sand was stronger. We have obtained a reduction of both yield % quantitative and selectivity of polyphenols; this shows that high pressures result in a loss of bioactive principles sensitive as polyphenols.

If we centrifuge the raw material (sample CULT_C_20D) to remove water, we had a good increase of the yield % of extraction and a little improvement on selectivity of polyphenols.

The yields % of extractions of the *OFI s.l.* were very low, ranging between 0,02 and 0,25 %; but the selectivity and purity of the extracts derived therefrom is really very high, as can be seen in Figure 3.5 for the sample SL_30E_20D.

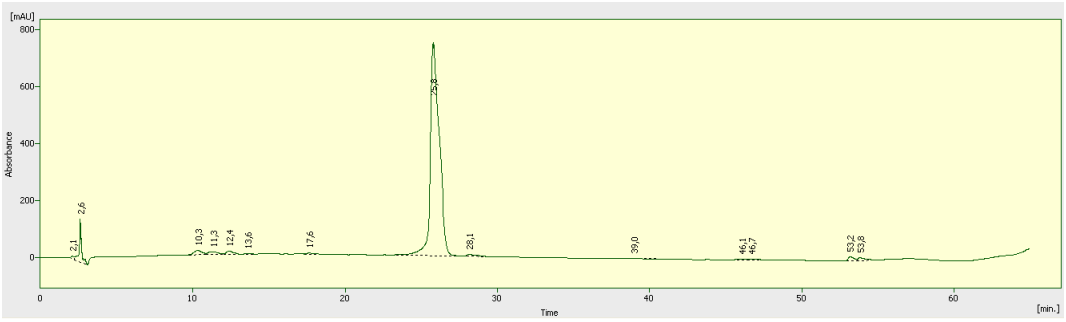

Figure 3.5: Chromatogram resulting from the analysis of the extract by SFE CO_2 of *OFI s.l.* dried to 30% and added 20% of Diatomee Sand.

CONCLUSIONS

There is a really global trend toward the use of natural flavonoids present in fruits, vegetables, oilseeds and herbs as antioxidants or functional foods. They are reported to play an important role in the control and prevention of a series of diseases. Supercritical fluid technology is a really innovative method to extract nutraceuticals from natural matrices for two important reasons; the extracts could be used directly as supplements for functional foods or in the pharmaceutical industry, because they are without any solvents traces and they are considered completely safe for the *American Food and Drug Administration*.

In this work we have studied two different ecotypes of *O. ficus-indica*, showing dissimilar phytochemicals profile. The total phenolic content in the extracts with solvents is almost unchanged between the fresh samples collected in June and in August. This indicates that the different maturation period, if the plant still did not produce the fruit, the content of secondary metabolites, such as phenols, is almost the

same. The polyphenol present in greater amount in all species is Rutin; the dried samples (Cult_AD and Sl_AD) contain greater amount than the fresh.

Being polyphenols polar compounds, yields % quantitative of extraction with SFE-CO_2 are not high even if the extractions are very selective and the extracts nearly pures as can be seen from the sample SL_30E_20D.

Concerning the SFE-CO_2 better results were obtained with the samples of *OFI cult.* spite to the *OFI s.l.* probably because the ecotype OFI sl was much mucilaginous and this prevented to complete some extractions.

The better SFE-CO_2 extraction results were obtained with samples preventively dried, as the sample CULT_90E_20O this means that, by drying up to 90% (by reducing the amount of water that could lead to degradation of active compounds in the matrix), leaving only a small part to exploit the action as a co-solvent, and adding a sand as Ottawa, which not going to bind water we can get highly selective extracts.

The treatment with Diatomee Sand implies a reduction of both quantity and selectivity of extraction of polyphenols, perhaps due to the fact that the Diatomee Sand is hygroscopic and it adsorb water from the surrounding environment, reduces the function of co-solvent of the water naturally present in the matrix.

The polyphenols are polar compounds, in order to increase the polarity of CO_2 we raised the pressure to 250 bar but we have obtained a reduction of both yield % quantitative and selectivity of extraction; this shows that high pressures result in a loss of bioactive principles sensitive as polyphenols.

The yields of extraction with SFE-CO_2 are lower than that one with methanol, but resulting extracts don’t need to be separated from solvent using expensive process such as distillation.

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CHAPTER 4

BIOACTIVE PIGMENTS FROM FRUIT OF *OPUNTIA FICUS INDICA* IN W/O AND O/W SYSTEMS; ANTIRADICAL ACTIVITY AND RHEOLOGICAL CHARACTERIZATION DURING OXIDATION

Introduction

Plant foods are well known sources of vitamins, such as vitamin C and folic acid, carotenoids and fiber, and they are naturally free of saturated fat and cholesterol. High consumption of fruits is associated with a lowered incidence of degenerative diseases including cancer, heart disease, inflammation, arthritis, immune system decline, brain dysfunction and cataracts (Zampini *et al.*, 2011).

The extraction and purification of phytochemicals from natural sources are needed since these bioactives are often used in the preparation of dietary supplements, nutraceuticals, functional food ingredients and additives, pharmaceutical and cosmetic products.

The cactus pear yields an edible fruit, called the prickly pear, that grows on the edges of the flat pads of the cactus, named cladones. The betalains content of the cactus pear fruit is found to be having an application in the low acid foods as natural colorants (Maran *et* Manikandan, 2012).

There has been an increasing trend, towards replacement of synthetic colorants by natural pigments, in the last 20 years, because of natural pigments' safety and health benefits, and strong consumer demand for more natural products.

Although natural pigments are generally less stable and have higher cost than synthetic colorants, their development and utilization is attracting more and more attention. In contrast to synthetic dyes, with an annual growth of 1% on the European market until 2008, colouring foodstuffs are forecasted to gain 10−15% in the same time range. Although not clearly legally defined, colouring foodstuffs are produced

by an unselective extraction of fruits, vegetables or spices using water- or oil-based solvents. Since their characteristic composition remains unchanged, the resulting extracts are considered as food and consequently do not require declaration with an E-number. Therefore, consumer- friendly labelling “coloured with fruit or vegetable extract” is possible giving them the character of an ingredient rather than an additive (Stintzing *et al.*, 2006).

4.1 BETALAINS

Betalains are water-soluble nitrogenous pigments. They can be divided into two major structural groups, the red to red-violet betacyanins (Latin *Beta*, beet and Greek *kyanos*, blue color) and the yellow betaxanthins (Latin *Beta*, beet and Greek *xanthos*, yellow). They are immonium conjugates of betalamic acid with cyclo-dopa and amino acids or amines, respectively (Strack *et al.*, 1993).

Betacyanins can be further classified by their chemical structures into four groups:

- betanin-group;
- amaranthin-group;
- gomphrenin-group;
- 2-Descarboxy-betanin group

instead betaxanthins are conventionally divided in:

- Amino acid-derived conjugates;
- Amine-derived conjugates;
- Semisynthetic structures

So far it has been found that betalains in nature comprise approximately 50 red betacyanins and 20 yellow betaxanthins (Strack *et al.*, 2003).

The structure scheme on Figure 4.1 shows betalamic acid (1), the chromophore of all betalains; betanidin (2), the aglycone of most of the betacyanins; and indicaxanthin

(3), a proline-containing betaxanthin. These were the first betalains identified by chemical means.

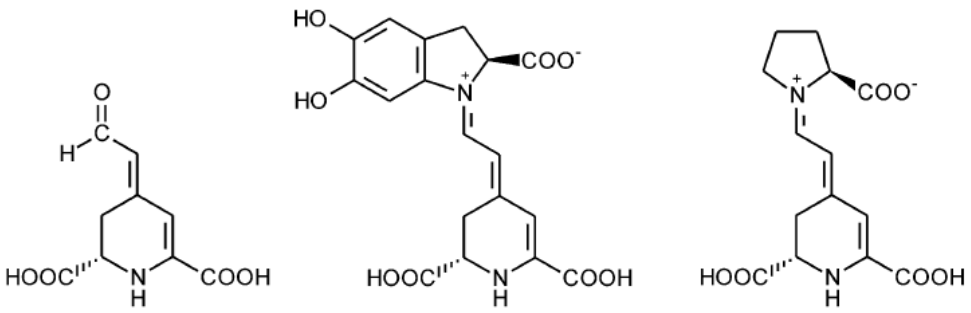

Figure 4.1: The chromophore of betalains (1), the aglycone of most of betacyanins (2), and a proline-containing betaxanthin (3).

Betalains accumulate in flowers, fruits and occasionally in vegetative tissues of plants belonging to most families of the *Caryophyllales*.

However, there are two exceptions: the *Caryophyllaceae* and the *Molluginaceae* accumulate instead anthocyanins, flavonoid-derived pigments occurring in all other families of flowering plants. The *Caryophyllales* specific accumulate betalains, this is a prominent example of the chemotaxonomic relevance of plant secondary products. Gain and loss of anthocyanins and betalains during plant evolution still remain a mystery. Molecular studies are needed to elucidate the evolutionary mechanisms leading to the mutual exclusion of the betalain and anthocyanin pathways in flowering plants; probably due to their divergent biosynthetic pathways on the argenate level, the precursor of phenylalanine (anthocyanins) and tyrosine (betalains), respectively, anthocyanins and betalains have never been found jointly in plant tissues (Stintzing *et al.*, 2006).

In food processing, betalains are less commonly used than anthocyanins and carotenoids, although these pigments, are well suited for colouring low acid food.

Betalains were also detected in some higher fungi, for example in the fly agaric (*Amanita muscaria*). Whereas the functions of betalains in plant flower and fruit colouration are obvious, their role in fungi is unknown.

The use of betalain pigments as food additives dates back to the turn of the 20th century, when juice from pokeberries was added to wine to impart a more advisable red colour. The most important source of betanin (Figure 4.2) as colouring agent is the red beet (*Beta vulgaris* subsp. *vulgaris*) root.

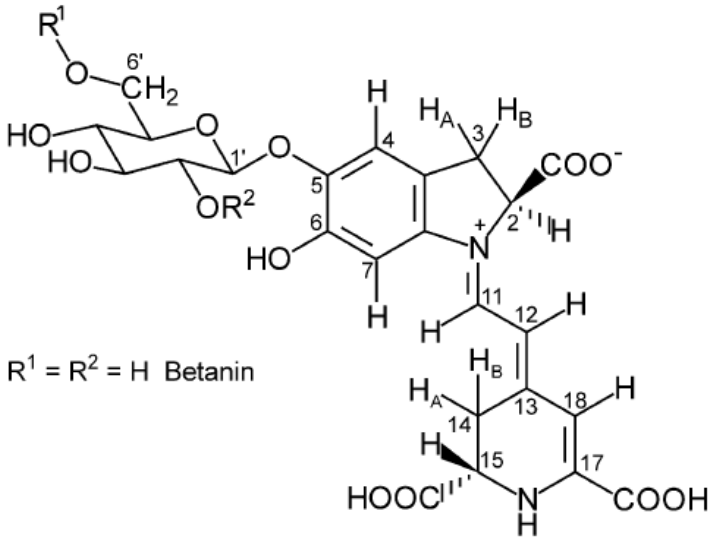

Figure 4.2: Molecular structure of betanin present in the red beet root.

Production of commercial betalains depends not only on efficient processing techniques (e.g. enzymatic control, extraction, purification, concentration, and drying operations), but also on a continuous availability of highly pigmented sources.

Besides betanin from red beet, also betacyanins from plants of the Amaranthaceae were tested concerning colour properties and pigment stability in model food systems. Red-violet betacyanin pigments from *Amaranthus* plants have involved interest as a potential alternative source of betalains, since some *Amaranthus* genotypes produce particularly high biomass and contain high levels of pigment.

Amaranthus red–violet betacyanins, like red beet betalains, are susceptible to temperature and also affected by pH, light, air, and water activity, with better pigment stability at lower temperatures (14-8°C) in the dark and in the absence of air over the pH range 5–7, being more stable pH 5.6 (Cai *et al.*, 1998).

Recent studies have reported about the antioxidant properties of betalains and have related them to the preventive action against several diseases like inflammatory (including cardiovascular disease, asthma and rheumatoid arthritis), chronic myeloid leukemia and cancer (Tesoriere *et al.*, 2004; Sreekanth *et al.*, 2007); they have antiulcerogenic, anti-genotoxicity, hepatoprotective and neuroprotective activity and some other beneficial effects to health (Allegra *et al.*, 2005)

4.1.1 *Opuntia ficus-indica* fruits

Opuntia ficus-indica fruit is a fleshy berry, varying in shape, size and colour and has a consistent number of hard seeds. The fruit is commonly called cactus fruit, cactus fig and indian fig or tuna in Spanish, is edible, although it has to be peeled carefully to remove the small spines on the outer skin before consumption.

Its fruit is a berry and varying in colour (Figure 4.3), it develop green (less sweet), with its maturity state to orange-yellow and then to reddish purple (very sweet) (Guesmi *et al.* 2012, Khatabi *et al.*, 2011).

The fruit of prickly pears is often used to make candies, jelly, or drinks such as vodka or lemonade and its pulp and juice have been used to treat numerous diseases, such as wounds and inflammations of the digestive and urinary tracts in folk medicine (Kuti, 2004).

Figure 4.3: Color changes in fruits of *Opuntia ficus-indica*

The aqueous extracts of cactus pear fruits exhibited a marked antioxidant capacity in several *in vitro* assays, including the oxidation of red blood cell membrane lipids and the oxidation of human LDLs induced by copper and 2,20-azobis (2-amidinopropane-hydrochloride). The fruit is characterized by a high sugar content (12-17%) and low acidity (0.03-0.12%). It has higher vitamin C, potassium, calcium and phosphorous and low sodium. The prickly pear contains betalains, betanin and indicaxanthin; that induce apoptosis in human chronic myeloid leukemia Cell line-K562 (Sreekanth *et al.*, 2007).

The prickly pears are considered as a rich source of yellow-orange betaxanthins and red-violet betacyanins and the red and purple coloured prickly pears contain high amounts of total phenols and purple skinned fruits contain the highest amounts of flavonoids which are responsible for the colour of *Opuntia* spp., and having radical-scavenging and reducing properties (Osorio-Esquivel *et al.*, 2011; Maran *et* Manikandan, 2012); they are a source of nutrients, vitamins and other bioactive constituents (Cayupán *et al.*, 2011).

Mexico and Italy account for most of the world-wide production and export. The marketing season in Italy begins in mid-August with fruit coming from the spring

flush (summer fruit) and lasts through November- December, due to late ripening fruit (autumn fruit) obtained through removal of the spring flush which promotes a second flush of flowers and cladones. Moreover, the prevailing environmental conditions that occur during the development of summer and autumn fruits greatly differ in terms of radiation, day length, temperature, relative humidity (RH) and rainfall occurrence. As with most tropical fruit species, cactus pear fruit is susceptible to chilling injury preventing low temperature storage. Without refrigeration, they deteriorate in a few days as a result of rapid ageing and rot development. Recommended storage conditions for cactus pears range from 6 to 8°C at 90-95% RH (Schirra *et al*., 1999).

The aim of this work was twofold; on one hand it was desired to evaluate the possibility of extracting through SFE-CO_2, the polar pigments, that no one has ever tried to do before; from another side to evaluate the antioxidant effectiveness of *Opuntia ficus-indica* fruit extract during oxidation of emulsions O/W and W/O and their rheological properties and stability.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Opuntia ficus indica (L.) Mill. fruits were collected between August and November 2012 in Calabria (Italy) and a third harvest in October 2013.

The plant, almost spineless, it's supposed to be a cultivar with hybrid origin often escape from cultivation and behave as an invasive species (Figure 4.4).

Figure 4.4: Collection and peel of *Opuntia ficus-indica* fruits

The fruits were peeled manually and the pulp was cut into small pieces, homogenized with a TYPE HR 2064 PHILIPS -600 W (Figure 4.5) and stored at -18°C until the analysis.

Figure 4.5: Pulp homogenized from fruits of *Opuntia ficus indica*.

4.2.2 Chemicals

Solvents as methanol and water (two both analytical grade and for HPLC), acetonitrile, acetic acid, ethanol and acetone were purchased from Sigma-Aldrich (USA).

Ottawa Sand and an Spe-ed^{TM} PSE Matrix - Hydroscopic Samples Dispersing Agent (Diatomee Sand), used like SFE-CO_2 accessories, were purchased from Labservice Analytica S.R.L. (Italy).

Betanin powder (Red Beet extract diluted with Dextrin) was purchased from CHEMOS GmbH (Germany); DPPH and ABTS radicals from Sigma-Aldrich (USA).

4.2.3 Preliminary analysis

The homogenised mix was prepared to measure the level of acidity of the primary source (pH 700, Eutech Instruments, Germany), to observe water activity (Novasina AW Sprint – TH 500, Switzerland) and humidity (Mettler Toledo Moisture Analyzer HB43-S, Switzerland). The measurement of water activity and of equilibrium relative humidity are a key parameter in the quality control of moisture-sensitive products or materials. Water activity is by definition the free or non-chemically bound water in foods and other products; instead humidity is refers to the total amount of water contained (Rockland *et.* Beuchat, 1987).

The measuring of brix degrees (ATAGO, Hand Refractometer N Type Series, Japan) was carried out to evaluate the sugar content. One degree Brix is 1 gram of sucrose in 100 grams of solution and it represents the strength of the solution as percentage by mass. When a sugar solution is measured by refractometer or density meter, the °Bx or °P value obtained represents the amount of dry solids dissolved in the sample if the dry solids are exclusively sucrose. This is seldom the case; grape juice, for example, contains little sucrose but does contain glucose, fructose, acids and other substances. In such cases, the °Bx value clearly cannot be equated with the sucrose

content, but it may represent a good approximation to the total sugar content (ICUMSA, 2009).

Colorimetric analysis was conduct with a colorimeter (CR-400, Germany). the colorimetric analysis has the advantage of quantitatively define the colour point of the object studied. The coordinate system intuitively simplest consists of the parameters (L, a, b) The parameter "L" defines the overall brightness of the object, the parameters "a" and "b" defined antipodes of colour, where high positive and negative values of "a" are associated with the colour red and the colour green, while high positive and negative values of "b" are respectively associated with the colours yellow and blue (Gonzalez de Cademartori *et al.*, 2013).

4.2.4 Different solvent extraction and HPLC analysis

Homogenized pulp of the collection of December 2012, after removal of seeds, was magnetically stirred for 20 min in darkness using a fruit/solvent ratio of 1:5 (m/v) following a modified procedure of Fernández-López *et al.* 2012.

Water, ethanol/water (60:40 by volume), ethanol and methanol were used as solvents. After stirring the samples were centrifuged at 3000 *rpm* at 24 °C for 10 min in centrifuge Type 5810, EPPENDORF, in order to remove the vegetal tissue residue. Supernatants (Figure 4.6) were filtered and then concentrated using a vacuum Büchi Rotavapor (Switzerland). Temperature was controlled at 40 °C and vacuum at 6 kPa.

Figure 4.6: Homogenized pulp after centrifugation

4.2.5 Spectrophotometric analysis of extracts with solvents

The visible spectra (300–600 nm) of the extracts were recorded using an CARY 60-UV-visible spectrophotometer (Agilent technologies). Betacyanins content was expressed as betanin (molar mass *M*=551,48 g/mol). Wavelength was setted to 527 nm.

4.2.6 Extraction at high pressure

The supercritical extractions by CO_2, on the fruits of *Opuntia ficus-indica* collected in December 2012 and October 2013, were performed on a Spe-ed SFE 4 extractor (Applied Separation, Allentown, PA, USA) and following the necessary steps: loading in the steinless steel vessel, pressurization with CO_2 and waiting a transitory state of temperature. When a balanced state was obtained, the system was kept closed for 20 min to promote the diffusion of CO_2 inside the matrix, then the micrometric valve was opened until a constant flow of 1.5 L/min was reach.

The extract left the vessel through a valve, which it was advance thermo stated to avoid obstructions, due to the possible solidification of CO_2. Experiments were carried out at 40°C and 110 bar by varying the preventive sample preparation with PSE accessories, as Ottawa Sand and an Spe-ed^{TM} PSE Matrix (Hydroscopic Samples Dispersing Agent).

The different methods to prepare the samples, temperature and pressure of the process are listed in the following Table:4.1.

All samples after collection have been previously cleaned and peeled, homogenized and frozen; at the time of the analysis, some of them were centrifuged (3500 rpm for 10 minutes) other partially dried (32°C in oven) in order to reduce the amount of water present in the vegetable matrix.

The extraction time was 1 h. The final extracts were collected in glass tubes covered with an aluminium foil (for the light sensibility) and frozen at -18°C until analysis.

Sample Fruit		Preventive preparation	T(°C)	P(bar)
December	2H_30E_25D	dried until 30% + 25 % Diatomee -	40	110
December	2H_30E_20D	dried until 30% + 20 % Diatomee	40	250
December	2H_C_40O	centrifughed + 40 % Ottawa	40	110
October	3H_30E_20D	dried until 30% + 20 % Diatomee	40	110
October	3H_C_40O	centrifughed + 40 % Ottawa	40	110
October	3H_20E_5D	dried until 20% + 5 % Diatomee -	40	110

Table 4.1: Different preparation of the samples before SFE

4.2.7 HPLC quantification

The analysis of all the extracts both from solvent extraction and belonged from SFE (*4.2.6*) was carried out by means of HPLC using a Smartline HPLC system (Knauer, Germany). Chromatographic separation was carried out using a 2,0 mm ID ×150 mm L, with precolumn, C-18 TSKgel ODS-100 V, 21810 (TOSOH BIOSCIENCE), both thermostated at 40°C. For the separation was used the below (Table 4.2) operative conditions (Fernández-López *et* Almela, 2001); absorbance spectra were recorded every 2 s, between 200 and 502 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 280 and 480 nm in according with absorption spectrum. Every HPLC analysis was performed in duplicate. The wavelength used for quantification was 480 nm.

Time (min)	Acetonitrile + 1% Acetic Acid[%]	Water + 1 % Acetic Acid [%]	Flow (mL/min)
0,00	0	100	0,2
30,00	12	88	0,2
35,00	0	100	0,2
45,00	0	100	0,2

Table 4.2: Mobile phase, flow rate and gradient of eluition utilized

4.3 EMULSION O/W AND W/O

An emulsion is a mixture of two or more liquids that are normally immiscible; emulsions are part of a more general class of two-phase systems of matter called colloids. Although the terms colloid and emulsion are sometimes used interchangeably, emulsion should be used when both the dispersed and the continuous phase are liquids; one liquid (the dispersed phase) is dispersed in the other (the continuous phase). Examples of emulsions include vinaigrettes, milk, mayonnaise etc. Two liquids can form different types of emulsions; as example, oil and water can form an oil-in-water emulsion, wherein the oil is the dispersed phase, and water is the dispersion medium; and they can form a water-in-oil emulsion, wherein water is the dispersed phase and oil is the external phase. Multiple emulsions are also possible, including a "water-in-oil-in-water" emulsion and an "oil-in-water-in-oil" emulsion (Khan *et al.*, 2006).

An emulsifier (also known as an "emulgent") is a substance that stabilizes an emulsion by increasing its kinetic stability. One class of emulsifiers is known as "surface active agents", or surfactants. Detergents are another class of surfactants, and will physically interact with both oil and water, thus stabilizing the interface between the oil and water droplets in suspension. This principle is exploited in soap, to remove grease for the purpose of cleaning. Many different emulsifiers are used in pharmaceutical and food industries to prepare emulsions such as creams or lotions. Common examples include emulsifying wax, cetearyl alcohol, polysorbate 20, and ceteareth 20 (Mc Clements, 2009).

4.3.1 Mounting of three biphasic systems

4.3.1.1 Preparation and purification of Cactus Pear Fruit Extract (CPFE)

Fruit homogenized with seeds was defrost and brought to environment temperature; then centrifuge to 3500 rpm for 15 minutes. Supernatant was filtered and precipitated in acetone following a 1 juice : 2 acetone ratio. The solution was decanted for 20 minutes in the dark; impurities of high molecular weight have been removed through two other filtrations with a PTFE Syringe Filter (2µm).

The obtained solution was dried under *vacuum* at 40°C through the use of a rotary evaporator (Buchi, Switzerland).

4.3.1.1 Emulsion oil in water (O/W)

Ingredients: The ingredients used for all samples preparation were:

- commercial extra virgin olive oil (De Santis, Italy, $20_{w/w}$%);
- Tween 60 (Sigma-Aldrich, Italy, $5_{w/w}$%) as emulsifiers,
- buffer solution prepared by dissolving in 350 g of demineralized water, 0.5 g of citric acid (Sigma Aldrich, Italy) and 0.6 g of disodium citrate (Sigma Aldrich, Italy) kept under stirring (AREX Magnetic Stirrer Velp Scientifica, Italy) for 15 min prior to use,
- $CaCl_2$ (Carlo Erba, Italy);
- pectin L.M. ($1{,}5_{w/w}$%) and ethanol ($3_{w/w}$%).

The two phases for the emulsion were obtained separately.

OILY PHASE: Heating oil at 70 ° C through a heating plate with the aid of magnetic stirrers, in order to ensure a uniform distribution of temperature in the sample; reached this temperature was added the emulsifier Tween 60 and after the plate was kept off of temperature but was be stirred. The system was then cooled to 25 ° C.

AQUEOUS PHASE: To the buffer (pH = 4.4) was added pectin previously dissolved in ethanol, the system is put to mix slowly (cold); is then added in the solution of calcium, required for pectins L.M. to gel.

The CPFE extract ($1_{w/w}$%) was added to aqueous phase instead the Vitamin E (A.C.E.F. spa, Italy, $1_{w/w}$%) to the oily phase of another two emulsions.

The emulsification was performed using a rotor stator system (Ultra Turrax T 50, IKA Germany); increasing gradually the mixing speed for 180 seconds.

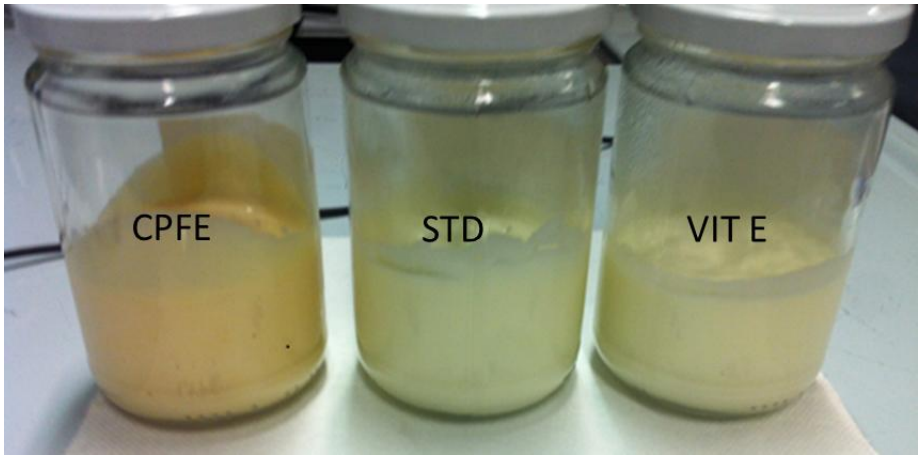

Figure 4.7: The three emulsion oil in water (O/W)

4.3.1.2 Emulsion water in oil (W/O)

Ingredients: The ingredients used for all samples preparation were:

- extra virgin olive oil (De Santis, Italy, $70_{w/w}$%) as main constituent commercial;
- mono- and diglycerides of fatty acids (Myverol 18-04, Kerry Group, Ireland, $10_{w/w}$%) as emulsifiers;
- NaCl (Panreac, Spain) 0,1 M was added to water ($20_{w/w}$%) in order to identify the mutual position of the two phases by an electric conductivity technique (emulsions W/O or O/W).

The two phases for the STD emulsion were obtained separately.

OILY PHASE: The oil was pre-heated at 70°C with a magnetic heater agitator and then the emulsifier was added to the oil. After the emulsifier was completely melted, the mixing was prolonged for a further 5 minutes. This phase was gently stirred and cooled applying a fast cooling rate in a thermostatic cold bath (at 0°C).

WATER PHASE: The phase was obtained dissolving NaCl in to the water at room temperature until complete dissolution.

The CPFE extract ($1_{w/w}$%) was addeg to aqueous phase instead the Vitamin E (A.C.E.F. spa, Italy $1_{w/w}$%) to the oily phase in another two emulsions .

The emulsification was performed using a rotor stator system (Ultra Turrax T 50, IKA Germany); increasing gradually the mixing speed for 120 seconds.

Figure 4.8: The three emulsion water in oil (W/O)

4.3.2 Process of oxidation

During storage of oils and fats, lipid oxidation is still a major cause of quality deterioration in spite of a wide use of several antioxidants. The most widely used synthetic anti-oxidants Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are quite volatile and decompose easily at high temperatures. There are some serious problems concerning the safety and toxicity of BHA and BHT related to their metabolism and possible absorption and accumulation in body organs and tissues. Therefore, the search for preparations of useful natural antioxidants is highly desirable. The antioxidant properties of many herbs and spices are reported to be effective in retarding the development of rancidity in oils (Abdalla *et* Roozen, 1999).

After preparation the emulsions were transferred into screw-capped glass bottles and covered externally with aluminium foils, were stored in refrigerator at 4 ° C for 24 h, in order to let the structure to stabilize, and then incubated in oven at 60° C for 15 days and subjected to accelerated oxidation in the dark.

4.3.3 Antioxidant activity

The antioxidant properties of all emulsions (O/W and W/O), at four different times after preparations (t_0= 1 day; t_1= 5 days; t_2= 10 days; t_3= 15 days), were evaluated by measurement of the scavenging activity towards DPPH and ABTS radicals, according protocol and with some modifications of Spizzirri *et al.*, 2013.

4.3.3.1 DPPH Assay

The emulsions were allowed to react with a stable free radical, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH). An amount of 200 mg of each emulsion were dispersed in 10 mL of a DPPH solution in ethanol. The tubes were vortexed for 2 minutes and then kept at room temperature for 30 minutes in the dark; then filtered with a PTFE Syringe Filter (4µm) and the residual concentration was colourimetrically determined at 517 nm.

The decrease (%) in DPPH absorbance was used to calculate the inibition according to the following equation:

$$\text{inhibition (\%)} = [(A_0 - A_1) / A_0] * 100 \qquad (1)$$

where A_0 is the absorbance of a standard prepared in the same conditions but without any emulsions, and A_1 is the absorbance of emulsions samples.

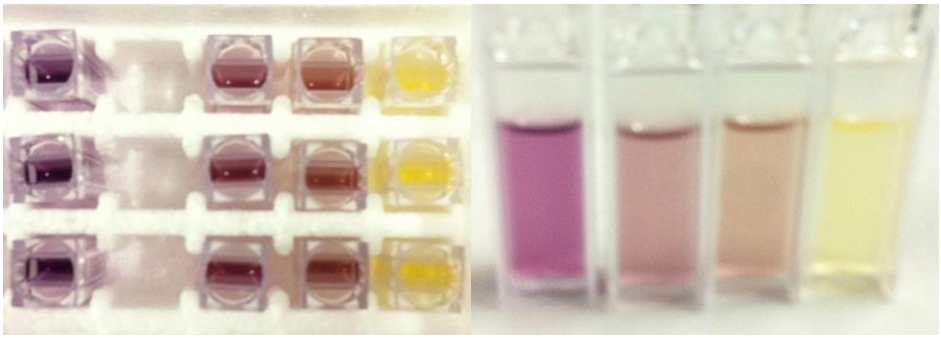

Figure 4.9: Spectrophotometric analysis for the evaluation of inhibition of DPPH

4.3.3.2 ABTS Assay

ABTS radical's, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), stock solution was generated by incubation of ABTS solution (7.0 mM) and potassium persulfate solution (2.45 mM) in the dark at room temperature for 16 h. The solution was further diluted with water to an absorbance of 1.00 ± 0.02. An amount of 200 mg of each emulsion were dispersed in 10 mL of a ABTS solution. The tubes were vortexed for 3 minutes, filtered with a PTFE Syringe Filter (4µm) and the residual concentration was immediately determined colorimetrically at 734 nm. The decrease (%) in ABTS absorbance was used to calculate the inhibition, according to the same equation (1) used for DPPH.

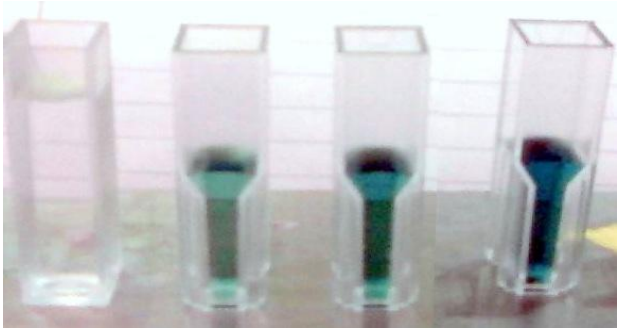

Figure 4.10: Spectrophotometric analysis for the evaluation of inhibition of ABTS

4.3.4 Rheological characterization

4.3.4.1 Small amplitude oscillation test (*SAOTs) and Steady test*

Tests were performed with two controlled stress rheometers: DSR-500 (Rheometric Scientific, USA) equipped with a parallel plate geometry (ϕ=25 mm, gap 2.0±0.2 mm) and a peltier system acting under the lower plate; and a rheometer NOVA (…..) with a parallel plate geometry (ϕ=25 mm, gap 2.0±0.2 mm), where the thermostating system is located on the cylindrical shell surrounding the geometry.

Preliminary stress sweep tests were performed, with DSR, at different temperatures (4°. 25°, 50°, 70 °C) to investigate the potential changes in linear viscoelastic region as a function of temperature and to be able to describe more finely the change of the linear viscoelastic conditions.

Temperature ramp tests were, then, carried out at the fixed frequency of 1 Hz, heating the sample from 4°C up to 70 or 100 °C, according to the sample, with a heating rate of 1 °C/min. For each test, the applied stress was modified with the temperature, applying different constant values in different temperature range, following the results obtained in the previously described stress sweep (Lupi *et al.*, 2013).

Steady tests (flow curves) were carried out with NOVA in order to evaluate flow behaviour of the material. Tests were conduct both at 25° C and at 37° C to mimic body temperature. The stress applied was in the range from 0.1 to 100 Pa.

4.4 RESULTS AND DISCUSSIONS

4.4.1 Preliminary analysis

The acidity level, water activity, Brix degree, humidity and colorimetric data of homogenized fruits were reported in Table 4.3.

***Opuntia ficus-indica* Fruits**	**First Harvesting (November 2012)**	**Second Harvesting (December 2012)**	**Third Harvesting (October 2013)**
pH	5,68±0,08	5,73±0,04	5,71±0,02
a_w	0,928±0,002	0,934±0,005	0,921±0,003
°Bx	15,0±0,1	14,8±0,2	15,2±0,1
W_b	83,98±0,17	84,37±0,24	83,62±0,08
L*	26,395±1,05	46,685±3,19	32,8±0,89
a*	19,34±1,39	23,27±0,35	12,28±0,52
b*	25,11±1,51	45,07±3,56	29,15±2,40
H	4,75YR ±0,21	5 YR±0,28	7,55 YR±0,64
C	31,70	50,73	31,63

Table 4.3: Data for pH, water activity, Brix degrees and colorimetric results for the three harvestings.

The pH value do not change a lot, we evaluated a slight reduction in acidity with the arrival of the winter season.

October's fruit, during their growth, had longer days and higher radiation as well as higher daily temperatures, lower rainfall than winter fruit; probably for these reasons from October to December we found an increase of both water activity and humidity and a decrease of brix degree.

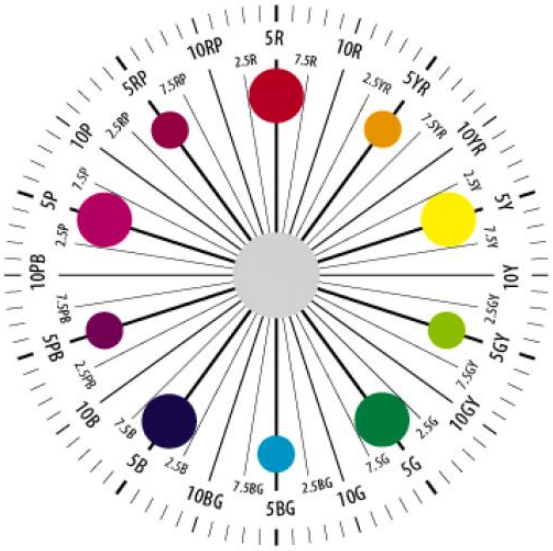

Figure 4.11: H values for Munsell escale

According with the scale of Munsell the values of H indicate that the colour is in the range of the Yellow-Red, and increases towards yellow in the collection of October while it remains almost unchanged in the collection of November and December

C was calculated following the equation below (Gonzalez de Cademartori, 2013):

(2)

4.4.2 Solvent extractions and spectrophotometric analysis

In the Table 4.4 are showed:

- the yield % of extraction calculated used the equation below:

(3)

- the mg of betacyanins for g of dry extract (Figure 4.12);
- the resulting Area (mAU) obtained with HPLC analysis (Figure 4.13)

Solvent	**Yield % extraction**	**mg of betacyanins /g of extract**	**Area (mAU)**
Water	6,94	435,36	3,51E+07

Ethanol/Water (3:2)	7,29	144,53	3,40E+07
Ethanol	8,93	122,37	1,81E+07
Methanol	6,09	181,27	4,65E+07

Table 4.4: Yield % extraction for the different solvents, mg of betacyanins /g of extract and Area (mAU) obtained with HPLC analysis

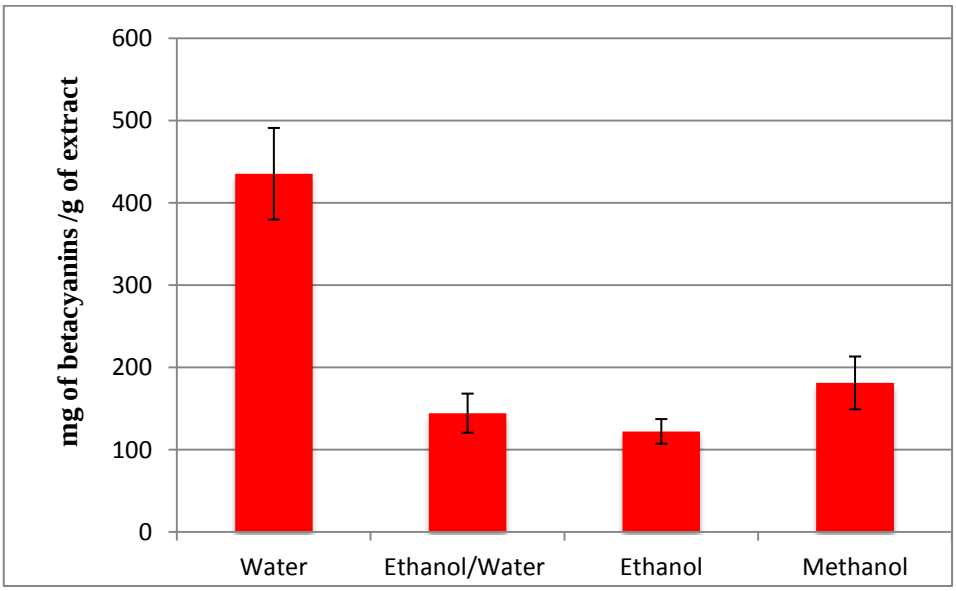

Figure 4.12: Mg of betacyanins for g of dry extract obtained with different solvents

Spectrophotometric analysis showed that the greater concentration of betacyanins were found in water extract probably because the betacyanins being slightly more polar than the betaxanthin they are able to pass more easily in a polar solvent such as water, which is among the solvents used is the more polar (Table 4.5).

The solvents are grouped into non-polar (NP), polar aprotic (PA), and polar protic (PP) and ordered by increasing polarity. The polarity is given as the dielectric constant (Wypych, 2001).

Solvent	**Ethanol (PP)**	**Methanol (PP)**	**Water (PP)**
Chemical formula	CH_3-CH_2-OH	CH_3-OH	H-O-H
Boiling point (°C)	79	65	100
Dielectric constant	24.55	33	80

Density (g/ml)	0.789	0.791	1.000

Table 4.5: Properties of solvents used for the extraction

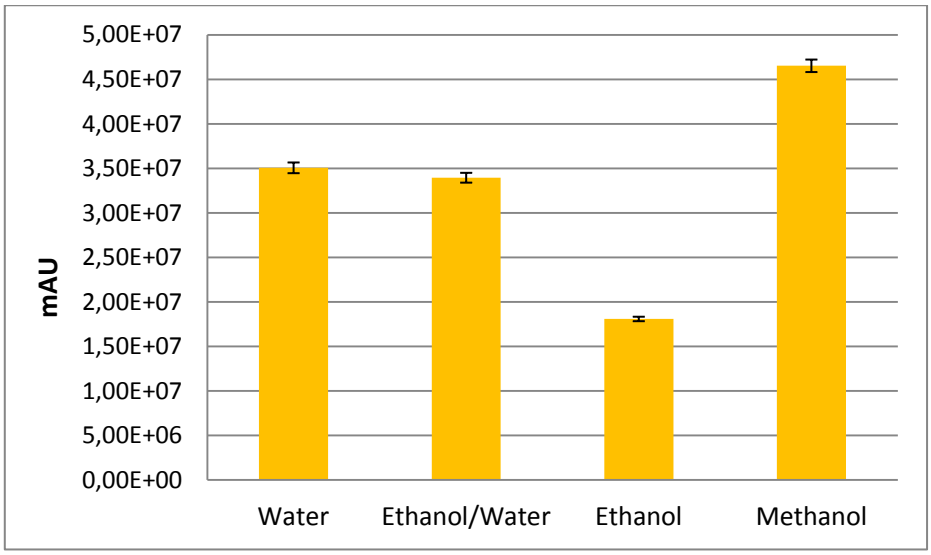

Figure 4.13: The resulting Area(mAU) obtained with HPLC analysis

High concentrations of total betalains (betacyanins and betaxanthin) are obtained using methanol as solvent of extraction, maybe because is both polar and small as solvent and can penetrate better than ethanol in the plants layers. It follows the extracts in water and ethanol/water, that are almost similar. Finally less quantities of betalains were found in the extract with ethanol, this probably because it is both the less polar that the largest between the solvents used.

4.4.3 Extraction at high pressure

In the following Table 4.6 are showed:

- the yield % of extraction (Figure 4.14) calculated using the equation below:

(4)

- the resulting Area (mAU) obtained with HPLC analysis (Figure 4.15)

Sample	**Yield % of**	**Area (mAU)**

	extraction	
2H_30E_25D	0,45±0,05	6,50E+07±1,40E+06
2H_30E_20D	0,96±0,11	4,90E+08±5,40E+06
2H_C_40O	1,99±0,23	3,90E+06±7,00E+05
3H_30E_20D	0,54±0,04	1,04E+09±1,67E+07
3H_C_40O	9,44±1,65	2,83E+07±2,30E+06
3H_20E_5D	4,05±0,43	1,09E+09±2,34E+07

Table 4.6: Yield % of extraction and the resulting Area (mAU)

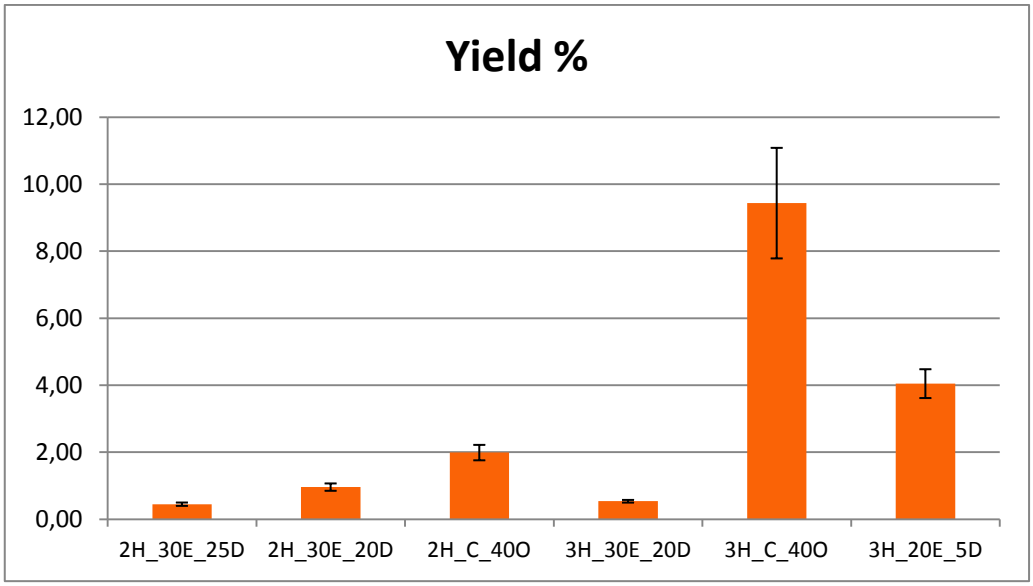

Figure 4.14: Yield of extraction of the sample extracted with SFE-CO_2

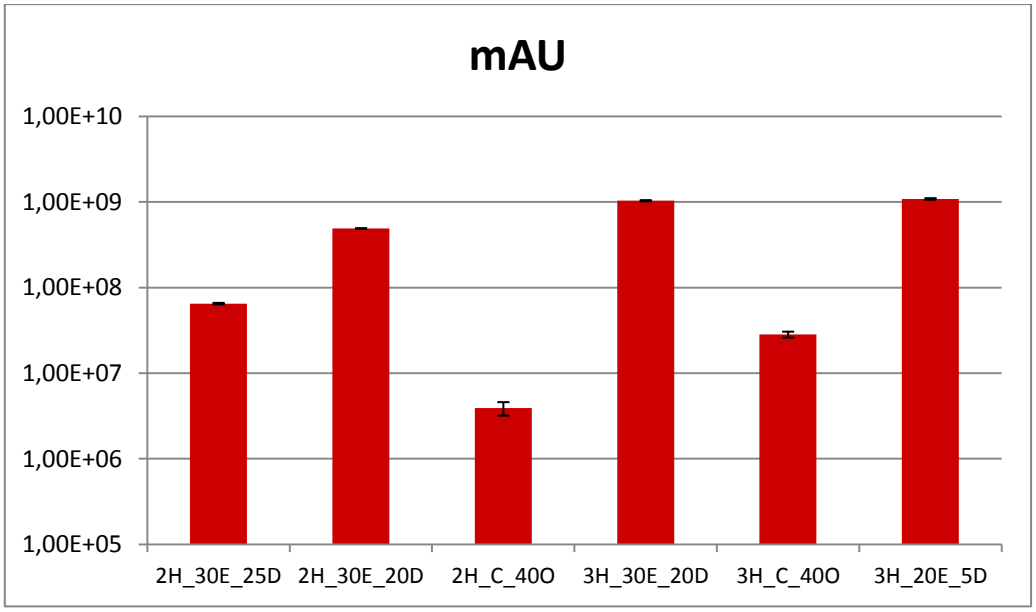

Figure 4.15: The resulting Area (mAU) obtained with HPLC analysis

The yield of extraction is not related to a greater amount of betalains present in the extracts obtained, often is inversely proportional. This is noticeable in the samples centrifuged 2H_C_40O and 3H_C_40O of both collections, second and third, to which was added Ottawa sand, that does not interfere with the residual water, but it is a dispersing agent and allows a better homogenization of the sample in the extraction column. In this samples the total yields quantitative of extraction are the highest, while the amount of extracted betalains is the lowest; this probably because removal of the water, which is polar, on one hand let that CO_2 can better spread in the vegetable matrix and to extract better quantitatively; on the other hand, however, the removed water carries with itself a rich portion of betalains that are polar.

It was therefore decided to partially drying the samples instead centrifuged and to add Diatomees sand that adsorbs water. Gradually reducing the amount of Diatomee we were able to increase both the extraction yields quantitative and the amount of total betalains extracted; this is evident in the sample 3H_20E_5D. In this sample we reached the maximum amount of betalains extractable, with a good yield %. It can be

said that this pre-treatment is optimal in terms of extraction of the product; the extract is highly pure and selective for betalains and devoid of any trace of organic solvent; the sample is not long dried (20%) and the amount of sand that are used are really minimal (5%), it is so also economical from the point of view of process.

Both quantitative best yields and larger quantities of betalains were obtained in the collection of October, probably the stage of fruit ripening and the onset of the winter season (cold and rain), contribute to a reduction of active ingredients within the matrix.

The amount of betalains obtained by the solvent extracts is comparable with that obtained by SFE-CO_2, this indicates that the SFE is an excellent technique for the extraction of colored pigments such as betalains.

4.4.4 Antioxidant activity of the three biphasic systems

4.4.4.1 DPPH and ABTS radicals inhibition in O/W emulsions

In the Table 4.6 are showed the % of inhibition (mean±S.D. of three determinations) of radicals DPPH and ABTS in O/W emulsions, calculated by following the equation (1).

SAMPLES	EMU STD	EMU CPFE	EMU VIT E
t(0) Inhib. DPPH (%)	11,589	20,695	95,075
t(1) Inhib. DPPH (%)	16,167	28,357	94,628
t(2) Inhib. DPPH (%)	15,000	19,947	91,436
t(3) Inhib. DPPH (%)	17,276	19,820	91,362
t(0) Inhib. ABTS (%)	6,857	20,673	98,023
t(1) Inhib. ABTS (%)	10,083	19,109	95,487
t(2) Inhib. ABTS (%)	10,649	15,361	96,297
t(3) Inhib. ABTS (%)	13,138	16,650	94,602

Table 4.7: % of inhibition of radicals DPPH and ABTS of O/W emulsions

The results are shown in Figures 4.16 and 4.17.

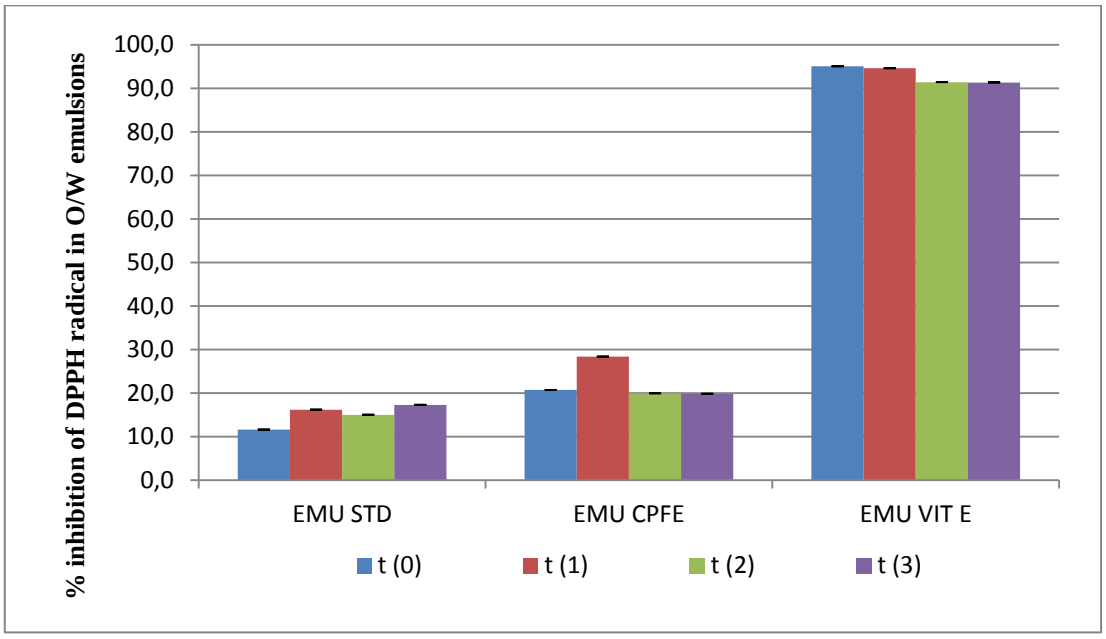

Figure 4.16: % of inhibition DPPH radical's in O/W emulsions

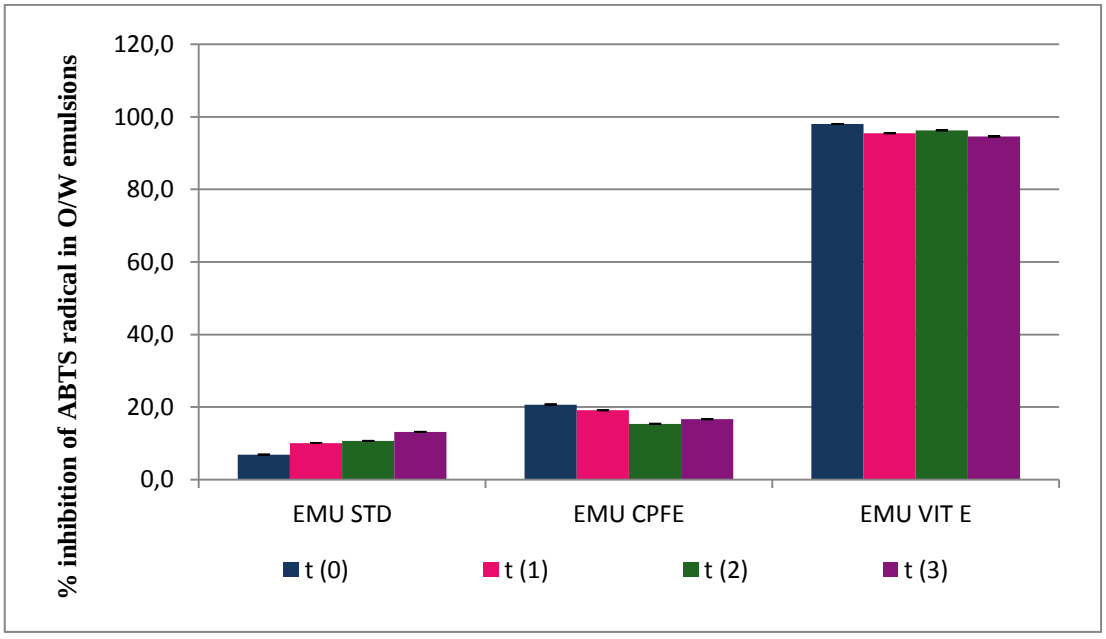

Figure 4.16: % of inhibition ABTS radical's in O/W emulsions

The % of inhibition is maximum in the emulsion with VIT E, used as positive control, being tocopherol one of the most powerful antioxidants existing (Packer *et al.*, 2001; Alam *et al.*, 2013).

Is immediately evident, however, that also the STD emulsion possesses a certain antiradical activity, on both the radicals DPPH and ABTS, this is because the oil phase was prepared using extra virgin olive oil that contains compounds with antioxidant activity such as polyphenols (Visioli *et* Bernardini, 2011).

The emulsion prepared with the CPFE, in the aqueous phase, reaches a good level of inhibition; 28% for the radical DPPH and 20% for the radical ABTS. Since water is the dispersing phase, this is most affected of oxidation induced by treatment in oven and the activity decreases slowly over time.

4.4.4.2 DPPH and *ABTS* radicals inhibition in W/O emulsions

In the Table 4.7 are showed the % of inhibition (mean±S.D. of three determinations) of radicals DPPH and ABTS in W/O emulsions, calculated by following the equation (1).

SAMPLES	EMU STD	EMU CPFE	EMU VIT E
t(0) Inhib. DPPH (%)	49,782	63,303	95,115
t(1) Inhib. DPPH (%)	39,525	55,377	94,111
t(2) Inhib. DPPH (%)	48,880	56,512	90,744
t(3) Inhib. DPPH (%)	49,645	58,263	90,865
t(0) Inhib. ABTS (%)	24,898	27,324	98,749
t(1) Inhib. ABTS (%)	36,675	67,558	95,869
t(2) Inhib. ABTS (%)	26,142	29,387	92,601
t(3) Inhib. ABTS (%)	18,454	28,946	90,749

Table 4.8: % of inhibition of radicals DPPH and ABTS of O/W emulsions

The results are shown in Figures 4.18 and 4.19.

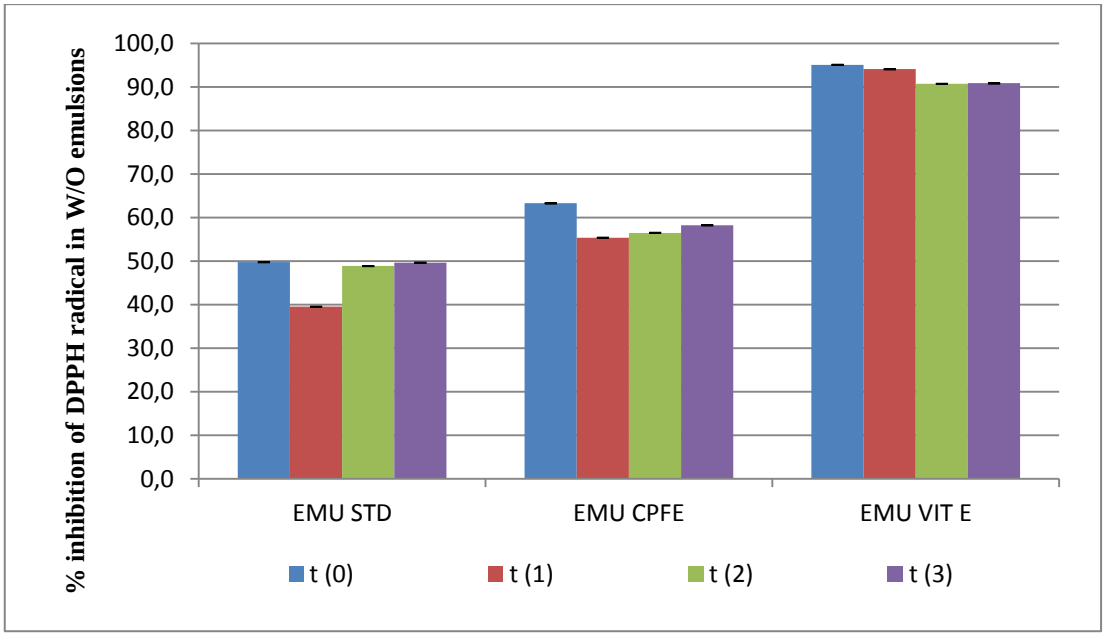

Figure 4.18: % of inhibition DPPH radical's in W/O emulsions

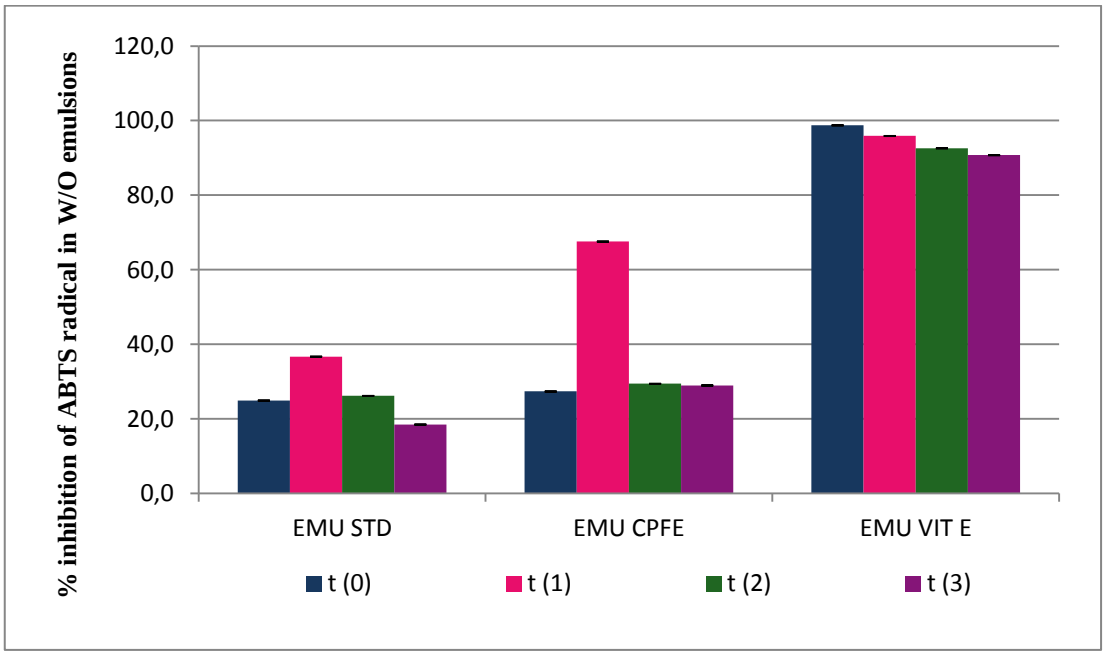

Figure 4.19: % of inhibition ABTS radical's in W/O emulsions

The trend is similar to that for the O / W emulsion but we reached much higher values of inhibition which almost 50% for STD emulsion and over 63% for CPFE emulsion on DPPH radical, and almost 25% for STD emulsion and 68% for CPFE emulsion on ABTS radical . This can be explained by two reasons:

1) the quantity of oil is higher and therefore it increases the effect of the polyphenols that it contains;

2) the aqueous phase, in which is dissolved the CPFE, is dispersed and less affected by the oxidation process, being within the oil phase it results more protected.

4.4.5 Rheological characterization of emulsions

Small amplitude oscillation test (*SAOTs)*

The data obtained are reported as terms of complex modulus with the rheological model of the weak-gel. The model of the weak-gel is valid for systems weakly structured as foods, cosmetics and pharmaceutical products.

The two dynamic modules, G' and G'', have a linear and almost parallel trend and are function of frequency. This trend, therefore, appears to be characteristic of the systems weakly structured or that set of materials whose structure is characterized by the presence of weak interactions between the various elements that constitute it.

Such systems can be considered as three-dimensional lattices in which various units interact one with each other through weak interactions (electrostatic forces or Van Der Valls bonds) holding the structure compact. As long as the stresses that we apply are small, we see that the behaviour is typical of a solid, but when the stress becomes high, break the bonds that form the structure and then the material begins to flow. The weak-gel also have other characteristics, such as:

- G' may be greater than G'' even of one order of magnitude;
- Not detects a crossover between the two modules in a wide temperature range.

The two dynamic modules can be described in terms of a single complex form that depends on the frequency via the following empirical relationship:

(5)

where A is a measure of the strength of the three-dimensional network and z is the coordination number and it is the number of flow units interacting with one another to give the observed flow response of material (Gabriele *et al.*, 2001).

When A increases the interaction forces within network are increasing whilst a high z value indicates a large number of interacting units cooperating and increasing the network connectivity. All data fitting is performed through Table Curve 2D Software (Jandel Scientific, USA).

This model was applied to frequency sweep tests at 25°C on all emulsions O/W and W/O at three different time of oxidation for both STD, CPFE and VIT E emulsions; the data are showed in the Table 4.9.

	O/W		**W/O**	
Samples	**A**	**z**	**A**	**z**
Emu STD (t_0)	559.95±0.73	10.42±0.11	117109±1338	6.42±0.33
Emu STD (t_1)	459.22±0.67	11.09±0.14	160876±2684	5.91±0.27
Emu STD (t_2)	358.41±0.99	10.49±0.24	254706±4839	3.92±0.19
Emu CPFE (t_0)	553.79±0.93	13.04±0.22	95790±965	6.04±0.30
Emu CPFE (t_1)	347.68±0.90	13.92±0.39	135211±3358	4,37±0,41
Emu CPFE (t_2)	331.97±0.85	12.59±0.31	140171±1183	5.36±0.17
Emu VIT E (t_0)	527.67±1.89	10.41±0.31	116317±1040	7.39±0.34
Emu VIT E (t_1)	454.69±1.21	10.94±0.25	141904±1674	7.08±0.41
Emu VIT E (t_2)	380.47±0.65	10.05±0.14	133437±3230	3,04±0.21

Table 4.9: Data for A and z for all emulsions analysed

The O/W emulsion present values of A much lower than those of the emulsions W/O, this is to indicate that in the second the interaction forces within network are

strong, but the z values in W/O is lower than that one in O/W emulsion maybe because there is a less number of interacting units cooperating. The higher values of z are showed in the CPFE emulsion O/W. The value of A is reduced with the progress of oxidation over time in the O/W emulsions, while increases in the emulsions W/O.

The temperature ramp tests, for three different time of oxidation for all emulsions O/W are showed below on Figure 4.20 (STD), 4.21 (CPFE), 4.22 (VIT E):

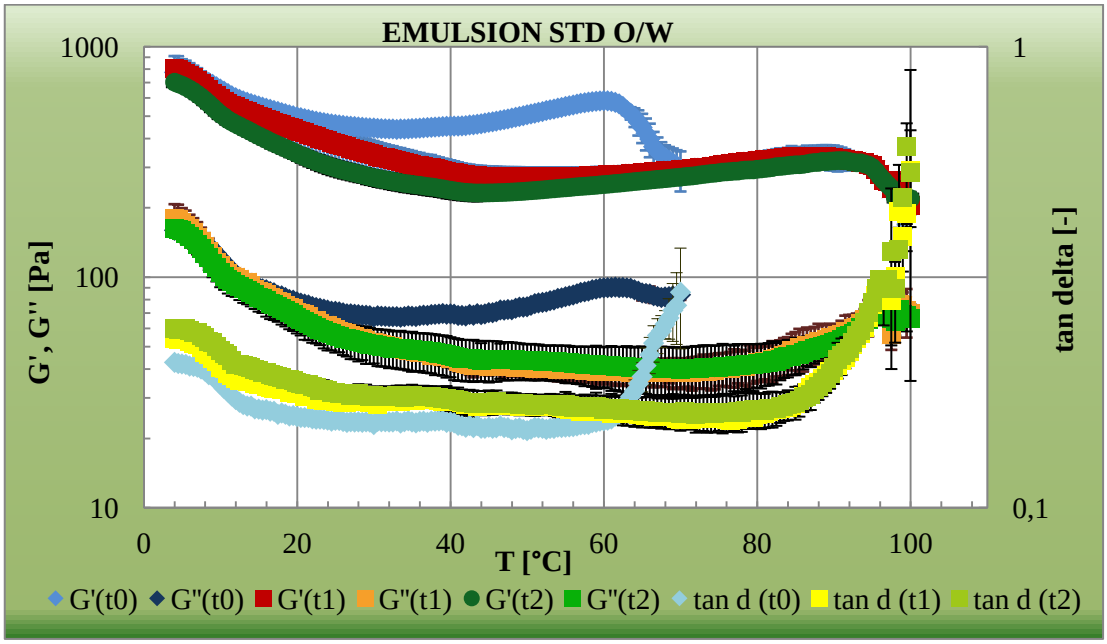

Figure 4.20: Time cure for three different time of oxidation for emulsion STD (O/W)

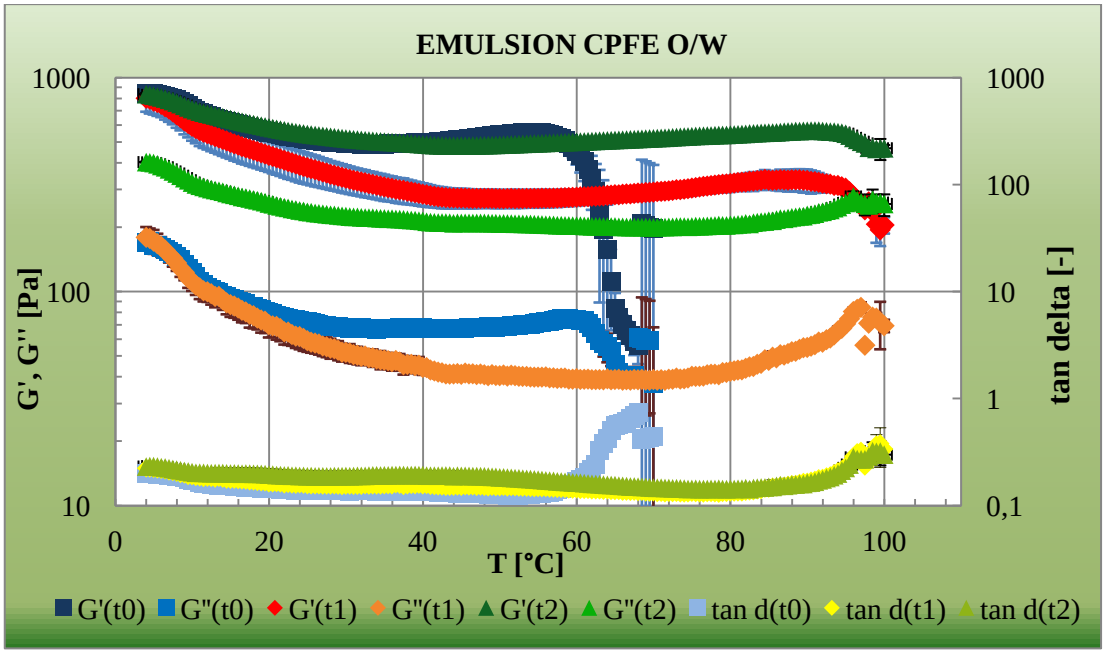

Figure 4.21: Time cure for three different time of oxidation for emulsion CPFE (O/W)

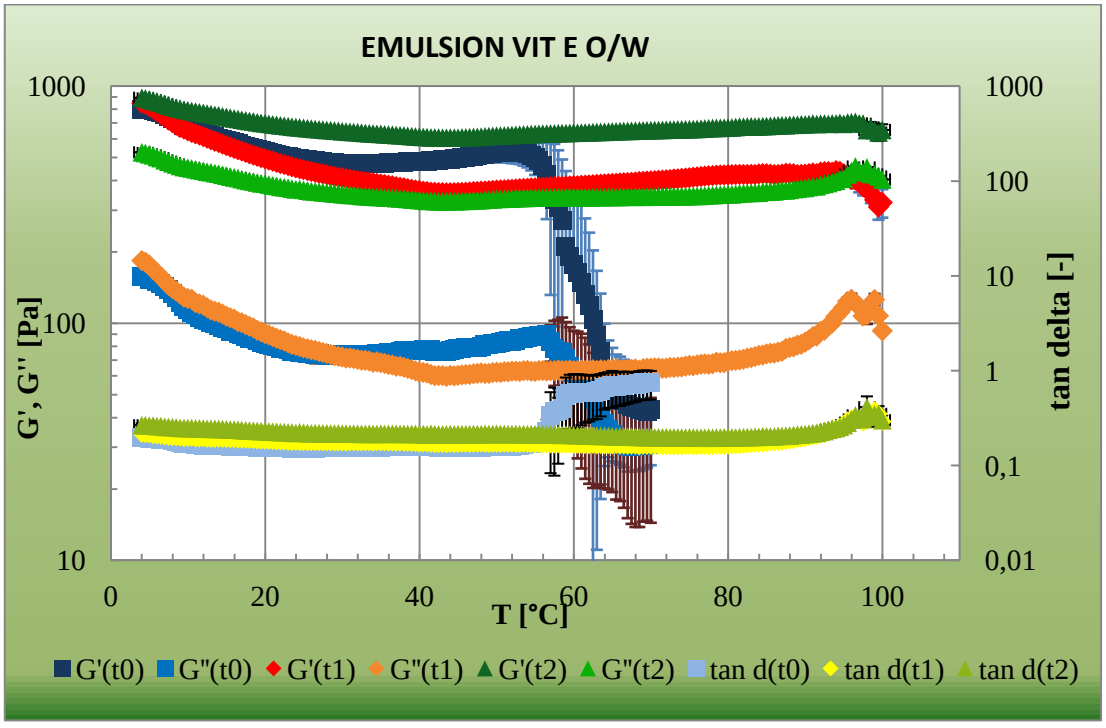

Figure 4.22: Time cure for three different time of oxidation for emulsion VIT E (O/W)

It’s immediately clear that in all three systems the modules G 'and G' 'collapse around 60° C, at time t0, while they are almost stable up to 100° C, at time t1 and t2. The modules are lowered slightly at time t1 and get up to the time t2. Apparently the emulsion with CPFE appears to have the most stable forms

Frequency sweep tests for three different time of oxidation for all emulsions O/W are showed below in Figure 4.23 (STD), 4.24 (CPFE); 4.25 (VIT E):

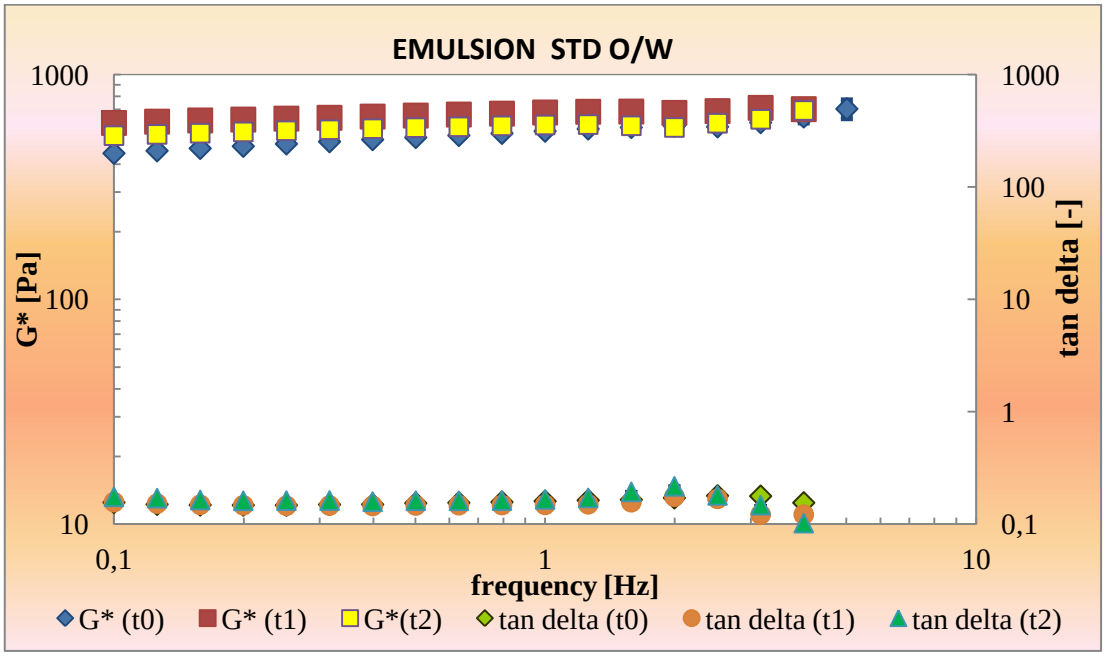

Figure 4.23: Frequency sweep tests for three different time of oxidation for emulsion STD (O/W)

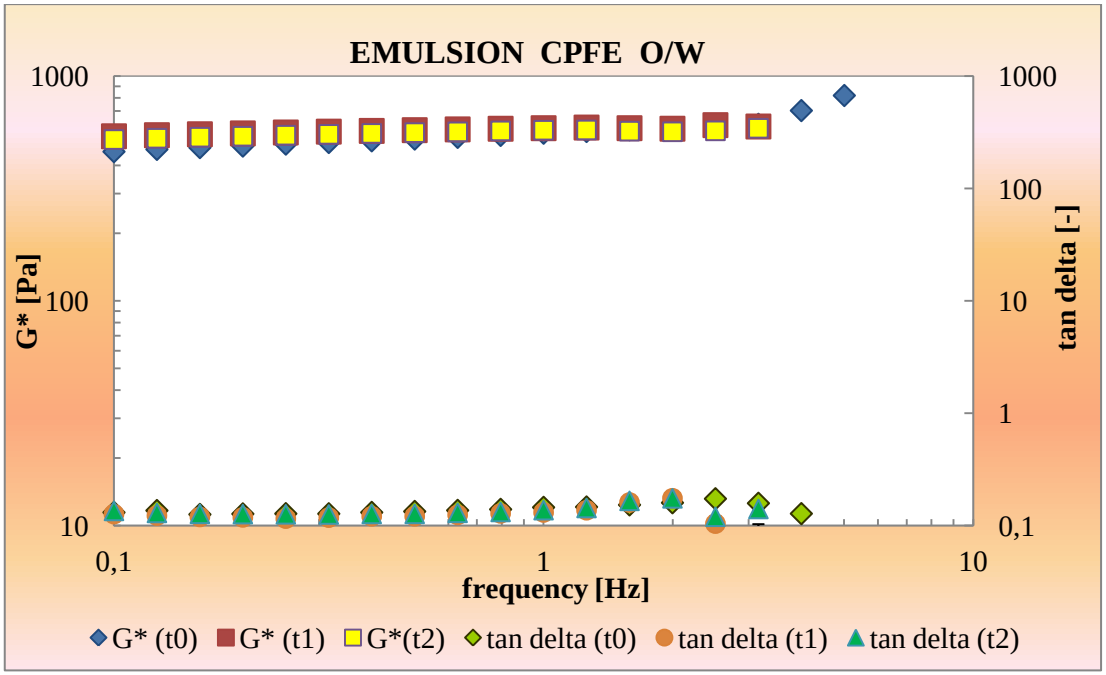

Figure 4.24: Frequency sweep tests for three different time of oxidation for emulsion CPFE (O/W)

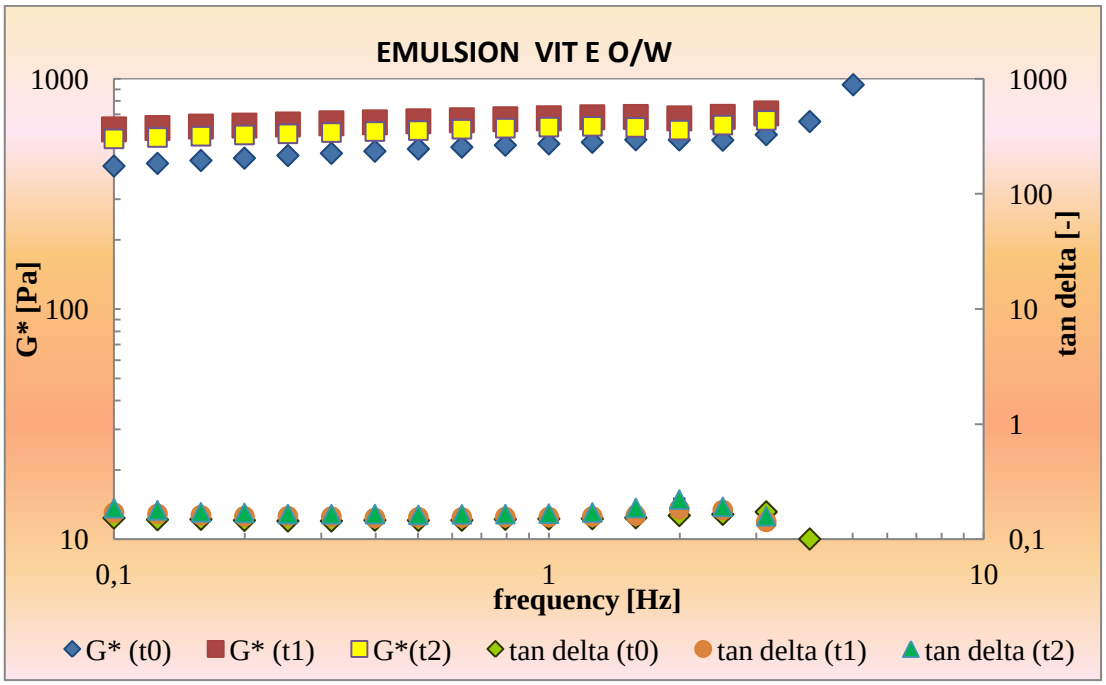

Figure 4.25: Frequency sweep tests for three different time of oxidation for emulsion VIT E (O/W)

The time cure for three different time of oxidation for all emulsions W/O are showed below in Figure 4.26 (STD), 4,27 (CPFE), 4.28 (VIT E):

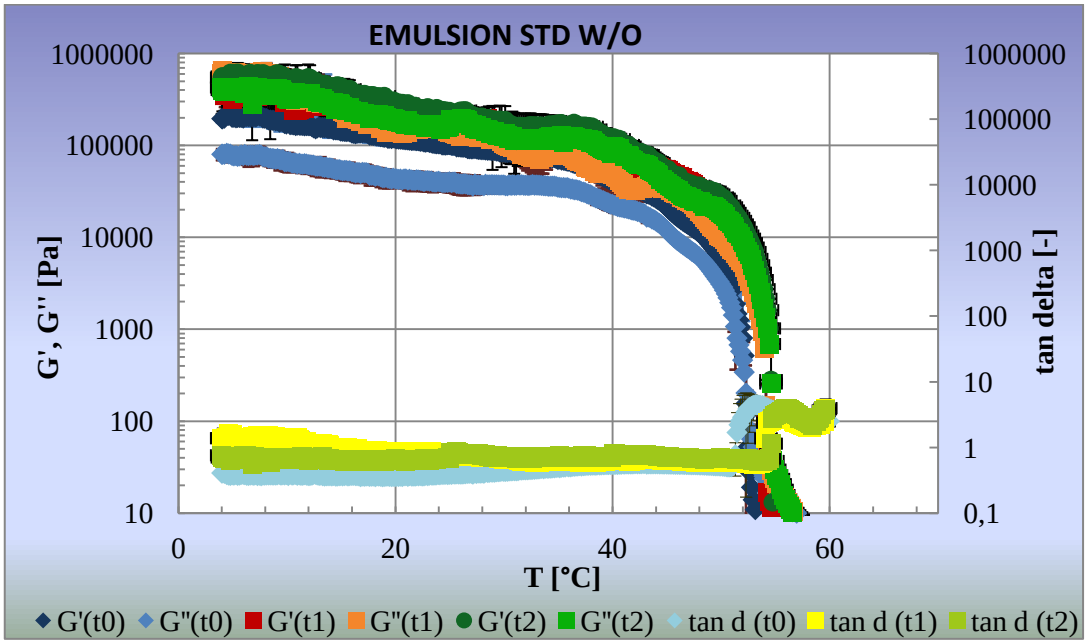

Figure 4.26: Time cure for three different time of oxidation for emulsion STD (W/O)

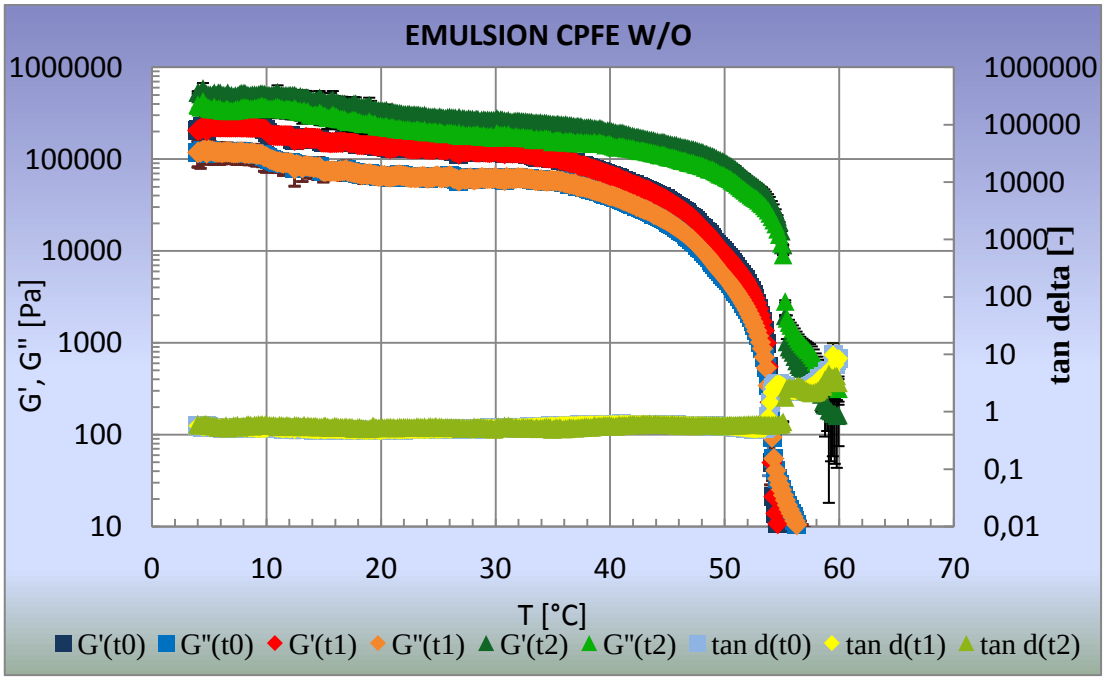

Figure 4.27: Time cure for three different time of oxidation for emulsion CPFE (W/O)

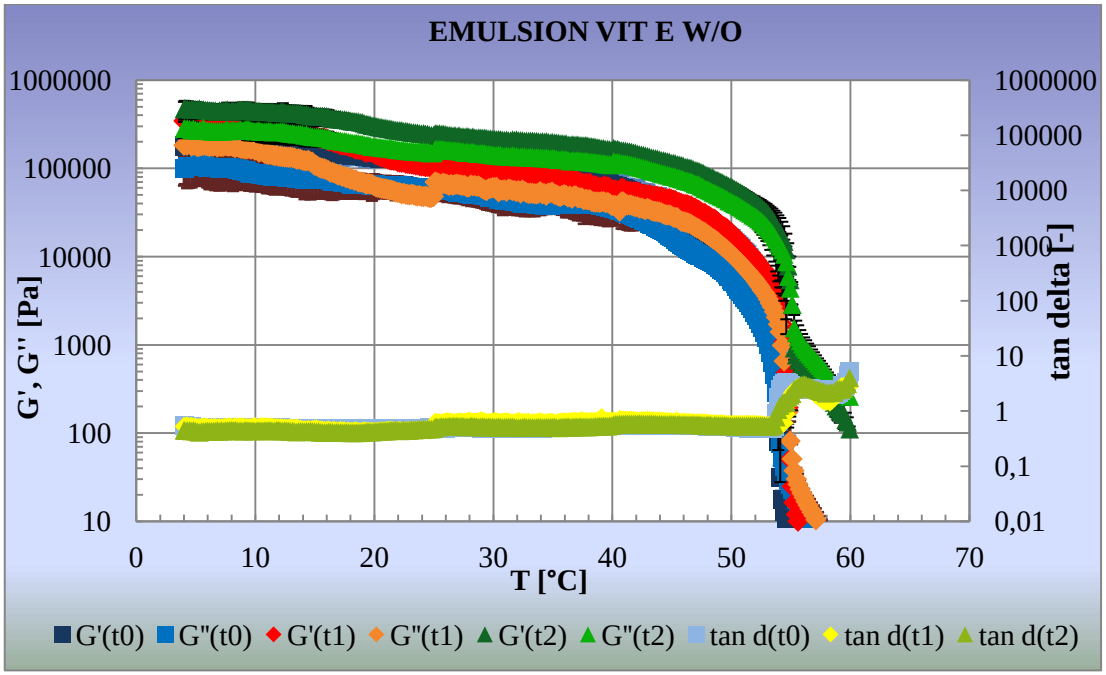

Figure 4.28: Time cure for three different time of oxidation for emulsion VIT E (W/O)

The modules are almost three orders of magnitude greater than the O/W emulsions, and they increase with increasing time. But unlike the O/W emulsion after the 55°C they start to crumble. Also in this case, however, as for the O/W, the modules at t2 are higher.

Frequency sweep tests for three different time of oxidation for all emulsions W/O are showed below in Figure 4.29 (STD), 4.30 (CPFE), 4.31 (VIT E):

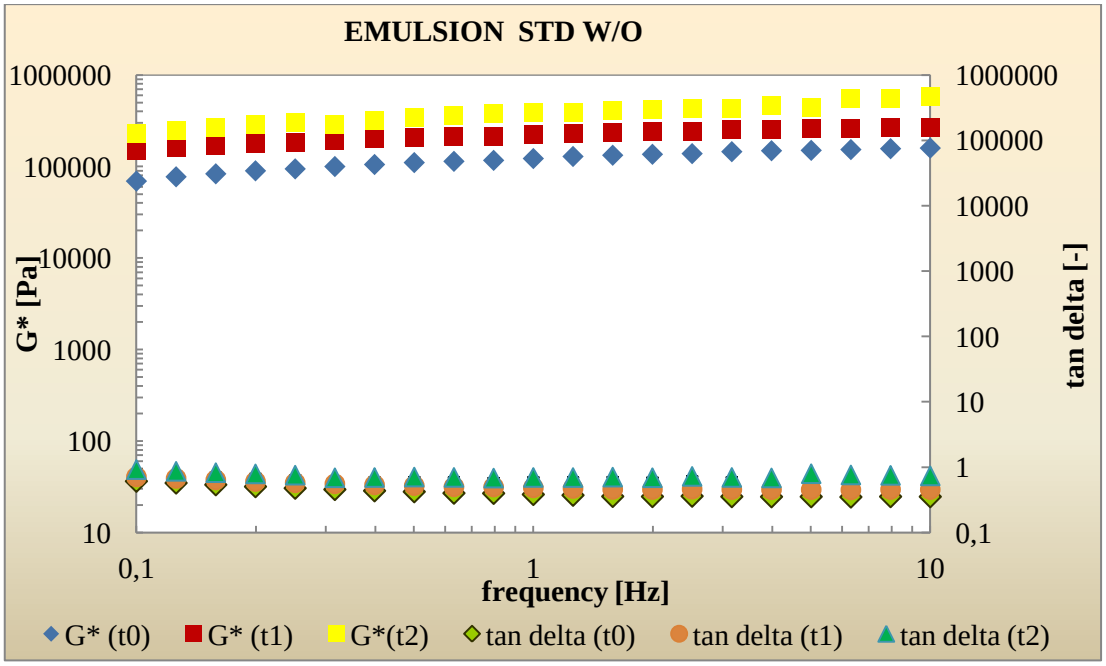

Figure 4.29: Frequency sweep tests for three different time of oxidation for emulsion STD (W/O)

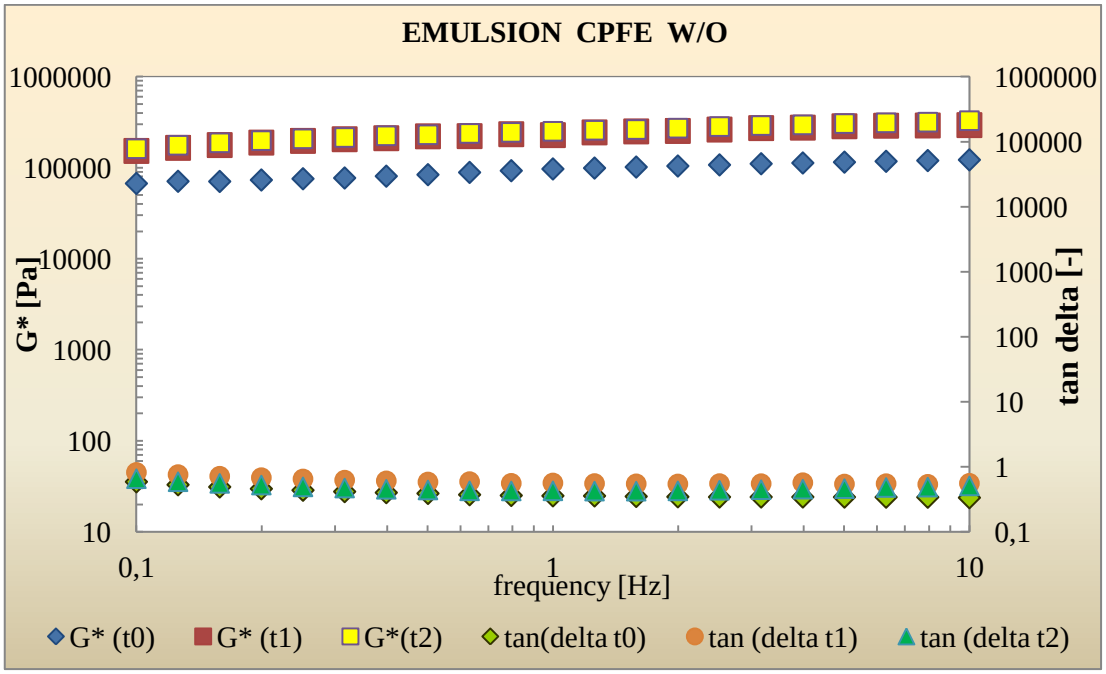

Figure 4.30: Frequency sweep tests for three different time of oxidation for emulsion CPFE (W/O)

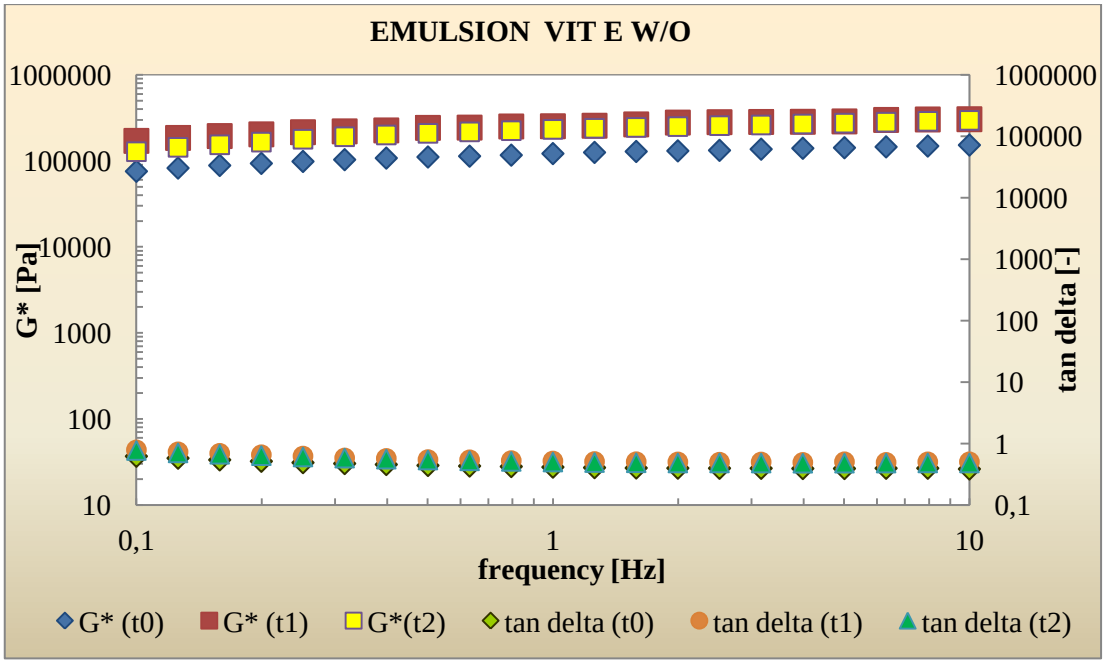

Figure 4.31: Frequency sweep tests for three different time of oxidation for emulsion VIT E (W/O)

Steady test

Flow curves at 25°C and 37°C test were done in order to evaluate the flow behaviour of all emulsions O/W. All data fitting was performed through Table Curve 2D Software (Jandel Scientific, USA) and was applied a Power Law, which describes quite well the behaviour of different polymer solutions. Moreover, when the exponent n = 1, the equation (6) describes a Newtonian fluid.

$$\eta = K_1 \dot{\gamma}^{n-1} \qquad (6)$$

Where η is viscosity, K is the consistency coefficient; γ is the shear rate and n is the flow behaviour index.

Below are showed the data for K and n for all emulsion O/W analysed:

Samples	**K**		**n**	
	25°C	**37°C**	**25°C**	**37°C**
Emu STD (t_0)	42.24±2.06	32.51±2.75	0.12±0.01	0.15±0.01

Emu STD (t_1)	32.99±1.64	17.54±2.16	0.16±0.01	0.19±0.03
Emu STD (t_2)	28.46±1.54	16.20±1.80	0.16±0.01	0.21±0.03
Emu CPFE (t_0)	48.96±2.78	36.60±4.46	0.09±0.01	0.13±0.03
Emu CPFE (t_1)	30.44±2.22	27.94±3.42	0.14±0.02	0.12±0.03
Emu CPFE (t_2)	29.54±2.11	20.24±1.81	0.13±0.01	0.15±0.02
Emu VIT E (t_0)	44.83±2.84	25.90±3.59	0.12±0.01	0.16±0.03
Emu VIT E (t_1)	33.30±3.28	20.06±1.84	0.15±0.02	0.23±0.02
Emu VIT E (t_2)	17.79±0.82	20.30±1.98	0.21±0.01	0.22±0.02

Table 4.10: Data for K and n for all emulsions O/W at three different time of oxidation

The values of K at 25° C are higher than those at 37° C but in both cases decrease with time. The values of n result very variable.

In the following figures are showed the flow curves for the emulsions O/W at three different stages of oxidation.

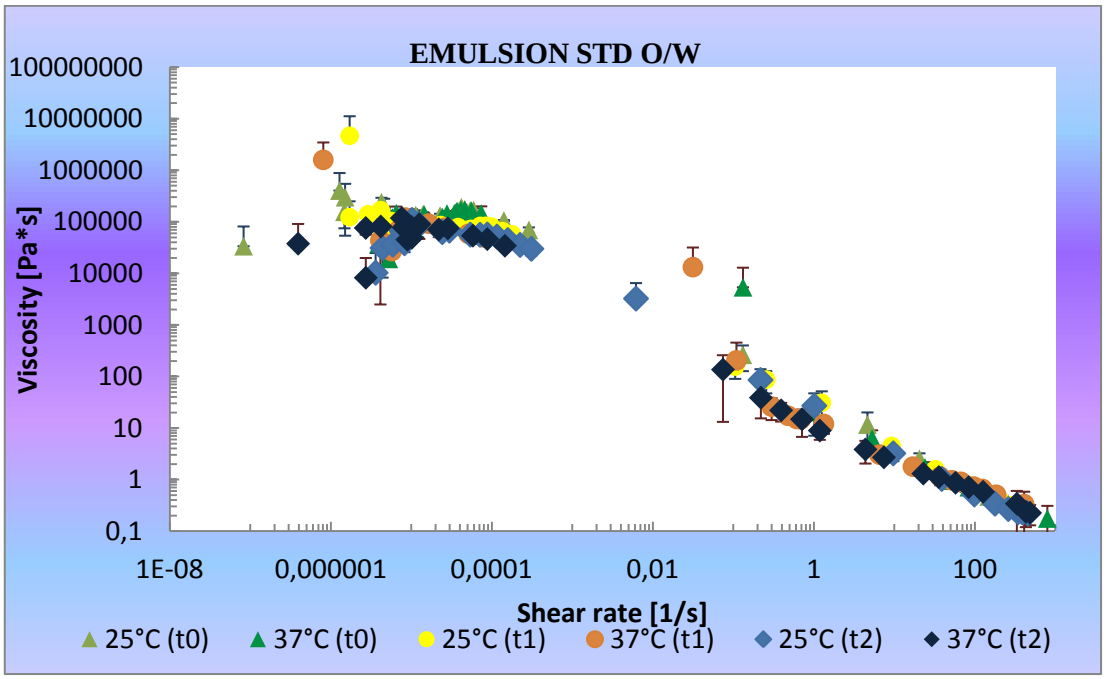

Figure 4.32:Flow curves for STD emulsions O/W at three different stages of oxidation

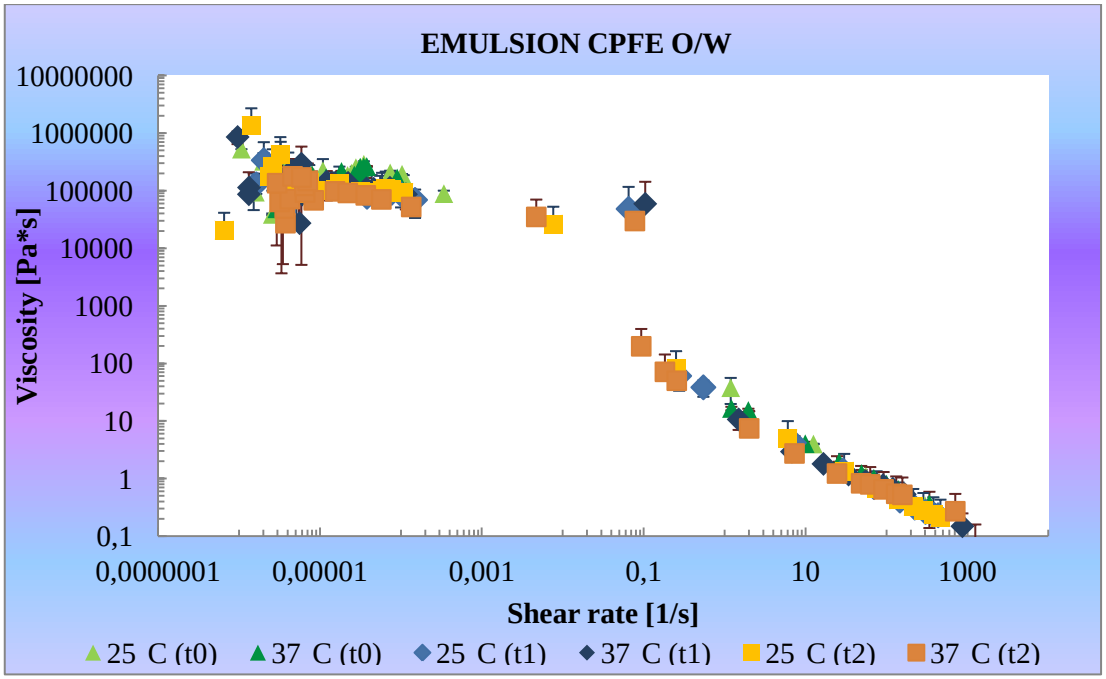

Figure 4.33:Flow curves for CPFE emulsions O/W at three different stages of oxidation

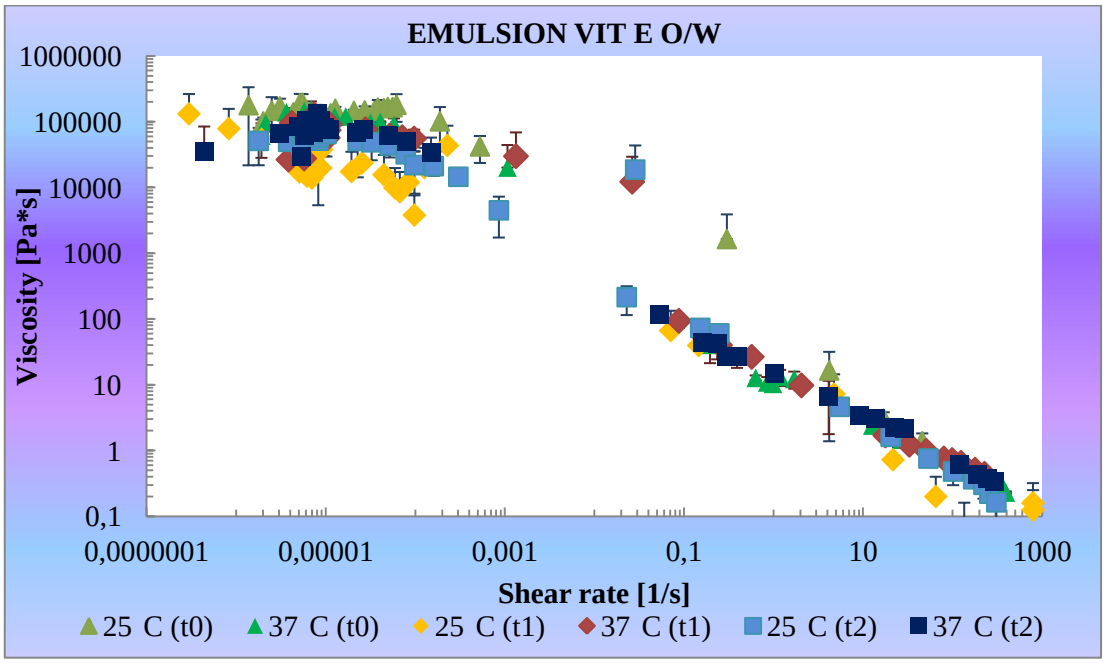

Figure 4.34:Flow curves for VIT E emulsions O/W at three different stages of oxidation

Below are showed the data for K and n for all emulsion W/O analysed:

Samples	K		n	
	25°C	37°C	25°C	37°C
Emu STD (t_0)	484.41±59.52	244.24±14.54	0.05±0.02	0.07±0.01
Emu STD (t_1)	254.87±16.29	208.79±38.27	0.11±0.01	0.13±0.02
Emu STD (t_2)	339.85±9.14	225.25±14.58	0.09±0.01	0.11±0.01
Emu CPFE (t_0)	465.01±58.41	203.59±18.20	0.04±0.01	0.11±0.01
Emu CPFE (t_1)	328.42±19.29	199.68±21.02	0.09±0.01	0.10±0.02
Emu CPFE (t_2)	506.21±89.05	180.37±10.73	0.05±0.02	0.12±0.01
Emu VIT E (t_0)	528.05±89.87	209.38±19.81	0.11±0.04	0.09±0.02
Emu VIT E (t_1)	321.87±30.41	190.65±17.18	0.07±0.01	0.10±0.01
Emu VIT E (t_2)	248.40±14.34	168.81±28.72	0.09±0.01	0.10±0.02

Table 4.11: Data for K and n for all emulsions W/O at three different time of oxidation

As in the O/W emulsions values of K at 25° C are higher than those at 37° C but here they are much higher than in O/W. and at t0 they reach the highest values. There are very variable values of n.

In the following figures are showed the flow curves for the three emulsions W/O at three different stages of oxidation.

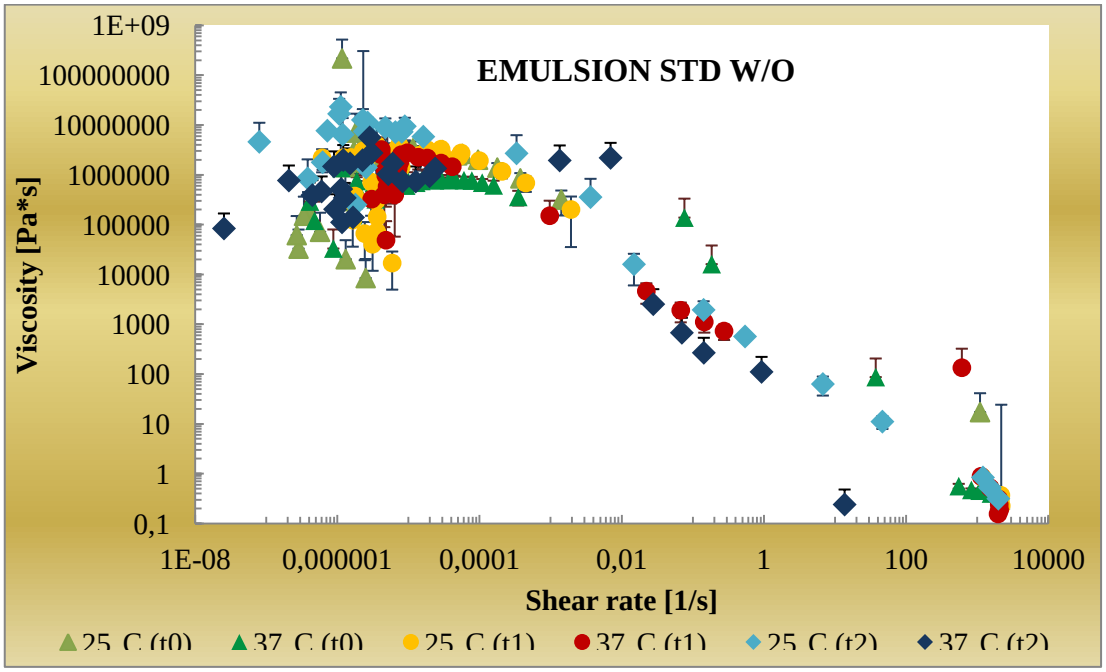

Figure 4.35:Flow curves for STD emulsions W/O at three different stages of oxidation

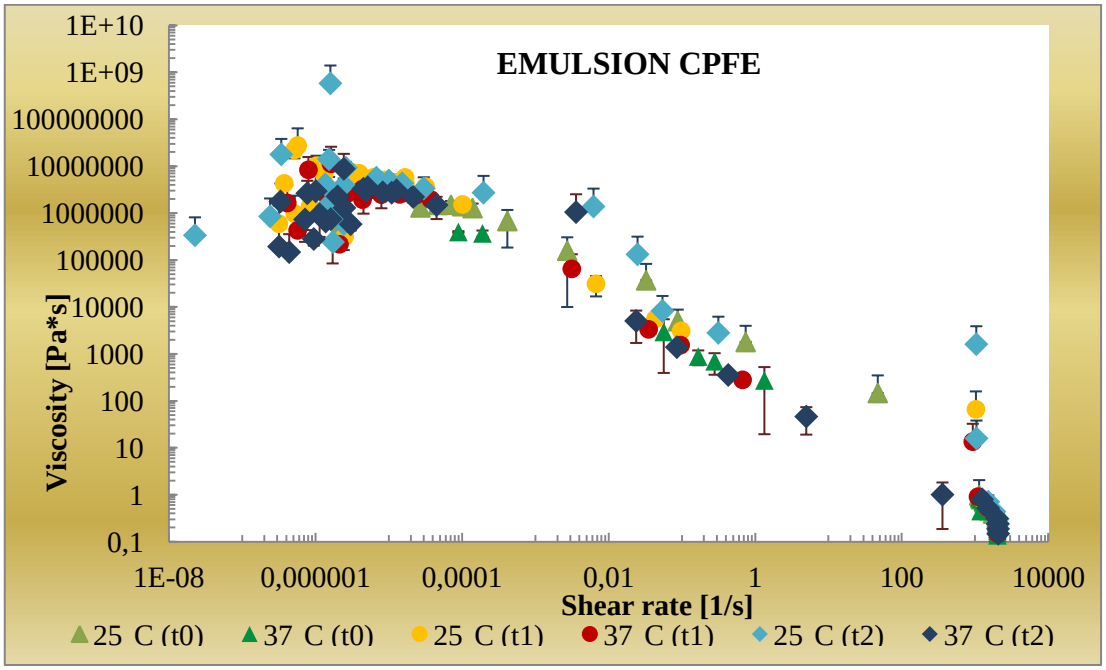

Figure 4.36:Flow curves for CPFE emulsions W/O at three different stages of oxidation

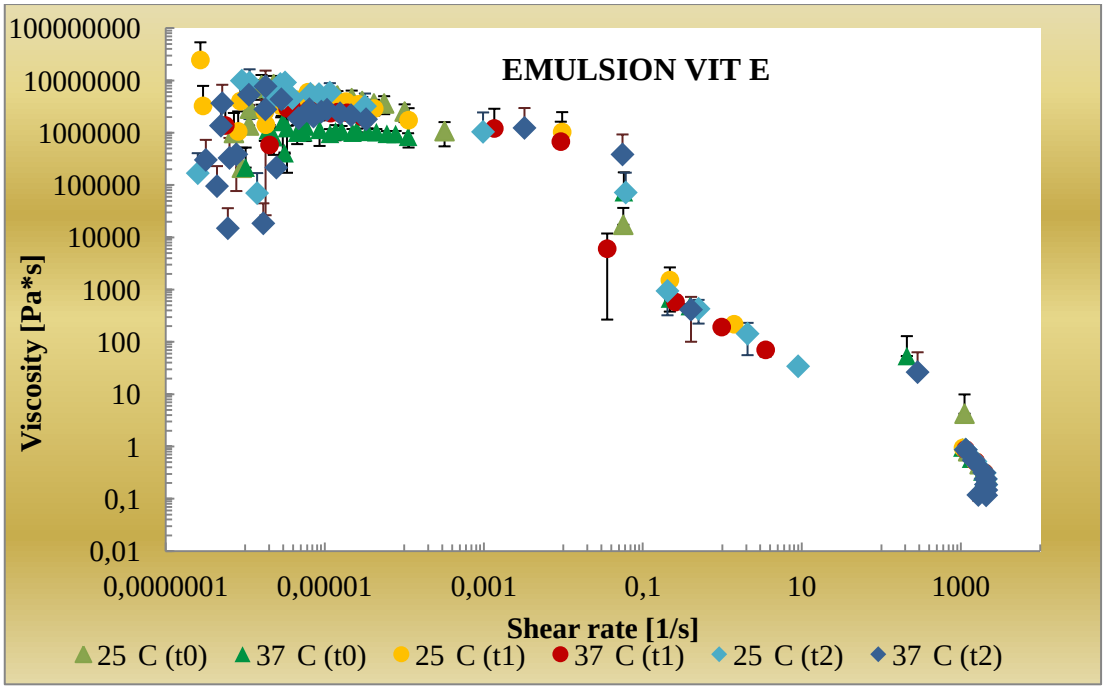

Figure 4.37:Flow curves for VIT E emulsions W/O at three different stages of oxidation

CONCLUSIONS

Exploring the edible fruits as sources of physiologically active compounds we have enormous opportunities for the development of novel foods or medical devices as well as conservation and propagation of plant species to commercial production.

The aim of this work was twofold; on one to evaluate the possibility of extracting through SFE-CO2, the polar betalains; the amount obtained by the solvent extracts is comparable with that obtained by SFE-CO_2, this indicates that the SFE is an excellent technique for the extraction of colored pigments such as betalains.

From October to December we found an increase of both water activity and humidity and a decrease of brix degree because October's fruits, during their growth, had longer days and higher radiation as well as higher daily temperatures, lower rainfall than winter fruits.

The greater concentration of betacyanins, in the spectrophotometric analysis of the extracted with solvents, were found in water extract probably because the betacyanins are more polar than the betaxanthin and they are able to pass more easily in a polar solvent such as water. The HPLC analysis of solvents extracts show an high concentrations of total betalains (betacyanins and betaxanthin) when was used methanol, maybe because is both polar and small and can penetrate better in the plants layers. Concerning the SFE- CO_2, the quantitative yield of extraction is not related to a greater amount of betalains especially in the samples centrifuged of both, second and third collections, to which was added Ottawa sand, that does not interfere with the residual water, but it is a dispersing agent and allows a better homogenization of the sample in the extraction column. In these samples the total yields quantitative of extraction are the highest, while the amount of extracted betalains is the lowest because the removed water carries with itself a rich portion of betalains that are polar. A partial drying of the samples and the addition of Diatomaee sand increase both the extraction quantitative yields and the amount of

total betalains extracted; the best results were obtained with the sample not long dried (20%), so waer can work as co-solvent, and the amount of sand minimal (5%), it is so also economical from the point of view of process.

Both quantitative best yields and larger quantities of betalains were obtained in the collection of October, probably the stage of fruit ripening and the onset of the winter season (cold and rain), contribute to a reduction of active ingredients within the matrix.

Other target was to evaluate the antioxidant effectiveness of *Opuntia ficus-indica* fruit extract during oxidation of emulsions O/W and W/O and their rheological properties. Also the STD emulsion possesses a certain antiradical activity, on both the radicals DPPH and ABTS, this is because the oil phase was prepared using extra virgin olive oil that contains polyphenols with antioxidant activity.

The trend is similar to both O/W and W/O emulsions but we reached much higher values of inhibition which almost 50% for STD emulsion and over 63% for CPFE emulsion on DPPH radical, and almost 25% for STD emulsion and 68% for CPFE emulsion on ABTS radical in the W/O systems. This because the quantity of oil is higher and therefore it increases the effect of the polyphenols that it contains and because the aqueous phase, in which is dissolved the CPFE, is dispersed and less affected by the oxidation process, being within the oil phase it results protected.

Regarding the rheology characterisation the small amplitude oscillation tests showed that the O/W emulsions present values of A much lower than those of the emulsions W/O, this is to indicate that in the second the interaction forces within network are strong. The z values in W/O is lower than that one in O/W emulsion maybe because there is a less number of interacting units cooperating. The higher values of z are showed in the CPFE emulsion O/W. The value of A is reduced with the progress of oxidation over time in the O/W emulsions, while increases in the emulsions W/O.

In all three systems O/W the modules G 'and G' 'collapse around 60° C, at time t0, while they are almost stable up to 100° C, at time t1 and t2.

The modules in W/O emulsions are almost three orders of magnitude greater than the O/W emulsions, and they increase with increasing time. But unlike the O/W emulsion after the 55°C they start to crumble.

In the steady tests both O/W and W/O emulsions showed higher values of K at 25° C than at 37° C but in both cases decrease with time. The values of n result very variable.

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CHAPTER 5

EXTRACTION AND QUANTIFICATION OF BIOACTIVES COMPOUNDS FROM HIGH PROTEIN NOVEL CROPS

The work presented on this chapter was carried out at the Rowett Institute of Nutrition and Health, University of Aberdeen, United Kingdom; from 27th January to 27th July 2014.

Figure 5.1: The main building of the Rowett Institute today and when was opened

The Institute was founded when the University of Aberdeen and the North of Scotland College of Agriculture agreed that an "Institute for Research into Animal Nutrition" should be established in Scotland.

The quality of the science and its applications was underlined by two Rowett scientists who have received nobel prizes: Sir John Boyd Orr, who was the recipient of the Nobel Prize for Peace in 1949 and Richard Synge, in 1952, jointly with John Porter Martin for discovery of partition chromatography.

During the late 1970s, the realization that all was not well with the health of Scotland's people started to turn the focus of the Institute's research back towards human health. The role of diet in chronic diseases such as heart disease and cancer, began to be investigated by Rowett scientists. During the late 80s and early 90s, major investments in technologies enabled Rowett scientists to attempt to unravel the

mechanisms whereby what we eat either promotes or protects us from the development of some of these diseases.

In 2008 the Institute merged with the University of Aberdeen, an event which has delivered several obvious benefits: There are significant complementary skills between the two organisations which have resulted in increased research capability, and wider access to funding streams. The Institute within the College of Life Sciences and Medicine now represents one of the UK's largest research centres for food and health-related research. Another benefit from the merger is the greater capability for interaction with Scotland's food and drink industry.

Much of the current researches are sponsored by the Scottish Government, including this work project called "NOVEL FOOD CROPS", and aims to address the big issues of our time, including food inequalities, food security and obesity, as well as the sustainable development of Scotland's food industry.

The research results will remain the property of the Rowett Institute.

Introduction

In recent years, food has been considered not only as a source of energy and nutrients for the maintenance and growth of the body but also as a source of bioactive compounds that may exert beneficial effects in humans.

Over the past decade, several noteworthy consumer trends have emerged, such as enhanced concern for the quality and safety of foods and medicines and regulations for nutritive and toxicity levels.

Food quality is a prerequisite for an optimal nutrition; regarding produced raw food, an optimal quality lies in tasty products, with high nutrient content and no/minimal contaminations by chemical toxicants. The products raised through the agro-ecological methods such as certified organic ones generally fit these two

requirements by improving the dry matter and some nutrients contents and minimizing chemical and nitrate contaminations.

One billion poor people still suffer from hunger and malnutrition while about 2 billion show under-nutrition and micronutrient deficiencies (FAO, 2011). At the same time, about 2 billion are overweight and/or obese, a steadily increasing number in all countries in the world (WHO, 2011).

It has been clearly shown that low-cost foods are those energy-dense (fat- and sugar-rich) and nutrient-poor, inducing both deficiencies and overweight consequences of inappropriate food choices, often driven by household income and education level.

The high energy content of most food consumed (meat, egs, butter, snacks etc.) can fit the important needs of people with a high energy expenditure, but is in excess for most urbanized sedentary people (Etiévant *et al.*, 2010); that are progressively less influenced by the local cultural heritage and a not suited integration in the local environment.

It is a well-established fact that the consumption of adequate amounts of dietary fiber reduces significantly the risk of degenerative diseases, including diabetes, obesity, coronary heart disease, bowel cancer and gallstones. Dietary fibers also have technological properties that can be used in the formulation of foods, resulting in texture modification and enhancement of the stability of the food during production and storage (Ayadi *et al.*, 2009).

It must be emphasized that although the texture and sensory qualities of the products made from refined flours have been improved, the nutritive values of these products have become lower because most of the nutritional compounds such as minerals, vitamins, dietary fiber, resistant starch and micro constituents have been removed during refining of flours (Graham *et al.*, 2007).

To satisfy this need for nutrients, there is the trend to trying more crops that contain both bioactive compounds, intended to improve the state of health, and which are high in fiber and protein necessary to the welfare of the organism.

Recent advances in cereal and pseudo-cereal based food researches have been reviewed; pseudo-cereals are broadleaf plants (non-grasses) that are used in much the same way as cereals (true cereals are grasses). Their seed can be ground into flour and otherwise used as cereals. New milling techniques have been applied to produce graded flours which contain large amounts of dietary fiber, vitamins and minerals and could be applied for substitution with the conventionally milled flour in bread-making to produce functional food products with high safety, palatability and nutrition. In addition, germination of cereal and pseudo-cereal grains helps to improve the chemical compositions, nutritive values and acceptability characteristics of the products (Morita *et al.*, 2013).

First focus of this work was the identification and quantification of important bioactive phytochemicals such as phenolic acids and flavonoids, anthocyanins, catechins and saponins, present in chia, quinoa, pumpkin, triticale, flaxseed and potato bean.

The second focus of this work was to evaluate how the main crops phytochemicals are metabolized and are available for different sites of the GI tract. This work was performed by specialised fractionated extractions (i.e. by varying the pH to mimic the process of absorption of phytochemicals in the GI tract and also to assess what phytochemical fraction is most bioavailable).

Phenolic acids can be divided into different fractions, depending on their potential interactions with other components in the food matrix. Soluble phenolic acids, directly measured in the supernatants of aqueous-organic extractions; esterified phenolic acids measured in the same supernatants after performing alkaline or acid hydrolysis; and insoluble phenolic acids, which remain in the residues following extraction and hydrolysis. Much research to date on phytophenols has focussed on the antioxidant potential of easily solubilised compounds. Recent evidence suggests that cereal grains could have a much more significant impact on total dietary intake

of phytophenols than previously considered and that their biological effects could be more extensive than just their ability to inhibit oxidation (Neacsu *et al*., 2013)
The esterified phenolic compounds represent a big fraction, which after consumption and metabolism, are likely to be bio-available and possibly bio-active in the gastrointestinal tract (Russell *et al.*, 2009).

5.1 Materials and methods

5.1.1 Plant material

The plant material used was from family of *Lamiaceae*, Chia (*Salvia hispanica* L.), organic seeds (OCS) and white organic seeds (WOCS); Quinoa (*Chenopodium quinoa* Willd.) family of *Chenopodiaceae*, normal grain (NQ), red organic grain (ROQ) and organic black (OBQ); Linseed (*Linumusitatissimum* L.) family of *Linaceae*, normal brown (NBL), organic golden (OGL) and organic brown (OBL); Pumpkin (*Cucurbita pepo* L.) family of *Cucurbitaceae* , normal seeds (PS) and organic seeds (OPS); Triticale (*Triticosecale* Wittm.) family of *Poaceae*, whole flour (WTF), cereal (TC) and rolled (TR); Potato Bean (*Apios americana* Medikus) family of *Fabaceae*, tubers (PBT) and peel (PBP).
All of the plant was commercial available and food grade. The samples were freeze-dried (lyophilised) (Heto Lab Equipment Allerød; Denmark) and then the samples were freeze-milled (Spex 6700; Edison; USA) and stored under vacuum until analysis.

5.1.2 Fractioned extraction of phenolic acids and flavonoids

This extraction procedure allows the isolation of free and bound (acid and alkali-labile) compounds, in order to give an indication about their potential availability in

the human gastrointestinal track. Free compounds are indicative of compounds absorbed in the stomach and small intestine and bound compounds of those likely to be delivered to the large intestine and colon.
All extractions were performed in triplicate following a modified procedure of Russell *et al*. 2009.

Free compounds (F1)

The samples (3 x 0.1 g) were suspended in HCl (0.2 M; 3 mL) and extracted with EtOAc (5 mL), shaked, vortexed and sonicated. The solvent solution layers were separate by centrifugation (3000 rpm; 5 minutes at 18 °C) and then filtered.
This process was repeated twice with final centrifugation longer (4000 rpm; 10 minutes at 18 °C).
The solvent was removed under reduced pressure with a rotary evaporator at 38°C, the extracts were resolved in Methanol LC/MS grade (1mL) and divided into two portions one was stored at -70°C until LC-MS analysis, the other portion was used for the measurement of the flavonoids (F2). The pH of the remaining aqueous fraction was neutralized with NaOH (4 M), the solutions were freeze-dried.

Acid hydrolysis of free fraction extract (F2)

Samples (0.5mL)of the free fraction (F1) were dry under nitrogen stream and bring to dryness. The hydrolysis was performed adding HCl (1 M; 4 mL) on a thermoblock for 1 hour at 90°C. The hydrolysis solutions were let to cool down and extracted with EtOAc (5 mL) for three times. The solvent solution layers were filtered and centrifuged (700 g for 5 mins at 18°C) . The EtOAC layers were pooled and the solvent was removed under reduced pressure, the extracts were resolved in LC/MS MeOH (0,5 mL) and stored at -70 °C until analysis.

Alkali-labile compounds (F3)

To the freeze dried aqueous fractions, was added NaOH (1 M; 3mL) and stirred at room temperature for four hours under nitrogen. The pH of the solutions were reduced to pH 2 with HCl (10 M) and extract with EtOAc as amended above. The aqueous fraction was neutralized with NaOH (4 M) and then freeze dried.

Acid-labile compounds (F4)

To the freeze dried aqueous fractions were added HCl (2 M; 3 mL) and incubated at 95 °C for thirty minutes with intermittent mixing. Cooled to room temperature and extracted with EtOAc same as above.

5.1.3 Extraction of anthocyanin aglycones

The sample (3 x 0,1 g) was suspended in 2 mL of an extraction mixture solution cointainig methanol, water and hydrochloric acid 37% (2N HCl) then vortexed and sonicated for 20 min. The supernatant was separated by centrifugation (5 minutes; 3000 rpm; 4 ºC). The extraction was repeated three times and the supernatants were combined and transferred to a vial with a Teflon-lined screw cap and placed on a thermoblock for acid hydrolysis (100 °C for 60 min). Hydrolyzed samples were then cooled to room temperature, filtered on 0.2µm filters and analysed by HPLC.

5.1.4 Extraction of catechins

The sample (3 x 0.25 g) was suspended in a solvent extraction mix solution, (acetone: water: acetic acid, 70: 28: 2, v :v:v), and placed in ultrasound bath for 10

minutes. The supernatant was separated by centrifugation (10 minutes; 4000 rpm; 4 ºC). The extraction was repeated three times and the supernatants were combined, and evaporated under reduced pressure at 38 °C. The residue was then re-dissolved in LC-MS methanol (2 mL, 50%) and stored at -70°C until LC-MS analysis. The pellets were freeze-dried and kept for further alkaline hydrolysis.

The freeze-dried pellet fractions were suspended in 6mL of NaOH (1 M) and stirred at room temperature for four hours under nitrogen. The pH was reduced to pH 2 with HCl (10 M) and the samples extracted with EtOAc (5 mL) for three times and the layers separated by centrifugation (10 minutes; 3500 rpm, 4°C); the supernatants combined and evaporated under reduced pressure at 38 °C and re-dissolved in LC-MS methanol (1 mL,50%) until analysis.

5.1.5 Extraction of saponins

The sample (3 x 0,5 g) was extracted in glass tubes with 5 mL of 70% ethanol, under magnetic stirring for 3 hours at room temperature. The mixture was then centrifuged (7 minutes, 700 g; 4 °C) and the supernatant was carefully decanted into new glass tubes and evaporated under nitrogen at room temperature. The dried residue was purified on a Strata C18-E cartridge (3 mL, 0,5 g from Phenomenex, USA). The cartridge was activated with 2 x 2 mL of technical methanol and conditioned with 2 x 2 mL of distilled water and then was added the residue solved in 2 mL of distilled water. After cartridge was washed with 2 x 2 mL of water and then the elution was performed using 100 mL of methanol. The solid phase elute was directly injected in HPLC-MS.

5.2 LC-MS/MS analysis of phenolic acids, flavonoids and catechins

5.2.1 Preparation of samples for LC-MS

To 100 µl sample from F1,F2, F3 and F4 extraction and to the catechins extract, was added 100 µl of internal standard 1 for negative mode mass spectrometry (IS1; ^{13}C Benzoic Acid, Isotec TJ2365; 2 ng μl^{-1} in 0.02% acetic acid in 75% methanol); and 100 µl of internal standard 2 for positive mode mass spectrometry (IS2; 2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline; Toronto research chemicals Inc. 12AZC-165-1, 0.5 ng μl^{-1} in 0.02% acetic acid in 75% methanol) and with 200 µl of LC-MS methanol. The samples were vortexed and centrifuged (JOUAN MR 18-22, 12500 g for 5 minutes at 4 ºC) and 65 µL aliquots transferred for analysis by LC-MS; each sample was prepared in triplicate.

5.2.2 LC-MS/MS analysis

The analyses of the extracts , with exception of anthocyanin aglycones and saponins, were performed on an Agilent 1100 LC-MS system (Agilent Technologies, Wokingham, UK) using a Zorbax Eclipse 5µm, 150 mm x 4mm column (Agilent Technologies). Three different gradients were used to separate the different categories of metabolites and the mobile phase solvents in each case were water containing 0.1% acetic acid and acetonitrile containing 0.1% acetic acid. In all cases the flow rate was 300 µL/min with an injection volume of 5 µL. The LC eluent was directed into, without splitting, an ABI 3200 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) fitted with a Turbo Ion Spray™ (TIS) source. For LC methods 1 and 2, the mass spectrometer was run in negative ion mode with the following source settings: ion spray voltage -4500 V, source temperature 400 ºC, gases 1 and 2 set at 15 and 40 respectively and the curtain gas set to 10. For LC method 3, the mass spectrometer was run in positive ion mode with the following source settings; ion spray voltage 5500, source temperature 400 °C, gases 1 and 2 set at 14 and 40 respectively and the curtain gas set at 10. All the metabolites were quantified using multiple reaction monitoring. Standard solutions

(10 ng/µL) for all analytes were prepared and pumped directly via a syringe pump. The ion transitions for each of the analytes were determined based upon their molecular ion and a strong fragment ion. For several categories of compounds, it was inevitable that their molecular ion and fragment ion would be the same, but this was overcome by their differing elution times. Their voltage parameters; declustering potential, collision energy and cell entrance/exit potentials were optimised individually for each analyte.

5.3 HPLC method for anthocyanins aglycones quantification

Liquid chromatography separation of the anthocyanin aglycones was performed on an Agilent 1200 HPLC system (Agilent Technologies, Wokingham, UK) equipped with binary pumps, thermostated autosampler (4 C), column oven at 35 °C and DAD detector (530nm). The following solvents were used: mobile phase A 2.125% formic acid, mobile phase B acetonitril/methanol 85/15 (v/v). The column used was Synergi 4u Polar-RP 80A, 250 x 4.6mm, the guard column Porar-RP 4x3mm, both from Phenomenex. The solvent program was isocratic 18% B for 45 minutes (flow 1mL/min). The injection volume 20µL.

Qualitative determination of the anthocyanin aglycones was done based on the anthocyanin aglycones standards and their HPLC retention time. For the quatification were used the following standards: delphinidin, cyanidin, peonidin, pelargonidin, petunidin and malvidin.

5.4 LC-MS/MS analysis of saponins

The LC-MS/MS analysis was conducted for soyasaponin I and soyasapogenol B and were carried out on an Agilent 6490 triple-quadrupole mass spectrometer with an Agilent 1290 Infinity LC. Metabolites were separated on a Phenomenex Kinetex C18 column (250x4.6mm, 5µ) with a total run time of 21 minutes. Solvent A was 0.25%

Acetic Acid and solvent B was Methanol with 0.25% Acetic. Starting conditions were 80% B rising to 95% over 13 minutes (linearly) then decreasing to 60% B for 5 minutes before returning to 80% in preparation for the following injection. The flow rate was 1mL/min which was split post-column before admittance to the mass spectrometer.

The ionisation was done with the Agilent Jet Stream (AJS) source in positive ion mode. Selected source conditions were as follows: Gas Temp 200^{o}C, Nebuliser 35psi, Capillary 3000V, Nozzle 2000V and Sheath Gas Temp 300^{o}C. For the first 8 minutes, data was collected in MS2 SIM mode (set at mass 944.2) for the analysis of soyasaponin I and then the scan type was changed to MRM mode (441.2 → 423.3) for the analysis of soyasapogenol B. The reasoning behind using selected ion monitoring (SIM) for the analysis of soyasaponin I was because of the poor fragmentation attained when pumping a 1ng/µL solution of the standard via a syringe pump. Sensitivity was greatly enhanced when analysing by SIM.

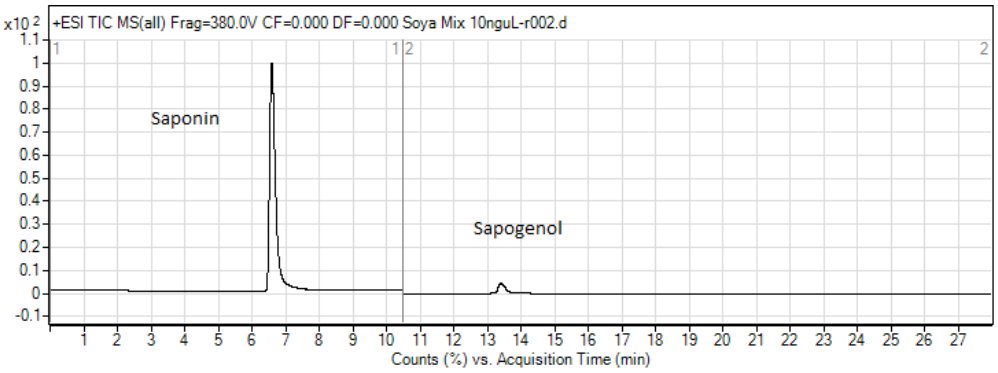

Figure 5.2: TIC of a 10ng/µL mix of soyasaponin I and soyasapogenol B

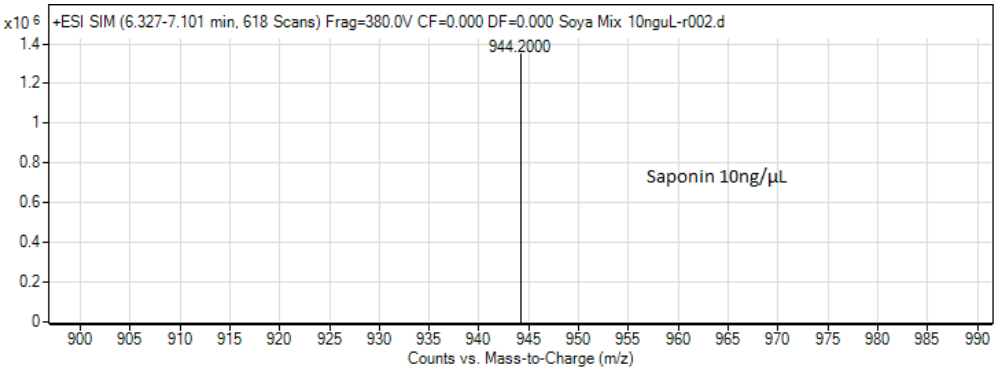

Figure 5.3: Extracted spectra of soyasaponin I

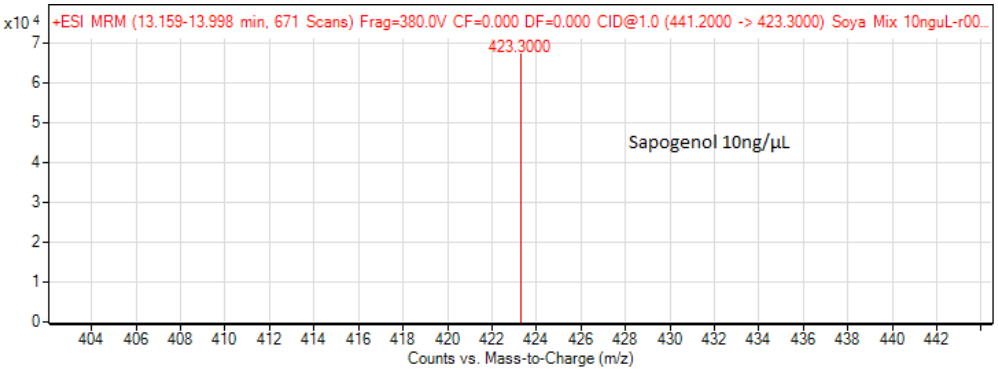

Figure 5.4: Extracted spectra of soyasapogenol B

5.5 RESULTS AND DISCUSSION

5.5.1 Qualitative and quantitative LC-MS/MS analysis of the phytochemicals present in F1, F2, F3 and F4 fractions

All the compounds detected and quantified by LC-MS/MS analysis from the F1, F2, F3 and F4 extractions reported in the tables below, expressed mg/kg of dry plant material, as mean ± standard deviations (n = 3); not detected = n.d.. Table 5.1 the phytochemicals detected in organic chia seeds; white organic chia seeds; pumpkin

seeds and organic pumpkin seeds; in the Table 5.2 the phytochemicals detected in red quinoa grain, black quinoa grain and quinoa grain; in the Table 5.3 the phytochemicals detected in brown linseed, organic golden linseed and organic brown linseed using LC-MS/MS analysis; the Table 5.4: for the phytochemicals detected in triticale glour, triticale cereal and triticale rolledand in the Table 5.5: The phytochemicals detected in potato bean tuber and potato bean peel using LC-MS/MS analysis

The results in the table are segregated based on species of each plant,

PLANT	Organic chia seeds				White organic chia seeds				Pumpkin seeds				Organic pumpkin seeds			
Fraction	**F 1**	**F 2**	**F 3**	**F 4**	**F 1**	**F 2**	**F 3**	**F 4**	**F 1**	**F 2**	**F 3**	**F 4**	**F 1**	**F 2**	**F 3**	**F 4**
salicylic acid	0.46± 0.09	0.49± 0.23	0.23± 0.04	1.12±0. 13	0.3±0. 06	0.32± 0.02	nd	0.98±0. 05	0.46± 0.06	1.41 ±0.1 6	nd	5.09±0. 3	0.49 ±0.1 6	0.98 ±0.1 8	0.32± 0.04	4.1±0.6 2
p-hydroxy benzoic acid	nd	0.51± 0.2	1.49± 0.38	2.16±0. 29	nd	0.26± 0.03	1.21± 0.15	1.7±0.2 7	1.04± 0.43	1.9± 0.2	4.36± 0.49	13.12± 0.84	1.33 ±0.5 1	1.27 ±0.2	5.86± 0.44	9.88±1. 21
2,4-dihydroxy benzoic acid	nd	nd	nd	0.13±0. 03	0.03± 0	nd	nd	0.12±0. 02	nd	nd	nd	Nd	Nd	nd	nd	nd
2,5-dihydroxy benzoic acid	0.13± 0.04	0.84± 0.2	0.01± 0.02	4.13±0. 33	0.06± 0.01	0.67± 0.01	0.03± 0.03	3.32±0. 14	0.03± 0.03	0.92 ±0.1 5	0.02± 0.01	7.87±0. 37	0.05 ±0.0 2	0.61 ±0.0 8	nd	7.02±0. 9
protocatechuic acid	1.22± 0.09	0.97± 0.33	3.08± 0.37	1.04±0. 1	0.87± 0.16	0.51± 0.01	2.32± 0.23	0.85±0. 03	0.11± 0.01	0.07 ±0.0 2	0.1±0 .03	0.3±0.0 5	0.14 ±0.0 4	0.08 ±0.0 1	0.09± 0.01	0.27±0. 01
o-anisic acid	0.74± 0.18	0.32± 0.28	nd	nd	nd	0.38± 0.12	nd	0.25±0. 44	0.59± 0.03	0.26 ±0.1 3	nd	0.7±0.1 8	0.54 ±0.3 5	0.38 ±0.0 9	nd	nd
vanillic acid	1.69± 0.13	1.67± 0.45	1.51± 0.33	9.17±1. 01	1.69± 0.33	1.28± 0.07	1.36± 0.15	8.65±0. 72	nd	0.12 ±0.0 1	0.47± 0.01	0.58±0. 02	0.07 ±0.0 6	0.09 ±0.0 3	0.61± 0.09	0.6±0.0 8
syringic acid	0.12± 0.04	0.18± 0.06	0.22± 0.04	0.82±0. 05	0.07± 0.01	0.1±0 .02	0.19± 0.01	0.73±0. 01	nd	nd	nd	Nd	Nd	nd	nd	nd
p-hydroxy benzaldehyde	0.42± 0.02	0.33± 0.06	4.98± 0.69	0.52±0. 01	0.42± 0.06	0.22± 0.03	5.12± 0.12	0.42±0. 02	0.56± 0.1	0.57 ±0.0 6	3.28± 0.27	3.17±0. 32	0.62 ±0.1 9	0.43 ±0.1	3.8±0 .2	2.4±0.6 9
protocatachaldehyde	1.09± 0.1	1.48± 0.45	4.58± 0.93	0.58±0. 09	1.69± 0.3	1.47± 0.08	5.88± 0.46	0.55±0. 09	nd	0.05 ±0.0 1	nd	0.06±0. 01	Nd	0.04 ±0.0 1	nd	0.05±0. 05
vanillin	1.37± 0.14	0.95± 0.33	4.65± 1.4	9.62±0. 77	1.92± 0.35	0.91± 0.01	4.89± 0.35	8.32±0. 47	nd	0.05 ±0.0	0.2±0 .03	0.84±0. 06	0.06 ±0.0	0.05 ±0	0.25± 0.05	0.73±0. 17

										1			1			
cinnamic acid	0.72±0.03	0.84±0.18	0.38±0.04	0.1±0.02	1.33±0.21	1.02±0.05	0.29±0.02	0.09±0.01	0.73±0.09	0.75±0.06	0.29±0.06	0.14±0.01	0.79±0.09	0.7±0.06	0.39±0.16	0.07±0.02
p-coumaric acid	nd	0.42±0.09	1.77±0.27	nd	nd	0.38±0.07	1.42±0.14	nd	0.4±0.11	0.28±0.02	0.41±0.08	Nd	0.38±0.1	0.13±0.12	0.38±0.06	nd
caffeic acid	1.94±0.18	25.1±6.95	18.76±4.73	8.93±0.67	1.12±0.2	28.93±1.37	12.11±1.86	7.59±0.41	0.09±0.01	0.15±0.02	0.06±0.11	0.25±0.06	0.14±0.01	0.08±0.01	nd	0.28±0.07
ferulic acid	0.46±0.07	1.56±0.64	4.66±1.13	0.42±0.03	0.34±0.05	1.48±0.11	2.95±0.31	0.36±0.03	0.03±0	0.07±0.01	0.2±0.02	0.08±0.01	0.06±0	0.06±0	0.3±0.04	0.08±0.03
sinapic acid	nd	nd	nd	0.3±0.04	nd	nd	nd	0.2±0.04	nd	nd	0.18±0.03	Nd	nd	nd	0.27±0.14	nd
phenol	nd	1.86±0.43	nd	41.43±3.97	nd	1.94±0.18	nd	33.06±4.02	nd	nd	nd	Nd	nd	1.49±0.1	nd	nd
4-hydroxy-3-methoxyacetophenone	0.07±0.01	0.08±0.03	0.09±0.02	0.32±0.02	0.06±0.01	0.05±0.01	0.12±0	0.28±0.01	nd	nd	nd	0.02±0	nd	nd	nd	0.03±0
phenylacetic acid	0.17±0	0.36±0.07	0.38±0.06	nd	0.13±0.02	0.32±0.01	0.44±0.03	nd	0.21±0.03	0.31±0.06	0.09±0.01	Nd	0.27±0.05	0.31±0.04	0.15±0.01	nd
3-hydroxyphenylacetic acid	0.86±0.08	0.74±0.27	1.69±0.13	1.41±0.13	0.66±0.04	0.53±0.04	2.56±0.1	1.62±0.1	0.8±0.09	0.97±0.12	0.45±0.09	7.8±0.33	0.93±0.3	0.91±0.13	0.61±0.04	9.23±1.53
3-hydroxymandelic acid	nd	0.54±0.18	nd	nd	nd	0.56±0.04	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
phenylpyruvic acid	2.07±0.1	0.25±0.02	2.12±0.13	2.14±0.21	1.99±0.09	0.09±0.01	1.78±0.04	2.01±0.12	2.16±0.01	0.2±0.05	1.86±0.14	1.9±0.39	1.77±0.2	0.1±0.01	1.61±0.22	0.84±0.4
4-hydroxyphe	18.76±0.58	18.85±4.1	16.11±1.91	22.17±7.1	18.65±2.11	23.45±0.64	15.8±0.26	40.99±16.4	10.19±0.71	3.68±1.3	11.87±1.24	14.11±4.32	14.43±2.	4.43±0.9	11±0.46	14.32±4.93

nyl pyruvic acid										1			9	9		
Benzoic Acid	1.82± 0.27	1.28± 0.2	2.53± 0.52	3.89±0. 61	1.81± 0.14	1.02± 0.12	2.73± 0.3	3.16±0. 24	2.88± 0.32	4.35 ±0.4 5	9.77± 0.97	3.54±0. 15	3.15 ±0.6 8	3.15 ±0.3 9	10.9± 0.21	3.32±0. 6
Biotinyl-Dodecanoyl-Cysteaminyl-Alprenolol	0.06± 0.01	nd	0.12± 0.01	0.16±0. 01	0.21± 0.03	nd	0.13± 0.03	0.11±0. 01	0.11± 0.03	nd	0.07± 0.01	0.06±0	0.04 ±0	nd	0.03± 0.01	nd
phenyllactic acid	0.4±0 .05	0.2±0 .05	0.32± 0.03	0.4±0.1	0.34± 0.04	0.16± 0.02	0.27± 0.04	0.37±0. 03	0.46± 0.06	0.17 ±0.0 3	0.34± 0.04	0.39±0. 02	0.53 ±0.0 7	0.24 ±0.0 2	0.16± 0.08	0.09±0. 02
4-hydroxyphenyl lactic acid	nd	1.51± 0.62	2.87± 0.26	1.01±0. 11	0.73± 0.2	2.02± 0.45	3.86± 0.17	1.52±0. 37	nd	nd	nd	Nd	nd	nd	nd	nd
anthranilic acid	nd	0.06± 0.02	0.11± 0.02	0.03±0	nd	0.07± 0.01	0.09± 0	nd	nd	nd	0.14± 0.01	Nd	nd	nd	0.15± 0.02	nd
chlorogenic acid	nd	0.38± 0.63	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
coniferyl alcohol	nd	5.47± 1.97	nd	nd	nd	7.61± 2.26	nd	nd	nd	0.9± 0.1	nd	Nd	nd	0.62 ±0.1 7	nd	nd
3OMe4OH CAlc	nd	nd	0.31± 0.05	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
p-cresol	nd	nd	nd	1.44±0. 38	nd	nd	nd	nd	nd	nd	nd	0.37±0. 04	nd	nd	nd	0.29±0. 08
4-ethylphenol	nd	nd	0.03± 0.05	0.22±0. 07	nd	nd	0.02± 0.04	0.22±0. 11	nd	nd	0.05± 0.01	0.06±0	0.02 ±0.0 4	nd	0.06± 0.02	0.07±0
tyrosol	0.7±0 .04	0.52± 0.15	nd	1.15±0. 02	0.6±0. 11	0.49± 0.04	nd	0.99±0. 04	nd	6.65 ±0.6 4	nd	26.9±1. 44	nd	3.84 ±0.4 6	nd	28.1±0. 82
Hydroxytyr	0.18±	0.25±	nd	0.13±0.	0.1±0.	0.21±	nd	nd	nd	0.04	nd	0.55±0.	nd	0.03	nd	0.47±0.

osol	0.04	0.06		06	02	0.01				±0.01		04		±0		11
indole	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
indole-3-acetic acid	0.07±0.01	0.1±0.01	0.31±0.07	nd	0.13±0.04	0.09±0.02	0.19±0.06	nd	nd	0.08±0.02	nd	Nd	nd	0.06±0.01	nd	nd
indole-3-carboxylic acid	0.13±0.01	nd	0.11±0.02	nd	0.12±0.03	nd	0.1±0.02	nd	0.05±0.02	nd	0.07±0.02	Nd	0.04±0.01	nd	0.08±0.01	nd
indole-3-pyruvic acid	62.97±8.71	10.97±0.96	62.28±15.27	387.75±86.02	41.41±11.02	6.18±1.14	76.55±11.04	414.58±147.42	10.73±0.37	2.78±0.41	13.62±1.07	121.19±16.62	9.71±2.49	2.5±0.14	16.18±1.21	104.47±35.21
spermidine	nd	0.38±0.02	nd	nd	nd	0.34±0.01	nd	nd	nd	0.25±0.01	nd	Nd	nd	0.28±0.03	nd	nd
putresine	nd	0.2±0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
Coumarin	nd	nd	0.04±0.04	0.09±0.01	nd	nd	0.04±0.03	0.1±0.01	nd	nd	nd	Nd	nd	nd	nd	nd
Cyanidin-3-Galactoside	0.04±0.01	nd	0.04±0	0.03±0	0.04±0	nd	0.04±0.01	0.04±0.01	0.04±0.01	nd	0.33±0.5	0.05±0	0.04±0.01	nd	0.05±0.01	0.04±0.01
Naringenin	nd	0.01±0.01	nd	nd	nd	0.01±0	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
Kaempferol	nd	0.04±0.03	nd	nd	nd	nd	nd	nd	nd	0.04±0.03	nd	Nd	nd	nd	nd	nd
Quercetin	nd	0.04±0.02	nd	nd	nd	0.02±0	nd	nd	nd	nd	nd	Nd	nd	nd	nd	nd
Genstein	nd	0.11±0.19	nd	nd	nd	nd	nd	nd	nd	nd	6.82±11.82	Nd	nd	nd	nd	nd
Umbelliferone	28.68±1.89	8.31±1.7	2.77±0.16	0.59±0.33	34.64±5.31	9.57±0.43	1.97±0.13	0.27±0.09	nd	nd	nd	Nd	nd	nd	nd	nd
Luteolin	0.03±0.01	0.04±0.02	nd	nd	0.01±0.01	0.02±0	nd	nd	0.01±0.01	0.01±0	nd	Nd	nd	nd	nd	nd

Isorhamnetin	nd	0.02±0.02	nd	nd	nd	nd	nd	nd	nd	0.01±0	nd	0.01±0.01	nd	0.01±0	nd	nd
Formononetin	0.34±0.37	0.03±0.03	0.07±0.01	0.04±0.03	0.16±0.07	0.01±0	0.15±0.1	0.03±0.03	0.9±0.57	0.02±0.01	0.12±0.11	0.04±0.01	5.56±5.72	0.03±0	0.14±0.15	0.05±0.01
Gossypin	2.46±0.31	0.4±0.04	0.52±0.26	nd	2.18±0.31	0.43±0.09	0.6±0.06	nd	nd	nd	nd	Nd	nd	nd	nd	nd
seco	0.21±0.04	0.08±0.02	0.04±0	0.2±0.04	0.05±0.01	0.06±0.01	0.03±0	0.12±0.01	0.02±0	nd	0.03±0.01	Nd	0.03±0	0.01±0	0.01±0.01	nd
mata	0.03±0	nd	0.03±0.01	0.03±0	0.03±0	nd	0.02±0.02	0.02±0	0.05±0	nd	0.04±0	0.04±0.01	0.04±0.01	nd	0.01±0.02	nd
Pino	0.28±0.02	0.04±0.01	1.68±0.34	0.34±0.03	0.27±0.04	0.03±0	1.79±0.09	0.29±0.02	0.04±0.01	nd	0.05±0.01	0.02±0.03	0.04±0	nd	0.01±0.01	nd

Table 5.1: The phytochemicals detected in Organic chia seeds; White organic chia seeds ;Pumpkin seeds; Organic pumpkin seeds, using LC-MS/MS analysis

PLANT	**Red Quinoa Grain**				**Black Quinoa Grain**				**Quinoa Grain**			
Fraction	**F 1**	**F 2**	**F 3**	**F 4**	**F 1**	**F 2**	**F 3**	**F 4**	**F 1**	**F 2**	**F 3**	**F 4**
salicylic acid	nd	0.19±0.04	nd	nd	nd	0.24±0.02	nd	nd	nd	0.16±0.03	nd	nd
p-hydroxy benzoic acid	1.84±0.38	1.1±0.07	4.27±0.49	2.43±0.26	2.59±0.93	1.67±0.26	4.96±0.13	2.67±0.17	2.08±0.44	1.55±0.29	4.89±0.27	3.25±0.52
2,4-dihydroxy benzoic acid	nd	nd	0.13±0.01	0.2±0.01	nd	nd	0.09±0.01	0.1±0.01	0.07±0.01	nd	0.17±0.01	0.19±0.04
2,5-dihydroxy benzoic acid	0.56±0.05	1.86±0.21	nd	0.93±0.13	0.87±0.2	2.28±0.38	nd	1.01±0.03	0.56±0.08	2.52±0.49	nd	0.63±0.12
protocatechuic acid	6.33±0.88	3±0.15	22.17±0.54	15.38±2.91	5.83±1.67	3.5±0.38	19.63±0.26	13.23±0.9	0.15±0.01	0.45±0.06	0.12±0.02	1.84±0.24
o-anisic acid	nd	0.46±0.04	nd	nd	nd	0.44±0.04	nd	nd	nd	0.46±0.05	nd	nd
p-anisic acid	nd	nd	0.69±0.	1.23±0.26	nd	nd	0.52±0.	0.86±0.12	nd	nd	nd	1.37±0.

			15				01					24
vanillic acid	9.22±1.28	8.97±0.52	18.72±1.56	26.62±2.26	5.43±1.5	5.67±0.97	11.49±0.52	13.38±1.06	6.22±0.51	7.76±1.18	16.63±1.3	19.58±2.58
syringic acid	nd	0.05±0	nd	0.21±0.03	0.09±0.05	0.05±0.02	0.09±0.02	0.27±0.04	nd	nd	nd	0.09±0.02
p-hydroxy benzaldehyde	0.16±0.02	nd	0.46±0.03	0.23±0.04	0.66±0.18	0.34±0.09	0.81±0.06	0.3±0.04	0.15±0.02	nd	0.36±0.01	0.06±0
Protocatach aldehyde	2.09±0.21	1.34±0.06	13.79±0.53	7.21±1.08	3.21±0.95	2±0.39	12.08±0.2	6.13±0.58	nd	0.04±0.01	0.02±0.03	nd
vanillin	0.18±0.02	0.07±0.01	0.3±0.04	0.23±0.02	0.27±0.06	0.12±0.01	0.35±0.03	0.21±0.02	0.14±0.02	0.08±0.01	0.23±0.04	0.18±0.02
cinnamic acid	0.35±0.01	0.35±0.05	0.31±0.03	0.34±0.02	0.6±0.13	0.4±0.05	0.38±0.06	0.43±0.04	0.42±0.02	0.32±0.02	0.39±0.08	0.42±0.02
p-coumaric acid	0.79±0.25	0.66±0.09	1.09±0.07	nd	0.97±0.29	0.73±0.11	1.16±0.12	nd	0.21±0.37	0.8±0.14	0.62±0.54	nd
caffeic acid	0.1±0.03	0.04±0	0.18±0.03	nd	0.13±0.03	0.07±0.02	0.6±0.02	0.2±0.02	nd	0.05±0.01	nd	nd
ferulic acid	1.71±0.29	1.5±0.19	21.46±1.9	0.39±0.04	1.71±0.42	1.41±0.26	26.91±0.7	0.39±0.04	1.33±0.23	1.7±0.29	17.82±0.83	0.44±0.11
sinapic acid	nd	0.07±0.01	0.85±0.17	nd	nd	0.08±0.03	1.49±0.04	nd	nd	0.05±0	0.54±0.17	nd
4-hydroxy-3-methoxyphenylpropionic acid	nd	nd	nd	0.28±0.02	nd	nd	nd	0.24±0.03	nd	nd	nd	0.06±0.06
phenol	nd	1.6±0.29	nd	2.91±5.04	nd	1.97±0.04	nd	6.37±11.03	6.96±0.7	2±0.12	nd	nd
4-hydroxy-3-methoxyacetophenone	0.04±0.01	0.08±0	nd	0.19±0.03	0.03±0.01	0.07±0	nd	0.14±0.02	0.03±0.02	0.1±0.01	nd	0.15±0.01
phenylacetic acid	0.4±0.01	0.32±0.01	0.27±0.24	nd	0.45±0.04	0.35±0.05	0.22±0.2	nd	0.41±0.09	0.28±0.02	0.25±0.22	nd
3-hydroxy phenylacetic acid	nd	0.44±0.04	2.62±0.56	1.25±0.25	nd	0.42±0.06	1.98±0.2	1.63±0.19	nd	0.41±0.02	nd	nd
3-hydroxymandelic	0.21±0.03	0.3±0.05	1.81±0.06	2.48±0.14	0.18±0.06	0.17±0.02	1.78±0.08	1.5±0.16	nd	nd	nd	nd

acid												
phenylpyruvic acid	nd	0.08±0.01	nd	nd	nd	0.05±0	nd	nd	nd	0.08±0.01	nd	nd
4-hydroxyphenyl pyruvic acid	0.67±0.02	5.59±1.94	0.7±0.07	0.64±0.07	0.67±0.07	6.37±1.36	1±0.03	0.64±0.03	0.59±0.11	6.72±0.83	0.64±0.13	0.49±0.09
Benzoic Acid	1.66±0.17	1.04±0.17	1.48±0.26	2.14±0.11	2.06±0.32	0.93±0.02	1.77±0.18	1.89±0.2	2.23±0.03	0.84±0.23	1.36±0.17	2.2±0.14
phenyllactic acid	0.27±0.05	0.24±0.03	0.16±0	0.17±0.04	0.32±0.04	0.23±0.05	0.16±0.02	0.17±0.03	0.23±0.03	0.24±0.02	0.12±0.01	0.14±0.03
anthranilic acid	nd	nd	0.21±0.02	nd	nd	nd	0.14±0.01	nd	nd	nd	0.11±0.01	nd
coniferyl alcohol	nd	0.05±0	nd	nd	nd	0.12±0.02	0.12±0.01	0.58±0.07	nd	0.08±0.01	nd	1.1±1
p-cresol	nd	nd	nd	nd	nd	nd	nd	1.48±1.32	2.1±0.02	nd	nd	1.65±0.21
4-ethylphenol	0.33±0.03	nd	nd	0.32±0.08	0.38±0.02	nd	nd	0.45±0.11	0.69±0.11	nd	nd	0.43±0.09
tyrosol	nd	nd	nd	nd	nd	nd	0.12±0.02	nd	nd	nd	nd	nd
Hydroxytyrosol	nd	nd	nd	0.03±0.01	nd	nd	nd	0.01±0	nd	nd	nd	nd
ferulic dimer (5-5 linked)	0.02±0	nd	0.74±0.07	nd	nd	nd	1.01±0.12	nd	nd	0.01±0	0.52±0.02	nd
ferulic dimer (8-5 linked)	nd	nd	4.18±0.34	nd	nd	nd	4.9±0.14	nd	nd	0.01±0	2.53±0.18	nd
indole	nd	nd	nd	nd	1.43±0.12	nd	nd	0.84±0.03	nd	nd	nd	nd
indole-3-acetic acid	0.57±0.07	0.1±0.01	0.74±0.01	0.58±0.03	0.63±0.02	0.11±0.01	0.66±0.03	0.53±0.04	0.54±0.03	0.11±0.01	0.63±0.05	0.51±0.02
indole-3-carboxylic acid	0.08±0.01	nd	0.12±0.03	nd	0.15±0.03	nd	0.18±0	nd	0.1±0.02	nd	0.08±0.01	nd
indole-3-pyruvic acid	56±7.64	3.89±1.9	35.25±3.08	186.14±10.13	124.72±23.06	9.64±0.15	65.88±3.24	145.88±20.03	53.25±8.19	5.35±1.28	15.96±1.66	89.86±22.5
spermidine	nd	0.26±0.01	nd	nd	nd	0.24±0	nd	nd	nd	0.24±0.01	nd	nd
Coumarin	nd	nd	nd	nd	0.2±0.01	0.06±0.06	nd	nd	nd	nd	nd	nd

Cyanidin-3-Galactoside	0.11±0.04	nd	0.08±0.01	0.04±0	0.11±0.02	nd	0.1±0.01	0.05±0.01	0.16±0.01	nd	0.13±0.03	0.05±0.01
Naringenin	0.02±0	0.16±0.02	0.01±0	0.02±0	0.02±0	0.14±0.02	0.01±0	0.01±0	0.01±0	0.18±0.01	nd	0.02±0
Kaempferol	0.08±0.02	1.14±0.17	0.13±0.01	7.44±0.81	0.07±0.02	1.9±0.49	0.15±0.01	8.53±0.86	0.17±0.08	5.48±0.86	0.23±0	20.66±1.98
Quercetin	0.19±0.03	6.49±0.64	0.41±0.04	19.97±2.09	0.26±0.1	7.33±2.47	0.62±0.05	22.44±2.03	0.25±0.23	6.53±1.2	0.16±0.01	14.38±1.24
Quercetin-3-Glucoside	0.3±0.03	nd	0.31±0.03	0.01±0	0.28±0.05	nd	0.48±0.05	nd	0.24±0.04	nd	0.31±0.01	nd
Genstein	nd	0.03±0.02	nd	nd	nd	nd	nd	nd	nd	0.01±0	nd	0.11±0.18
Luteolin	nd	0.02±0	nd	nd	nd	0.02±0	nd	nd	nd	0.01±0	nd	nd
Isorhamnetin	nd	0.34±0.04	nd	0.91±0.07	nd	0.26±0.07	nd	0.39±0.03	nd	0.47±0.11	nd	1.53±0.21
Formononetin	0.04±0.02	0.01±0	0.02±0	0.01±0.01	0.03±0.02	0.01±0	0.01±0	0.01±0	0.08±0.06	0.01±0	0.01±0	0.12±0.19
seco	nd	nd	nd	nd	nd	nd	nd	0.03±0.01	nd	nd	nd	nd
Syrg	nd	0.14±0.01	nd	nd	nd	0.06±0.05	nd	nd	nd	0.06±0	nd	nd

Table 5.2: The phytochemicals detected in Red Quinoa Grain, Black Quinoa Grain and Quinoa Grain using LC-MS/MS analysis

	Brown Linseed				**Organic Golden Linseed**				**Organic Brown Linseed**			
	F 1	F 2	F 3	F 4	F 1	F 2	F 3	F 4	F 1	F 2	F 3	F 4
salicylic acid	0.86±0.13	0.61±0.1	0.5±0.49	1.76±0.37	0.3±0.02	0.46±0.17	nd	1.33±0.25	Nd	0.43±0.08	nd	0.74±0.16
p-hydroxybenzoic acid	2.92±0.56	0.85±0.06	4.73±0.32	15.19±3.55	2.8±0.13	1.07±0.31	10.69±1.3	22±1.71	2.24±0.3	1.19±0.13	5.56±0.50	12.54±3.94
2,5-dihydroxybenzo	0.48±0.16	0.2±0.08	0.06±0.11	5.15±1.61	0.18±0.03	0.04±0.07	nd	1.92±0.19	0.18±0.04	0.13±0.03	nd	1.11±0.71

ic acid												
protocatechuic acid	0.48±0.21	0.13±0.01	0.27±0.06	0.59±0.05	0.2±0.03	0.1±0.02	0.2±0.03	0.6±0.09	0.44±0.04	0.18±0.02	0.32±0.04	0.44±0.15
o-anisic acid	nd	0.44±0.02	nd	nd	nd	0.37±0.02	nd	nd	Nd	0.36±0.07	nd	nd
p-anisic acid	nd	nd	nd	nd	nd	nd	nd	1.29±0.07	Nd	nd	0.69±0.07	nd
gallic acid	1.37±0.25	0.19±0.05	1.37±0.37	1.92±0.34	nd	nd	nd	nd	2.08±0.31	0.42±0.04	1.09±0.64	1.45±0.59
vanillic acid	0.68±0.27	0.2±0.04	0.42±0.05	1.83±0.39	0.94±0.13	0.4±0.09	0.66±0.1	3.37±0.39	0.95±0.22	0.4±0.07	0.68±0.04	1.90±0.52
p-hydroxy benzaldehyde	0.45±0.13	nd	0.47±0.08	0.75±0.2	1.06±0.15	0.43±0.05	3.39±0.3	2.46±0.26	0.46±0.07	0.13±0.03	0.69±0.21	0.64±0.19
Protocatachaldehyde	0.08±0.02	0.05±0.01	0.2±0.03	0.33±0.03	0.09±0	0.07±0.02	0.19±0.04	0.28±0.05	0.13±0.01	0.1±0.03	0.34±0.12	0.25±0.06
3,4,5-trihydroxy benzaldehyde	1.14±0.17	0.19±0.05	4.25±1.02	2.16±0.37	nd	nd	nd	nd	1.52±0.4	0.38±0.04	3.57±2.16	1.57±0.78
vanillin	2.03±2.35	0.19±0.01	1.01±0.19	2.01±0.41	1.69±0.04	0.58±0.14	1.7±0.11	3.48±0.31	1.51±0.22	0.55±0.09	1.59±0.32	1.85±0.16
cinnamic acid	0.6±0.08	0.29±0.03	0.47±0.03	0.54±0.04	0.56±0.04	0.3±0.03	0.68±0.09	0.64±0.1	0.61±0.09	0.28±0.02	0.67±0.15	0.59±0.06
p-coumaric acid	1±0.1	0.37±0.04	12.44±0.36	28.55±6.66	nd	nd	9.63±0.9	30.58±4.29	Nd	0.23±0.07	10.32±0.85	18.77±6.09
caffeic acid	0.21±0.08	0.09±0.01	6.07±0.36	9.93±2.39	nd	0.03±0	1.9±0.23	9.57±1.33	Nd	0.09±0.02	2.75±1.77	5.68±1.96
ferulic acid	1.51±0.19	0.35±0.05	60.31±5.29	27.07±4.52	0.96±0.07	0.28±0.06	82.67±9.73	38.41±4.21	1.39±0.23	0.31±0.06	62.98±3.37	17.18±5.31
sinapic acid	0.06±0.1	nd	1.23±0.35	0.7±0.17	nd	nd	8.55±5.7	0.67±0.04	Nd	0.06±0.07	6.78±3.23	0.19±0.33
4-hydroxy-3-methoxy phenylpropionic acid	0.16±0.03	0.05±0.04	0.1±0.08	1.87±0.31	nd	nd	nd	1.95±0.2	Nd	nd	0.17±0.01	1.32±0.40
phenol	10.87±0.87	1.47±0.15	5.12±8.87	19.36±4.24	11.84±0.91	1.42±0.01	nd	23.96±2.91	10.09±0.67	1.37±0.12	9.35±2.25	22.33±6.76

4-hydroxy-3-methoxyacetophenone	0.06±0.1	0.02±0	0.07±0.01	0.37±0.09	nd	0.03±0	0.13±0.03	0.58±0.07	0.03±0.03	0.03±0	0.10±0.00	0.29±0.03
phenylacetic acid	0.76±0.08	0.29±0.01	0.5±0.1	nd	0.5±0.09	0.22±0.03	0.53±0.05	nd	0.66±0.03	0.32±0.01	0.57±0.07	0.29±0.26
3-hydroxyphenylacetic acid	nd	0.4±0.04	nd	2.23±0.35	0.05±0.09	0.47±0.05	nd	2.64±0.14	Nd	0.41±0.03	nd	1.31±1.14
phenylpyruvic acid	1.54±0.08	0.18±0.04	nd	nd	1.2±0.14	0.19±0.06	nd	nd	1.65±0.14	0.28±0.03	nd	nd
4-hydroxyphenyl pyruvic acid	1.78±0.21	6.22±0.59	0.72±0.03	1.2±0.21	1.29±0.19	5.65±0.44	0.59±0.05	1.37±0.27	1.45±0.2	5.37±0.17	0.54±0.12	0.71±0.25
Benzoic Acid	2.9±1.37	0.91±0.14	2.57±0.31	2±0.23	1.88±0.18	0.97±0.1	2.02±0.1	2.38±0.19	2.3±0.07	0.88±0.06	2.30±0.21	2.26±0.36
phenyllactic acid	0.22±0.04	0.18±0.01	0.12±0.01	0.18±0.01	0.16±0.03	0.12±0	0.15±0.03	0.19±0.05	0.22±0.04	0.14±0.01	0.14±0.03	0.15±0.03
anthranilic acid	nd	nd	0.05±0.01	nd	nd	nd	0.04±0.04	nd	Nd	nd	0.06±0.01	nd
chlorogenic acid	0.13±0.03	0.02±0.01	nd	nd	nd	nd	nd	nd	0.15±0.03	0.09±0	nd	nd
coniferyl alcohol	1.07±0.12	0.28±0.13	1.12±0.16	2.11±0.88	4.1±0.9	0.15±0.01	1.13±0.08	2.01±0.46	2.54±0.53	0.23±0.08	2.24±0.17	1.43±1.24
3OMe4OHCAlc	0.88±0.16	nd	nd	nd	1.99±0.15	nd	1.43±0.22	nd	1.01±0.19	nd	2.81±0.54	nd
p-cresol	2.33±0.51	nd	1.9±0.67	nd	3.73±0.21	nd	1.35±1.18	0.7±1.21	2.42±0.17	nd	2.38±0.21	nd
4-ethylphenol	0.57±0.12	nd	0.6±0.06	1.32±0.2	0.88±0.17	nd	0.67±0.1	1.28±0.03	0.68±0.13	nd	0.71±0.08	1.18±0.18
tyrosol	nd	nd	nd	0.08±0.02	nd	nd	nd	0.08±0.01	Nd	nd	nd	0.07±0.03
Hydroxytyrosol	0.01±0	nd	0.01±0	0.39±0.03	nd	nd	nd	0.24±0.05	Nd	nd	nd	0.19±0.05
ferulic dimer (5-5 linked)	nd	nd	0.37±0.04	nd	nd	nd	0.41±0.06	nd	Nd	nd	0.56±0.03	nd
ferulic dimer (8-5 linked)	nd	nd	1.29±0.22	nd	nd	nd	1.51±0.28	nd	Nd	nd	1.74±0.10	nd

indole-3-acetic acid	0.61±0.01	0.12±0	0.55±0.01	0.5±0.03	0.52±0.03	0.12±0	0.67±0.07	0.74±0.22	0.49±0.06	0.11±0.01	0.65±0.07	0.68±0.10
indole-3-carboxylic acid	0.03±0.03	nd	0.06±0.01	nd	nd	nd	nd	nd	Nd	nd	nd	nd
indole-3-pyruvic acid	67.82±21.1	4.92±0.54	118.06±26.09	104.11±13.05	32.17±2.47	4.32±0.24	148.28±21.82	130.58±15.3	32.9±5.15	3.62±0.43	80.92±3.96	55.08±19.08
spermidine	nd	0.23±0.01	nd	nd	nd	0.22±0.01	nd	nd	Nd	0.21±0.01	nd	nd
Coumarin	nd	0.01±0	nd	nd	nd	0.01±0	nd	0.16±0.02	0.09±0.02	0.02±0	nd	nd
Cyanidin-3-Galactoside	0.06±0.03	nd	1.18±0.07	0.58±0.1	0.05±0.01	nd	1.49±0.16	0.74±0.22	0.05±0.02	nd	1.13±0.28	0.61±0.09
Naringenin	0.01±0	0.04±0.01	nd	0.01±0	0.01±0	0.02±0	nd	0.01±0	0.03±0	0.29±0.01	0.01±0.00	0.02±0.01
Kaempferol	0.09±0.05	0.07±0.01	0.02±0	0.69±0.06	nd	0.14±0.07	0.04±0.01	5.21±0.6	Nd	0.08±0.02	0.03±0.00	1.43±0.47
Quercetin	0.07±0.05	0.06±0.03	0.02±0	0.04±0	nd	0.02±0	nd	0.12±0.02	Nd	0.03±0.01	nd	0.10±0.10
Quercetin-3-Glucoside	0.01±0	nd	0.23±0.03	nd	0.01±0.02	nd	0.71±0.17	nd	0.01±0	nd	0.56±0.16	nd
Genstein	nd	nd	nd	nd	0.01±0.01	nd	nd	nd	Nd	0.01±0.01	nd	nd
Luteolin	nd	0.02±0.01	nd	nd	nd	nd	nd	nd	Nd	0.01±0	nd	nd
Isorhamnetin	nd	0.01±0.01	nd	nd	nd	nd	nd	0.04±0.01	Nd	0.01±0	nd	nd
Formononetin	0.02±0.01	0.01±0.01	0.02±0.01	0.01±0	0.01±0.01	0.01±0	0.01±0	0.01±0	0.01±0	0.01±0	0.02±0.01	0.01±0.00
Gossypin	nd	nd	0.11±0.01	nd	nd	nd	0.09±0.02	nd	Nd	nd	0.10±0.03	nd
Seco	0.15±0.02	0.1±0.04	0.04±0	153.15±28.98	0.04±0	0.03±0	0.03±0.01	234.54±39.35	0.09±0.03	0.05±0.01	0.08±0.00	199.36±51.98
Mata	nd	0.01±0	nd	0.62±0.06	nd	nd	nd	0.11±0.01	Nd	0.01±0	nd	0.66±0.18
Pino	0.09±0.03	0.04±0	0.07±0.01	0.23±0.03	nd	nd	nd	nd	Nd	nd	nd	nd

Table 5.3: for the phytochemicals detected in Brown Linseed, Organic Golden Linseed and Organic Brown Linseed using LC-MS/MS analysis

	Triticale Flour				**Triticale Cereal**				**Triticale Rolled**			
	F 1	F 2	F 3	F 4	F 1	F 2	F 3	F 4	F 1	F 2	F 3	F 4
salicylic acid	0.21±0.02	0.07±0.02	0.47±0.03	0.38±0.02	0.23±0.04	0.05±0.01	0.37±0.04	0.38±0.05	0.31±0.16	0.05±0.01	0.33±0.06	0.28±0.03
p-hydroxybenzoic acid	nd	nd	1.32±0.12	1.31±0.18	0.11±0.03	nd	0.98±0.11	1.41±0.21	0.11±0.01	nd	0.87±0.16	1.18±0.06
2,5-dihydroxy benzoic acid	nd	nd	nd	0.18±0.04	nd	nd	nd	0.18±0.06	Nd	nd	nd	0.14±0.01
protocatechuic acid	0.25±0.02	0.18±0.02	0.29±0.01	0.24±0.03	0.24±0.05	0.17±0.01	0.23±0.02	0.26±0.02	0.29±0.02	0.23±0.02	0.28±0.02	0.23±0.03
o-anisic acid	nd	0.32±0.08	nd	0.44±0.04	0.14±0.24	0.38±0.06	0.12±0.21	0.46±0.04	Nd	0.39±0.01	nd	nd
gallic acid	nd	0.06±0.02	nd	nd	nd	0.04±0.03	nd	nd	Nd	0.06±0.01	nd	nd
vanillic acid	0.46±0.09	0.29±0.04	2.20±0.38	1.29±0.21	0.37±0.04	0.29±0.07	1.59±0.11	1.36±0.17	0.42±0.08	0.28±0.04	2.14±0.32	1.04±0.10
syringic acid	0.13±0.04	0.1±0.03	0.92±0.08	1.27±0.12	0.13±0.03	0.11±0.02	0.75±0.07	1.46±0.14	0.14±0.03	0.19±0.01	0.93±0.16	1.15±0.19
p-hydroxybenzaldehyde	nd	nd	0.32±0.07	0.14±0.01	0.05±0.01	nd	0.23±0.04	0.12±0.04	0.2±0.03	nd	0.26±0.06	0.10±0.01
protocatachaldehyde	0.16±0.05	0.16±0.01	0.39±0.05	0.15±0.02	0.12±0.02	0.14±0.02	0.29±0.03	0.19±0.01	0.2±0.02	0.22±0.01	0.27±0.02	0.13±0.02
vanillin	0.54±0.06	0.32±0.05	1.26±0.28	0.68±0.07	0.4±0.07	0.24±0.12	0.83±0.11	0.66±0.08	1.43±0.49	0.59±0.07	0.87±0.14	0.44±0.03
cinnamic acid	0.41±0.19	0.29±0.03	0.27±0.02	0.23±0.01	0.25±0.02	0.23±0.03	0.28±0.05	0.32±0.02	0.33±0.01	0.29±0.01	0.33±0.05	0.27±0.01
p-coumaric acid	nd	0.45±0.12	4.65±0.76	0.34±0.06	nd	nd	2.72±0.32	nd	Nd	nd	2.82±0.39	nd

caffeic acid	0.18±0.04	0.09±0.01	1.00±0.10	0.13±0.03	0.16±0.02	0.1±0	1.08±0.23	0.16±0.02	0.07±0	0.09±0	1.78±0.25	0.13±0.01
ferulic acid	0.68±0.15	0.97±0.09	98.38±9.52	2.98±0.29	0.43±0.02	0.37±0.1	79.11±5.67	3.14±0.30	0.99±0.17	0.68±0.1	95.12±10.7	2.42±0.31
sinapic acid	0.1±0.03	0.08±0.01	17.48±1.47	1.13±0.14	0.09±0.01	0.11±0.05	16.20±2.85	1.36±0.17	0.11±0.01	0.13±0.02	19.05±2.55	1.10±0.08
4-hydroxy-3-methoxyPhenyl propionic acid	nd	nd	nd	0.39±0.03	nd	nd	nd	0.39±0.05	Nd	nd	nd	0.29±0.02
phenol	3.23±0.4	1.38±0.26	3.71±0.13	5.79±0.91	2.41±0.37	1.38±0.37	2.98±0.32	4.54±0.63	2.75±0.46	1.1±0.13	3.32±0.28	3.29±0.80
4-hydroxy-3-methoxyacetophenone	nd	0.04±0	0.16±0.03	0.14±0.02	nd	0.02±0.01	0.12±0.01	0.14±0.01	0.04±0.03	0.04±0	0.13±0.03	0.12±0.02
phenylacetic acid	0.46±0.21	0.28±0.02	0.49±0.08	nd	0.27±0.03	0.34±0.08	0.44±0.07	nd	0.18±0.02	0.35±0.04	0.57±0.13	nd
3-hydroxyphenylacetic acid	0.23±0.2	0.38±0.12	0.49±0.03	0.87±0.02	0.39±0.08	0.31±0.06	0.54±0.06	0.96±0.11	0.41±0.05	0.45±0.05	0.56±0.12	0.89±0.17
phenylpyruvic acid	0.01±0	0.01±0	0.02±0.00	0.03±0.01	0.01±0	0.01±0	0.02±0.00	0.04±0.01	0.1±0	0.03±0	0.03±0.01	0.05±0.01
4-hydroxyphenylpyruvic acid	3.98±0.53	4.52±0.51	6.93±0.37	4.85±2.37	3.98±1.08	4.21±0.22	4.51±1.10	3.91±1.69	4±0.44	4.66±0.09	6.63±1.11	4.22±0.32
BA	0.63±0.06	0.75±0.09	1.10±0.17	1.39±0.19	0.71±0.09	0.67±0.1	1.01±0.05	1.35±0.09	0.73±0.2	0.79±0.07	1.28±0.35	1.35±0.09
phenyllactic acid	0.07±0.01	0.08±0.02	0.07±0.01	0.21±0.01	0.1±0.03	0.08±0.01	0.09±0.03	0.26±0.03	0.16±0.01	0.1±0	0.11±0.01	0.22±0.02
4-hydroxyphenyllactic acid	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd
anthranilic acid	nd	nd	0.07±0.01	nd	nd	nd	0.06±0.00	nd	Nd	nd	0.05±0.01	nd
coniferyl alcohol	0.03±0	0.13±0.03	nd	0.07±0.02	0.04±0.01	0.24±0.16	nd	0.08±0.02	0.12±0.02	0.17±0.02	nd	0.06±0.03

3OMe4OHCAlc	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd
p-cresol	nd	nd	nd	1.19±0.30	nd	nd	nd	1.38±0.12	Nd	nd	nd	2.01±0.83
Tyrosol	nd	nd	nd	0.23±0.01	nd	nd	nd	0.32±0.10	Nd	nd	nd	0.42±0.02
Hydroxytyrosol	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd
ferulic dimer (5-5 linked)	nd	nd	14.33±1.00	0.22±0.05	0.01±0	nd	12.72±1.07	0.38±0.04	Nd	nd	16.90±3.26	0.19±0.00
ferulic dimer (8-5 linked)	nd	0.01±0	17.52±0.96	0.48±0.11	0.01±0.01	0.01±0	13.95±1.28	0.75±0.04	0.07±0.01	0.05±0.01	31.60±6.66	0.67±0.03
Indole	0.74±0.09	nd	2.13±0.13	nd	0.8±0.1	nd	2.06±0.09	nd	0.54±0.04	nd	2.25±0.41	nd
indole-3-acetic acid	0.21±0.12	0.13±0.05	1.17±0.16	nd	0.06±0.01	0.15±0.03	1.27±0.18	0.05±0.02	0.11±0.02	0.12±0.04	1.08±0.24	0.05±0.04
indole-3-carboxylic acid	0.1±0.02	nd	0.31±0.05	nd	0.11±0.01	nd	0.30±0.02	nd	0.08±0.01	nd	0.33±0.05	nd
indole-3-pyruvic acid	7.96±1.18	3.43±0.32	8.66±1.31	18.76±17.11	4.96±1.03	2.68±0.55	6.57±1.14	20.93±18.16	7.44±1.36	3.89±0.45	7.42±1.32	23.27±3.82
Spermidine	0.41±0.01	0.22±0.01	0.50±0.02	0.42±0.02	0.4±0.01	0.21±0.01	0.51±0.02	0.45±0.02	0.41±0.02	0.22±0.01	0.47±0.02	0.36±0.02
Putresine	nd	nd	0.52±0.01	nd	nd	nd	0.36±0.01	nd	Nd	nd	0.40±0.07	nd
Naringenin	nd	nd	nd	0.01±0.00	nd	nd	nd	0.01±0.00	Nd	nd	nd	0.01±0.00
Kaempferol	nd	nd	nd	0.11±0.03	nd	nd	nd	0.12±0.02	Nd	nd	nd	0.10±0.01
Quercetin	nd	nd	nd	0.09±0.03	nd	nd	nd	0.11±0.00	Nd	nd	nd	0.10±0.02
Quercetin-3-Glucoside	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd
Genstein	nd	0.06±0.1	nd	0.14±0.02	nd	0.01±0.01	nd	0.23±0.25	Nd	nd	nd	0.11±0.01
Luteolin	nd	0.05±0.08	nd	0.16±0.02	nd	0.01±0	nd	0.20±0.07	Nd	0.01±0	nd	0.09±0.01
Isorhamnetin	nd	0.01±0	nd	0.75±0.14	nd	0.01±0	nd	0.84±0.09	nd	0.02±0	nd	0.62±0.0

												7
Formononetin	0.03±0.01	0.01±0.01	0.02±0.00	0.03±0.03	0.06±0.04	0.01±0	0.06±0.05	0.01±0.00	0.01±0	0.01±0	0.07±0.08	0.04±0.03
Gossypin	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	nd	nd
Seco	nd	nd	nd	0.04±0.01	nd	nd	nd	0.07±0.03	Nd	nd	nd	0.05±0.00
Mata	nd	0.02±0.01	nd	0.04±0.01	nd	0.01±0	nd	0.06±0.01	0.01±0	0.01±0	nd	0.07±0.01
Syrg	0.08±0.14	0.05±0	0.32±0.02	0.40±0.05	0.19±0.04	0.03±0.05	0.26±0.01	0.55±0.11	0.47±0.04	0.06±0.01	0.27±0.05	0.42±0.07
Pino	0.04±0.01	nd	0.01±0.01	nd	nd	nd	nd	nd	0.04±0.01	nd	nd	nd

Table 5.4: for the phytochemicals detected in Triticale Flour, Triticale Cereal and Triticale Rolled using LC-MS?MS analysis

PLANT	**Potato Bean Tuber**				**Potato Bean Peel**			
Fraction	**F1**	**F2**	**F3**	**F4**	**F1**	**F2**	**F3**	**F4**
salicylic acid	0.79±0.15	0.5±0.02	0.34±0.03	5.18±0.44	2.67±0.08	1.86±0.6	0.77±0.16	10.52±0.71
p-hydroxybenzoic acid	0.07±0.06	0.95±0.08	0.55±0.06	2.64±0.24	nd	0.77±0.03	1.15±0.29	4.23±0.29
2,3-dihydroxybenzoic acid	0.02±0.01	0.06±0.01	0.07±0.02	0.09±0.01	0.02±0	0.12±0.02	0.03±0.01	0.19±0.02
2,4-dihydroxybenzoic acid	nd	0.05±0.01	0.06±0.00	0.07±0.01	nd	0.1±0.02	0.17±0.04	0.26±0.05
2,5-dihydroxybenzoic acid	nd	1.59±0.06	nd	1.62±0.26	0.08±0	5.39±0.67	0.05±0.02	5.39±0.57
protocatechuic acid	0.06±0.01	0.08±0.01	0.13±0.01	0.17±0.02	0.21±0.03	0.35±0.03	1.34±0.28	2.09±0.18
o-anisic acid	nd	0.43±0.1	nd	nd	nd	0.35±0.0	nd	nd

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p-anisic acid	nd	0.16±0	0.23±0.03	0.07±0.13	nd	0.4±0.02	1.02±0.27	0.36±0.05
gallic acid	nd	nd	nd	nd	0.15±0.02	0.15±0.03	0.33±0.07	0.56±0.23
vanillic acid	nd	0.44±0.07	0.15±0.02	1.54±0.19	0.29±0.02	2.1±0.18	2.23±0.53	16.11±1.26
syringic acid	nd	nd	nd	0.28±0.03	nd	0.14±0.03	0.33±0.04	1.40±0.15
p-hydroxybenzaldehyde	0.07±0.02	nd	0.40±0.02	0.25±0.04	0.07±0.03	Nd	1.37±0.28	0.51±0.08
protocatachaldehyde	nd	0.08±0.01	0.06±0.01	nd	0.33±0.04	0.7±0.07	3.93±1.21	1.77±0.16
3,4,5-trihydroxybenzaldehyde	nd	nd	nd	nd	0.15±0.01	0.16±0.03	3.09±0.90	0.89±0.15
vanillin	nd	0.12±0.02	0.27±0.00	0.34±0.02	0.53±0.03	0.53±0.1	5.50±1.31	4.79±0.40
syringin	nd	0.05±0.01	0.05±0.01	0.09±0.01	0.05±0	0.1±0.01	0.26±0.07	0.28±0.02
cinnamic acid	0.36±0.03	0.37±0.05	0.37±0.05	0.45±0.05	0.45±0.06	0.42±0.03	0.61±0.10	0.39±0.05
p-coumaric acid	nd	nd	0.28±0.02	0.89±0.12	nd	0.32±0.02	2.46±0.76	2.07±0.34
caffeic acid	nd	nd	nd	nd	0.08±0.01	0.22±0.03	0.52±0.12	0.35±0.03
ferulic acid	nd	0.05±0	0.57±0.06	0.21±0.04	0.22±0.05	0.43±0.03	7.55±2.13	3.55±0.29
sinapic acid	nd	nd	0.15±0.02	nd	Nd	0.23±0.03	1.59±0.45	0.15±0.00
3,4-dihydroxyphenylpropionic acid	nd	nd	nd	nd	nd	0.63±0.08	nd	nd

phenol	3.55±0.66	nd	2.66±0.16	7.81±0.41	4.11±0.67	nd	5.36±0.62	6.94±0.79
4-hydroxyacetophenone	nd	0.02±0	0.04±0.01	0.02±0.00	nd	0.02±0	0.37±0.09	0.07±0.01
4-hydroxy-3,5-dimethoxyacetophenone	nd	nd	nd	nd	nd	nd	0.11±0.03	nd
3,4,5-trimethoxyacetophenone	nd	0.02±0	nd	0.08±0.01	0.02±0	0.05±0	0.01±0.00	0.14±0.00
phenylacetic acid	nd	0.45±0.03	nd	nd	nd	1.55±0.22	0.14±0.03	nd
3-hydroxyphenylacetic acid	0.34±0.06	0.46±0.03	0.51±0.10	3.24±0.77	0.38±0.04	0.46±0.03	1.04±0.22	1.73±0.11
3-hydroxymandelic acid	nd	nd	nd	nd	nd	0.14±0.05	0.33±0.03	0.34±0.04
4-hydroxy-3-methoxymandelic acid	nd	nd	nd	nd	nd	nd	0.83±0.06	nd
phenylpyruvic acid	0.04±0	0.02±0	1.45±0.39	0.23±0.04	0.08±0.01	0.06±0.01	0.39±0.14	0.21±0.01
4-hydroxyphenylpyruvic acid	1.9±0.16	4.66±0.33	3.97±0.23	3.57±1.10	2.56±0.2	4.42±0.2	3.54±0.13	2.66±0.66
BA	0.69±0.04	0.82±0.14	0.76±0.13	1.66±0.29	0.99±0.11	1±0.05	1.41±0.28	1.64±0.09
phenyllactic acid	0.09±0.01	0.09±0.01	0.12±0.00	0.12±0.01	0.1±0.02	0.14±0.01	0.09±0.01	0.10±0.01
anthranilic acid	nd	nd	nd	nd	nd	nd	0.17±0.04	nd
coniferyl alcohol	0.04±0	0.04±0	0.03±0.01	0.03±0.02	0.11±0.01	0.1±0.01	0.09±0.01	0.06±0.00
p-cresol	nd	nd	nd	2.76±0.38	nd	nd	nd	3.51±0.56
4-ethylphenol	nd	nd	nd	0.10±0.01	nd	nd	0.04±0.03	0.11±0.03
tyrosol	nd	nd	nd	nd	nd	nd	nd	0.23±0.03
ellagic acid	0.08±0.02	0.25±0	nd	nd	1.45±0.05	0.76±0.0	0.69±0.09	nd

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ferulic dimer (8-5 linked)	nd	nd	nd	nd	nd	nd	0.40±0.11	nd
reservatrol	nd	nd	nd	nd	0.14±0.02	0.2±0.07	nd	nd
indole	0.46±0.11	nd	0.62±0.05	nd	nd	nd	2.52±0.70	nd
indole-3-carboxylic acid	0.04±0	nd	0.06±0.01	nd	nd	nd	0.30±0.06	nd
indole-3-pyruvic acid	28.33±5.91	12.4±1.52	22.33±4.84	25.37±25.35	172.21±33.81	40.77±3.6	204.91±26.93	121.63±106.75
I-acrylic	nd	nd	0.02±0.00	nd	nd	nd	0.02±0.00	nd
spermidine	0.41±0.02	0.35±0.01	0.45±0.00	0.60±0.02	0.45±0.01	0.4±0.02	0.44±0.00	0.54±0.01
Naringin	0.19±0	nd	nd	nd	0.15±0.01	nd	nd	nd
Glycitein	nd	0.05±0	0.03±0.05	0.08±0.02	0.04±0.03	0.26±0.03	0.09±0.15	0.19±0.17
Isorhamnetin	0.18±0.04	2.59±0.21	1.18±0.16	16.22±2.03	0.56±0.09	7.7±1.58	3.18±0.92	26.75±2.58
Formononetin	0.05±0.05	0.01±0	0.12±0.15	0.01±0.00	0.01±0	0.01±0	0.02±0.01	0.04±0.04
Apigenin	0.02±0	0.84±0.05	0.06±0.01	4.84±0.25	0.12±0.01	1.25±0.16	0.10±0.02	4.86±0.19
seco	0.02±0.02	0.03±0	0.03±0.00	0.06±0.01	0.15±0.01	0.14±0.02	0.08±0.00	0.16±0.01
mata	0.26±0.03	2.55±0.05	0.05±0.01	0.31±0.03	2.17±0.11	9.04±1.16	0.65±0.08	1.49±0.20

Table 5.5: The phytochemicals detected in Potato Bean Tuber and Potato Bean Peel using Lc-MS/MS analysis

The figures from below represents the main 10 compounds found in each food crop analysed (calculated as sum of F1,F3 and F4 fractions); for flavonoids the values were sum of F2, F3 and F4. In the Figure 5.5 are show the ten main compounds (mg/kg dry material) identified and quantified in organic chia seeds and white organic chia seeds; in Figure 5.6 for pumpkin seeds and organic pumpkin seeds, in Figure 5.7 for red organic quinoa grain, black organic quinoa grain and quinoa grain, in the Figure 5.8 for brown linseed, organic golden linseed and organic brown linseed, in the Figure 5.9 for triticale flour, triticale cereal and triticale rolled, in the Figure 5.10 for potato bean tuber and potato bean peal.

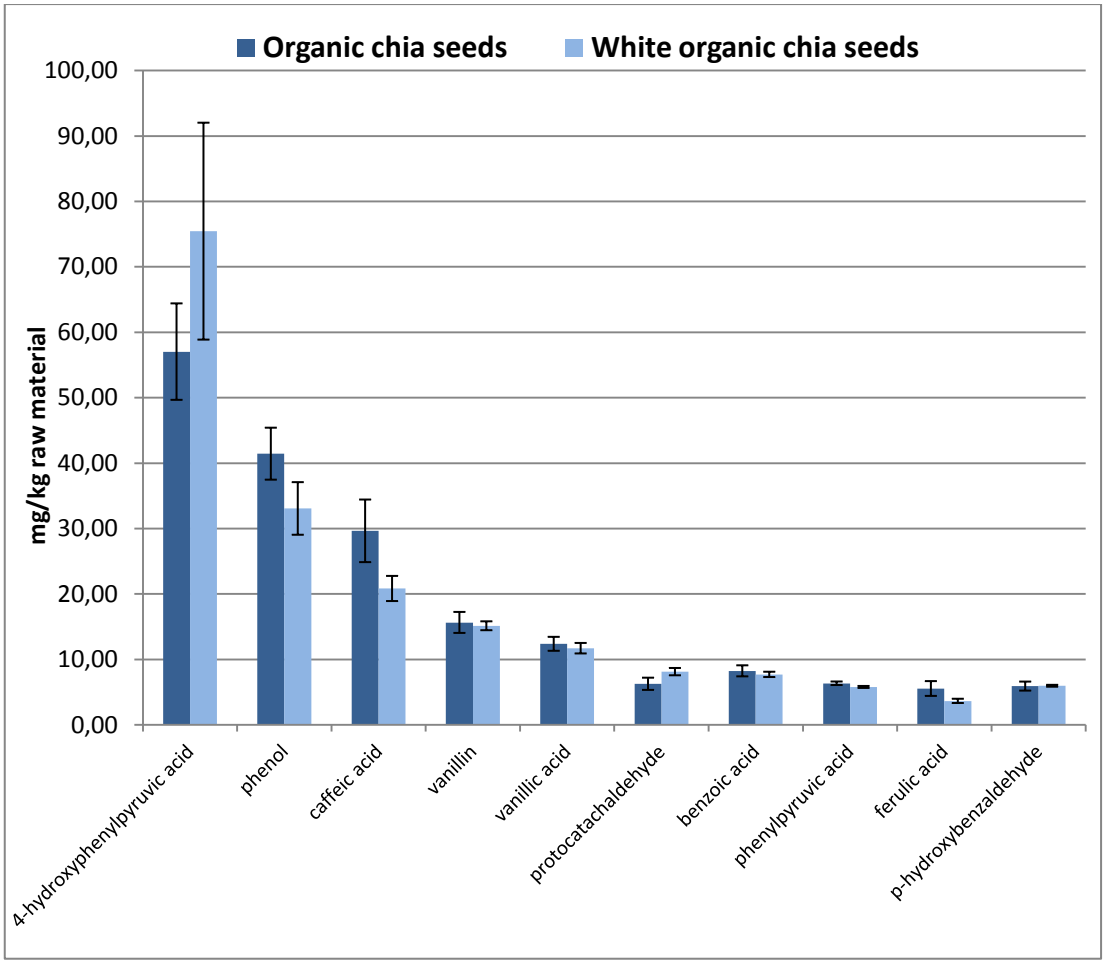

Figure 5.5: The ten main compounds (mg/kg dry material) identified and quantified in organic chia seeds and white organic chia seeds

The 4-hydroxyphenylpyruvic acid (4-HPPA), was the main compound found in both species of chia and pumpkin (see figure 5.6). Only the protocatachaldehyde was found in higher amounts in WOCS than in OCS, all other compounds were found in higher amounts in OCS.

The 4-HPPA is an intermediate in the metabolism of the essential amino acid phenylalanine; this is a precursor for tyrosine, the monoamine signalling molecules dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline), and the skin pigment melanin (Brand *et* Harper, 1974). Phenylalanine is used in the manufacture of food and drink products and sold as a nutritional supplement for its reputed analgesic and antidepressant effects. In plants phenylalanine is the starting compound used in the flavonoid biosynthesis. Lignan is derived from phenylalanine and from tyrosine (Nelson and Cox, 2000).

Caffeic acid is normally found in all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal components of plant biomass and its residues. It is an antioxidant *in vitro* and *in vivo* and also it shows immunomodulatory and anti-inflammatory activity. It has recently been established inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line (Prasad *et al.*, 2011).

Vanillin is a phenolic aldehyde, Vanilla was cultivated as a flavouring by pre-Columbian Mesoamerican people; at the time of their conquest by Hernán Cortés, the Aztecs used it as a flavouring for chocolate. Europeans became aware of both chocolate and vanilla around 1520. The largest use of vanillin is as a flavouring, usually in sweet foods, it is also used in the fragrance industry, in perfumes, and to mask unpleasant odours or tastes in medicines, livestock fodder, and cleaning products (Kermasha *et al.*, 1995).

Vanillic acid is a dihydroxybenzoic acid derivative used as a flavouring agent, and it an oxidized form of vanillin. The highest amount of vanillic acid in plants known so

far is found in the root of *Angelica sinensis*, a herb indigenous to China, which is used in traditional Chinese medicine (Duke, 1992).

Phenol was widely used as an antiseptic, especially as carbolic soap, from the early 1900s to the 1970s. It is a component of industrial paint strippers used in the aviation industry for the removal of epoxy, polyurethane and other chemically resistant coatings. Today is produced on a large scale (about 7 billion kg/year) from petroleum and its major uses involve its conversion to plastics or related materials. Phenol is also a versatile precursor to a large collection of drugs, most notably aspirin but also many herbicides and pharmaceutical drugs. Phenol is also used as an oral anaesthetic/analgesic in products such as Chloraseptic® or other brand name and generic equivalents, commonly used to temporarily treat pharyngitis.

Injections of phenol were used as a means of individual execution by the Nazis during the Second World War. The Nazis learned that extermination of smaller groups was more economical via injection of each victim with phenol. Phenol injections were given to thousands of people, especially at Auschwitz-Birkenau (Lifton R.J., 1986).

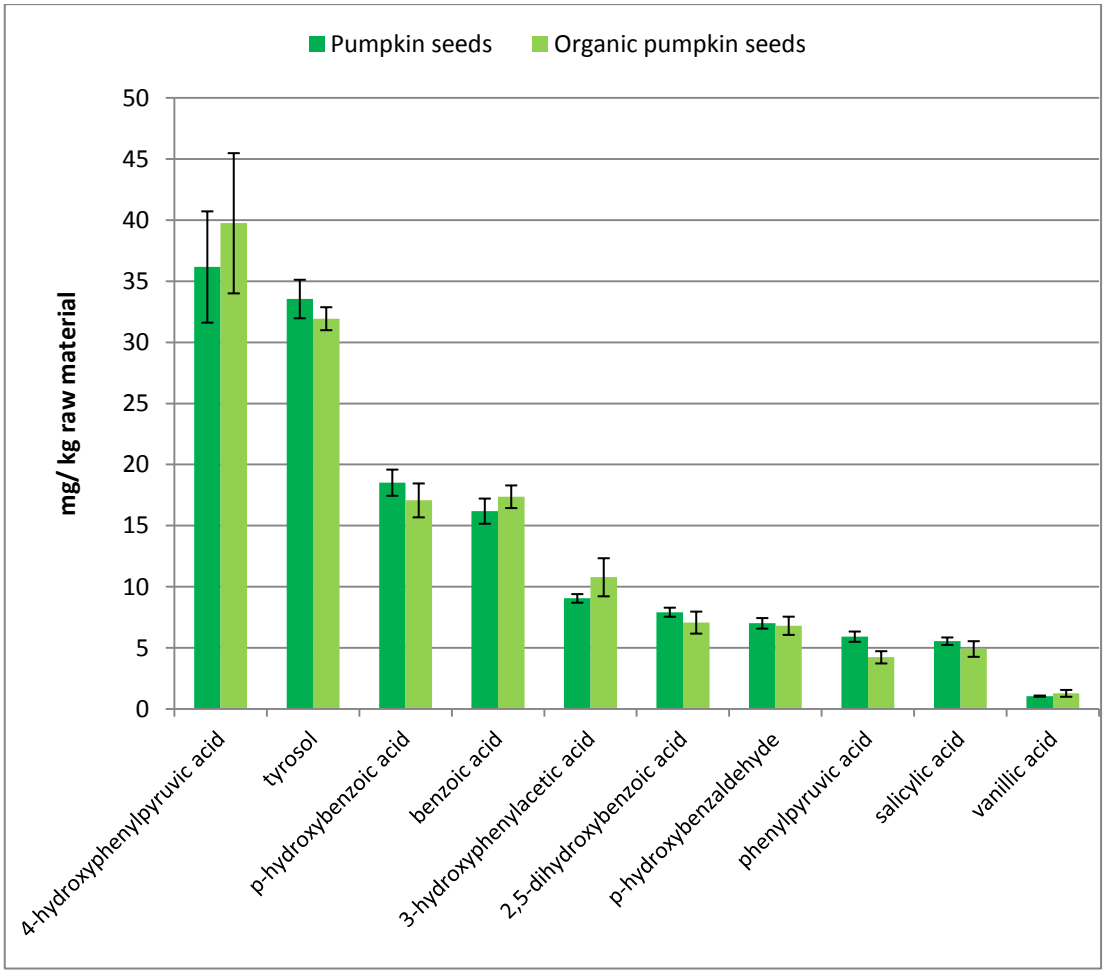

Figure 5.6: The ten main compounds (mg/kg dry material) identified and quantified in pumpkin seeds and organic pumpkin seeds

The 4-HPPA represents also the component detected. There were nosignificant differences between the organic normal chia in terms of the phytochemicals content.

A good amount of tyrosol, was higher in the organic chia. Tyrosol is an antioxidant present in a variety of natural sources. The principal source in the human diet is olive oil. As an antioxidant, tyrosol can protect cells against injury due to oxidation. Although it is not as potent as other antioxidants present in olive oil, its higher concentration and good bioavailability indicate that it may have an important overall effect (Giovannini et al., 1999).

This effect may contribute significantly to the health benefits of olive oil commonly used in the Mediterranean diet.

The benzoic acid and p-hydroxybenzoic acid occurs naturally in many plants and they serve as intermediates in the biosynthesis of many secondary metabolites (Qualley *et al.*, 2012). Salts of benzoic acid are used as food preservatives. Benzoic acid was discovered in the sixteenth century. The dry distillation of gum benzoin was first described in 1556 by Nostradamus. In 1875 Salkowski discovered the antifungal abilities of benzoic acid, which was used for a long time in the preservation of benzoate-containing cloudberry fruits. Benzoic acid is a constituent of Whitfield's ointment which is used for the treatment of fungal skin diseases such as tinea, ringworm, and athlete's foot (Wilson *et al.*, 2004). For humans, the *World Health Organization's International Programme on Chemical Safety* (IPCS) suggests a provisional tolerable intake would be 5 mg/kg body weight per day (WHO, 2000).

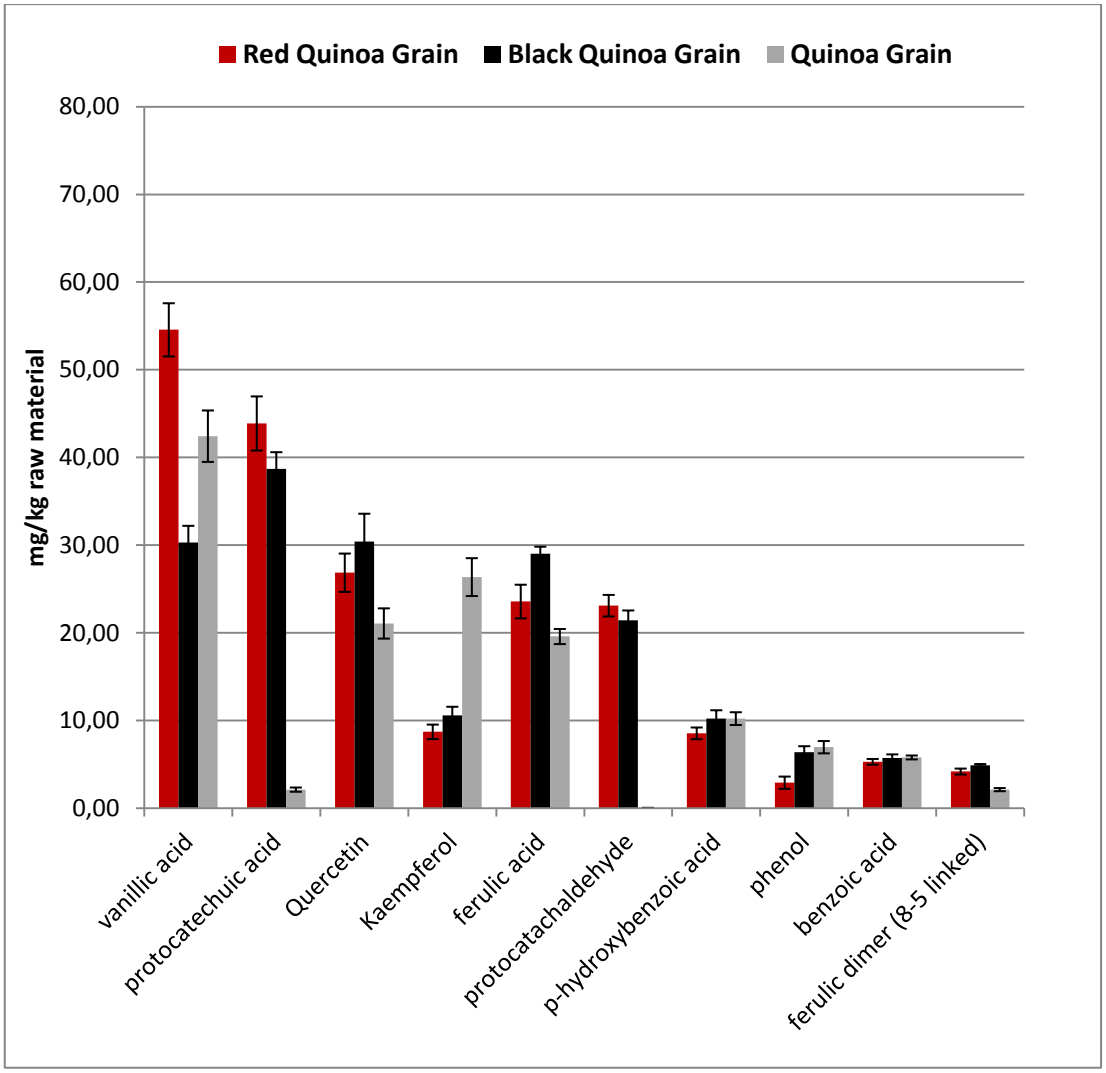

Figure 5.7: The ten main compounds (mg/kg dry material) identified and quantified in red organic quinoa grain, black organic quinoa grain and quinoa grain.

The vanillic acid, was found as main phytochemical in quinoa. Except for the kaempferol, all the the other compounds were found in higher quantities in organic red and black quinoa than in the normal one.

The quercetin is a flavonoid widely distributed in nature. The name has been used since 1857, and is derived from *quercetum* (oak forest). The quercetin has reported to have antitumor effect of quercetin in SW480 colon cancer cells, related to the

inhibition of expression of cyclin D(1) and survivin as well as the Wnt/beta-catenin signaling pathway (Shan et al., 2009) and also it induces insulin secretion in pancreatic beta cells (Bardy *et al.*, 2013).

The kaempferol is a natural flavonoid isolated from tea, broccoli, cabbage, leek, tomato, strawberries, grapes, apples and other plant sources; and was identified in many plant species commonly used in traditional medicine. Some epidemiological studies have found a positive association between the consumption of foods containing kaempferol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases. Numerous preclinical studies have shown that the kaempferol and some glycosides of kaempferol to have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anxiolytic, analgesic, and antiallergic activities (Calderon-Montaño *et al.*, 2011).

The ferulic acid is an abundant phenolic phytochemical found in plant cell wall components such as arabinoxylans as covalent side chains. Ferulic acid, like many natural phenols, is an antioxidant *in vitro* in the sense that it is reactive toward free radicals such as reactive oxygen species (ROS). Animal studies and *in vitro* studies suggest that the ferulic acid may have direct antitumor activity against breast cancer and liver cancer (Ibtissem *et al.*, 2012)

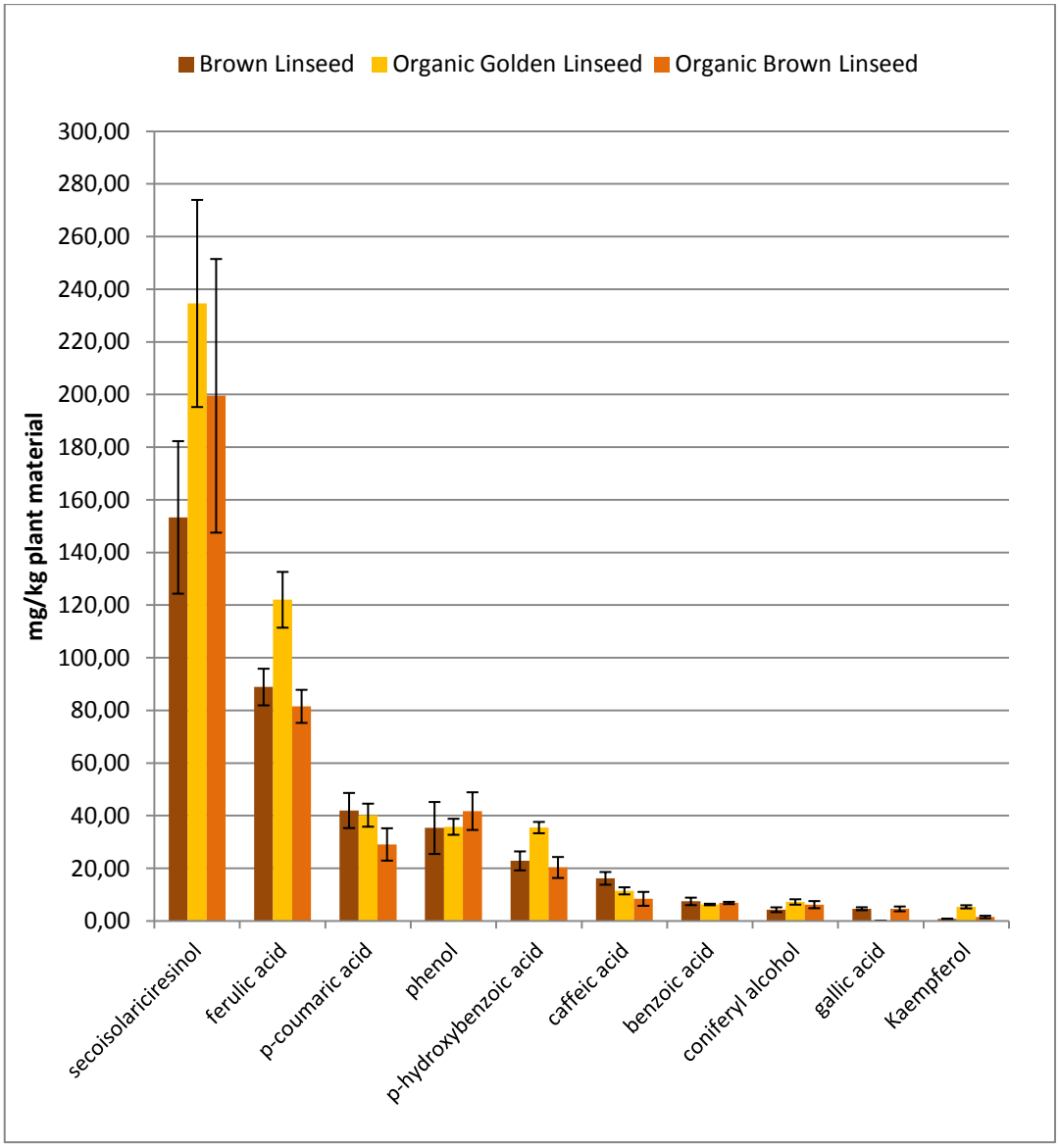

Figure 5.8 The ten main compounds (mg/kg dry material) identified and quantified in Brown linseed, Organic Golden Linseed and Organic Brown linseed.

The secoisolariciresinol was the main lignan in all three varieties of flaxseed,. The lignans are abundant in superior plants and have properties particularly interesting to the health sector. The food and cosmetic fields could also potentially exploit their antioxidant activity (Blecker *et al*., 2012).

The ferulic acid, p-coumaric acid were found also in higher quantities in flaxseed; together with sinapyl alcohol and coniferyl alcohols, p-coumaric acid is a major component of lignin. Gallic acid was found in brown and organic brown linseed and not detected in the golden variety. Gallic acid is commonly used in the pharmaceutical industry. It is used as a standard for determining the phenol content of various analytes by the *Folin-Ciocalteau assay* (Waterhouse, 2001).

The kaemperol was detected in golden one but not in the brown and organic brown. It was identified also phenol. Golden variety shows a different phytochemicals profile than brown and organic brown.

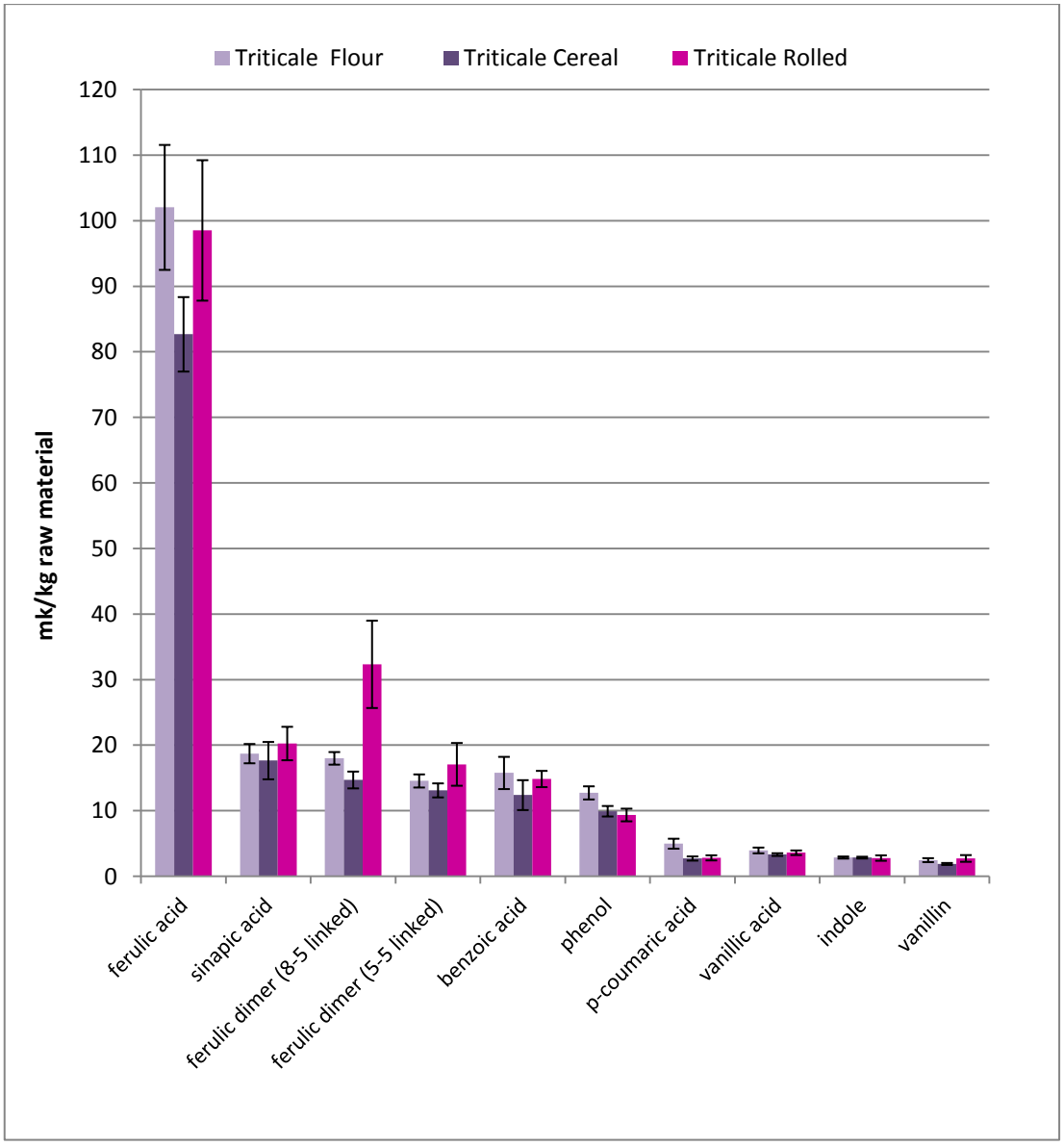

Figure 5.9: The ten main compounds (mg/kg dry material) identified and quantified in Triticale Flour, Triticale Cereal and Triticale Rolled.

High amounts of ferulic acid and its dimers were found in all three triticales samples, followed by sinapic. Sinapic acid is a naturally occurring hydroxycinnamic acid a member of the phenylpropanoid family and commonly used matrix in MALDI (matrix-assisted laser desorption/ionization) mass spectrometry. It is a useful matrix

for a wide variety of peptides and proteins. It serves well as a matrix for MALDI due to its ability to absorb laser radiation and to also donate protons (H+) to the analyte of interest. Sinapic acid can form dimers with itself and ferulic acid in cereal cell walls and therefore may have a similar influence on cell-wall structure to that of the diferulic acids (Bunzel *et al.*, 2003).

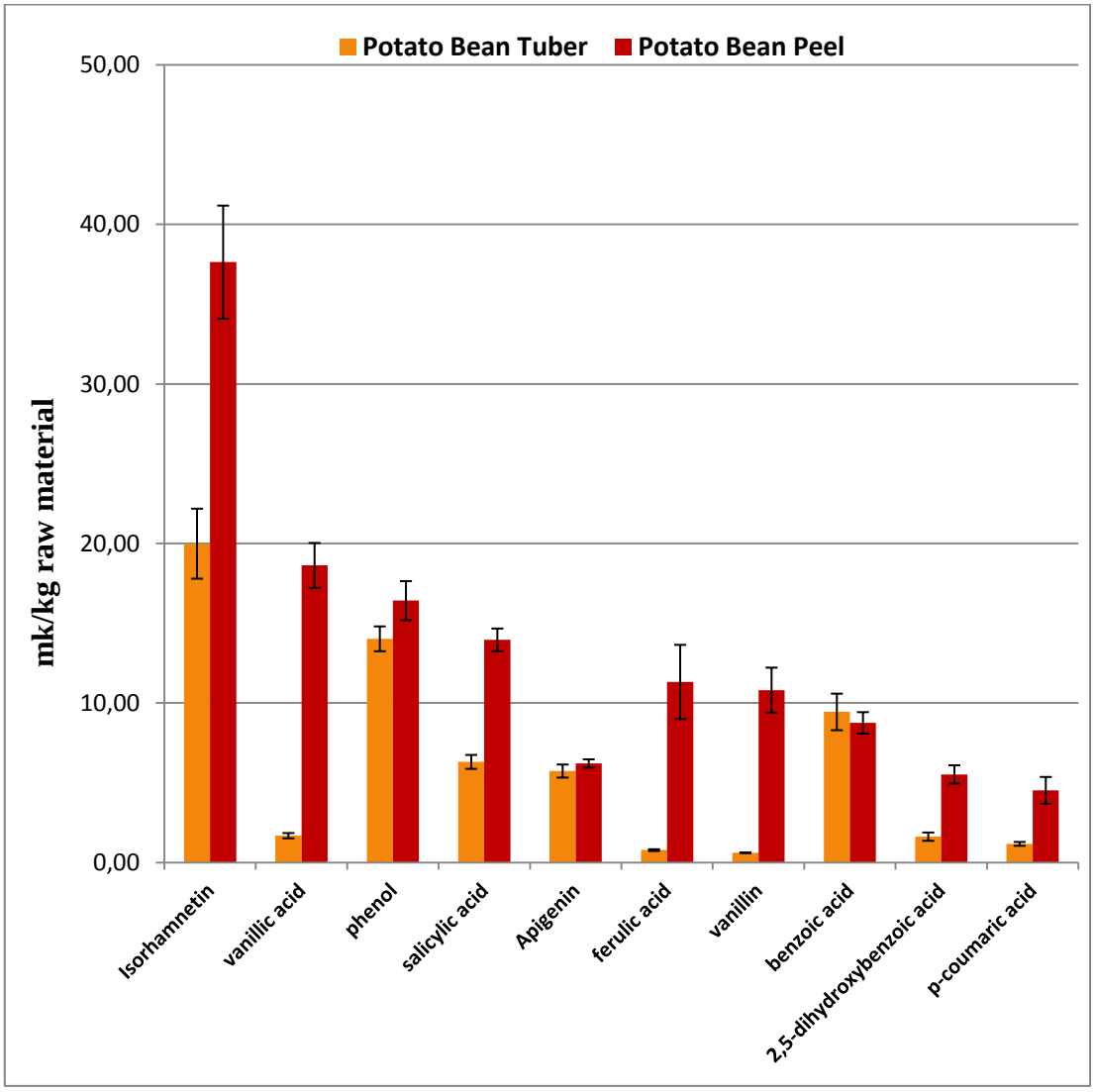

Figure 5.10: The ten main compounds (mg/kg dry material) identified and quantified in Potato Bean tuber and Potato Bean Peal.

Overall, the potato peel was found to be richer in the phytochemicals that the tuber with the exception of benzoic acid. Isorhamnetin (methylated quercetin) is the main compound found in both peel and tuber, it is a flavonoid also found in the sunflower. Isorhamnetin is less studied than quercetin,but indicate that it has similar health benefits: it may reduce the risk of cancer, improve heart health and attenuate diabetes complications (Bohm *et* Stuessy, 2007).

Good amounts of ferulic acid and salicylic acid were also found in the peel. Salicylic acid is a phenolic acid widely used in organic synthesis and functions as a plant hormone. It is derived from the metabolism of salicin. In addition to being an important active metabolite of aspirin (acetylsalicylic acid), which acts in part as a pro-drug to salicylic acid, it is probably best known for its use as a key ingredient in topical anti-acne products and for its ability to ease aches and pains and reduce fevers. These medicinal properties, particularly fever relief, have been known since ancient times, and it is used as an anti-inflammatory drug (Madan *et* Levitt, 2014) .

5.5.2 Qualitative and quantitative HPLC analysis of the antocyanins

The anthocyanins found in the plant crops studied are shown in the Table 5.6 (A, B) detected with HPLC analysis, expressed mg/kg of dry plant material, as mean ± standard deviations (n = 3); not detected = n.d..

A	*Organic chia seeds*	*White organic chia seeds*	*Pumpkin seeds*	*Pumpkin seeds organic*	*Red quinoa grain*	*Black quinoa grain*	*Quinoa grain*
mg/Kg ± SD							
Delphinidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Cyanidin	n/d	n/d	n/d	n/d	468.83 ± 19.83	233.69± 22.04	n/d
Petunidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Pelargonidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Peonidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Malvinidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d

B	*Brown linseed*	*Organic golden linseed*	*Organic brown linseed*	*Triticale grain flour*	*Triticale cereal*	*Triticale rolled*	*Potato bean tubers*	*Potato bean peel*
mg/Kg ± SD								
Delphinidin	34.53± 2.19	n/d	54.64 ± 8.04	n/d	n/d	n/d	n/d	98.02± 4.12
Cyanidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d	10.05± 0.15
Petunidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Pelargonidin	n/d	74.53± 8.53	n/d	n/d	n/d	n/d	n/d	n/d
Peonidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Malvinidin	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d

Table 5.6 (A, B): Anthocyanins aglycones detected with HPLC analysis,expressed mg/kg of dry plant material, as mean ± standard deviations (n = 3); not detected = n.d.

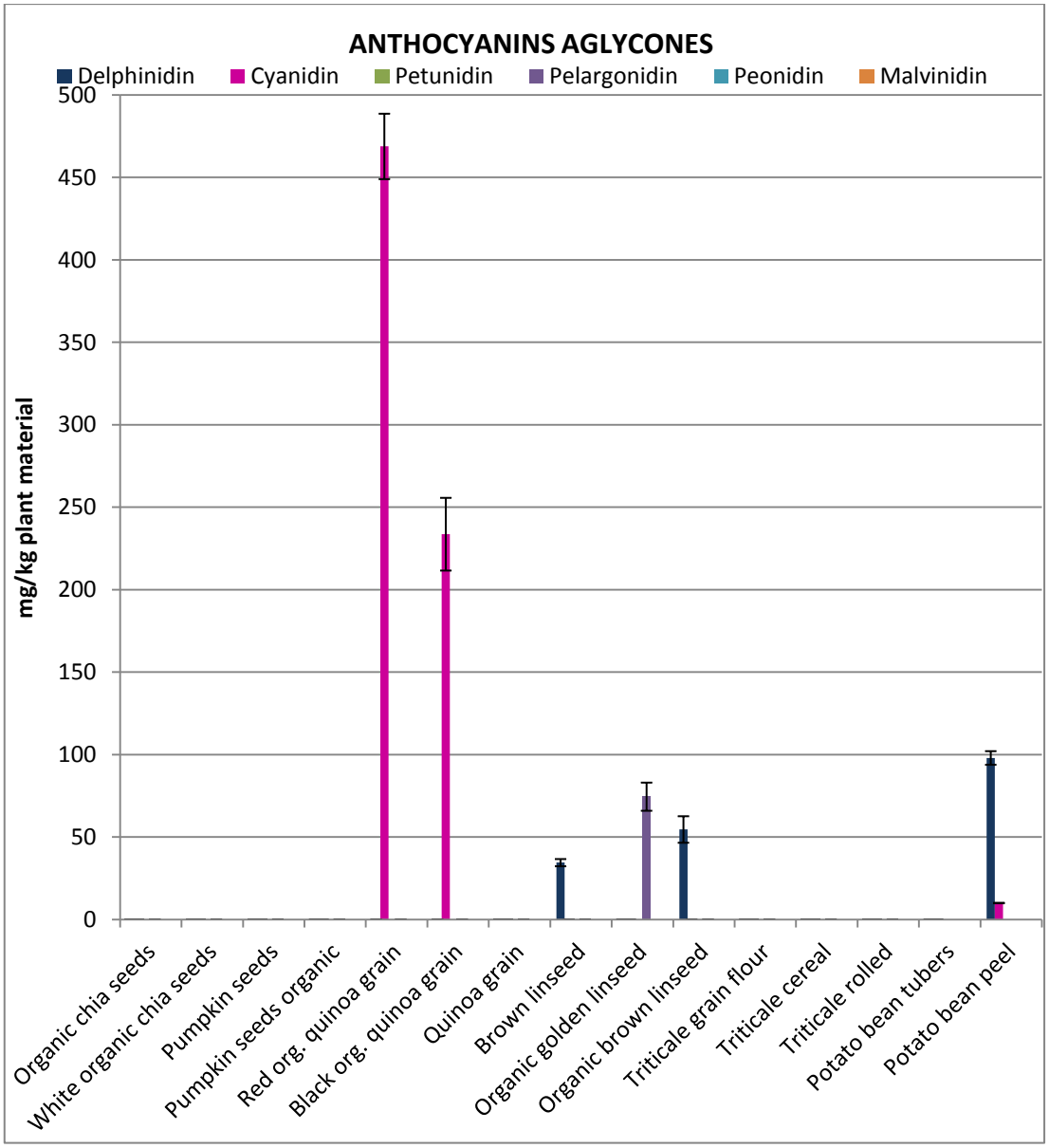

Figure 5.11: The main anthocyanin aglycones in the food plant crops

As seen in the figure 5.11, no anthocyanins were detected in chia, pumpkin and triticale. There were big differences in anthocyanins quantities found in quinoas, the cyanidin, was present in the red and black organic only. The pelargonidin was only

found in in golden organic linseed. While delphinidin was detected in brown linseed (34,53 mg/kg) with much higher quantities in organic brown linseed (54,64 mg/kg). Only in the potato bean peel were found anthocyanins, such as delpfinidin (98,02 mg/Kg) and a small quantities of cyaniding (10,05mg/Kg).

5.5.3 Qualitative and quantitative Lc-MS/MS analysis of cathechins

In the following Table 5.7 are show the contents of catechins detected with LC-MS analysis, expressed mg/Kg of plant material. The results are reported as free catechins (F1 extract) and bound catechins (F3 and F4 extracts); The different extraction procedures liberate free and bound (acid and alkali-labile) compounds, which give an indication about their potential availability in the human gastrointestinal tract.

	Catechin	**Epi catechin**	**Gallo catechin**	**Epigallo catechin**	**Epigallo Catechin gallate**
	mg/Kg ± SD	mg/Kg ± SD	mg/Kg ± SD	mg/Kg ± SD	mg/Kg ± SD
Organic chia seeds	0,151±0,034	0,131±0,030	0	0	00
Free	0	0	0	0	0
Bound	0,151±0,034	0,131±0,030	0	0	0
White organic chia seed	0	0	0	0	0
Free	0	0	0	0	0
Bound	0	0	0	0	0
Pumpkin seeds	0	0	0	0	0
Free	0	0	0	0	0
Bound	0	0	0	0	0
Pumpkin seeds organic	0	0	0	0	0
Free	0	0	0	0	0
Bound	0	0	0	0	0
Red quinoa grain	2,796±1,101	3,326±0,522	0	0	0
Free	0	1,439±0,168	0	0	0
Bound	2,796±1,101	1,888±0,495	0	0	0

Black quinoa grain	0,177±0,128	0,605±0,175	0	0	0
Free	0	0,445±0,117	0	0	0
Bound	0,177±0,128	0,160±0,130	0	0	0
Quinoa grain	0	0	0	0	0
Free	0	0	0	0	0
Bound	0	0	0	0	0
Brown linseed	0,160±0,034	0,083±0,031	2,483±0,193	1,389±0,254	0
Free	0	0	0,861±0,038	0,784±0,232	0
Bound	0,160±0,034	0,083±0,031	1,622±0,189	0,605±0,103	0
Organic golden linseed	0	0	0	0	0
Free	0	0	0	0	0
Bound	0	0	0	0	0
Organic brown linseed	0,230±0,069	0,135±0,039	4,238±1,065	2,066±0,411	0
Free	0	0	2,015±0,366	1,366±0,021	0
Bound	0,230±0,069	0,135±0,039	2,223±1,000	0,700±0,411	0
Triticale grain flour	0,078±0,042	0	0	0	0
Free	0	0	0	0	0
Bound	0,078±0,042	0	0	0	0
Triticale cereal	0,081±0,003	0	0	0	0
Free	0	0	0	0	0
Bound	0,081±0,003	0	0	0	0
Triticale rolled	0,103±0,051	0	0	0	0
Free	0	0	0	0	0
Bound	0,103±0,051	0	0	0	0
Potato bean tubers	0	0	0	0	0
Free	0	0	0	0	0
Bound	0	0	0	0	0
Potato bean peel	1,312±0,076	0	3,776±0,622	0	0
Free	1,312±0,076	0	3,776±0,622	0	0
Bound	0	0	0	0	0

Table 5.7: Catechin content (mg/kg of dry plant material) quantified with LC-MS analysis, mean ± standard deviations (n = 3); not detected = n.d..

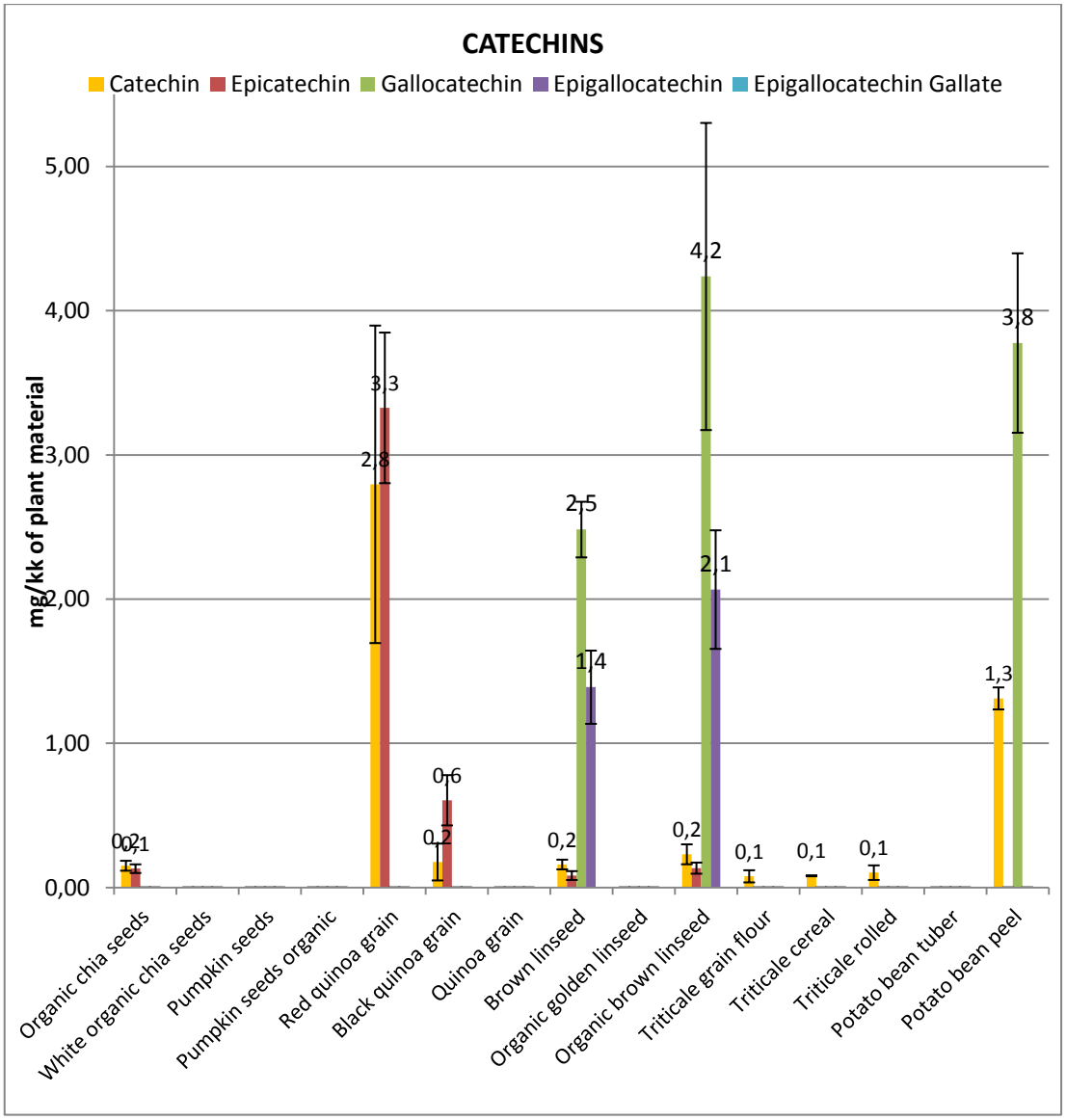

Figure 5.12: Catechin content (mg/kg of plant material) analysed with LC-MS/MS, mean ± standard deviations (n = 3)

In the both, the brown and organic brown linseed are found low quantities of catechin (0,16 and 0,23 mg/kg respectively) and epicatechin (0,08 and 0,13 mg/Kg); they contain also gallocatechin (2,5 and 4,2 mg/kg) and epigallocatechin (1,4 and 2,1

mg/kg), larger quantities in the organic one. No catechins were found the golden variety.

The catechin and epicatechin were found in the chia (only in organic and not in white organic) and in quinoas, (only in organic red and black varieties) with higher amounts in the red variety (catechin 2,8 mg/kg and epicatechin 3,3 mg/kg). Traces of catechin were also found in all three varieties of triticale. The catechin and gallocatechin were detected in potato bean peel but not in potato bean tuber

5.5.4. Qualitative and quantitative LC-MS/Ms analysis of saponins

In the Table 5.8 and Figures 5.13 and 5.14 are show the quantities (mg/Kg plant material) for soyasaponin I and soyasapogenol B in food plant crops analysed by LC-MS/MS. Values mean ± standard deviations (n = 3)

Plant	**soyaaponin I**	**soyasapogenol B**
	mg/kg of dry plant material	
Organic chia seeds	0	0
White organic chia seeds	0	0
Pumpkin seeds	0	0,12±0,05
Pumpkin organic seeds	0	2,66±0,28
Red quinoa grain	0	0
Black quinoa grain	0	0
Quinoa grain	0	0
Brown linseed	1,04±0,16	0,12±0,02
Organic golden linseed	0,50±0,01	0
Organic brown linseed	0,69±0,16	0
Triticale grain flour	0,05±0,03	0,11±0,02
Triticale cereal	0	0,08±0,01
Triticale rolled	0	0,05±0,01
Potato bean tuber	18,64±1,09	0
Potato bean peel	196,14±18,97	1,97±0,48

Table 5.8: Data for saponin and sapogenol founded in the plants with LC-MS analysis

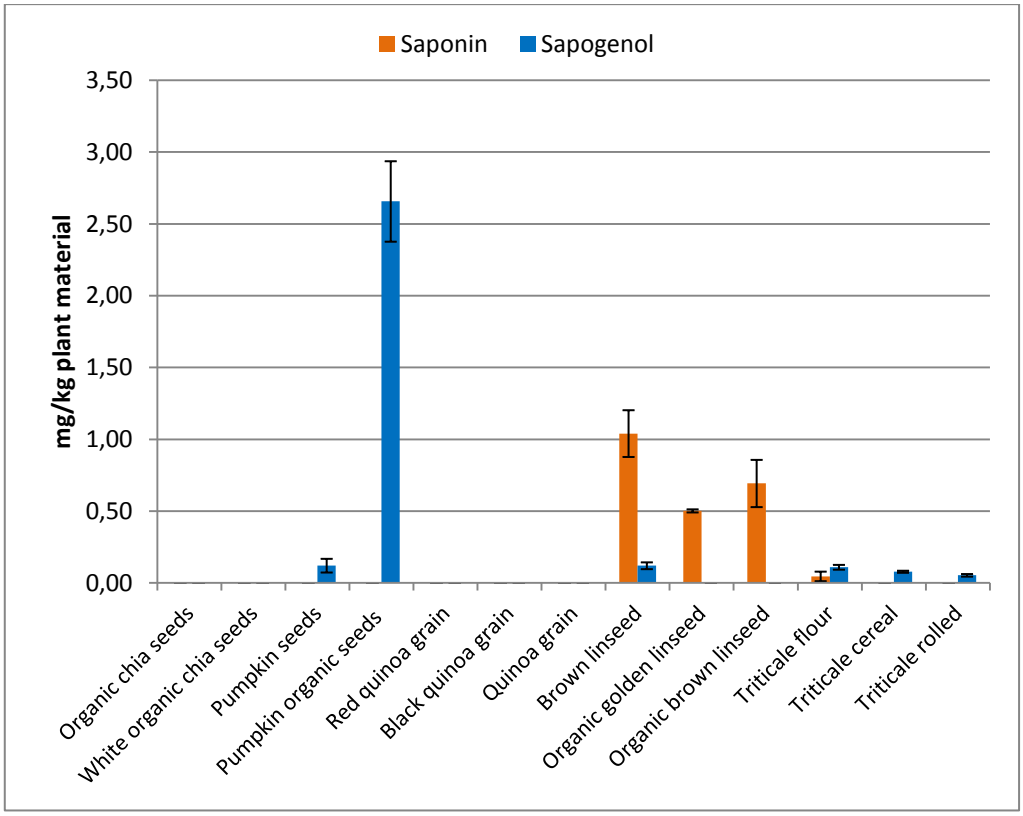

Figure 5.13: Soyasaponin I and soyasapogenol B in food plant crops analysed by LC-MS/MS. Expressed mg/kg plant material, values mean ± standard deviations (n = 3)

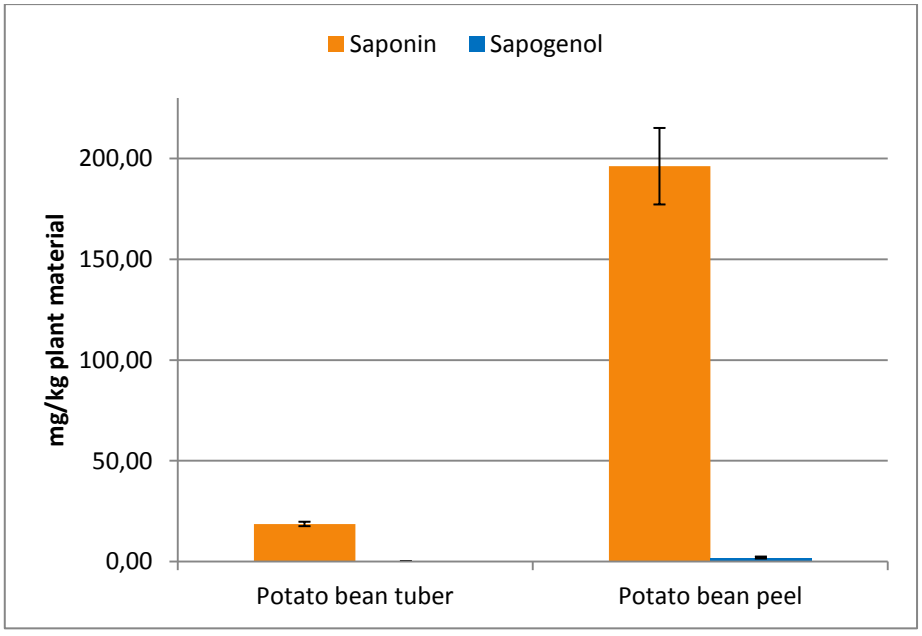

Figure 5.14: Figure 5.13: Soyasaponin I and soyasapogenol B detected in potato bean tuber and peel.Expressed mg/kg plant material, values mean ± standard deviations (n = 3)

The soyasaponin I was found in very low quantities in in all three varieties of linseed species (1,04 mg/kg for brown, 0,5 mg/kg for organic golden and 0,69 for organic brown), and as well triticale flour (0,5 mg/kg). Higjer content of saponins was found in potato bean tuber (18,64 mg/kg), with largest amount in peel (196,14 mg/kg). Small amounts of soyasapogenol B were found in potato bean peel (1,97 mg/kg); in triticale flour (0,11 mg/kg), triticale cereal (0,08 mg/kg) and triticale rolled (0,05 mg/kg). Was found in pumpkin species, in greater quantities in the organic variety (2,66 mg/kg) than the normal pumpkin (0,12 mg/kg).

5.6 Estimation of total phytochemicals

In order to estimate the total phytochemicals content found in the food plant crops analysed the individualcompounds identified was summed , see Figure 5.15.

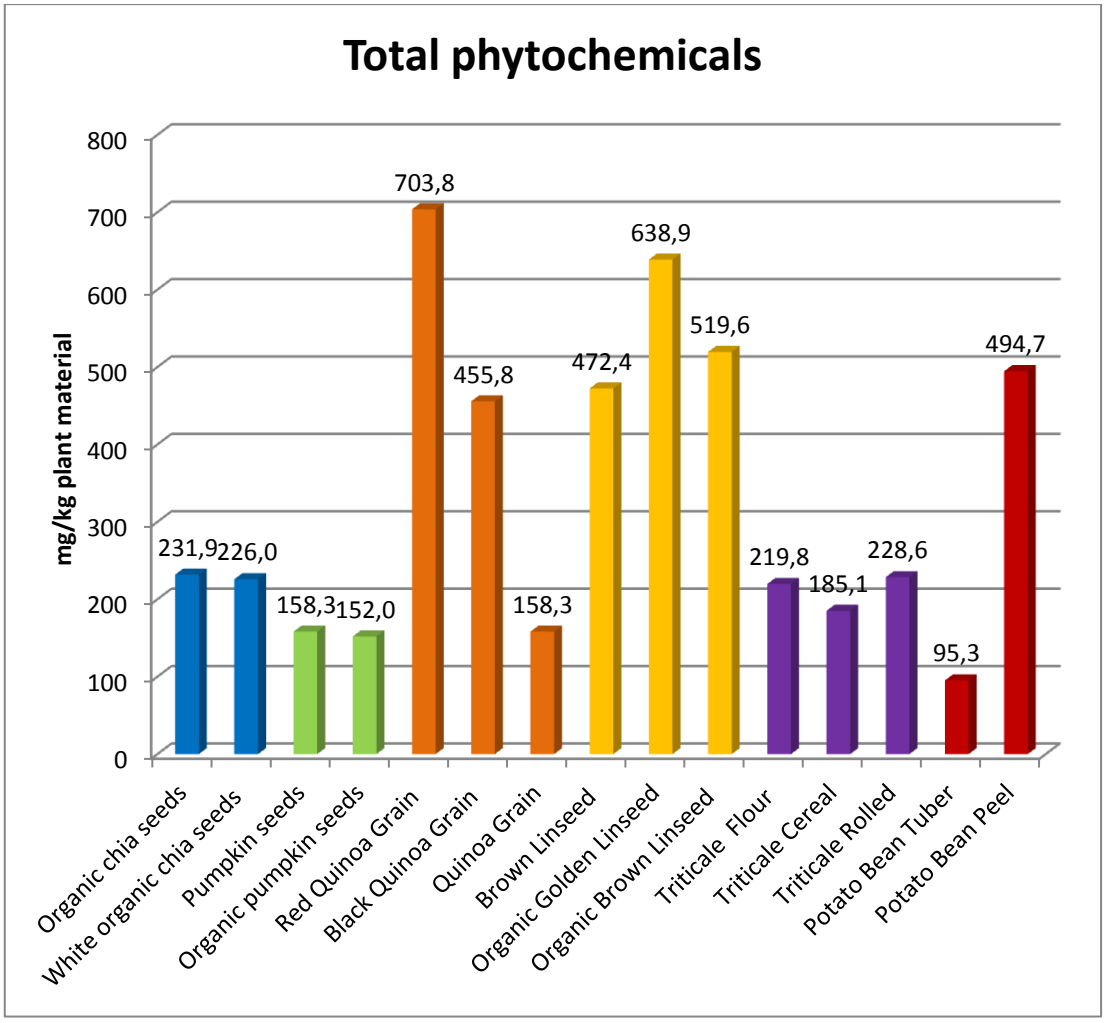

Figure 5.15: The sum of individual compounds identified (mg/kg dry plant material)

Overall, the linseed and quinoa species were the highest in the phytochemicals followed by potato bean. Comparative amounts of total phytochemical around 200 mg/kg were in chia and triticale. Pumpkin seeds were amongst the lowest total phytochemicals analysed.

CONCLUSIONS

It has been argued that changes in agricultural production systems from diversified cropping systems towards ecologically more simple cereal based systems have contributed to poor diet diversity, a potential source of nutrients for the animal feed industries, micronutrient deficiencies and resulting malnutrition in the developed as well as developing world (Graham *et al.*, 2007).

As a trend, with small exceptions, is that the organic varieties in general have greater amounts of phytochemicals compared with not organics crops. This could speculate that is probably related to the fact that in organic crops is reduced the use of pesticides or artificial fertilizers that can cause a loss or reduction of phytochemicals.

The main component detected in chia and pumpkin was 4-hydroxyphenylpyruvic acid, an intermediate in the metabolism of the essential amino acid phenylalanine. In chia plants were also found in good amounts caffeic acid (normally found in all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal components of plant biomass and its residues), vanillin, vanillic acid and phenol

No significant differences were found between pumpkin organic normal.

The vanillic acid was the major components of quinoa species the quercetin and ferulic acid were found more abundant in organic red and black quinoa respect to the normal one, exception was the kaempferol.

The Secoisolariciresinol was the main compound founded in all three varieties of linseed. Other major compounds detected in linseed were ferulic acid and p-coumaric acid; gallic acid (in brown and organic brown linseed but no detected inin the golden variety); expection was again the kaemperol which was detected in golden one but not in the other varieties.

High amounts of ferulic acid and its dimers were also found in all three varieties of triticale. With the exception of benzoic acid; a greater amount of phytochemicals were found in potato bean peel potation comparison with the tuber. Isorhamnetin (methylated quercetin) is the main compound found in both the tuber and peel.

No anthocyanins were detected in chia, pumpkin and triticale. The red and black organic quinoa has large amounts of cyanidin, The pelargonidin was found only in in golden organic linseed; while delphinidin was detected in brown organic brown linseed. In the potato bean peel were found delphinidin and a small amount of cyanidin.

In both brown and organic brown linseed were found small quantities of catechin and epicatechin, they also contain epigallocatechin and gallocatechin, with larger quantities present in the organic variety. No catechins were found instead in the golden variety.

The catechin and epicatechin were also found in chia (only organic and not in white organic) and in quinoa (in organic varieties with higher amounts in the red variety). Traces of catechin were also found in all three varieties of triticale,. In the potato peel bean has been detected catechin and gallocatechin, but not in potato bean tuber.

Traces of soyasaponin I were found in all three varieties linseed species , and in triticale flour. The highest content of soyasaponin I was found in potato bean, with largest amounts in the peel. Soyasapogenol B was found in all triticale samples and pumpkin species, with greater quantities in the organic varieties. Small amounts were also found in potato bean peel.

Different varieties of the same species show very different phytochemical profiles, this is quite evident in organic linseed, which phytochemical profile is different from the brown, red organic quinoa and from the black.

The use of plant foods rich is proyeins and rich in bioactive phytochemicals, it would be the beneficial for health; and also ould be beneficial for enviromentreducing the impact of food on greenhouse gas emissions, alterations of ecosystems such as biodiversity loss, deforestation,

The consumption of food plants rich in bioactive phytochemicals like cereal and pseudoceral has been considered to have many physiological benefits such as

coronary heart disease, colon cancer and diabetes: germination of cereal and pseudo-cereal grains helps to improve the chemical compositions, nutritive values and acceptability characteristics of the products.

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