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CITRUS LIMONOIDS:
FUNCTIONAL CHEMICALS IN AGRICULTURE AND FOODS

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Chapter 1

Introduction

The search for limonoids started long back when scientists started looking for the factor responsible for bitterness in citrus. The term limonoids was derived from limonin, the first tetranortriterpenoid obtained from citrus bitter principles (Roy *et al*, 2006). In 1938, Highby first isolated limonin from Washington navel orange and showed it as bitter principle of navel orange juice in 1949. Ongoing studies show that limonoids are highly oxygenated, modified terpenoids and have recently attracted attention because compounds belonging to this group have exhibited a range of biological activities like insecticidal, insect antifeedant and growth regulating activity on insects as well as antibacterial, antifungal, antimalarial, anticancer, antiviral and a number of other pharmacological activities on humans (Hasegawa *et al*, 1996, 2000) (Tundis *et al*, 2014). Interest in limonoids research has become greater than before also because some of them are responsible for producing bitterness in citrus fruits, which has negative impact on citrus fruit and juice industry world wide.

Juice is the primary product obtained from citrus fruits (Braddock, 1999) which account for 40-45% of the weight of the raw material and it is also one of the most important commodities. The juices produced from the citrus fruits are either in the form of single-strength or concentrated juices (Ting and Rouseff, 1986).

Another important product of citrus fruits is the essential oils extracted mainly from the peel (around 3%). In order to obtain the oil, the oil-bearing sacs need to be punctured by either abrasion or scraping the surface of the peels (Redd and Hendrix, Jr., 1996). For its recovery, the oil is washed away from the peel as an aqueous emulsion and then separated from the water by centrifugation (Ohloff, 1994).

During the process of juice concentration, some of the natural flavor compounds are also removed together with the water juice (1%), including the small amounts of peel oil

remaining in the juice. The volatiles recovered during the production of juice concentrates are called essence (Redd and Hendrix, Jr., 1996). The water-soluble portion of the essence is known as aqueous essence or aroma while essence oil or oil phase essence refers to the oil-soluble portion. Aroma and essence oil are commonly used as natural flavorings for citrus juice products as they contain many volatile compounds found in cold-pressed oil (Shaw, 1977).

However the main by-products of citrus processing are the peel, pulp and seeds (pastazzo), which account for 40-60% of the weight of the raw material (Licandro and Odio, 2002). These residues can be further processed into 3 main categories: animal feed, raw material used for further extraction of marketable products and food products. Although most of the citrus by-products are used for animal feed (Ting and Rouseff, 1986), there are many useful by-products made from different portions of the citrus fruits, such as pectin, dried pulp, molasses, marmalades, candied peel, peel seasoning, purees, beverage bases, citrus alcohol, bland syrup, citric acid, seed oil, flavonoids and other products (Kesterson and Hendrickson, 1958; Braddock and Cadwallader, 1992; Braddock, 1995; Hendrix, Jr. and Hendrix, 1996; Braddock, 1999; Licandro and Odio, 2002). In the past, by-products became the source of additional revenue for many citrus processors with low juice values (Braddock, 1995). Hence, the utilization of citrus by-products to produce more valuable products is getting increasingly important as future world citrus production increases and then surpasses the demand for citrus juices and beverage products. Furthermore, the future uses of citrus by-products will also need to expand beyond the current major use as low-value animal feed.

1.1. STRUCTURE OF THE PROJECT BASED ON TASK GROUPS

Based on this premise this paper has focused on technological, healthful and chemical aspects of the limonoids.

1.1.1. TECHNOLOGICAL APPROACH:

Based on a project titled “Enhancement of bioactive compounds isolated from agro-

industrial wastes") financially supported by the Italian Ministry of Education, University and Research (MIUR) (Project of High National Interest PRIN 2009), a Sicilian juice company (Ortogel S.P.A.) wanted to assess the possibility of transforming the waste by-product of citrus processing (pastazzo) in a resource trying to turn it into dietary fiber. To do that, Ortogel has inserted a debittering line to the plant using an alkaline aqueous solution in order to extract flavanones and limonoids.

In the present paper the operational conditions of debittering were evaluated and optimized by determining the limonin content of samples from various stages of fiber production; It was also verified if the recovery of limonin extracted was economically viable.

1.1.2. HEALTH AND ORGANOLEPTIC CHARACTERISTICS:

It has been established that U.S. producers are turning to organic farming system as a potential way to lower input costs, decrease reliance on nonrenewable resources, capture high-value markets at premium price, and boost-farm income (USDA-ERS, 2006 http://www.ers.usda.gov/Amberwaves/ April06/Findings/organic.htm).

Organic production agriculture is characterized by inputs of biologically (non-synthetic) based fertilizers and pest management practices that are sustainable (National Organics Standard Board, 2006; http://www.ams.usda.gov/ NOSB/index.htm).

Much of the U.S. organic farm sector expansion has occurred since the U.S. Department of Agriculture's establishment of uniform organic standards in 2000.

In order to understand if the market source contributes to differences in bio-actives content, the bio-actives content of fruits obtained from farmers' markets was compared to the content found in fruit purchased from retail grocery stores. Organoleptic properties, including Brix, TTA, color and pH were measured.

Limonin, ascorbic acid and flavanoid contents were also determined.

1.1.3. SYNTHESIS AND CHARACTERIZATION OF FUNCTIONAL COMPOUNDS:

Ehrlich's reagent, p-dimethylaminobenzaldehyde (Feigl, F. 1954, Stahl, E. 1962., Fieser L. F.and Fieser, M. 1967) in hydrochloric acid, has a long history and is known as the coloring reagent of pyrrole. 2,3.
Nomura and Saito (1966) determined limonin by a spectrophotometric method using Ehrlich's reagent.

Burnham (1970) used Ehrlich's reagent to determine indole. The difference between those methods was the composition of the reagent and the acidic environment required for the reaction. Vask and Lifshitz (1981) achieved a sensitive method by modification of Burnham reagent for indole and applying it to limonin. Also Breksa and Ibarra (2007) achieved a colorimetric method for the estimation of total limonoids content in citrus juices and Maier and Grant (1970) and Fong and Hasegawa (1993) used the Ehrich's reagent for a specific Thin-Layer-Chromatography assay for limonoids.

When a solution of limonoids is treated with p-dimethylaminobenzaldehyde in acid environment the solution immediately change to red-purple until dark blue. This reaction has named Ehrlich's reaction and the purple coloring is probably due to the presence of an adduct compound with an electron-rich trisubstituted furan ring (Kuroda *et al,* 2004).

In this paper, in order to determine the structure of the limonin-DMBA and limonin glucoside-DMBA adducts, both compounds have been synthesized, purified and characterized. This project involves synthesis of the target compounds. MS analysis were conducted for the characterization of the isolated products.

Chapter 2

Literature Review

2.1. Citrus fruits

Citrus fruits have been cultivated for over 4000 years (Davies and Albrigo, 1994) and are the most produced fruit crops in the world (FAOSTAT). Citrus fruits belong to the family Rutaceae, in which the leaves usually possess transparent oil glands and the flowers contain an annular disk (Kale and Adsule, 1995). The place of origin of citrus fruits is believed to be south eastern Asia and these were subsequently brought to the Middle East and Southern Europe, and further distributed to many other countries by the assistance of travelers and missionaries following the paths of civilization (Samson, 1990; Ruberto, 2001; Calabrese, 2002). The production of citrus fruits, particularly the sweet oranges, continues to show a tremendous growth with Brazil being the largest producer, followed by the United States of America; both sharing more than a third of total production of sweet oranges in the world (FAOSTAT).

2.1.1. Fruit Morphology

In general, citrus fruits are composed of 3 main sections (**Figure 2.1**):

a. The outer peel

The outer peel of citrus fruits is also known as flavedo due to the presence of flavonoid compounds (Ortiz, 2002). It consists of the cells containing the carotenoids, which give the characteristic color to the fruits according to the species or cultivar. The color ranges from deep orange or reddish to light orange, 3 yellow or greenish. The carotenoid pigments are located inside the chromoplasts in the flavedo (Kefford, 1955). The oil glands, which contain the citrus essential oils, are also found in the flavedo. The glands are spherical in shape and have different sizes.

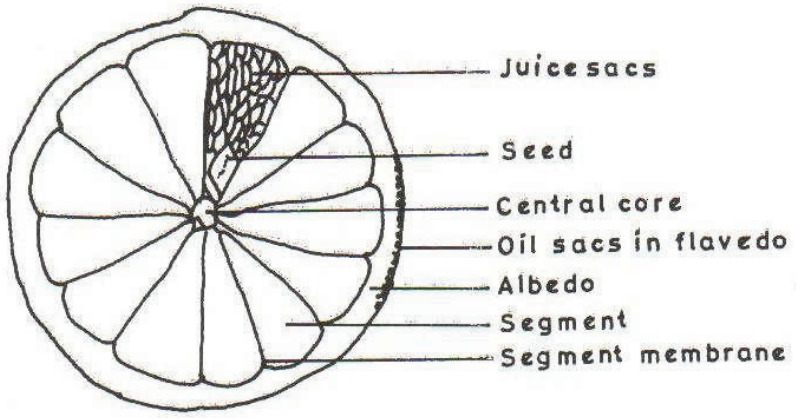

Figure 2.1. *Section of citrus fruit (Ranganna et al, 1986)*

b. The inner peel

Also known as albedo, the inner peel is located underneath the flavedo. It is typically a layer of spongy and white parenchyma tissue that is rich in sugars, pectic substances, celluloses, hemicelluloses and pentosans (Ranganna *et al*, 1986). The thickness of the albedo varies with the species. For example, mandarins generally have very thin albedo while the one in citrons is very thick. Both flavedo and albedo form the non edible part of the fruit called the pericarp, and they are commonly known as the rind or peel.

c. The endocarp

Beneath the albedo of citrus fruits is the edible portion or also known as endocarp. It is composed of many segments or carpels, usually around 8-12 in most citrus. Each segment is surrounded by a fairly tough, continuous membrane and covered by vascular bundles that transfer nutrients for growing of the fruit. The interior of a segment consists of 2 major components, the juice vesicles and the seeds (Soule and Grierson, 1986). The juice vesicles are thin-walled and they constitute the juice within the vacuole of the cell.

2.1.2. Chemical Composition

The chemical composition of citrus fruits may vary as affected by many factors such as growing conditions, maturity, rootstock, cultivar and climate (Ranganna *et al*, 1986). The chemical profiles that are characteristic of particular citrus species can be used to detect the authenticity of citrus juices in quality control (Sass-Kiss *et al*, 2004). Some important chemical constituents in citrus fruits are:

a. Sugars

The main sugars present in citrus fruits are glucose, fructose and sucrose, which determine the sweetness of the juices (Kefford, 1966). Maturity is the main factor that affects the sugar content in citrus juices (Izquierdo and Sendra, 1993). The concentration of sugars in citrus fruits may range from less than 1% in certain limes up to 15% in some oranges.

b. Polysaccharides

The main polysaccharides present in citrus fruits are cellulose, hemicelluloses and pectic substances. Even though they are found in relatively small quantity, these polysaccharides play a role in adding to the body of the juice and hence, contributing to a desirable juice quality (Nagy and Shaw, 1990). Pectins present in citrus juice are important as a colloidal stabilizer in protecting juice cloud (Croak and Corredig, 2006)

c. Organic acids

The sourness of citrus fruits is imparted by the presence of organic acids, mainly citric and malic acids (Kefford, 1955). Other organic acids found in smaller quantities in citrus fruits are succinic, malonic, lactic, oxalic, phosphoric, tartaric, adipic and isocitric acids (Izquierdo and Sendra, 1993). The acid concentration in citrus fruits can be affected by maturity, storage, climate and temperatures

(Vandercook, 1977). The organic acids in citrus fruits are mainly measured as titratable acidity, which is expressed as grams of citric acid as per 100mL of juice (Ranganna *et al*, 1986). The concentration of citric acid in oranges may decrease with maturity and results in the decrease of acidity (Geshtain and Lifshitz, 1970).

d. Lipids

The lipids present in citrus fruits include simple fatty acids in the seed, phospholipids and complex lipids in the juice and the components of cuticle. They constitute about 0.1% of orange juice (Moufida and Marzouk, 2003). Some major fatty acids commonly found in citrus juices as reported by Nagy (1977a) are palmitic, palmitoleic, oleic, linoleic and linolenic acids. As different citrus varieties consist of different types of fatty acids, its profile can also be used as markers for various citrus species (Nordby and Nagy, 1971). The breakdown of lipids in citrus juices may contribute to the development of off-flavor (Nagy and Nordby, 1971).

e. Carotenoids

The colors of citrus fruits are mainly imparted by the presence of carotenoids (Stewart, 1977). It ranges from deep orange in red tangerines to light yellow in lemons. The complex mixture of carotenoids is located in the plastids of the flavedo and of the internal juice vesicles. Recent study on carotenoid composition of various citrus species by Agócs *et al* (2007) revealed that most citrus species, except lime, contain β-cryptoxanthin and lutein in considerable amounts. The carotenoids present in lime are mainly β-carotene and lutein (Agócs *et al*, 2007).

f. Vitamins

The main vitamin present in citrus fruits is ascorbic acid. The juice typically contains one quarter of the total ascorbic acid present in the fruit. Other vitamins present in citrus juices in various quantities include thiamine, riboflavin, niacin, pantothenic acid, inositol, biotin, vitamin A, vitamin K, pyridoxine, p-

aminobenzoic acid, choline and folic acid (Kefford, 1955; Ting and Attaway, 1971).

g. Inorganic elements

Generally, citrus fruits are rich in potassium and nitrogen, which accounts for about 80% of the total minerals (Izquierdo and Sendra, 1993). Other major inorganic elements found in citrus juices are calcium, iron, phosphorus, magnesium and chlorine (Nagy, 1977b). The concentration of these elements may vary depending on the geographical condition, maturity, seasonal variation and level of fertilization. Thus, the presence of these inorganic elements has been proposed of tracing the geographic origin of the citrus fruits.

h. Flavonoids

The flavonoids in citrus fruits are present in high concentrations and easily isolated. Some of them are useful for taxonomic markers while some have distinct taste properties and can be utilized as valuable by-products. The main 3 groups of flavonoids are flavanones, flavones and anthocyanins (Ranganna *et al*, 1986). Generally flavanones are mainly found in higher amounts while flavones and anthocyanins are relatively present in trace amounts. Hesperidin is the main flavonoid found in sweet oranges and lemon, while naringin is the flavonoid responsible for bitter flavor in grapefruit (Nagy and Shaw, 1990).

i. Volatile compounds

The volatile compounds present in citrus fruits impart the flavor of the citrus significantly. Their individual contribution and concentrations, as well as interactions among them, give characteristic odor to individual species (Izquierdo and Sendra, 1993). They are mainly present in the juice vesicles and in the oil sacs of the flavedo. Limonene is the major volatile compound found in citrus fruits.

j. Limonoids

Limonin is the only limonoid found in significant amount in citrus fruits and it imparts bitter flavor (Kefford, 1955). Limonin is not found in fresh fruits and is produced by acid and enzyme catalyses of limonoid acid A-ring lactone (Nagy and Shaw, 1990). This conversion normally takes place during juice storage or with heat treatment.

2.1.3. Uses of Citrus Fruits

2.1.3.1. Juices

Juice is the primary product obtained from citrus fruits (Braddock, 1999) and it is also one of the most important commodities. The juices produced from the citrus fruits are either in the form of single-strength or concentrated juices (Ting and Rouseff, 1986). The single-strength juice can be obtained directly from the fruit by adding water to the citrus concentrate, while in concentrated juice, water is removed from the juice in order to reduce the cost of transportation and storage. The citrus juices contain vitamins, minerals, carotenoids, sugars, organic acids, aminoacids, phenolics, nucleotides, enzymes, limonoids, lipids, proteins, pectins and other soluble and insoluble solids. The technology and choice of juice recovery methods play an important role in juice processing. Various extraction methods in juice processing are discussed profoundly by Braddock (1999). Among the citrus fruits, oranges and grapefruits are commonly extracted for their juices and they are widely consumed for their health benefits due to the content of nutrients and other bioactive compounds (McGill *et al*, 2004).

2.1.3.2. Essential oils

Another important product of citrus fruits is the essential oils extracted mainly from the peel. In order to obtain the oil, the oil-bearing sacs need to be punctured by either abrasion or scraping the surface of the peels (Redd and Hendrix, Jr., 1996). For its recovery, the oil is washed away from the peel as an aqueous emulsion and then separated from the water by

centrifugation (Ohloff, 1994). Hence, expression or cold- pressing method is frequently applied in extracting the oil, and the oil is commonly known as cold-pressed oil. The oil can also be extracted from the peel by other means, such as distillation by steam or water as well as extraction with supercritical or liquid CO_2. Cold pressed oils have finer aromas and greater stability than distilled oils due to the absence of heat during process and the inclusion of components, such as anti- oxidants (Wright, 2004). The types of citrus fruits from which their peel oils are recovered commercially are orange, grapefruit, tangerine, lemon and lime (Shaw, 1977). Some oil is also present in the juice, but in a relatively small quantity. The amount of oil in the processed juice should not exceed 0.015-0.02% by volume (Redd and Hendrix, Jr., 1996). Hence, excess oil will be removed from the juice by steam distillation in order to lower the juice's oil content for optimal citrus flavor. The essential oils contain many volatile compounds, mainly aldehydes, ketones, esters, alcohols and terpenes, which give the characteristic aromas and flavors of the citrus fruits (Kefford, 1955; Braddock, 1999). Citrus essential oils are greatly utilized as the flavorings in the food and beverage industries (Colombo *et al*, 2002), and as fragrance materials in the perfumery, toiletries, fine chemicals and cosmetic products (Buccellato, 2002; Baser and Demirci, 2007). Furthermore, citrus essential oils can also be used, to some extent, as a traditional medicine (Imbesi and De Pasquale, 2002).

2.1.3.3. Essence oil and aroma

During the process of juice concentration, some of the natural flavor compounds are also removed together with the water, including the small amounts of peel oil remaining in the juice. The volatiles recovered during the production of juice concentrates are called essence (Redd and Hendrix, Jr., 1996). The water-soluble portion of the essence is known as aqueous essence or aroma while essence oil or oil phase essence refers to the oil-soluble portion. Aroma and essence oil are commonly used as natural flavorings for citrus juice products as they contain many volatile compounds found in cold-pressed oil (Shaw, 1977).

2.1.3.4. Other citrus by-products

The main by-products of citrus processing are the peel, pulp and seeds, which account for 40-60% of the weight of the raw material (Licandro and Odio, 2002). These residues can be further processed into 3 main categories: animal feed, raw material used for further extraction of marketable products and food products. Although most of the citrus by-products are used for animal feed (Ting and Rouseff, 1986), there are many useful by-products made from different portions of the citrus fruits, such as pectin, dried pulp, molasses, marmalades, candied peel, peel seasoning, purees, beverage bases, citrus alcohol, bland syrup, citric acid, seed oil, flavonoids and other products (Kesterson and Hendrickson, 1958; Braddock and Cadwallader, 1992; Braddock, 1995; Hendrix, Jr. and Hendrix, 1996; Braddock, 1999; Licandro and Odio, 2002). In the past, by-products became the source of additional revenue for many citrus processors with low juice values (Braddock, 1995). Hence, the utilization of citrus by-products to produce more valuable products is getting increasingly important as future world citrus production increases and then surpasses the demand for citrus juices and beverage products. Furthermore, the future uses of citrus by- products will also need to expand beyond the current major use as low-value animal feed.

On the whole, the current rapid growth of the citrus industry is largely due to population increase and improved economic conditions in the consuming nations of the world, together with the rapid advance of agricultural sciences and technology for the production of by-products. The fact that citrus fruits is a rich source of essential minerals, vitamins and dietary fibers with its distinctive natural flavor and that the consumers are nowadays more nutrition-conscious, have also contributed to the increased demand for citrus fruits and their by-products.

2.2. Citrus variety

2.2.1. General classification

As a result of massive hybridization, there are literally thousands of citrus cultivars in the

world. Consequently, the taxonomic classification of citrus becomes quite complex with many diversities and is not universally agreed upon (Young, 1986). However, in general, citrus can be categorized into five major groups that are significant economically:

a. Sweet oranges (*Citrus sinensis* Osbeck)

Sweet orange, like most other citrus fruits, probably originated in the region between south-west China and north-east India and has been cultivated in southern China for several thousand years. There is speculation that the sweet orange is a natural hybrid of pummelo and mandarin but its true identity may never be known with certainty.

Sweet orange is grown throughout the world and provides the greatest fresh fruit production of any citrus groups (Young, 1986). It is round to oval in shape, orange colored, tight skinned and has a juice and sweet flesh. It can be eaten out-of-hand easily and is used as fresh ingredients in salads, in fresh juice and for juice concentrate. It can be sub-divided into four categories – round or common oranges, navel oranges, acidless oranges and blood oranges (Ortiz, 2002). Some popular cultivars of sweet oranges are Valencia, Jaffa, Mosambi, Pineapple, Hamlin, Washington navel and Shamouti.

b. Mandarins (*Citrus reticulata* Blanco)

like that of other citrus species the precise origin of the mandarin is far from certain but is believed to be either north-east India or south-west China. The mandarin has probably been cultivated in China for several thousand years, and the earliest reference to this fruit dates back to the 12th century BC.

From its region of origin, the mandarin spread throughout much of south-east Asia, and to other parts of India. By the tenth century AD the mandarin was widely cultivated in the southern prefectures of Japan.

Nowadays, mandarin ranks second in the citrus production worldwide and China is the largest producer of mandarins (FAOSTAT). Although the name tangerine is

used interchangeably with mandarin, tangerine usually refers to those varieties producing deep orange colored fruits (Webber, 1948). Mandarin is round in shape, sweet in taste, loose skinned and orange in color. Its segments are easily separable. It is used primarily for eating out-of-hand, in fresh juice, and to a limited extent for processing. It into four classes – Satsuma
group, Mediterranean mandarin, Tangerine or Clementine group and other mandarins, such as King mandarin (Ortiz, 2002). Some important commercial cultivars of mandarin groups are Dancy, Ponkan, Mikan, Owari and Temple.

c. Grapefruits (Citrus paradisi Macfadyen)

The origin of grapefruit is uncertain but recent studies suggest it is a natural cross between sweet orange and pummelo (or Shaddock) which occurred in the 1700s on Barbados in the west Indies (Morley-Bunker, 1999). It is believed pummelo seeds were taken to Barbados by an English man, captain Phillip Shaddock, around 1649, but it was not until 1823 that mention of the grapefruit was first recorded. Known locally as "the forbidden fruit", it was given the species name Citrus Paradisi in 1830.

Of equal uncertainty is the origin of the name Grapefruit: someone suggest the flavor resembles that of some grape, while others believe it is the way the fruits are borne in small clusters on the tree, rather than individually as with pummelos.

It is sweet, juicy, medium to large in size and has thick and spongy rind. It has few cultivars – white-fleshed, pink-fleshed and red-fleshed (Young, 1986). The commercial cultivars are prized as breakfast fruit and for salads and juice due to their refreshing flavor and mild bitterness. Examples of popular grapefruit cultivars are Marsh, Star Ruby, Ruby Red and Foster.

d. Lemons (Citrus limon Burmann)

Lemon constitutes an important fresh fruit group even though it is not eaten fresh as mandarins and oranges. They usually have high acid content although acidless

cultivars also exist (Ortiz, 2002). It is used primarily for drinks and fresh juice or lemonade, cooking and flavoring especially in the making of lemon pies, lemon cakes, candies, jams and marmalades, and also for medicinal purposes due to its high content of vitamins (Webber, 1948). The fruit is generally oval to elliptical with characteristic necks and nipples. The peel is yellow at maturity and has prominent oil glands. The flesh is pale yellow in color and very sour. There are three major groups of lemons: the Femminello, the Verna and the Sicilian groups (Morley-Bunker, 1999).

e. Limes (*Citrus aurantifolia* Swingle)

Lime is commonly used in limeade and carbonated beverages, and as a constituent of alcoholic drinks. They can also be used for pickling; for culinary purposes, such as flavoring for jellies, jams and marmalades; as a garnish, especially with

meats and fish; for medicinal purposes, especially in the treatment and prevention of scurvy; as well as a source of lime oil (Webber, 1948; Young, 1986). It is greenish-yellow in color and thin skinned. The juice is highly acidic. The two major groups include the acid and acidless limes of which the acid limes are of commercial importance (Davies and Albrigo, 1994). Two popular acid lime cultivars are Tahiti and Key (Mexican) limes.

On top of these 5 major groups, there are other citrus groups that are widely cultivated and important for various purposes, such as sour or bitter oranges (*Citrus aurantium* Linnaeus), pummelos (*Citrus grandis* Osbeck), citrons (*Citrus medica* Linnaeus), calamondins (*Citrus mitis* Blanco), bergamot (*Citrus bergamia* Risso), Kaffir lime (*Citrus hystrix* DC.) and kumquats (*Fortunella sp*. Swingle). Moreover, the feasibility of hybridization across various groups of citrus results in the emergence of many novel cultivars, and in some cases are difficult to identify (Ortiz, 2002). Some of these hybrids are tangelos (hybrids of grapefruits and mandarins), tangors (hybrids of mandarins and sweet oranges), orangelos (hybrids of sweet oranges and grapefruits), citranges (hybrids of trifoliate oranges and

sweet oranges), citrangors (hybrids of citranges and sweet oranges), limequats (hybrids of limes and kumquats) and other hybrid varieties.

2.2.2. Selected citrus cultivars grown in California

2.2.2.1. Navel orange

Navel oranges (figure 2.2) have the distinctive feature of having a small secondary fruit embedded in the apex of the primary fruit, and although this characteristic is sometimes found in other oranges and particularly in mandarins, it is never consistent and varies depending upon climatic factors (Saunt, 1990).

Generally speaking, navels are the earliest maturing of orange varieties, producing seedless fruit of larger size than most others, with deep orange, easily peeled rinds, and a rich, sweet and pleasant flavor.

However, there are serious limitations to their production since the trees are less vigorous and less productive than those of many other varieties, and they are far more specific in their climatic adaptability. For example, navels thrive and produce superior quality fruit only in sub-tropical, mediterranean-types climates and are unsuited to many regions where other orange varieties perform well (Saunt J., 1990).

In contrast with Valencia oranges, which are grown in many citrus-producing environments and are one of the mainstays of production in tropical as well as semi- and sub-tropical regions, navels are far more restricted in their distribution. However, they are important in many countries worldwide and form a significant proportion of the citrus production of Spain, Marocco, Turkey, South africa, California, Australia, Uruguay and Argentina.

While navels are rarely equalled, and never surpassed, by other oranges as a dessert fruit, they also have characteristics other than poor climatic adaptability which prevent their more widespread production. Although navel oranges yield less juice than most other oranges, it is the development of delayed bitterness in the juice which makes them unsuitable for processing. Unlike the bitterness in grapefruit caused by the compound naringin or in sour oranges by neohesperidin, bitterness in navel orange juice becomes evident only when the fruit is juiced and the bitter factor limonin is released from other closely related

compounds. Although navel juice contains only extremely low levels of limonin, it is a very bitter compounds which most people are able to detect at levels of no more than around 5-6 ppm. For this reason navel orange juice cannot usually be used for the preparation of juice products unless it is first blended with juice of other varieties of low limonin content (Saunt, 1990).

For some time it was widely believed that the navel orange we know today originated as a limb sport on a tree of the old-established Salata variety at Cabulla near Bahia (now Salvador), Brazil, some time prior to 1822. There is now much evidence to disprove this theory, for navel oranges are known to have grown in Spain and Portugal for many years prior to 1822 and it seems more likely that they were first brought to Portugal from China and thence to Brazil much earlier than this. However, the worldwide expansion in navel orange growing started only after the Bahia navel was sent in 1870 to the United States Department of Agriculture's facilities at Washington DC, for propagation in glasshouses before being sent to California and Florida in 1873. It was from this importation that the Washington navel spread to the other citrus areas.

Navels are genetically far more unstable than other leading orange varieties, with the result that countless selections have been made by growers and others in many parts of the world during the past century. Many have fruit characteristics which are almost indistinguishable from the Washington navel but a few have markedly different traits particularly with respect to time of maturity. It is now possible in many navel-growing regions to extend the season from the normal two or three months to six months and sometimes longer. In California, for example, the harvesting season normally extends over a four- to five-month period with the Washington variety because of the several climatic zones in which navels are grown (Saunt, 1990).

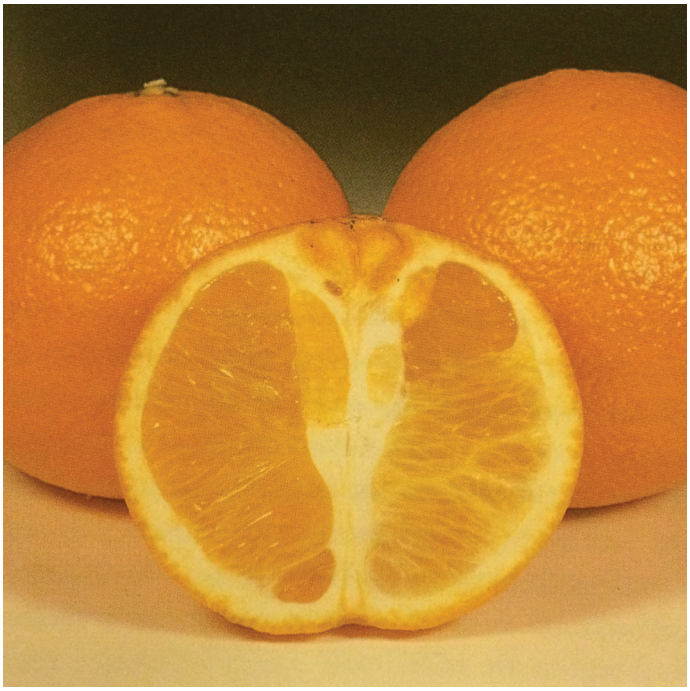

Figure 2.2. *Navel oranges*

2.2.2.2. Valencia orange

It is commonly assumed, perhaps understandably considering the name, that the Valencia is of Spanish origin. However, the variety first became of interest in the Azores and is almost certainly of old Portuguese origin (Saunt, 1990).

No other citrus variety has a more fascinating and improbable history than that of the Valencia, which is now the world's most important orange (**figure 2.3**). Sent from the Azores in the early 1860s to Thomas Rivers, a nurseryman at Sawbridgeworth, England, it was first named Excelsior. Rivers has recognized its late maturing characteristics and believed it suitable for growing in containers in the fashionable orangeries of country houses.

He sent trees of the Excelsior and other varieties to S.B. Parsons, Long Island, USA, in 1870, who in turn supplied both A.B. Chapman of San Gabriel, California, in 1876 and F.H. Hart, Federal point, Florida, the following year. Chapman named the variety Rivers Late, while Hart's trees were initially designated as Hart's Tardif (or Hart Late).

In 1887 Rivers Late was renamed Valencia late at a suggestion of a Spanish citrus expert visiting California who believed that it bore great similarity to a late maturing orange grown in the Valencia region. It was a decade or so later than authorities recognized that the Hart Late and Valencia Late were in fact the same variety (Saunt, 1990).

Its outstanding qualities were soon recognized and the Valencia Orange was to change the face of citrus production on a world scale so that today it is the leading variety in many citrus-producing countries and an important one in others. There is no other citrus variety more widely grown and on such an extensive scale. The Valencia leads production in Argentina, Australia, California, Florida, Morocco, southern Africa, Uruguay and other countries and is an important variety in Brazil, Israel and elsewhere. Somewhat surprisingly, it has not been extensively planted in Spain, but Valencia production is now increasing and has replaced the Berna as the principal late maturing variety, although it still accounts for only 8% of the country's annual orange crop (Saunt, 1990).

Valencia trees are vigorous, upright, large and very prolific but have some tendency to alternate-bearing.

Fruit size is medium to large, roundish-oblong in shape, with a well-colored, moderately thin rind of smooth, but sometimes finely pebbled, texture. Valencia rind is prone to creasing particularly on some rootstocks. Not difficult to peel when fully mature, the rind is thin and leathery and the flesh well colored, with a very high juice content of outstanding color and good flavor although sometimes slightly acidic except when fully mature. Seeds typically number two to four per fruit. It is the latest maturing of all sweet orange varieties (with the exception of Natal in Brazil) and often hangs late into the summer of the following season without losing quality except that the rind may regreen somewhat while still on the tree.

Moreover, the later it is picked, the smaller the next year's crop because of the "two crops on the tree at one time" phenomenon.

In tropical region, the rind, like that of other citrus varieties, never attains good color and is often greenish, extremely thin and tightly adhering while the flesh and juice are a paler orange than that of Valencia fruit produced in sub-tropical Mediterranean climates. Valencia

juice has excellent processing characteristics, including a deep orange color, and the fruit ships and stores exceptionally well.

There are several clones of the Valencia, some of which have been given separate variety names. The most common improved selections are all thought to be nucellar in origin; they are Olinda, Cutter, Frost. Other selections have been evaluated but have not gained more than local popularity – Armstrong and Campbell in California, Lue Gim Gong in Florida, Ksiri in Morocco and Harwood in New Zealand (Saunt, 1990).

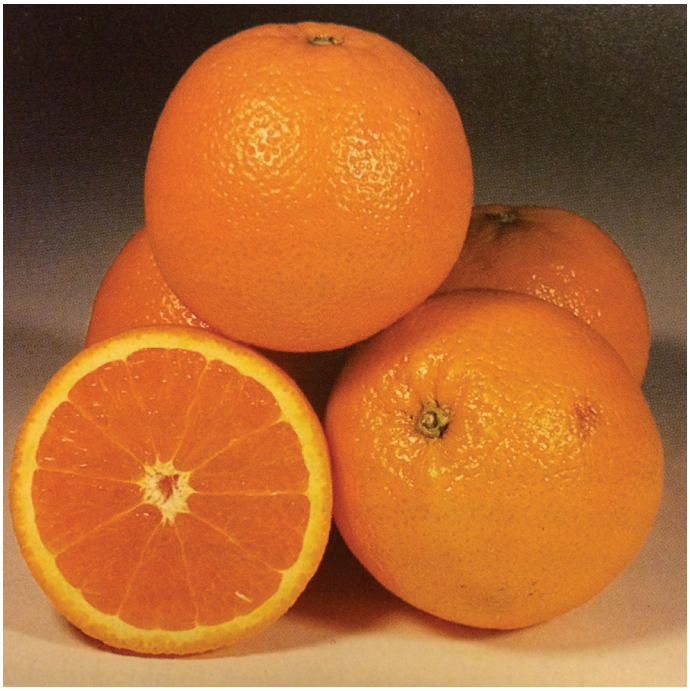

Figure 2.3. *Valencia oranges*

2.2.2.2. Pink Star Ruby grapefruit

This variety was produced by irradiating seed from the Hudson variety by R. A. Hensz, Texas A&I University, Weslaco, Texas, in 1959. Flesh of the Star Ruby is slightly redder

than Hudson and it remains the most heavily pigmented grapefruit yet developed. Furthermore, external pigmentation was also enhanced and is unsurpassed by more recent

selections. Although the internal color fades during the season, it remains outstandingly strongly pigmented until the end of the season (figure 2.4) (Saunt, 1990).

In addiction to these two good and important characteristics, Star Ruby is almost invariably seedless, rarely having more than one or two seeds in a minority of fruits. It has an extremely thin rind, a very high juice content and a sweeter, less bitter flavor than Marsh and other pigmented varieties. It is the standard pigmented grapefruit against which all others are measured.

However, it is evident that the irradiation had deleterious as well as beneficial effects on the genetic make-up of the variety, since it has been found under many conditions worldwide to be the most problematic of all grapefruit trees to grow. It is slow growing and develops a rather compact, stunted, bushy growth habit. In addiction, it is extremely susceptible to foot rot and is herbicide-sensitive, as well as developing stem-pitting disease (CTV) at a far earlier age than Marsh and Ruby. It exhibits excessive sunburn-sensitivity in hot desert areas. Whereas most grapefruit varieties may successfully be stored for several months, Star Ruby is particularly prone to Diplodia stem-end rot after no more than a few weeks. Because Star Ruby trees are often lacking in vigor, fruit size is affected, with the result that small fruit predominate. This is a serious disadvantage since the significant premiums paid for this and other well-pigmented grapefruit varieties are restricted almost entirely to large size fruit (Saunt, 1990).

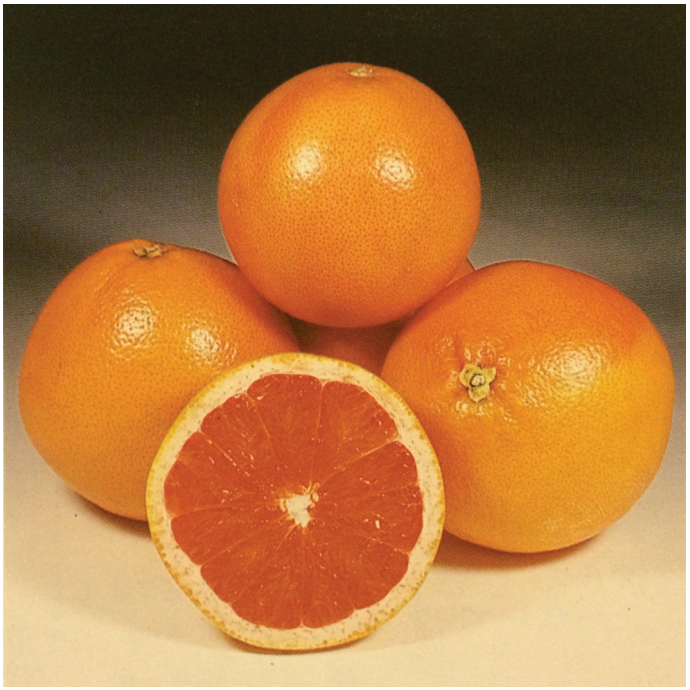

Figure 2.4. *Star Ruby grapefruits*

2.3. Limonoids

2.3.1. General characteristics

Limonoids are highly oxygenated triterpenoids present in the rutaceae and Meliaceae family plants (Hasegawa, 2000). Limonin, the first characterized compounds of this group of phytochemicals, has been known as a constituent of Citrus since 1841(Bernay,1841). It was isolated from Navel orange juice in 1938 and shown to be the bitter principle in Navel orange juice in 1949 (Highby, 1938; Emerson, 1949). The structure of limonin remained unknown for more than 120 years after its discovery. Its structure was finally determined by a combination of chemical methods and X-ray crystallography in the 1960s (Arigoni, 1960; Barton, 1961, Arnott and Watson,1960-1961).

Limonin's chemical composition is $C_{26}H_{30}O_8$ with a molecular weight of 470 (Yuan, 2009a,b).

Limonoids are important quality constituents of citrus fruits and have been shown to possess biological activities. One of the roles limonoid aglycone may play in the plant is as pest deterrent. They are abundant in young leaves and fruit when these tissue need to be protected from pathogen attack.

In 1973, a comprehensive study of the taste thresholds of limonin was performed by Guadagni *et al* Where a carefully screened panel of judges were able to detect a bitterness taste threshold level at 6 ppm of limonin.

Limonin bitterness is especially acute in juice obtained from early season fruits, such as Navel oranges. Juice from early to mid-season navel oranges can contain as much as 25 ppm of limonin. Grapefruit also has significant levels of limonin, an average of 15 ppm or more in the early season (Maier, 1977).

Research on the chemistry and biochemistry of citrus limonoids has made significant progress in recent years, providing new information about limonoids.

Progress is being made on a genetic engineering solution to the problem of limonin bitterness in citrus juices. The limonoids are also proving to be important compounds in the human diet (Hasegawa, 2000).

2.3.2. Analysis of limonoids

Dreyer made several initial significant contributions to the field of limonoid analysis, including a TLC analysis for limonin detection and the use of NMR for determination of limonoid structure. These two methodologies were used by Hasegawa and Bennett (1989) to isolate and identify 30 additional limonoid aglycones and 20 limonoid glucosides from citrus and its allied species.

The major analytical techniques for the detection and quantitative analysis of limonoids are HPLC introduced by Fisher, radioimmunoassay introduced by Mansell and Weiler, and HPLC-MS by Manners and Hasegawa.

2.3.3. Delayed bitterness in citrus juice

Citrus fruits are accepted for their nutritive and medicinal value as well as for providing distinctive flavor to a wide variety of food products, making it very popular among food product designers. But, a major problem in the citrus industry worldwide is the formation of bitterness in citrus juice and products within hours after extraction of juice. This bitterness occurs in certain varieties of oranges, grapefruits and lemons having a significant negative impact commercially.

Cause for bitterness of citrus fruits has been attributed to the presence of limonin, nomilin and to some extent to ichangin.

Navel oranges in general do not taste bitter if eaten fresh or if juice is squeezed from the fruit and consumed immediately. However, the juice becomes bitter within a few hours after juicing at room temperature or overnight if stored in a refrigerator. This delayed bitterness differentiates limonoids bitterness from flavanone neohesperidoside bitterness which occurs in citrus cultivars related to pummelo. Among 36 limonoid aglycones isolated from citrus and its hybrids, only six are bitter. Limonin is the major limonoid found in most citrus fruit juices and is the major cause of delayed bitterness. Nomilin is also involved, but its role is very minor.

The mechanism of the delayed bitterness was not fully understood until 1968. The precursor theory was first put forth by Higby (1938), after he first isolated limonin from Washington navel orange juice. Over the years evidence to support the precursor theory has accumulated (Emerson, 1949). Mayer and Beverly (1968) finally identified limonoate A-ring lactone as the precursor of limonin in citrus fruit. A ring-closing reaction proceeds under acidic conditions below pH 6.5 and is accelerated by the enzyme, limonin D-ring lactone hydrolase. (Fig. 2.5).

The delayed bitterness is an important economic problem in commercial citrus juice production. It lowers the quality and value of commercial juices and has significant negative economic impact to the citrus industry.

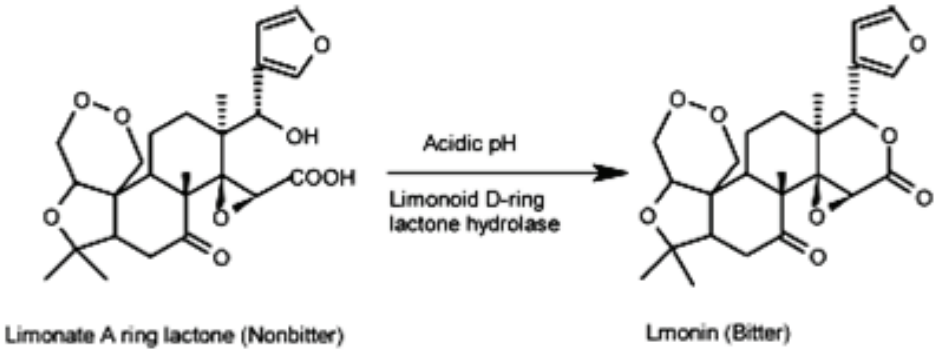

Figure 2.5 *Mechanism of delayed bitterness (Mark et al, 2000)*

2.3.4. Properties

Limonoids are of moderate polarity, insoluble in water and hexane but soluble in hydrocarbons, alcohol and ketone; they are mostly bitter in taste and account for the scent of fresh peels of citrus fruits. Limonoids are present in neutral (noncarboxylated aglycon) as well as acidic (carboxylated glucoside) forms, the former are insoluble and bitter while latter are soluble and tasteless. Chemically they are highly oxygenated triterpenes, classed as tetranorterpenoids. They present, perhaps the most extreme examples of oxidation of triterpenes in nature (Waterman P.G., 2001).

2.3.5. Distribution

Although hundreds of limonoids have been isolated from various plants but, their occurrence in the plant kingdom is confined to only plant families of order Rutales and that too more abundantly in Meliaceae and Rutaceae, and less frequently in Cneoraceae and *Harrisonia* sp. of Simaroubaceae. The limonoids occurring in Meliaceae are also known as meliacins. Out of over 300 limonoids known to day, about one-third is accounted by neem (*Azadirachta indica*) and Chinaberry (*Melia azedarach*) alone (Waterman, 2001) (Suarez, 2002).

Citrus fruits and its closely related genera contain about 36 limonoid aglycones and 17 limonoid glucosides. (Hasegawa, Miyake, 1996). Citrus limonoids and their glucosides, the water-soluble triterpenoid compounds that occur naturally in citrus fruit and citrus juice in

amounts comparable to vitamin C, can be reclaimed from citrus processing and citrus seeds as by-products in large quantities. Limonin glucoside is the most abundant of the limonoid glucosides in citrus. (Manners, 2003) Azadirachta indica (Neem tree) a species of meliaceae family is a storehouse of limonoids containing more than hundred different limonoids and their derivatives in its different plant parts. (Kraus, 1995) Other important sources of limonoids in meliaceae family are *Cedrela* sp., *Khaya* sp., *Melia azedarach*, *Sandoricum koetjape*, *Swietenia mahogany*, *Trichilia* sp. and *Turraea* sp (Suarez, 2002).

In mature fruit tissue, glucosides, predominantly limonin glucoside, accumulate while in seeds both aglycones and glucosides, predominantly nomilin glucoside, are found (Figure 2.6). The limonin glucosides stored in the fruit tissue are very stable. Hence fresh tissue and freshly squeezed juice do not taste bitter. However, tissues and seeds were crushed during commercial juice processing release bitter aglycones and glucosidases hydrolyzing limonoid glucosides to bitter aglycones or crashing may release this P-glucosidase activity in the juice (Mark *et al*, 2000). These may increase the level of the bitter aglycones, such as limonin, by hydrolyzing the tasteless limonoid glucosidase.

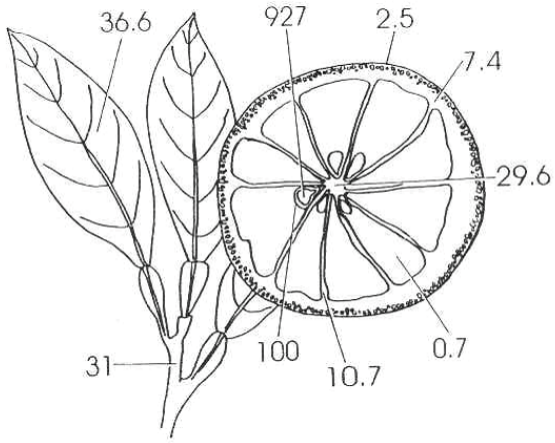

Figure 2.6. *Distribution of limonin in a fruit and vegetable part as µm/100 mg (Nagy et al, 1977)*

2.3.6. Chemistry and biosynthesis

Limonoids are stereochemically homogenous compounds, with a prototypical structure either containing or derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton; all naturally occurring citrus limonoids contain a furan ring attached to the D-ring, at C-17, as well as oxygen containing functional groups at C-3, C-4, C-7, C-16 and C-17 (Okamura *et al,* 1997)

Most of the biogenetic proposals are tentative as they are not supported by valid biosynthetic studies and there is only one instance of biosynthetic investigation in neem that of nimbolide in neem leaves. The triterpenes containing a C, side chain at C-17 are supposed to be biogenetic precursors of meliacins and hence are known as protolimonoids or protomeliacins or melianes. Meliantriol was the first tetracyclic triterpenyl alcohol biogenetically related to 20 (*S*)-tirucallol, isolated from both neem oil and the fresh fruits of *Melia azedarach*.

Endo *et al* (2002), Bagge D (1998), Waterman (2001) and Suarez *et al* (2002) have illustrated the biosynthesis of limonoids showing that limonoids are synthesized *via* terpenoids biosynthetic pathway, starting with cyclization of squalene, which results into a tetracyclic ion. Oxidative degradation at the C-17 side chain of either of these nucleus results in loss of four carbon atoms and formation of bi-substituted furan, further oxidations and skeletal rearrangements in one or more of the four rings, which are designated as A, B, C and D (as shown in Fig. 2.7), gives rise to different groups of limonoids and each group consist of number of limonoids possessing a variety of biological activity into their triterpene skeleton. It may be mentioned here that only plants belonging to the family Meliaceae specialize in the production of C-seco meliacins.

Major citrus species accumulate limonin, nomilin, obacunone and deacetylnomilin; *Citrus ichangensis* and relatives accumulate ichangensin; *Fortunella* and related species accumulate calamine group limonoids such as calamine and cyclocalamin. Limonoid aglycones are endogenously converted into tasteless limonoid glucosides during fruit maturation (Endo *et al,* 2002). Recently a method combining solid-phase extraction and

reversed-phase high-performance liquid chromatography has been described for the isolation of two key metabolites, limonoate and nomilinoate A-ring lactones, in the limonoid biosynthetic pathway critical to citrus quality (Breksa *et al,* 2005).

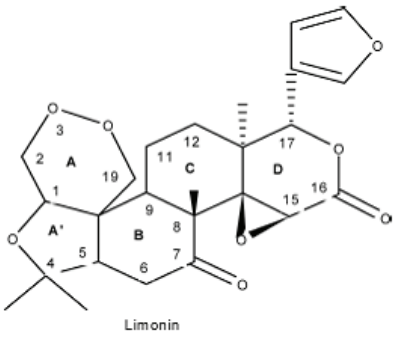

Figure 2.7 Structure of limonin (Maier *et al,* 1980)

2.3.7. Bioavailability of limonoids

Manners *et al* (2003) utilized liquid chromatography/mass spectrometry (LC-MS) to analyze the plasma of four groups of four healthy male and female subjects, administered high doses of pure limonin glucoside, for the presence of limonin to establish the absorption, metabolism, and bioavailability of citrus limonoids to humans. The plasma analysis revealed increasing amounts of limonin associated with increasing doses of limonin glucoside among the subject groups in mean maximum concentration amounts ranging from 1.74 to 5.27 nmol/l. They also observed a high degree of variability in the analyzed limonin concentration within the subject groups. The mean time to maximum concentration was 6 h. A second compound with MS/MS characteristics identical to limonin was detected in amounts up to 5.13 nmol/l and is hypothesized to be a limonin epimer. Post-study health evaluation established no ill effects among study subjects consuming high doses of limonin glucoside.

2.3.8. Anticancer, antiplasmodial, antiviral, and antimicrobial activities

Many experimental evidences have revealed that limonoids present in citrus fruits and their

juice have cancer chemopreventive property, limonoids have been shown to inhibit the growth of estrogen receptor-negative and -positive human breast cancer cells in culture, limonoids have also been found to target and stop neuroblastoma cells (Jacob *et al,* 2000; Poulose *et al,* 2005, 2006; Miller *et al,* 2004; Tian *et al,* 2001). Hesperidin, other flavonoids, limonin 17-beta-D-glucopyranoside, and other limonoid glucosides are potential chemopreventive agents in orange juice that could account for the decreased colon tumor-genesis associated with feeding orange juice (Miyagi, 2000).

The citrus limonoids obacunone, limonin, nomilin and their glucosides and some aglycones inhibit chemically induced carcinogenesis and a series of human cancer cell lines, with remarkable cytotoxicity against lung, colon, oral and skin cancer in animal test system and human breast cancer cells (Silalahi, 2002; Berhow *et al,* 2000; Tanaka *et al,* 2000, 2001). Pure limonin glucoside and limonin, its water insoluble relative lacking glucose, have been found to possess significant anti-tumor properties in animal tests and with human cells (Manners *et al,* 2000, 2003). All these studies have reported the lack of toxicity of the limonoids in mammals and also have presented their modifying effect on the development of aberrant cryptofoci, as well as ability of these compounds to induce specific carcinogen-metabolizing enzymes, glutathione S- transferace and quinine reductase in the liver and mucosa of the small intestine to detoxify chemical carcinogenesis.

Nutritional research on health benefits of chemicals present in plant foods advocate that citrus limonoids possess substantial anticancer activity and they are also free of any toxic effects in animal models (Jacob *et al,* 2000).

Guthrie *et al,* (2001) were awarded a patent, recently, for proposing composition and methods for treatment of neoplastic diseases with limonoids in combination with flavonoids and tocotrienols.

A large number of studies demonstrated that nonnutritive dietary bioactive compounds derived from fruits and vegetables showed antiproliferative activities through different mechanisms of action.

Among them, limonoids have attracted the scientists' interest. A recent study (El-Readi *et al*, 2010) investigated the P-gp reversal activities of limonin and deacetylnomilin, isolated

from *C. jambhiri* and *C. pyriformis* (Rutaceae) in human leukemia cells, and their potential cytotoxicity against this cell line, its parental cell line CCRF-CEM and Caco-2, which is used as a model for intestinal epithelial cells with a relatively high expression of *P-gp/ MDR1* gene.

Limonin, nomilin, deacetylnomilin, and obacunone, and their glucosides were tested for the potential effects against two human cancer cell lines, (Kim *et al*, 2009). Neuroblastoma cells were more sensitive than colon carcinoma cells. Although micromolar levels of both aglycones and glucosides arrested cell growth, biochemical and morphological data showed that the glucosides induced a more rapid cell death.

Malaria is one of the major parasitic diseases in the tropical and subtropical regions of the world, and its etiological agents are protozoans of the genus *Plasmodium*. Several classes of natural products, including limonoids, were studied for the treatment of malaria.

Four naturally occurring limonoids, including limonin, were isolated from *K. anthotheca* and tested for their potential antimalarial activity against *P. falciparum* (Lee *et al*, 2008).

All isolated limonoids influenced parasite development. In fact, the result of an assay of development that measures the formation of new ring-stage parasites after 48 hours of incubation with anthothecol, gedunin, limonin, and obacunone exhibited antimalarial activity, with IC_{50} values ranging from 2.7 to 0.14 mM.

2.3.9. Structure Activity Relationships (SAR)

Madyastha and Venkatakrishnan, (2000). have described the studies carried out on the structure–activity relationships amongst limonoids, showing that limonoids with an intact apoeuphol skeleton, a 14, 15 b epoxide, and a reactive site such as either a 19—28 lactol bridge or a cyclohexanone 'A' ring are biologically very active and absence of these structural features results in reduced activity; *C*-seco limonoids with an enone system in ring 'A' are potent cytotoxic and anti-malarial agents, in some of these (*e.g*. nimbolide 5,28-deoxonimbolide and gedunin) a ,b -unsaturated ketone in ring 'A' has been proposed as common feature that is primarily responsible for their biological activity. They further say that the *C*-seco limonoids are two to three times more active than other limonoids and they

are highly active against herbivorous insects. Data from the studies conducted by Miller *et al* (2004), have suggested that certain rings in the limonoid nucleus may be critical to anti-neoplastic activity. Changes in the A ring of the limonoid nucleus can lead to a loss of anti-cancer activity, whereas changes in the D ring can be tolerated without any apparent loss of biological activity (fig. 2.7).

Studies carried out on azadirachtin and some of its derivatives as insect feeding deterrents that revealed that neither hydrogenation of Δ^{22} double bonds nor deacetylation caused any change in effect but blocking of hydroxyl group affected the feeding inhibitory activity, while acetylation of azadirachtin caused a decrease in the activity to 75%, etherification with a bulky trimethylsilyl group eliminated it altogether. Thus, the stereochemical environment around hemiacetal region seemed to be critical for its activity (Devakumar, 1996).

On structural modifications and screening the new products for insect feeding deterrent action following conclusions were derived: even a simple analogue retaining the hydroxy-dihydrofuran portion of the molecule was 50—60% as active as azadirachtin. Compounds showing gross structural rearrangements of this portion were less active. Considering the structural homology with salannin, the uniquely high level of activity of azadirachtin apparently stems from the hydroxydihydrofuran portion of the molecule (Devakumar, 1996).

In structure–activity studies of limonin, it has been determined that the furan ring and epoxide groups in the citrus limonoid structure are critical for the antifeedant activity of the limonoids against Colorado potato beetle larvae (Danielson, 1996).

Ruberto *et al* (2002), evaluated the antifeedant activity of citrus-derived limonoids limonin, nomilin, and obacunone and their semisynthetic derivatives against a commercially important pest, *Spodoptera frugiperda*. These conversions focused on functional groups considered being important for the biological activity, namely the C-7 carbonyl and the furan ring. In particular, reduction at C-7 afforded the related alcohols, and from these their acetates, oximes, and methoximes were prepared. Hydrogenation of the furan ring was also performed on limonin and obacunone and on comparison with previously reported data it

showed that insect species vary in their behavioral responses to these structural modifications. Highly significant antifeedant activity for two natural (limonin and obacunone) and three semisynthetic limonoids (Umonol, Umonin-7-oxime, and Limonin-7-oxime acetate) was observed against *S. frugiperda*.

2.3.10. Additional Findings

The health benefits of limonoids have made the scientists to find methods to synthesize them in laboratory (Fernandez-Mateos *et al*, 2002). Patents have been obtained for industrial scale method for manufacturing limonoid glucosides contained in citrus fruit (Hasegawa and Miyake,). Scientists are trying to design food products, fortified with limonoids to provide prophylactic benefits against cancer and many other diseases (Charleston, 2002). Methods are also been established to purify limonoids (Sunthanont *et al*, 2002) and increase their yields through better extraction procedures but caution has been advocated in consumption of limonoids as they may interfere with activity of other drugs (Lienet *et al*,) or may even produce harmful effects if consumed in very high quantities (Gibbins *et al*, 2004). But except for a few exceptions in most of the studies long term consumption of limonoids have produced no adverse effects and have been found to be safe (Manners et al, 2003). It has also been suggested that limonoids may interact with other bioactive components present in fruits and vegetables and may reduce the risk of degenerative diseases, hypertension, cataract, and stroke and in particular cancers (Silalahi, 2002).

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PAPERS IN PRESS

Chapter 3

Bitter Compounds from Citrus Byproducts

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A local citrus industry (Ortogel S.P.A.) has entered a line of debittering in the cycle of production of dried fibers in order to make them suitable for human consumption using a simple, fast and environmentally friendly method.
Preliminary tests carried out in laboratory and the consecutive industrial scale-up, established the proper dwell time and ratio between peels and extractive alkaline solution.
This paper describes the performance of the debittering line of the Ortogel plant by analysis of the polyphenols and limonin contents.
In order to verify the extraction degree of bitter compounds, the final fiber and the main liquid fractions from the straining processes after contact with the alkaline solution were analyzed.
The limonin extraction method was fast, effective and solvent-free in the extraction of limonoids from orange by-products. Furthermore, it was possible to produce good dietary fiber from oranges, characterized by good color and neutral taste.
Since the knowns medical activities of studied bioactives, this paper also investigated about the possibility and advantage to recovery the extracted limonoids.

KEYWORDS: Human nutrition, blood oranges, byproducts, limonin, debittering, HPLC, fiber.

1. INTRODUCTION

Limonoids are triterpene derivatives from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton found in plant families such as Rutaceae, in particular in *Citrus* species, and Melicaceae.

Citrus limonoids were considered a major problem for the *Citrus* juice industry as they cause delayed bitterness of the juices at room temperature, thus lowering the quality and value of the commercial juice (Pifferi *et al*, 1993), (Tundis *et al,* 2014).

Bitterness in citrus fruits is mainly attributed to the presence of limonoids (triterpenes) and flavanone glycosides (flavonoids) such as limonin and naringin, respectively (Ribeiro *et al,* 2002). Bitterness from limonin develops gradually in fruit juices, a phenomenon referred to as "delayed bitterness" (Hasegawa, 1989). citrus bitterness, which was a major factor contributing to losses of up to $90 million in California from 1992 to 2006, limits marketability; certain commercial citrus varieties (e.g., navel orange) are primarily used as table fruits instead of juice sources (Manners, 2007). In Sicily, the main producer of citrus products and byproducts in Italy, the limonin content of blood orange cultivars is approximately 18 ppm (Scordino *et al,* 2005), which constitutes a problem for cattle feed that contains citrus byproducts. Furthermore, citrus byproducts (approximately 350,000-420,000 ton/yr) represent an environmental and economic problem because pulp, pulp wash, and yellow water are difficult to dispose of. Additionally, citrus byproducts are relatively resistant to microbial degradation (high COD and BOD_5 indexes) due to their high content of bioactive compounds with antimicrobial activity, e.g., ascorbic acid, limonoids, and polyphenols (Todaro *et al,* 2013).

Several studies have focused on the extraction of limonoids from citrus juice and citrus pulp (for human and animal consumption, respectively) to reduce bitterness; few studies have focused on the in vitro recovery of limonoids from peel waste. Citrus byproducts are good sources of limonoids, especially limonin and nomilin. Limonin synthesis takes place at low pH in a reaction catalyzed by limonin D-ring lactone hydrolase (Manners, 2007). At 6 ppm, limonin confers a bitter taste to juices and citrus byproducts (Guadagni *et al,* 1973). researchers have attempted to remove limonin from juices and molasses (Pifferi *et al*, 1993; Bianchi *et al*, 1995). Currently, the main limonin extraction method involves the use of an organic solvent. Recently, Liu *et al* (2012) extracted limonin using an alkaline solution; however, the authors did not report the optimum extraction conditions such as pH, solid-solvent ratio, and temperature.

In order to product a fiber suitable for human nutrition, a local citrus industry (Ortogel S.P.A.) has entered a line of debittering in the cycle of production.

This paper describes the performance of the debittering line of the Ortogel plant by analysis

of the polyphenols and limonin contents.

2. MATERIALS AND METHODS

2.1. Set-up of debittering line.

As already specified, this research project is based on the possibility to convert the by-products of industrial processing of citrus fruits in a resource trying to turn it into dietary fiber.

Assuming the water can eliminate the sugar content but it is not enough to remove the bitter compounds (flavonoids and limonoids), it was decided to modify the already existing Ortogel plant based on debittering treatment of peels by an aqueous alkaline solution.

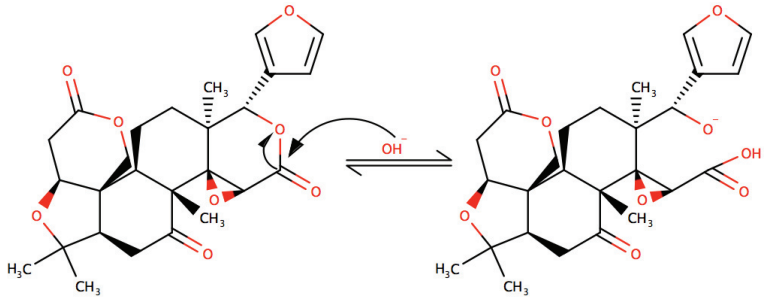
It is a known fact that the responsible compounds for the bitter taste of citrus peels are soluble in alkaline environment based on the D ring opening of the limonin (scheme 1).

According to some test performed before to insert the debittering stage, it was needed to increase the peels washing stages to remove the still too high contents of pectin and sugar in order to moderate viscosity and gelation issues.

The debittering stage consisted of:

- dosage systems of alkalizing solution;
- dwell reservoirs equipped with an improved stirring system;
- a solid-liquid separation system consisting of refiners and presses to drain as much as possible the bitter alkaline solution from solid.
- an automatic acid dosing to bring back the peels to a initial pH value.

In order to set-up the debittering line, preliminary dwell time and peel–to-water (pH=10) ratio tests and the consecutive industrial scale-up carried out. The dwell time was determine by alkaline treatments a pH=10 for different times (5, 10, 15, 20, 25, 30, 35, 40 min.), while alkaline solution-to-solid ratios were tested for 1:2, 1:3, 1:4, 1:5, 1:10 ratios.



Scheme 1. Alkaline extraction of the Limonin: Predicted reaction mechanism based on the D ring opening

2.2. Samples. Different samplings were supplied from a continuos line of debittering of a citrus local industry (Ortogel S.P.A.). Samples were analyzed for pH, polyphenols and limonin in order to verify the extraction level. Unless otherwise specified, all reagents were obtained from Sigma.

2.3. pH. The portions derived from debittering line were centrifuged at 15000 rpm for 15 min and the supernatant was analyzed for pH (data not reported);

pH misure is most important because the following analysis of the limonin and polyphenol content requires an adsorption step through a C18 cartridge that is dependent of solubility of the bitter compounds (scheme 1); for this reason the collected fractions have to be corrected at a final pH of 5 using the minimum quantity of HCl or KOH to avoid excessive dilution.

pH was analyzed using a Beckman 720 pHmeter in combination with a glass-body pH electrode with BNC connection.

2.4. Limonin extraction and analysis. The extraction of the limonin from citrus peel, pulp and seeds (pastazzo) was previously conducted directly at the Ortogel during the set up of debittering stage (Todaro *et al,* 2013) by an extraction in alkaline solution.

For the analysis of limonin, each sample after pH correction at 5, was centrifuged and filtered; an aliquot of supernatant was passed through a C18 Sep-Pak cartridge previously activated with CH_3OH and H_2O (Waters Milford, MA) to accumulate limonoids and polyphenols.

The cartridge was washed three times with water and pushed with $CH_3CN:H_2O$ (30:70) in order to obtain the flavanones first and subsequently with $CH_3CN:H_2O$ (50:50) to obtain limonoids, in particular limonin.

The limonin was quantified by HPLC according to a modified method reported by Van Beek and Blaakmeer (1989). The HPLC system was comprised of a Shimadzu LC-10A (Japan) in series with a Shimadzu photodiode detector (SPD-M-10A).

Standards and samples (20 µL) were injected on a C18 Altima ODS Hypersil column 250 × 4.6 mm I.d. (Milan, Italy) equipped with a guard column of the same material and maintained at 30°C.

The flow rate was 1.0 mL/min and an isocratic solvent composition of acetonitrile, water and isopropyl alcohol (60:30:10) was used.

The samples were filtered through PTFE filters (0.45µm) prior to HPLC analyses. The determinations were carried out in triplicate and the identification of limonin was performed by comparing the retention times of the sample (RT) with those of standards (Breksa *et al*, 2008; 2009). The method used allows to discriminate limonin and limonoids, from flavanones (Fig.1).

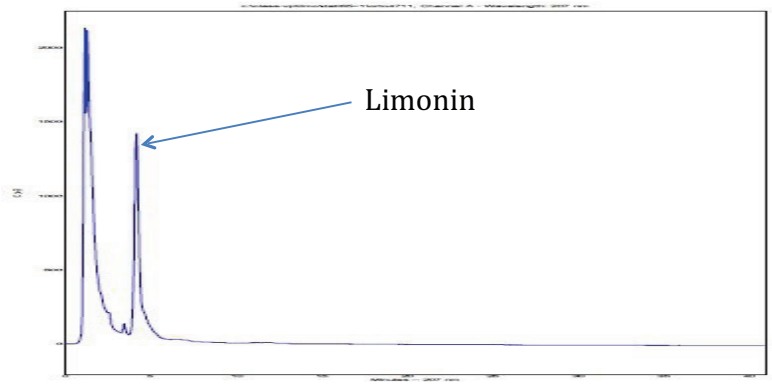


Fig. 1. HPLC chromatogram of the limonin in the sample named cloudy detected at 210 nm.

2.5. Polyphenols assay. The colorimetric assay is used to determine the concentration of simple and complex soluble phenolics in extracted solutions from fruits and vegetables and citrus juices. This analysis was based on the Folin-Ciocalteu (FC) method (Singleton

and Rossi, 1965) with some adjustments made to adapt the procedure to the sample under investigation.

The reaction mixture was composed of 0.1 ml of diluted citrus juices, 1.5 ml distilled water, 0.1 ml of Folin–Ciocalteu's reagent, and 0.3 ml of a 7.5% sodium carbonate anhydrous solution (added 5 min after the Folin–Ciocalteu's reagent). After initial mixing the tubes were allowed to stand for 2 h. The optical density of the blue-colored samples was measured at 765 nm. The total phenolic content was determined as gallic acid equivalents (GAE) and values are expressed as mg of Hesperidin/L of sample.

2.6. Statistical Analysis. All experiments were performed in triplicate and mean values with standard deviations are reported. Differences between variables were not tested for significance.

3. RESULT AND DISCUSSION

The most important citrus byproducts in Sicily is called "pastazzo", which is used in cattle feed. However, following the removal of bitter compounds, dried peel can be used as sources of fiber for human consumption (Todaro *et al,* 2013). The limonin extraction method used in this study it can be applied in the food/citrus industry because it is solvent-free and the extraction temperatures are compatible with industrial processes.

Alkaline solution-to-solid ratios were previously tested by Todaro *et al* (2013) for 1:2, 1:3, 1:4, 1:5, 1:10 ratios. Results demonstrated that the ideal peel:water (pH=9) ratio was 1:2 to 1:3 (data not showed).

Figure 2 shows both limonin and polyphenols contents in the orange pastazzo treated with an alkaline solution (pH 10); the analyses established an optimum dwell time of 25 min for the limonin extraction and 30 min for polyphenols.

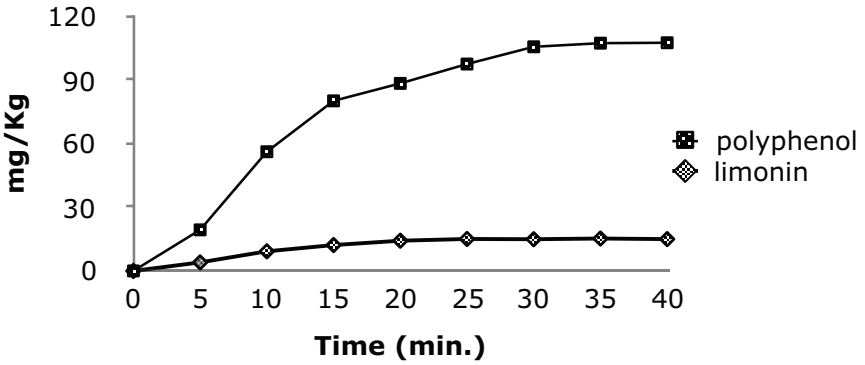


Fig. 2. Limonin and polyphenol content in orange peel with a solution at pH=10 at different time of extraction.

The samples analysis showed that both limonin that polyphenols are distributed between the solid phase and liquid phase according to the washing degree and, simultaneously, the liquids are enriched in bitter chemicals according to the considered stage (table 1).

In the present study, in order to verify the extraction degree of bitter compounds, only the final fiber and the liquid fractions from the straining processes after contact with the alkaline solution were analyzed in triplicate.

Table 1 – Limonin and Polyphenols contents of different fractions of debittering line[a]

Portions	Limonin (mg/L)	Polyphenols (mg/L Hesp.)
B	12.00±0.47	297.00±3.33
C	11.32±0.09	182.07±2.21
D	8.40±0.16	90.24±0.85
E	4.14±0.09	98.47±1.62
F	2.16±0.33	45.54±2.58
G	5.14±0.14	10.22±0.16
X	33.70±2.47	462.49±12.83

[a]Values are expressed as the mean ± standard deviation (n=3).

Table 1 shows that the highest values have been found in the samples named "*X*" with a limonin content of 33.70 ppm, followed by the "*B*" with a content of 12.00 mg/L and "*C*" 11.32 mg/L.

Chromatographic profiles with a higher concentration in limonin, showed that the purification was effective on the isolation of limonin from flavanones.

In fact, the peaks obtained suggest they are flavonoids in the same wash water, such as hesperidin and naringin based on the retention times at which the peaks were obtained.

Furthermore, the Folin Ciocalteu analysis of polyphenols showed a correlation between limonin and total polyphenols content, demonstrating that the alkaline extraction was effective for both categories (Fig. 3).

In particular, "*X*" had a polyphenols content of 462.49, "*B*" 297, and "*C*" 182.07. All other fractions have a much lower polyphenols content between 10 and 100 mg/L.

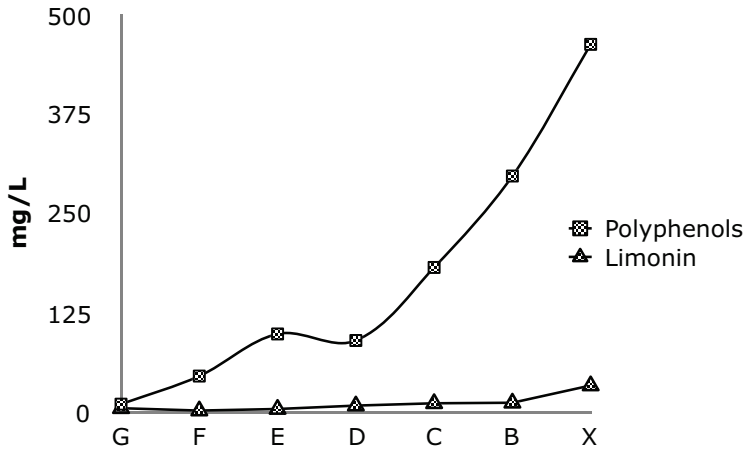


Fig. 3. Correlation between Limonin and polyphenols content.

4. CONCLUSION

Taking into account the results describe above, the trend of the system as a function of time and the distribution of bitter compounds in the washing, debittering and acidification stages were studied in order to verify pros and cons in the recovery of this compounds without affect the organoleptic characteristics of the final fiber.

Analyses showed that is not economically viable to recover bitter compounds; indeed the most important ones, the limonoids are contained in small quantities in the blood oranges by-products that are the main raw material in Ortogel plant.

In those circumstances, considering the cost of recovery and the market value, the bioactives recovery was not considered advantageous.

The limonin content of final fibers was 2 mg/Kg, that is lower than the bitterness taste threshold (6 ppm) while total polyphenols expressed as hesperidin were always below 40 mg/Kg, an acceptable value from a sensory point of view.

In conclusion, as demonstrated by the work carried out by a local food industry (DAIS S.P.A.), it is possible to produce good dietary fiber from oranges, characterized by good color, neutral taste and excellent ability to bind water.

The fiber is suitable to be used in recipes for baked goods with an increased shelf-life and with the possibility to decrease the amount of fat in recipes.

On the contrary, although the extracted limonoids may be used as a functional ingredient in the food and medical industry, the low concentrations of limonin obtained in the liquid phase and the high flow-rates of the continuos plant, make the investment unacceptable.

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Chapter 4

Farmers' markets versus retail grocery stores: How the market source contributes to differences in bioactive content of selected citrus grown in California

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Organic production agriculture is characterized by inputs of biologically based natural fertilizers practices that are sustainable (National Organics Standard Board, 2006) and exclude the use of synthetic chemicals in order to respect the environment and avoid the over-exploitation of natural resources such as water, soil and air. Besides, several studies show that the nutritional value of organic products is often higher than that of conventional products. In this study the bioactive content of selected fruits grown in California obtained from farmers' markets was compared to the content found in fruit purchased from retail grocery stores. Organoleptic properties, including Brix, TTA, color and pH were measured. Carbohydrates, limonin and flavanones, ascorbic acid contents were determined by HPLC with diode array detection and the antioxidant activities of juice were measured using both DPPH, TSP and ABTS assays. Significant differences were observed in bioactive contents and the antioxidant capacities of fruits from farmers' market was found to be higher than that from grocery stores.

KEYWORDS: Juice, grapefruit (*Citrus Paradisi*), Valencia orange, ascorbic acid, limonin, naringin, DPPH, bioactive content, HPLC, antioxidant.

1. INTRODUCTION

During last century, trends in food production changed from local farms to large enterprises. The large production system, favored by technological advances, turned to chemical solution to control pests and diseases and optimize soil productivity, obtaining at the same time an enhancement of yield and the external quality of fruit and vegetables

products; on the other hand, pollution problems and food contamination by chemicals became more frequent. The organic farming practices offered an alternative to industrial practices. Consumers, driven by environmental and and health concerns, emphasized this change (Davis *et al*, 1995) and caused an increased demand of organically produced food.

The word "organic" refers to the way farmers grow and process agricultural products, such as fruits, vegetables, grains, dairy products and meat. Organic farming practices are designed to encourage soil and water conservation and reduce pollution using natural fertilizers and crop rotation or mulch to manage weeds.

Much of the U.S. organic farm sector expansion has occurred since the U.S. Department of Agriculture's establishment of uniform organic standards in 2000 (USDA-ERS, 2006).

Sicily has a long tradition in citrus fruit cultivations that with vineyard and olive tree represent the main Mediterranean tree crops. In a recent paper (Sgroi *et al*, 2015), the economic and financial sustainability of lemon production, both in organic farming and in conventional farming was evaluated; Results, which referred to one hectare area located Sicilian northwestern coast one hectare area, showed both a higher economic and financial sustainability of organic farming respect to conventional farming. The higher profitability of organic farming was due to minor labor requirement and to greater market appreciation for organic products that granted a premium price respect to conventional prices. Moreover, greater profitability of organic farming and use of environmentally friendly inputs in production process make farms competitive and eco-friendly.

In addition, consumption of foods grown organically is often perceived to reduce risk by reducing exposure to pesticide residues (Williams and Hammit, 2001).

Surveys indicate that consumers consider organic food to be more positive for the environment and human health and more flavorful than their conventionally-grown counterparts (Bourn and Prescott, 2002) and also for its supposed greater nutritional quality.

Of particular interest is the determination of the content of secondary metabolites in fruit and vegetables with the aim to discriminate organic versus conventional plants products (Young *et al*, 2005; Dimberg *et al*, 2005; Hajšlová *et al*, 2005).

Findings showed that organic food production methods resulted in higher levels of

nutritionally desirable compounds including vitamins, antioxidants and poly-unsaturated fatty acids such as omega-3 and CLA, and lower levels of nutritionally undesirable compounds such as heavy metals, mycotoxins, pesticide residues and glyco-alkaloids (Niggli, 2009).

However, the nutritional quality of food grown by organic and conventional methods is the subject of much controversy (Woese *et al*, 1997; Brandt and Mølgaard, 2001; Bourn and Prescott 2002; Williams 2002; Magkos *et al*, 2003). The data on nutritional quality of organic versus conventional products are often inconclusive.

There are reported trends in higher content of chlorogenic acid in organically grown potatoes versus conventional ones (Hajšlová *et al*, 2005). Contrary to these results there is no difference in content of secondary metabolites in oat grains (Dimberg *et al*, 2005) as well as vegetables (Young *et al*, 2005).

Objective of the present work was to examine the differences in the bioactives content and antioxidant activity in Navel and Valencia orange fruits and in the Pink Star Ruby cultivar grapefruit obtained from farmers' market and purchased in retail grocery stores in order to verify if the market contributes to differences.

2. MATERIALS AND METHODS

2.1. Plant Material. The study was conducted in March 2015 on citrus fruits, i.e., Navel and Valencia oranges (*Citrus sinensis Osbeck*) and Pink Star Ruby grapefruits (*Citrus paradisi Macfadyen*) with fresh appearance, free of rotting and bruising or any other signs of deterioration.

The citrus fruits were purchased from 4 sources (2 farmers' markets and 2 retail grocery stores) located in the Californian bay area (Table 1). Both sources were chosen to obtain the same environment conditions. After purchasing the samples were kept at 4 °C until the time of preparation, which was within 24 hours of the purchase.

Table 1 – Analyzed sample

Cultivars Farmer's Markets	Sampling date	Location	Price/Lb ($)
Navel orange	2/28/15	Downtown Berkeley	2
Valencia orange	2/28/15	Downtown Berkeley	0.90
Pink star ruby grapefruit	2/28/15	Downtown Berkeley	2
Navel orange	3/3/15	South Berkeley	2
Valencia orange	3/3/15	South Berkeley	0.90
Pink star ruby grapefruit	3/3/15	South Berkeley	2

Cultivars Grocery Stores	Sampling date	Location	Price/Lb ($)
Navel orange	2/28/15	Downtown Berkeley	0.59
Valencia orange	2/28/15	Downtown Berkeley	0.69
Pink star ruby grapefruit	2/28/15	Downtown Berkeley	0.8
Navel orange	3/3/15	South Berkeley	0.59
Valencia orange	3/3/15	South Berkeley	0.69
Pink star ruby grapefruit	3/3/15	South Berkeley	0.8

2.2. Sample preparation. Samples of 30 fruits were purchased from each of the 4 sources at commercial maturity. Each sample was divided into three subsamples and the fruits were washed, dried and squeezed. The juice content, total acidity (TA), total soluble solids (TSS) and color were right away determined from fresh juice (AOAC, 2007). Vitamin C, flavanones, limonin, sugars and the antioxidant activities by ABTS, TSP and DPPH assays were determined from frozen juice samples stored at -20 °C.

2.3. Chemicals, materials and equipment. Analytical grade standards were purchased from Sigma-Aldrich (St. Louis, MO). Water (HPLC Grade) was prepared in house purifying it in a Milli-Q system from Millipore (Bedford, MA, USA).

Acetonitrile (HPLC grade) and methanol, formic acid, O-phosphoric acid, m-phosphoric acid and acetic acid (analytical grade) were purchased from Fisher Scient. (Pittsburgh, PA).

Special reagents were ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich), Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (Sigma- Aldrich), Folin-Ciocalteu's phenol reagent (M.P. Biomedicals, Inc.), Dithiothreitol (Sigma-Aldrich).

HPLC mobile phases were filtered through 0.45 lm HV filters before use (Millipore Corporation, Bedford, MA, USA). HPLC columns and guard columns were purchased from Phenomenex (Torrance, CA, USA) and Water corporation (Milford, MA, USA).
Citrus fruit samples were purchased from local Farmer's markets and grocery stores (Berkeley, CA).

2.4. Experimental.

2.4.1. Determination of pH, total soluble solids (TSS) and titratable acidity (TA)

A portion of fresh citrus juice were centrifuged at 15000 rpm for 15 min and the supernatant was analyzed for pH, TSS and TA; pH was analyzed using a Beckman 720 pH-meter in combination with a glass-body pH electrode with BNC connection. The percentage of total soluble solids (TSS) was measured by a Rudolph J257 automatic bench Refractometer (Hacketts town, NJ, USA), and acidity as citric acid (TA) was determined by titration of the juice samples to a target pH of 8.10 +- 0.1 using a 0.1 N sodium hydroxide solution according to AOAC (2000) using a Metrohm 730 Sample Changer in conjunction with the 751GPD Titrino automatic titrator (Methrom AG, Switzerland). All measurements were carried out in triplicate.

2.4.2. Color measurement

In fresh juice, bright orange color is determined by the composition and concentration of its naturally occurring pigments, carotenoids (Meléndez-Martínez,Vicario, Heredia, 2009).
Color is an important characteristic of food. The deliverance of a good impression through color will determine consumers' acceptability and their purchase decision. Also, color plays an important role as a quality indicator. According to Van Boekel (2008), different chemical and biochemical reactions which occur in a food product can be detected visually by its color.
The color of citrus juice was analyzed using a Konica Minolta CM700d colorimeter (Konica Minolta inc, Japan). The instrument (45°/0° geometry, Illuminant D65, 10° observer) was calibrated with a black and white ceramic tile (X = 78.66, Y = 83.31, Z = 88.40) before the measurement. Subsequently, juice samples were placed in a glass cell and

the color right away measured. Color measurements were carried out in triplicate with five readings for each sample. The recorded XYZ tristimulus values were then converted to CIE L^*, a^* and b^* color values. The L^* values represent lightness, ranging from 0 (black) to 100 (white). The a^* values indicate greenness (negative) to redness (positive) and the b^* values quantify blueness (negative) to yellowness (positive).

Two other parameters were determined by the following equations:

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$h_{ab} = \arctan {b^{*2}}/{a^{*2}} \quad (2)$$

2.4.3. Flavonoids determination

The content of the flavonoids narirutin, hesperidin, naringin (for grapefruit) and didymin was determined using high-performance liquid chromatography (HPLC).

Frozen juice samples were thawed in a 20°C water bath for 20 min and mixed prior to processing. A portion sample was transferred to 15mL conical vial and clarified by centrifugation using the Sorval model RC 5C Plus centrifuge (15 min, 15000 rpm, 4°C).

Clarified liquid is collected, diluted with the mobile phase and then filtered through 0.45 μm PTFE membrane filter prior to HPLC analysis. Juice samples were to be diluted 10:1.

HPLC analysis was performed with a Waters 2695 LC (Milford, MA) in series with a Waters PDA 996. Instrument control and data acquisition is accomplished using Masslynx (Version 4.0). Separation was performed on a 5 µm Luna C18 column (50 × 2 mm i.d.) (Phenomenex, Torrence, CA) operating in gradient with a solution 0.01 N of acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.6 ml/min. Flavonoids were detected at a wavelength of 280 nm; the values provided are the average of three replicates.

2.4.4. Limonin content

Determination of Limonin content was accomplish by HPLC.

A 1.0 mL aliquot of orange juice sample was clarified by the same protocol used for the determination of flavanones, and extracted twice with 2 mL of chloroform

The chloroform layer was evaporated to dryness with nitrogen gas and reconstitute with

500 uL of 10 mM formic acid in 30% ACN.

Quantification was performed based on external standard calibration curve covering the linear concentration range from 0.05-100 ppm.

A 5 and 10 ppm limonin controls and the 5 ppm limonin spiked water control were also analyzed. The HPLC system was comprised of a Waters 2695 LC (Milford, MA) in series with a Waters PDA 996. Instrument control and data acquisition is accomplished using Masslynx (Version 4.0).

Standards and samples (20 µL) are injected on a 50 x 2.0 mm Phenomena Phenosphere-Next-5µ Phenyl Column (Torrance, CA), equipped with a guard column of the same material and maintained at 30°C. The flow rate was 1.0 mL/min and an isocratic solvent composition of 70% of 10 mM formic acid, 30% acetonitrile was used. Total run time was 5.5 minutes.

2.4.5. Chromatographic determination of Ascorbic Acid

Vitamin C is the most important water-soluble antioxidant. Both, ascorbic acid (AA) and its oxidation product, dehydroascorbic acid (DHAA), have vitamin C activity.

AA, DHAA and TAA were analyzed using a modification of the subtraction method (Mazurek A. *et al*, 2015), (Kranthi K. *et al*, 2012), (Inga Klimczak I. *et al*, 2015).

The frozen juice samples were thawed in a 20 °C water bath and a portion sample was clarified by centrifugation using the Sorval model RC 5C Plus centrifuge for 15 min at 15000 rpm at 4 °C.

To determine the AA, the clarified liquid was diluted with a solution of meta-phosphoric acid 10% and then filtered through 0.45 μm PTFE membrane filter prior to HPLC analysis. Juice samples were diluted 5:1.

To determine the TAA, the same clarified juice sample was combined with DL-DiThioThreitol (DTT) Solution (10% w/v) up to obtain a 1% final concentration in DTT. The solution was vortexed on a VWR multi-tube vortexer (West Chester, PA) for 10 sec at speed #5 and incubated for 15 minutes. After this time, the sample was diluted 5:1 with meta-phosphoric acid 10% and then filtered through 0.45 μm PTFE membrane filter prior to HPLC analysis.

No preparation regarding the DHAA content was used; DHAA was obtained by subtraction between TAA and AA content (TAA content is the sum of AA and dehydroascorbic acid (DHAA) after its reduction to AA).

HPLC determination of the ascorbic acid was achieved using a Thermo Hypersil-Keystone BDS C18 (250 x 4.6 mm id, 5 μm) (Waters Milford, MA) and a guard column of the same material maintained at 35 ˚C

A gradient of mobile phase composed of 0.02 M o-phosphoric acid (solvent A) and acetonitrile (solvent B) was used according to the following program:

0-4 min 0% B (isocratic); 4-6.5 min a linear increment up to 7%B; 6.5-8, 7%B (isocratic) and 8-9.5 return to the initial conditions 0% B and then isocratic until 15 min. The eluate was detected using a Waters 996 photodiode array detector set at 245 nm (Gliszczyn´ ska-S´ wigło , 2006). The injection volume was 20 μL.

Quantification was performed based on external standard of L-AA purchased from Sigma (St. Louis, MO). Calibration curves of the standard ranging from 5 to 150 mg/mL was used with good linearity and R2 values exceeding 0.99 (peak areas vs concentration).

The values provided are the average of three replicates.

2.4.6. Estimating of antioxidant activity

DPPH Radical Scavenging Activity Assay. DPPH is a method to determine the antioxidant activity of fruits or vegetables by decolorization applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids. The DPPH assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in comparison to Trolox, a water-soluble tocopherol analog. By quantifying the cumulative effect of all antioxidants present it is possible to have relevant biological information.

The scavenging effects of the phenolic compounds toward the stable free radical DPPH were measured according to the procedure by Brand-Williams *et al*. Hamburger *et al* and Bouaziz *et al* with some modifications. Briefly, samples juices properly diluted with methanol to block the PPO action, along with positive (BHT, ascorbic acid) and negative (cinnamic acid) controls (50 μL) prepared in methanol (0.001-1 mg/mL), were combined in

triplicate with 155 μM methanolic DPPH (200 μL). Following incubation at room temperature for 30 min, the absorbance at 517 nm was read on a Molecular Devices Spectromax 384-Plus plate reader (Sunnyvale, CA).

ABTS Radical Cation Decolorization Assay (TEAC). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS in comparison to Trolox. Antioxidant capacity as assessed by the ABTS radical cation (ABTS•+) decolorization assay was accomplished following the methods of Sellappan *et al*, 2002 and Re *et al,* 1999 with some modifications. Briefly, ABTS•+ was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate for 16 h in the dark at room temperature. The ABTS•+ solution was diluted with MeOH to an absorbance of 0.70±0.01 at 734 nm. Citrus juice samples properly dilute with methanol along with positive (BHT, ascorbic acid, Trolox) and negative (cinnamic acid) controls (20 μL, 1 mg/mL, 0.02-1.0 mg/mL for Trolox) prepared in methanol were combined in triplicate with the ABTS•+ solution (400 μL, absorbance 0.70±0.01. After a brief incubation (6 min, 30 °C), the absorbance at 734 nm was read on a Molecular Devices Spectromax 384-Plus plate reader.

Total Soluble Phenolics Assay (TSP). This colorimetric assay is used to determine the concentration of simple and complex soluble phenolics in extracted solutions from fruits and vegetables and citrus juices. This analysis is based on the Folin-Ciocalteau (FC) method (Singleton and Rossi, 1965) with some adjustments made to adapt the procedure to the sample under investigation.

The reaction mixture was composed of 0.1 ml of diluted citrus juices, 1.5 ml distilled water, 0.1 ml of Folin–Ciocalteu's reagent, and 0.3 ml of a 7.5% sodium carbonate anhydrous solution (added 5 min after the Folin–Ciocalteu's reagent). After initial mixing the tubes were allowed to stand for 2 h. The optical density of the blue-colored samples was measured at 765 nm. The total phenolic content was determined as gallic acid equivalents (GAE) and values are expressed as mg of Gallic acid/g of extract (in GAE).

2.5. Statistical Analysis. All experiments were performed in triplicate and mean values with standard deviations are reported. Differences between variables were tested for

significance by using a one way analysis of variance procedure, using a level of siginificance of $p < 0.05$.

3. RESULT AND DISCUSSION

3.1. phisiochemical differences

Organic plant foods are produced without synthetic pesticides and mineral fertilizers, but with compost, green manure and diversified rotation. Certification in organic farming means that a control unit examines the product according to the accepted rules and production system.

Final rule of the organic foods production act defined the term "organic" and set standards for U.S. production and handling of agricultural products.

USDA's National program (http://www.ams.usda.gov/about-ams/programs-offices/national-organic-program) provides access to program standards, information on trade issues, materials on organic certification and accreditation including a listing of USDA accredited certifying agents, and resources for producers, handlers, processors and retailers regarding organic production and marketing.

In the present study, the physicochemical characteristics were evaluated and compared for the 3 cultivars; the analyses showed a significantly higher difference in the pH value and the titrable acidity of juices of all cultivars from farmers' source compared with conventional (Table 2); Although this is in contrast to what observed by Candir *et al*, 2013, Koneru (2013), observed an higher TA content in organically grown peaches compared with conventional farming.

The total solid soluble content (SST) showed the same trend for Navel and Pink Star Ruby but Organically grown Valencia oranges had lower SST than conventionally grown even though not statically significant (Table2).

The solid soluble content determines the taste of fruit and vegetable juices. An higher TSS content was reported in oranges, lemons and mandarin grown under organic production system (Duarte *et al*, 2010). Consistent with our discordant results, no significant differences in TSS percentage were found for citrus and strawberries fruits between organic

and conventional systems (Nunes *et al*, 2010; Camin *et al*, 2011 and Roussos, 2011).

Table 2 – Physicochemical characteristics of different citrus cultivars[a]

Cultivars	pH	TA (%ascorbic acid)	SST (°Brix)	SST/TA
Navel orange Farmer	3.85±0.19a	2.00±0.41a	13.90±0.59a	7.14±1.10b
Navel orange Grocery	3.92±0.18a	1.76±0.38b	12.15±0.36b	7.21±1.59b
Valencia orange Farmer	3.33±0.05a	4.11±2.62a	10.72±0.49b	2.62±0.15b
Valencia orange Grocery	3.80±0.07b	2.62±0.34b	11.33±0.33b	4.32±0.12a
Pink star ruby Farmer	3.12±0.04a	4.66±0.25a	11.47±0.39a	2.47±0.14b
Pink star ruby Grocery	3.30±0.07b	3.93±0.17b	10.24±0.20b	2.61±0.12a

[a]Mean values with different letters (a–b) within the same cultivar are statistically different *($p < 0.05$)*
Values are expressed as the mean ± standard deviation (n=3).

Table 3 – Color CIE *L*, a*, b** values, chroma *(C*ab)* and hue *(hab)* of citrus cultivars.

Cultivars	*L**	*a**	*b**	*C*ab*	*hab*
Navel orange Farmer	20.76±1.78a	2.63±0.43a	19.39±2.22b	19.94±2.49a	1.55±0.00b
Navel orange Grocery	18.85±1.55b	1.26±0.75b	19.45±2.22b	19.23±2.16a	1.57±0.00a
Valencia orange Farmer	21.51±4.89a	2.39±0.39a	18.34±1.56a	18.50±1.54a	1.55±0.01b
Valencia orange Grocery	18.84±1.48b	-0.06±0.87b	16.77±1.04b	16.79±1.05b	1.57±0.00a
Pink star ruby Farmer	13.31±1.59a	4.48±0.39a	4.54±0.32a	6.38±0.43a	0.80±0.08a
Pink star ruby Grocery	12.94±1.19a	4.29±0.69a	3.76±0.93b	5.75±0.78b	0.65±0.28b

Mean values with different letters (a–b) within the same cultivar are statistically different *($p < 0.05$)*
[a]Values are expressed as the mean ± standard deviation (n=3).

Color is defined as the impact of the wavelengths of light in the visible spectrum (390–760nm) that can be detected by human eyes (Francis, 1995) and it is one of the main attributes that is strongly associated with the concept of quality (Wibowo *et al*, 2015). Some studies have revealed that the color of orange juice is related to the consumer's perception of flavor, taste (sweetness, sourness) and, thus, overall acceptability (Wei, Ou, Luo, & Hutchings, 2012).

In this study, at least 3 of 5 measured parameters showed significant differences especially for lightness and yellowness (table 3). Color difference, chroma (Cab) and hue (hab) were calculated to provide additional information about the color characteristics of the orange juice samples. Chroma difference is insignificant just for the Navel cultivars but all samples organically grown showed a hue significantly different compared to conventional cultivars.

Recently, Wei *et al*, (2012) defined an ideal orange juice color with a lightness of 67°, a chroma of 62° and a hue of 88°. Orange juice will more likely to be accepted when the color difference against the ideal color is smaller. Results obtained in this study are much lower than suggested values.

3.2. Differences in bioactive compounds

The data in Tables 4 shows ascorbic acid levels. Analysis was conducted in order to determine both L-AA, TAA by reduction of DHAA. Many investigators reported an increase in ascorbic acid content in the organic products such as Duarte *et al* (2010) on 'Valencia late' and 'Baia' oranges, Lester *et al* (2007), on 'Rio Red' grapefruit.

The possible interpretation for this finding is that nitrogen fertilizers under high rates seems to decrease the concentration of ascorbic acid content in fruits and vegetables (Lee and Kader, 2000) Besides Lee and Kader (2000) reported that the use of agrochemicals and pesticides may affect the nutritional quality of fruits and vegetables.

In discordance to this, in this study, the differences of ascorbic acid levels for the Navel and Valencia cultivars from Farmers and retail grocery stores were not significant (table 4). In the other hand, ascorbic acid concentration in Pink star ruby juice was higher in fruits produced in the organic orchards.

However, Duarte *et al* (2012), demonstrated that the higher ascorbic acid content in citrus fruit juice from organic production system depend on species and cultivar.

The concentration of DHAA, being very close to zero, has not contributed to TAA value. It was reported that phenolic compounds protect AA from degradation but this protection depends on the type of compound (Miller & Rice-Evans, 1997; Özkan, Kırca, & Cemeroglu, 2004).

Citrus juice phenolic acids were recognized as the best efficient protectors of AA than flavonoids of other fruit juice. (Miller & Rice-Evans, 1997). On based of this, the oxidation process of AA in citrus is only partly responsible for the loss of AA because the concentration of DHAA doesn't compensate the decrease of AA in fresh juice.

Table 4 – Vitamin C (mg/100mL juice) and Limonin content of different citrus cultivars[a]

Cultivars	L-AA	DHAA	TAA
Navel orange Farmer	56.37±7.20a	-0.28±1.80a	56.08±6.89a
Navel orange Grocery	57.21±3.06a	0.91±1.14a	58.12±3.19a
Valencia orange Farmer	52.11±4.34a	0.23±1.19a	52.33±4.54a
Valencia orange Grocery	50.90±3.09a	0.62±0.95a	51.52±3.23a
Pink star ruby Farmer	51.18±3.01a	0.31±1.79a	51.48±2.56a
Pink star ruby Grocery	33.25±1.69b	0.22±0.50a	33.47±1.48b

[a] Mean values with different letters (a–b) within the same cultivar are statistically different *($p < 0.05$)* Values are expressed as the mean ± standard deviation (n=3).

Figure 4.1, show an HPLC chromatogram of the limonin detected at 210 nm.

In 1973, a comprehensive study of the taste thresholds of limonin was performed by Guadagni *et al* where a carefully screened panel of judges were able to detect a bitterness taste threshold level at 6 ppm of limonin.

Limonin bitterness is especially acute in juice obtained from early season fruits, such as Navel oranges. Juice from early to mid-season navel oranges can contain as much as 25 ppm of limonin. Grapefruit also has significant levels of limonin, an average of 15 ppm or more in the early season (Maier, 1977).

Progress is being made on a genetic engineering solution to the problem of limonin bitterness in citrus juices.

In this study, we also wanted to verify how the market source contributes to differences in the limonin content. This is important because the limonoids are proving to be important compounds in the human diet (Hasegawa, 2000) and, on the other hand, the bitterness of most limonoids is a problem because it contributes to drive the consumers in fruits purchase.

Table 5 shows no actual correlation between the citrus purchase source and the limonin content; Even though the limonin content in Valencia cultivar organically grown is higher than that conventional, we obtained the opposite trend for the Navel cultivar; the difference in limonin content for the Star Ruby grapefruit was not significant.

By the way, according to a study of Breksa and Manners, 2006 the limonoids have no

contribution in the antioxidant activity of citrus juices. In fact, limonoids were unable to quench the DPPH radical; besides the limonoid contents found in Citrus fruits are greatly influenced by variety (Breksa *et al*, 2009).

Table 5 – Limonin content (mg/L juice)[a]

Cultivars	Limonin
Navel orange Farmer	3.75±2.08b
Navel orange Grocery	5.27±1.17a
Valencia orange Farmer	5.16±0.54a
Valencia orange Grocery	2.86±1.86b
Pink star ruby Farmer	9.36±1.45a
Pink star ruby Grocery	9.21±1.83a

[a] Mean values with different letters are statistically different *($p < 0.05$)*
Values are expressed as the mean ± standard deviation (n=3).

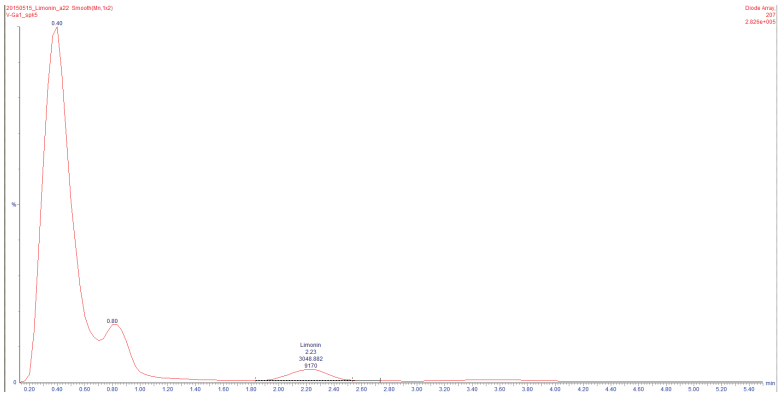

Fig. 4.1. HPLC chromatogram of the limonin in Valencia cultivar detected at 210 nm.

The content of phenolic compounds detected in 3 citrus fruits is shown in Table 6. Hesperidin, Narirutin, Didimin and Naringin were analyzed in the study.

Apart from naringin which was the predominant phenolic compound present just in the Star Ruby grapefruit (Fig. 4.2), the same phenolic compounds were present in each cultivar, but there were net differences in relative levels.

Table 6 – Flavanones content (mg/100mL juice) of different citrus cultivars[a]

Cultivars	Narirutin	Hesperidin	Naringin	Didimin	Tot Flavan
Navel orange Farmer	14.87±2.75	31.59±1.37	-	3.70±1.67	46.87±9.57
Navel orange Grocery	11.98±1.13	13.47±1.08	-	2.70±0.45	28.18±1.49
Valencia orange Farmer	5.29±1.15	29.52±1.43	-	1.76±0.85	36.57±8.22
Valencia orange Grocery	9.01±1.64	16.58± 1.74	-	2.51±0.42	28.10±1.37
Pink star ruby Farmer	37.95±6.92	2.72±0.42	100.10±15.25	1.57±0.33	142.34±21.01
Pink star ruby Grocery	17.21±6.75	1.08±0.32	46.37±13.96	1.03±0.24	65.70±21.12

[a]The estimate of all mean values results statistically significant *($p < 0.05$)*
Values are expressed as the mean ± standard deviation (mean=3).

In the present study, analyses of cultivars purchased in the farmer's market showed a significant increase in the concentration of flavanones compared with conventional (Fig. 4.3, Fig. 4.4).

According with Brandt and Molgaard (2001), biosynthesis of phenolic compounds in plants is strongly affected by the cultivator techniques, environmental conditions and the fertilizers used. It has previously been reported that the phenol concentration is influenced by level of available nitrogen. Besides, increase in phenolic compounds is related to the defense role they play in plants under stressed conditions (Dixon and Paiva, 1995). In the absence of pesticides, plants could contain higher levels of antioxidant components as a result of enhanced synthesis of active phytochemicals produced in defense against biotic and abiotic stress (Tarozzi *et al*, 2006).

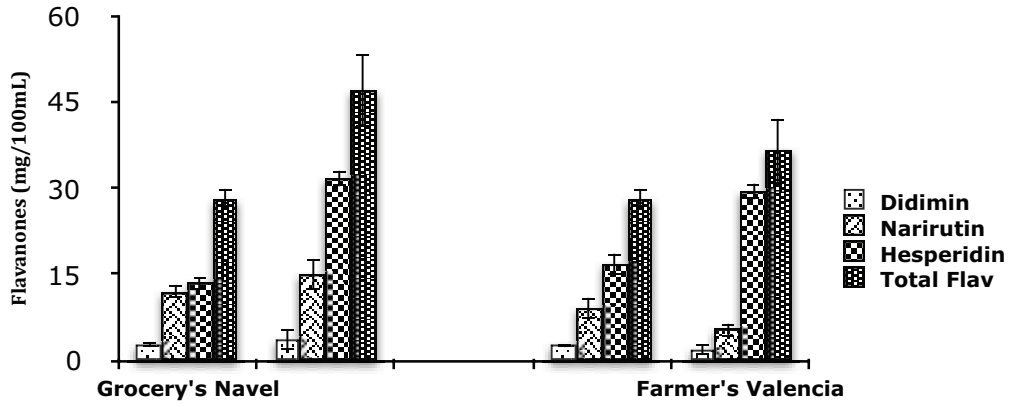

Fig. 4.2. Istogram graph of the flavanones content of Navel and Valencia cultivar.

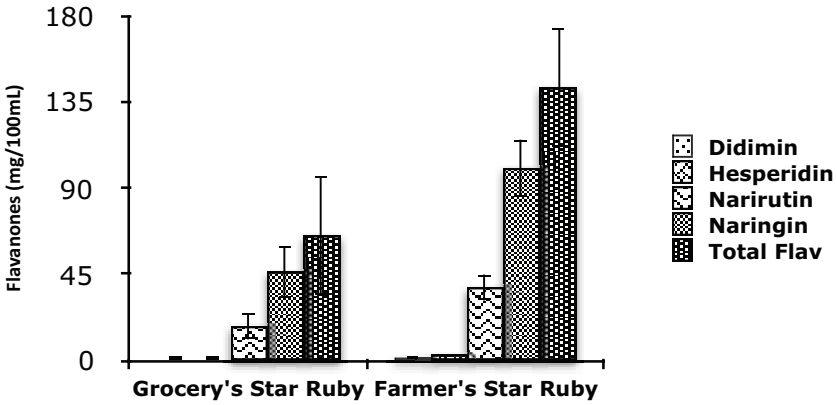

Fig. 4.3. Istogram graph of the flavanones content of Pink Star Ruby grapefruit.

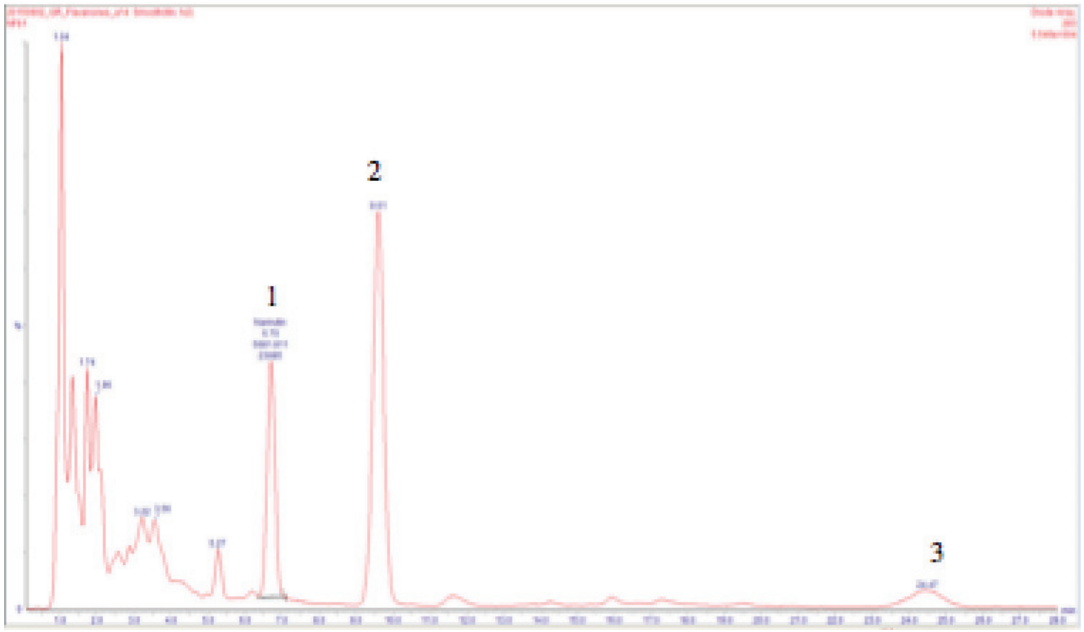

Fig. 4.4. HPLC chromatogram of the flavanones in Navel cultivar detected at 243 nm. (1) Narirutin; (2) Esperidin; (3) Didimin.

3.3. Antioxidant activity by DPPH, ABTS, and TSP assays

Antioxidant activities were measured three times in all cultivars after dilution of the juice with methanol using DPPH, ABTS and TSP assays (table7).

DPPH and ABTS assays showed some differences among determinations (Table 7). This is

in agreement with the findings of other authors. Aaby *et al* (2004) assert that the different results of ABTS and DPPH assays, depend on the conditions used in the assays because the reaction rates are functions of both reaction time and temperature.

Table 7 – Antioxidant Capacity of different citrus cultivars[a]

Cultivars	DPPH (Tr Eqv mmol/100mL FW)	ABTS (Tr Eqv mmol/100mL FW)	TSP (GA Eqv micromol/100mL FW)
Navel orange Farmer	355.09±33.02a	391.28±42.00b	598.39±7.09a
Navel orange Grocery	306.28±21.71b	408.08±13.73b	587.77±19.14b
Valencia orange Farmer	300.83±46.67b	358.27±34.94b	595.91±26.83a
Valencia orange Grocery	303.08±26.17b	374.72±12.56b	542.34±19.48b
Pink star ruby Farmer	322.04±28.58a	379.47±16.94a	570.75±36.95a
Pink star ruby Grocery	228.34±12.09b	250.16±26.94b	451.96±46.21b

[a] Mean values with different letters within the same cultivar are statistically different *($p < 0.05$)*
Values are expressed as the mean ± standard deviation (n=3).

However, both assays show the same trend of the ascorbic acid contents (table 4.).
Navel and Valencia cultivars from Farmers and retail grocery stores were not significantly different unless for Navel cultivar analyzed with DPPH assay; whereas antioxidant activity of samples from farmer source measured with TSP assay resulted significantly higher than samples purchased in the grocery stores for all cultivars (Fig. 4.5.). This was in accordance with the flavanones content obtained by HPLC (Table 6.).

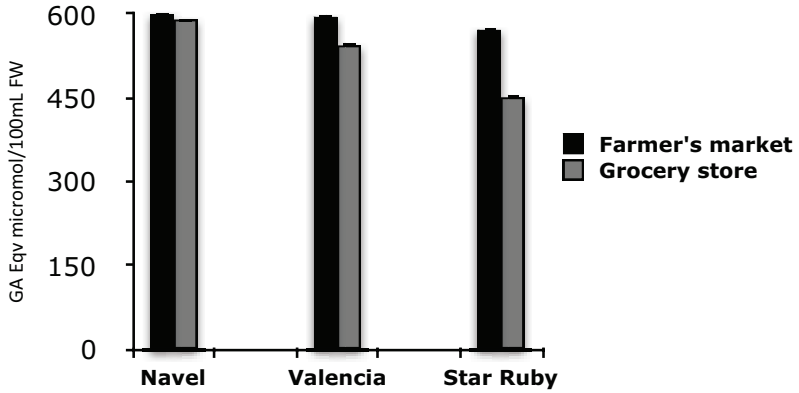

Fig. 4.5. Histogram graph of Antioxidant activity measured using TSP assay for the cultivars Navel, Valencia and Star Ruby grapefruit. The estimate of all mean values ± standard deviation results statistically significant *($p < 0.05$)*.

4. CONCLUSIONS

The farmers' markets are very popular in the United States even though the price of the products purchased is strongly higher (25-70% more) compared to that of retail grocery stores. However, this doesn't keep the consumers away because they seem to perceive that there is a difference in flavor, taste and healthy components.

This study showed that the fruits purchased from a farming source, regarding the cultivars under investigation, had more soluble solids and organic acids, a better color and a lower maturation index. Also the antioxidant activity and polyphenol content was higher. Contrary to this, vitamin C and limonin analysis gave discordant results, maybe because the response depend on different production systems, species, cultivars or environmental conditions. However, the success of farmer's markets seems to be justified.

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Chapter 5

Ehrlich's Reaction of Limonoids: Synthesis and Characterization

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This study describes the reactions between both limonin (Lim) and limonin glucoside (LG) (Fig. 1) with the Ehrlich's reagent, p-dimethylaminobenzaldehyde (DMAB) under acidic conditions in a 2:1 ratio.
Different reaction conditions were tested; both limonin and limonin glucoside generated the same products: 1:1 and 2:1 Lim:DMAB adducts.
1:1 and 2:1 LG-DMAB adducts was not obtained because of LG's hydrolysis due strongly acidic conditions.
Reactions produced several isomers in low yield not characterized in this study; However, it was deduced that the colored compound in Ehrlich's test is the cationic species of the intermediate 1:1 adduct affords in 45% yield and that the concentration of the uncolored 2:1 adduct in the reaction mixture was 25% utilizing trifluoroacetic acid as a reaction medium.
Furthermore, working with an excess of DMAB in 36% chloridric acid or in a mixture of perchloric acid:acetic acid 45:55, the 2:1 adduct Lim: DMAB was not obtained.

KEYWORDS: Ehrlich, limonin, limonin glucoside, p-dimethylaminobenzaldehyde, purple, TLC, adduct, yield, Mass-Spectrometry, furan.

1. INTRODUCTION

Dreyer (1965a, b) made several initial significant contributions to the field of limonoid analysis, including a chromophoric TLC analysis for limonoid detection.

Nomura and Saito (1968) and Maier and Beverly (1968) determined limonin by a spectrophotometric method using Ehrlich's reagent.

They spotted and developed the limonoids samples on silica gel TLC plates and after drying, sprayed moderately with Ehrlich's reagent, and the color developed in a chamber of HCI.

The intensities of the purple limonin spots were estimated by visual (or spectrodensitometric) comparison with the knowns after color formation with a spray of Ehrlich's reagent (Maier and Grant, 1970).

Several other investigators used the reaction between Ehrlich's reagent (DMAB) and limonoids as a chromatographic detection system (Hasegawa, 2000), (Hasegawa *et al*, 2000) (Vaks *et al,* 1981), (Ohta *et al,* 1993). Burnham (1970) used Ehrlich's reagent to determine indole.

However, the method is specific for limonin since the TLC system separates limonin from other limonoids (Maier and Beverly, 1968) and Ehrlich's reagent gives a characteristic color with limonoids (Dreyer, 1965a, b).

Breksa and Ibarra (2007) developed a colorimetric method for the estimation of total limonoid aglycones and glucoside contents in citrus Juices and another method for the identification and quantification of citrus limonoid glucosides in juices, based upon high performance liquid chromatography (HPLC) separation coupled to post-column reaction with Ehrlich's reagent in the presence of perchloric and acetic acids, has been developed by Breksa *et al* (2015).

Although this reaction is very famous and widely used, we do not still have adequate information concerning the chemical structure of the reaction products.

In this study in order to determine the structure of the limonin-DMAB and limonin glucoside-DMAB adducts, both compounds were synthesized, purified and characterized.

This project involves synthesis of the target compounds.

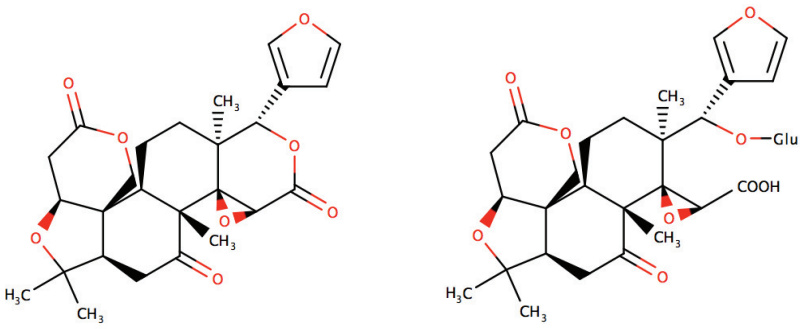

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Figure 1. Limonin (1) and Limonin Glucoside (2) with their chemical structures.

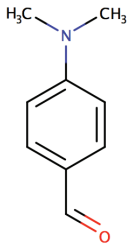

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Figure 2. Chemical Structure of p-dimethylaminobenzaldehyde (DMAB).

2. MATERIALS AND METHODS

2.1. Materials. Water was distilled and deionized. Solvents (Fisher, Pittsburgh, PA) were HPLC grade. Perchloric acid (70%, ACS Reagent) and glacial acetic acid (ACS PLUS), chloridric acid, trifluoroacetic acid, methanol and dichloromethane were purchased from Fisher, and DMAB (Ehrlich's Reagent) was purchased from Sigma (St. Louis, MO). Limonin and limonin glucoside were isolated and evaluated for purity as previously described by Breksa and Manners (2006). All other reagents were analytical grade.

2.2. Ehrlich's reaction conditions:

2.2.1. Reaction 1:

Compound **1** (50 mg, 0.100 mmol) was dissolved in CH_3CN (2 mL), and to this was added compound **3** (500 mg, 3.35 mmol) and 10 mL 36% HCl.

Compound **2** (70 mg, 0.100 mmol) was dissolved in 70:30 CH_3CN:H_2O (2 mL), and to this was added compound **3** (500 mg, 3.35 mmol) and 10 mL 36% HCl.

2.2.2. Reaction 2:

Compound **1** (50 mg, 0.100 mmol) was dissolved in CH_3CN (2 mL), and to this was added compound **3** (500 mg, 3.35 mmol) and 10 mL 55:45 $HClO_4$:CH_3COOH.

Compound **2** (70 mg, 0.100 mmol) was dissolved in 70:30 CH_3CN:H_2O (2 mL), and to this was added compound **3** (500 mg, 3.35 mmol) and 10 mL 55:45 $HClO_4$:CH_3COOH.

For both reaction, mixture became red purple, and slowly changed to dark blue. After being

stirred at room temperature for 1 day, an aqueous 10 N NaOH was added until obtaining of a precipitate purple colored (pH=9). Therefore, the solid was washed with brine, water and diethiletere to eliminate the inorganic salts and the excess of DMAB and then dried under vacuum. The residue was chromatographed on silica gel (3g) using dichloroethane/CH_3OH (96:4) as the eluent. The chromatography was repeated using dichloroethane/CH_3OH (95:5 and 94:6) until **4** was obtained.

2.2.3. Reaction 3:

Compound **1** (50 mg, 0.100 mmol) was dissolved in CH_3CN (2 mL), and to this was added compound **3** (8 mg, 0.0054 mmol) and 2 mL CF_3COOH.

Compound **2** (70 mg, 0.100 mmol) was dissolved in 70:30 CH_3CN:H_2O (2 mL), and to this was added compound **3** (8 mg, 0.054 mmol) and 2 mL CF_3COOH.

After being stirred at 50°C for 1 day, the reaction mixture was evaporate under vacuum. Evaporation of the solvent afforded an oily residue which was chromatographed on silica gel (3g) using dichloroethane/CH_3OH (96:4) as the eluent. The chromatography was repeated using dichloroethane/CH_3OH (95:5 and 94:6) until **4** and **5** were both obtained.

2.3. Thin-Layer Chromatography. Silica gel thin-layer plates or sheets with 0.25-mm. layer thickness, 20 cm. long and 5 to 20 cm. wide were used. The developing solvent was the upper phase of dichloromethane and methanol (96:4).

2.4. Spectrofotometric analysis. For the reaction rates study, 450 to 750 nm absorbance was read on a Molecular Devices Spectromax 384-Plus plate reader (Sunnyvale, CA).

2.5. LC-MS System Parameters. MS analysis was conducted on a Micromass LCZ single quadrupole mass spectrometer equipped with an APCI probe. The mass spectrometer was operated in the positive ion mode, with a probe temperature of 120 °C, cone voltage of 50 V, and corona voltage of 3.50 kV.

Protonated molecules of all adducts were monitored operating in the single ion monitoring (SIM) mode. In the analysis the SIM was conducted at m/z 602.7 (Lim:DMAB), 1072.2 (Lim:DMAB:Lim), 782.86 (LG:DMAB) and 1431.19 (LG:DMAB:LG).

3. RESULTS AND DISCUSSION

Reaction of **1** and **2** with **3** was achieved at different stoichiometric ratios and using different reaction conditions.

When an acetonitrile solution of **1** was treated with p-dimethilamino-benzaldehyde (1:10 ratio) and 36% HCl at room temperature, the solution immediately changed to red-purple and then slowly changed to dark blue.

After 1 day of stirring, the reaction was quenched by the addition of 10N aqueous of sodium hydroxide until pH 8 and the product was purified from inorganic salt and unreacted DMAB by washing with water and diethiletere and then separated by thin layer chromatography.

Same reaction was carried out with a mixture of perchloric acid:acetic acid 45:55 (Breksa *et al*, 2007) in place of HCl.

In both cases, although many products were detected on TLC, only the major product was isolated and identified as the 1:1 adduct **4** (34% yield using HCl, 40% yield using perchloric acid/acetic acid) from its spectral data (Figure 6).

When **2** was treated with **3** under the same reaction conditions, although the reaction was kinetically faster, the same adduct **4** was afforded with an higher yield (43% yield using HCl, 60% yield using perchloric acid/acetic acid); however, the 2:1 adduct was obtained by any of the above reactions.

Same reactions were carried out again using an excess of **1** or **2** in order to facilitate the 2:1 adduct (**5**) production, but only **4** was obtained as a major product.

This could be consistent with the fact that the 2:1 adduct (**5**) is bulky and not stable under strong acid conditions and it decomposes during purification and handling.

Besides, the **4** and **5** synthesis is achieved by two different mechanisms; the second reaction step is not an addition but a condensation (Scheme 3). It's a known fact that a condensation reaction achieves with the resulting loss of molecules of water or other small molecules like methanol, ammonia or chloridric acid.

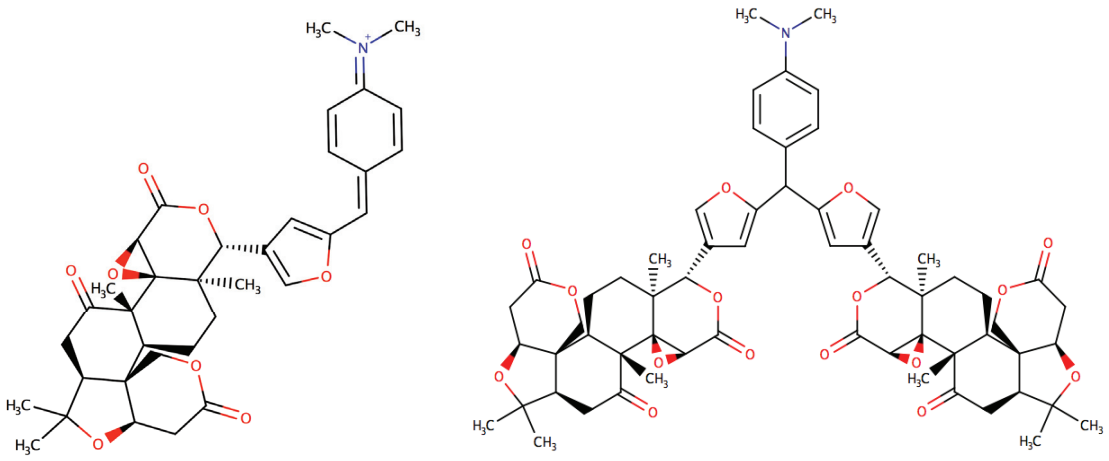
In this case the condensation as a second step of the reaction produces a molecule of water because of the double protonation of the carbonyl group. Therefore the entry of the second

molecule of **1** or **2** in **4** is penalized by the presence of water in the reaction environment (Le Chatelier-Brown principle).

Besides, such as showed in scheme 2, although the first reaction step is an addition, the acidic environment causes the loss of a molecule of water in order to produce the colored dehydrated cationic intermediate **3** which is resonance-stabilized (scheme 1). Therefore, the presence of water retards also the formation of the colored cationic intermediate.

For the reasons above specified and in order to obtain the adduct **5**, the synthesis of **1** and **2** with **3** were carried out using trifluoroacetic acid (TFA) in a stechiometric ratio (two limonin or LG and one p-dimethylaminobenzaldehyde). The TFA is stronger than acetic acid by inductive effect of fluorine atoms and weaker than chloridric acid and perchloric acid; additionally it is easy to remove without an excessive handling.

After 1 day of stirring at 50°C (temperature was set at 50°C because of the lower chemical kinetic), the solvent was removed by rotovap and the reaction mixture was separated; the adducts **4** (yield 45%) and **5** (yield 25%) were isolated and identified from its spectral data (Fig. 6, 7 and 8).



4 **5**

Figure 3. Chemical Structure of 1:1 and 2:1 Limonin:DMAB adducts.

Breksa and Ibarra (2007), observed for limonin and limonin glucoside that these limonoids likely follow a similar reaction mechanism and lead to the hypothesis that the freedom afforded at C-17 by the open lactone ring of limonin glucoside facilitates the formation of the 2:1 adduct, whereas in the case of limonin, rigidity of the lactone ring may impose steric constraints that retard the formation of the 2:1 product.

On that basis, the formation of red to purple adduct resulting from the treatment of the limonin standard with DMAB under acidic condition for 36% chloridric acid was kinetically studied.

Vaks and Lifschits (1981) reported a 503 nm absorbance maximum for limonin and Breksa and Ibarra (2007) reported an 470 nm.

In this study, using chloridric acid in place of the mixture acetic and perchloric acids, the measured visible spectrum showed after 5 hours an absorbance maximum for limonin derivatives at 510 nm plus another 670 nm absorbance (Figure 5).

According to what was said by Breksa and Ibarra (2007), possibly the differences in the limonin absorbance maximums are a result of the nature of the solutions, homogeneous versus biphasic, or caused by the presence of chloroform. Additionally, it was noticed that, using high concentration of limonin and DMAB, the maximum absorbance shifts from 490 to 510 nm dependent of time. This is consistent with the fact that the density and polarity of the reacting medium increases with time and this causes a change of spectral band position toward a longer wavelength (bathochromic shift).

Figure 4 shows the formation of the colorimetric 1:1 adduct (**4**) reaction time dependent; utilizing 36% chloridric acid such as an acidic medium (see materials and methods section), it is possible to notice that, after a gradual increase, the absorbance signal stabilized around 20-23 hours.

On the basis of this results it was chosen a day reaction endpoint for limonin products and for convenience it was utilized the same endpoint for limonin glucoside.

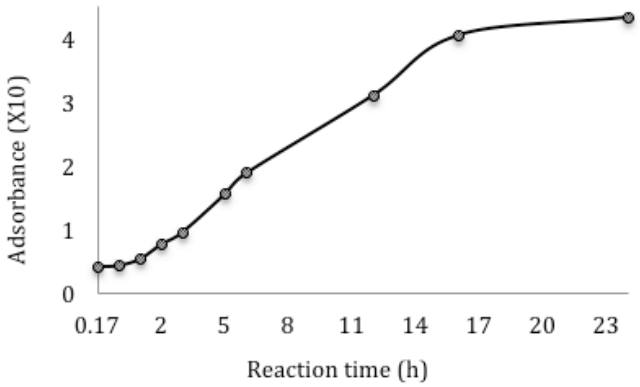


Figure 4. Absorbance vs time (hours) of limonin reaction products.

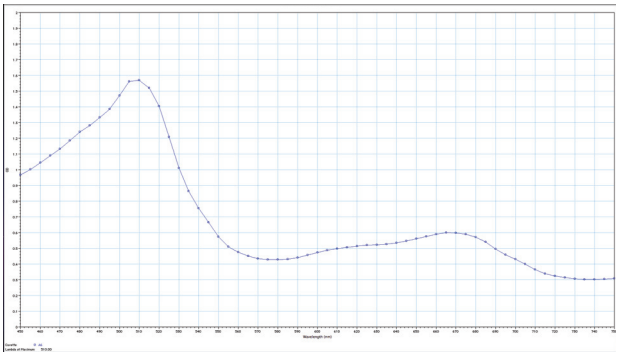
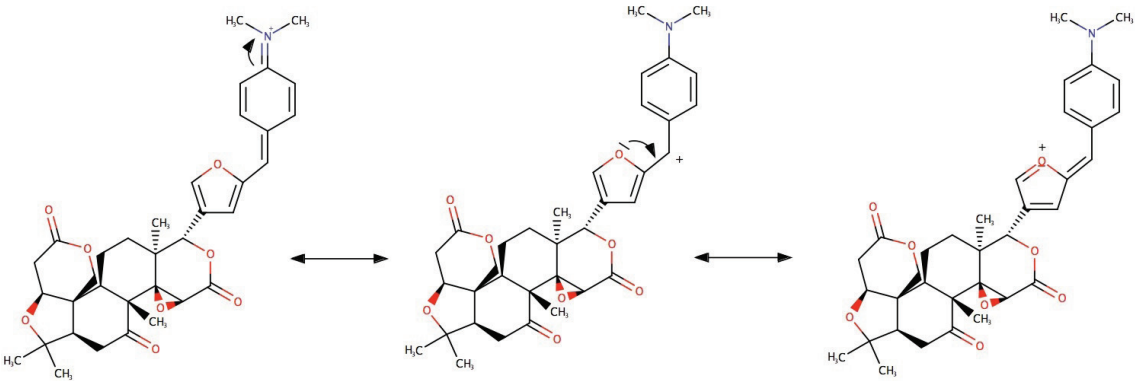
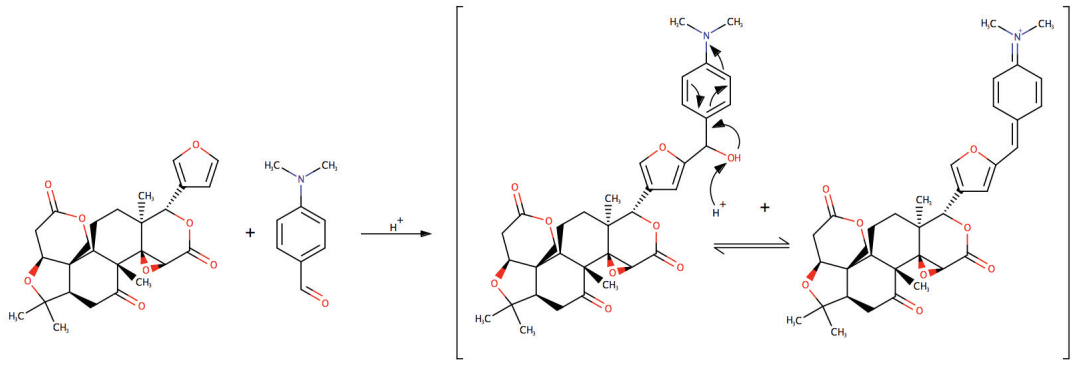


Figure 5. Visible spectrum (450-750 nm) of limonin reaction products with DMAB after a 5 hours



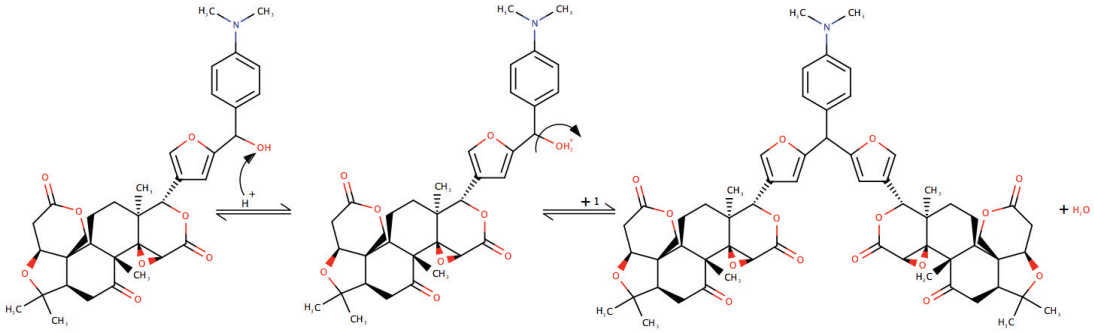
Scheme 1. Resonance structures of 1:1 Lim:DMAB adduct



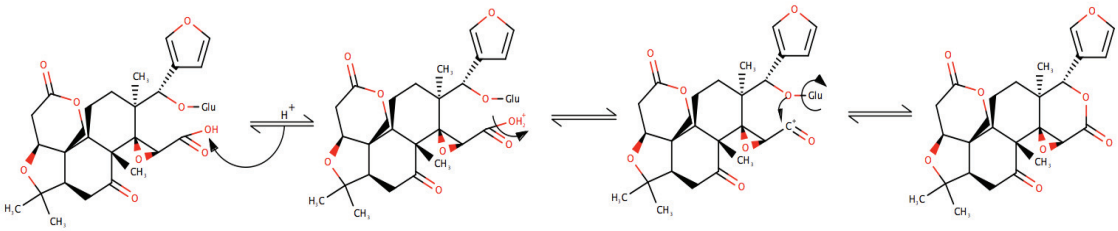
Scheme 2. Synthesis of 1:1 Lim:DMAB adduct (**4**): Proposed reaction mechanism.

Figure 8 shows the predicted mass spectra and the relative isotopic abundances of **4** and **5** adducts; by comparing with experimental mass spectra it was possible to notice a match of the signals even regarding the isotopic abundances. Furthermore, based on the proposed reaction mechanisms (scheme 2 and 3), production of the adducts was achieved through a direct bond on the carbonyl group and it does not cause the limonin ring opening which could generate different spacial arrangements; additionally, in the considered acidic conditions, limonin glucoside turns in limonin by hydrolysis (scheme 4) before or during the condensation reaction. This is consistent with the fact that the reaction afforded the same **4** and **5** adducts regardless of the utilizing of **1** or **2**; add to this, DMAB has no chiral centers which could generate different conformers. For all these reasons, it was not difficult predict the adducts structures, later confirmed by the spectral data.

On the contrary, the achievement of a complex product mixture can be attributed to the handling the strong acidic conditions which could generate a derivatives fragmentation.



Scheme 3. Synthesis of 2:1 Lim:DMAB adduct (**5**): Proposed reaction mechanism.



Scheme 4. Hydrolysis of Limonin Glucoside: Proposed reaction mechanism.

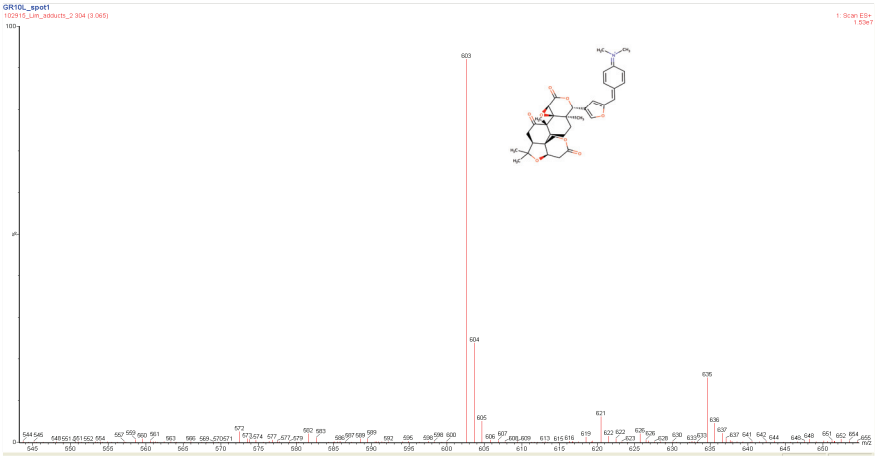


Figure 6. Molecular Ion Peak of Adduct **4**.

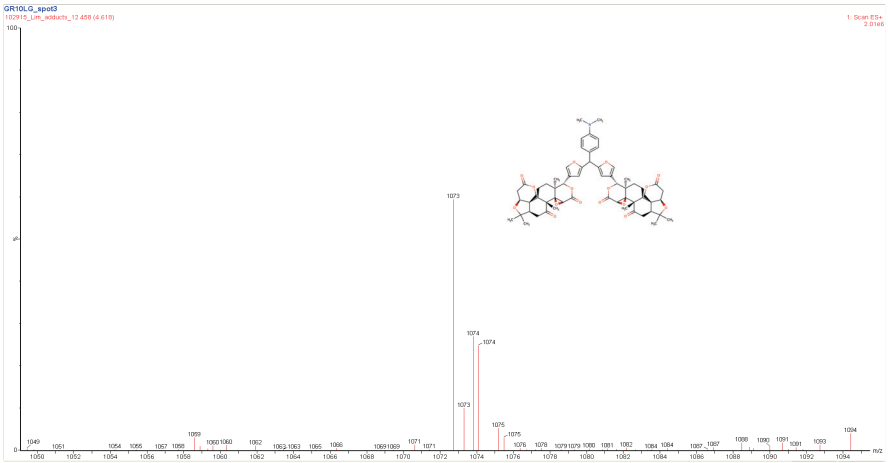


Figure 7. Molecular Ion Peak of Adduct **5**.

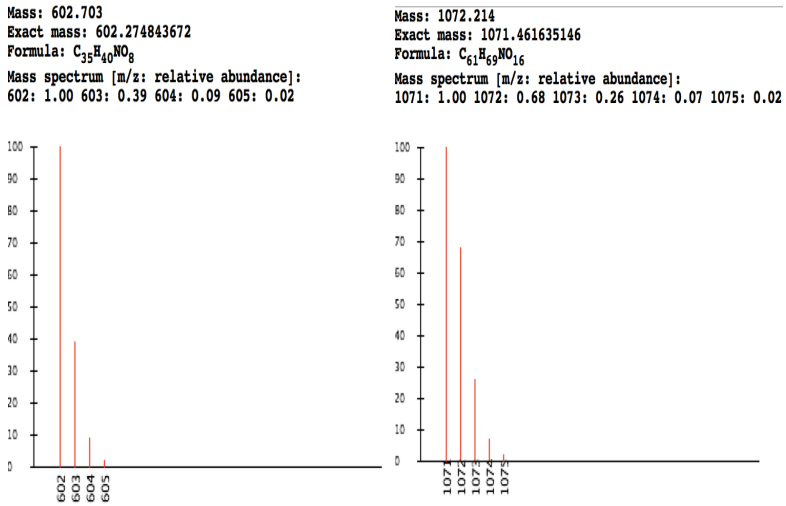


Figure 8. Predicted mass spectra of 1:1 and 2:1 Lim:DMAB adducts.

4. CONCLUSION

In conclusion, the primary result of this study is that the Ehrlich's coloring reaction of **1** and **2** gives a complex products mixture for the reason above specified.

Although a low handling of the products and weaker conditions were used in the reaction 3 (see materials and methods section), the 2:1 adduct was obtained with low yield (25%).

Because of the acidic conditions, LG (**2**) lose the glucose functional group by hydrolysis (scheme 4) and the reaction with **3** produces the same adducts **4** and **5**.

It could be deduced that the coloring compound is a dehydrated cationic intermediate 1:1 adduct (**4**) (Kuroda *et al*, 2004) resonance-stabilized (Scheme 1).

Aim of this study was the synthesis and characterization of the target compounds **4** and **5**; although, the mass spectra for both adducts has confirmed their actual presence by the molecular ion masses and the relative abundances, elucidation of the exact identity of the limonoid condensation products by NMR analysis would be essential to confirm their spacial structures; additionally the reaction of both **1** and **2** with **3** produces other minor compounds which were not characterized in this paper, and should be a subject of future studies.

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EXTRAS

CRA
CONSIGLIO PER LA RICERCA E LA SPERIMENTAZIONE IN AGRICOLTURA

CRA-SFM
UNITÀ DI RICERCA PER IL RECUPERO E LA VALORIZZAZIONE DELLE SPECIE FLORICOLE MEDITERRANEE

Fatty acids characterization of Chamaerops humilis L. seed and its relation with the environment

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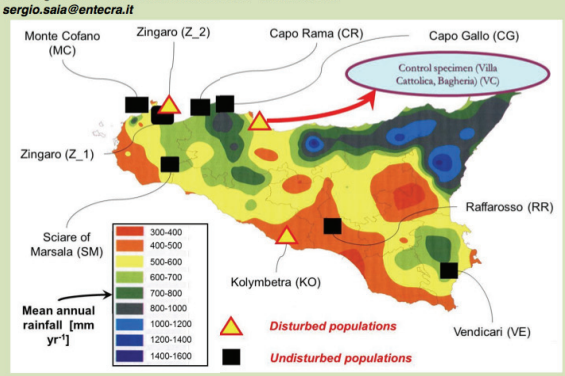
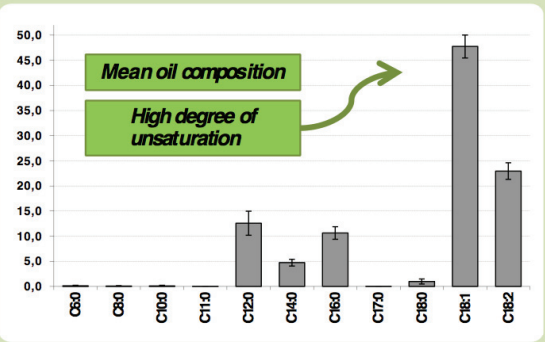
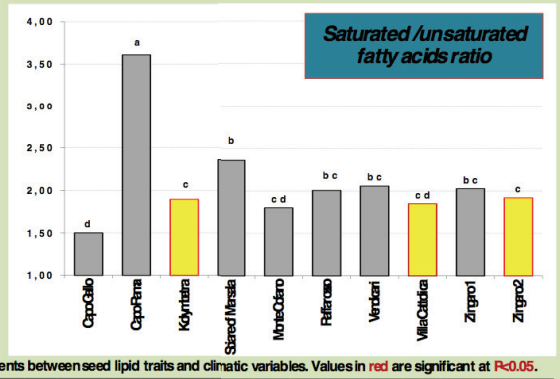
European fan palm (*Chamaerops humilis*) is widespread in the Mediterranean semiarid environments and produce lipid-rich seeds, which can play a role in wildlife feeding during winter and summer and are seldom used for human nutrition. ***Aim of the present research was evaluating the lipid content and fatty acid composition of the seeds from natural populations of European fan palm and the relation among fatty acid composition and climatic traits of the collection sites.***



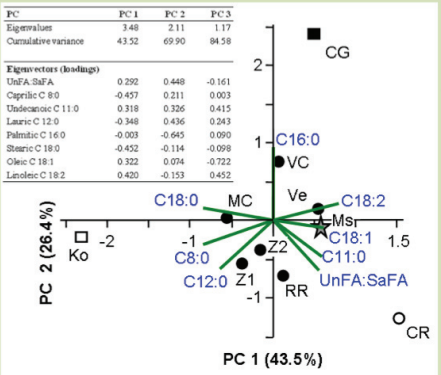


European fan palm seed showed a mean lipid content of 5.5%, *which importance of this species for feeding wild life during winter and summer*



Content in oleic acid negatively correlated with year ETP and could emerge from an environmental adaptation of the genotypes. The present results suggest that seed extracts of the European fan palm could be used as fatty acid in diet supplementation. However, further studies are needed to identify the content of both anti-nutritional and other nutraceutic compounds.

PC	PC 1	PC 2	PC 3
Eigenvalues	3.48	2.11	1.17
Cumulative variance	43.52	69.90	84.58
Eigenvectors (loadings)			
UnFA:SaFA	0.292	0.448	-0.161
Caprilic C 8:0	-0.457	0.211	0.003
Undecanoic C 11:0	0.318	0.326	0.415
Lauric C 12:0	-0.348	0.436	0.243
Palmitic C 16:0	-0.003	-0.645	0.090
Stearic C 18:0	-0.452	-0.114	-0.098
Oleic C 18:1	0.322	0.074	-0.722
Linoleic C 18:2	0.420	-0.153	0.452



Correlation coefficients between seed lipid traits and climatic variables. Values in red are significant at P<0.05.

		Lipids	UnFa:SaFa	O/L	C6:0	C8:0	C10:0	C11:0	C12:0	C14:0	C16:0	C17:0	C18:0	C18:1	C18:2
Mean rainfall (R)	Fall	0.64	0.46	-0.04	0.45	-0.36	-0.44	0.24	**-0.90**	-0.56	0.47	0.39	-0.27	**0.63**	0.48
	Winter	0.62	0.42	0.23	0.16	-0.32	-0.19	0.07	**-0.73**	-0.43	0.45	0.44	-0.07	**0.61**	0.17
	Spring	0.56	0.33	0.39	-0.10	-0.19	-0.11	-0.06	-0.49	-0.35	0.25	0.32	-0.06	0.56	-0.04
	Summer	0.55	0.42	-0.02	0.40	-0.37	-0.42	0.30	**-0.85**	**-0.62**	0.31	0.28	-0.26	**0.68**	0.49
	Year	0.64	0.43	0.20	0.20	-0.39	-0.27	0.10	**-0.76**	-0.48	0.39	0.41	-0.14	**0.65**	0.23
Temperature (T, expressed in °C degrees)	Mean Fall	-0.26	-0.22	-0.01	-0.09	0.53	0.50	-0.29	0.40	**0.62**	0.11	0.13	0.46	-0.56	-0.37
	Mean Winter	-0.32	-0.14	-0.05	0.04	0.16	0.10	-0.24	0.16	0.31	0.09	-0.03	0.11	-0.27	-0.13
	Mean Spring	0.21	0.03	-0.04	0.15	-0.06	-0.10	-0.18	-0.13	0.07	0.23	0.09	-0.05	-0.01	0.03
	Mean Summer	-0.30	0.03	**-0.81**	**0.62**	-0.27	-0.40	0.49	-0.08	-0.05	0.23	0.07	-0.34	-0.38	**0.62**
	Max Fall	-0.19	-0.10	-0.20	0.12	0.29	0.23	-0.11	0.19	0.42	0.22	0.19	0.25	-0.48	-0.10
	Max Winter	0.07	-0.08	0.00	0.07	0.17	0.16	-0.28	0.04	0.30	0.17	0.07	0.21	-0.20	-0.14
	Max Spring	0.58	0.39	-0.04	0.46	-0.38	-0.25	0.13	**-0.81**	-0.35	0.53	0.46	-0.06	0.46	0.36
	Max Summer	-0.13	0.20	-0.35	0.34	-0.20	-0.13	0.54	-0.28	-0.25	0.16	0.25	-0.07	0.02	0.41
	Min Fall	-0.29	-0.26	0.07	-0.18	**0.61**	**0.59**	-0.36	0.47	**0.68**	0.05	0.09	0.56	-0.59	-0.47
	Min Winter	-0.09	-0.18	-0.09	0.01	0.35	0.65	-0.24	0.25	0.30	0.02	-0.11	0.03	-0.31	-0.12
	Min Spring	0.06	-0.09	-0.03	0.02	0.05	-0.03	-0.24	0.10	0.19	0.09	-0.05	-0.03	-0.15	-0.08
	Min Summer	-0.15	-0.11	-0.40	0.26	-0.09	-0.23	0.03	0.13	0.12	0.07	-0.12	-0.22	-0.31	0.21
Evapo-transpiration (ETP)	Mean	-0.06	-0.02	-0.36	0.31	-0.03	-0.12	0.03	-0.01	0.15	0.23	0.09	-0.07	-0.29	0.19
	Min	-0.08	-0.18	-0.08	0.01	0.16	0.07	-0.25	0.24	0.32	0.03	-0.10	0.06	-0.31	-0.13
	Max	-0.53	-0.39	-0.11	-0.24	0.48	0.32	-0.24	**0.75**	0.58	-0.24	-0.26	0.20	**-0.69**	-0.35
Climatic indexes	Long Index	0.62	0.42	0.26	0.12	-0.37	-0.24	0.16	**-0.73**	-0.50	0.33	0.37	-0.13	**0.68**	0.19
	De Martonne Index	0.65	0.43	0.23	0.15	-0.42	-0.29	0.16	**-0.75**	-0.53	0.34	0.36	-0.18	**0.70**	0.22
	Emberger Index	**0.70**	0.39	0.32	0.10	-0.33	-0.20	-0.06	**-0.72**	-0.41	0.38	0.37	-0.09	**0.66**	0.10
	Thornthwaite Index	0.64	0.41	0.31	0.09	-0.36	-0.22	0.06	**-0.72**	-0.50	0.31	0.35	-0.12	**0.70**	0.15
	Aridity Index	0.64	0.42	0.30	0.09	-0.37	-0.23	0.07	**-0.72**	-0.50	0.31	0.35	-0.13	**0.71**	0.16

Materials and Methods: Seeds were collected in late summer (19th to 23th of September) 2011. In the Zingaro collection sites, two putative populations, differing for morphological traits, were found, thus they were taken separately and named as Zingaro 1 and Zingaro 2. Seeds were ground and dried under vacuum. Seed lipids were extracted by Soxhlet with n-hexane (15 g dried seeds with 400 ml solvent) for 24 h at 50°C. Fatty acid methyl esters (FAMEs) were obtained by trans-esterification with sodium methoxide; 1-Brome Tetradecane was used as an internal standard. Three biological replicates were adopted for each accession. Analyses were performed in duplicate using a gas chromatographer (GC) Agilent (6890 series) coupled with a single quadrupole (SQ-5973 inert). Injector was set in split-mode (split ratio 1:100) at 250 °C. 1 µl of FAMEs in n-hexane was injected into a WAX column (Varian, VF WAXms 30 m ×, 0.25 mm × 0.25 µm). Oven temperature was held at 50°C for 2 minutes, then increased at a rate of 4°C min-1 to 220°C and held at 220°C for 15 minutes. The transfer line was held at 290°C. Ion source was at electronic ionization (230°C and 70eV). Quadrupole was kept at 150°C. Acquisition was made as total ion current (TIC). Acquisition ranges were from 40 to 300 atomic mass units (amu) until 25 minutes run time and from 40 to 400 amu from 25 min to the end of the run. Chemical structure was assigned by comparison of each retention time with a standard FAMEs mix and confirmed comparing the mass spectra with those in the NIST 2002 database. Relative percentage of every single fatty acid, unsaturated (UnFA) to saturated fatty acids (SaFA) ratio and oleic to linoleic ratio (O/L) were computed. All variables corresponding to proportions were arcsine-transformed to better fit with the Gaussian law distribution. Data were subjected to ANOVA and treatment means were compared using Tukey's test (P values) at 5% probability level. A principal component analysis (PCA) with previously standardised data was performed to establish the importance of different traits in explaining multivariate polymorphisms. Correlations among variables were calculated and whereas two or more variables were highly correlated (r>0.70) one of these was discarded from analysis to avoid weighting distortion as suggested by Pengelly & Maass (2001). Only those principal components (PCs) with eigenvalue >1 were retained for cluster analysis (Kaiser 1960), adopting Euclidean distance as a measure of dissimilarity and Average method as clustering algorithm. Number of clusters were estimated by means of pseudo F and t2 statistics (Milligan & Cooper 1985). Correlation between the variables and the climatic or weather traits of the collection sites were calculated.

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NEW APPLICATION OF AGRO-INDUSTRIAL WASTE TO PREVENT MELANOSIS OF MEDITERRANEAN PINK SHRIMP

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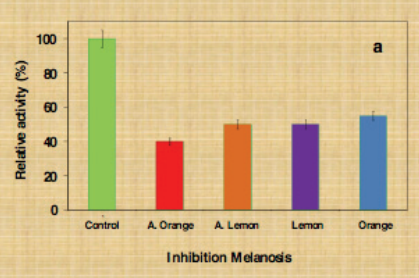
INTRODUCTION

The citrus are high nutritional value fruits and they are widely used for the production of juice. The citrus byproducts represent an environmental and economic problem because peel, pulp, and yellow water are difficult to dispose of.
Additionally, citrus byproducts have a high content of bioactive compounds with antimicrobial and antioxidant activity e.g., ascorbic acid, limonoids, and polyphenols [1]. Obtaining these compounds can make more sustainable the production chain of the citrus industry and their properties can be used in the melanosis preservation of crustaceans.
Polyphenol oxidase (PPO, EC 1.14.18.1) is primary enzyme which causes enzymatic browning in shrimp [4, 5]. Different anti-browning agents are used in seafood products to control its undesirable effects [2, 3].
Aims of work is test how natural extracts obtained from vegetable waste, in particular orange and lemon peel, may inhibit the process of melanosis in pink shrimp species (*Parapenaeus Longirostris*).

MATERIAL AND METHODS

The pink shrimp were purchased from a local market of Catania (Sicily, Italy). The crustaceans were kept in ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the DISPA laboratory within 1.5 h. Upon arrival, the shrimp were washed in cold tap water, air-dried to remove the water in excess present on the surface, peeled of cephalothoraxes. The enzyme extraction and the spectrophotometric assay were carried out according to the method of Espin (1996) with some modifications. The potential melanosis inhibitors (lemon, orange) at the same concentration (1%) were individually mixed with crude PPO extract to obtain the final concentration of 0.5% (w/v). Sensory analysis was conducted by a panel of 10 trained assessors, in accordance with the Quality Index method.

Figure.1: Effect in vitro of anti-browning treatments (albedo orange, albedo lemon, lemon, orange) the inhibition of PPO enzyme from pink shrimp (a: extraction in hot water; b: extraction in ethanol). Bars represent the standard deviation, the experiment were conduced in triplicate



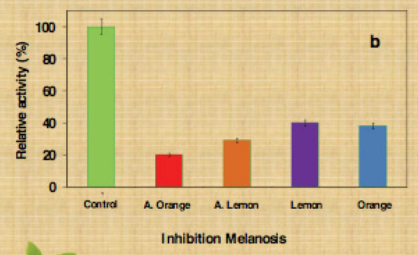
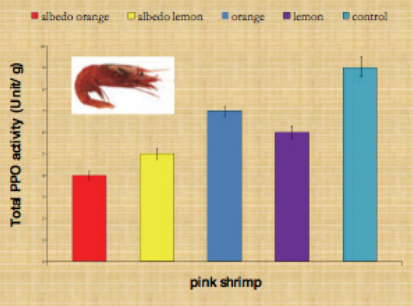


Figure.2 - Effect in vivo of anti-browning treatments (albedo orange, albedo lemon, lemon, orange) the inhibition of total PPO enzyme from pink shrimp extraction in hot water during conservation at 4 °C. Bars represent the standard deviation, the experiment were conduced in triplicate



RESULTS

The treatment with orange peel, extracted in hot water, significantly reduced ($p<0.05$) the enzymatic activity of the PPO at the level of the cephalothorax of the shrimp (Fig.1 a-b). Ethanolic extract of lemon peels was also effective for Melanosis inhibition. The Fig.2 shown the effect in vivo of anti-browning treatments (albedo orange, albedo lemon, lemon, orange) the inhibition of total PPO enzyme from the pink shrimp extraction in hot water during conservation at 4 °C. The extract of albedo orange has significantly reduced the enzymatic activity of the PPO at the level of the cephalothorax in all species analysed also in vivo. Unlike the treatment with orange peel extracts has been ineffective in species analysed.

CONCLUSIONS

These results were related to the evaluation of the polyphenol quality index (QI) that confirmed the efficiency of the treatment of orange and lemon peel extracts. The addition of these natural extracts in pink shrimp samples can be considered a valid alternative to the use of agro-industrial waste.

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DRY CHERRY TOMATO: INNOVATION FROM STUDIES ON ISOTHERMS

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ABSTRACT
The drying of vegetables is a very ancient practice for food preservation still in use nowadays. Tomatoes cut into two parts, added with salt and dried are an important element of the food tradition in south of Italy. The tomato's drying in Sicily is still carried out with a sun-drying process, with an empiric method. The final product obtained has a high quality which it is not homogeneous. In addition to dried products, the market offer even semidry products, to limit the loss of the nutritional characteristics of the dried product. The semidry product is a special category of dehydrated products that have a residual moisture content about 30%; for this reason, nutritional product characteristics may be are best preserved.
Drying experiments of tomatoes were conducted at different final moisture content. The changes in the chemical parameters of tomatoes and principal drying parameters were recorded during all drying process. Isotherms were carried out with the AquaLab Vapor Sorption Analyzer.
The aim of this study is:

- the optimization of the drying process in a pilot plant fold under different temperatures conditions;
- the study of adsorption and desorption isotherms to better understand the drying process;
- to obtain semidry product and dry products comparable for nutritional and sensorial characteristics, monitoring some of the basic chemical parameters, the trend of the main nutrients of tomato during the drying process and the sensory parameters.

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ORIGINAL ARTICLE

Fatty acid composition of the seed lipids of *Chamaerops humilis* L. natural populations and its relation with the environment

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Abstract
Seed lipids composition is a tool to discriminate among plant *taxa* and is related to phylogeny and biogeographic distribution. Aim of the present study was to evaluate the fatty acid (FA) composition of the seed lipids from nine natural *Chamaerops humilis* L. population and its relation with the climatic traits of the collection sites. The average seed lipids content was 54.8 g kg^{-1} and the most represented FAs were oleic (478 g kg^{-1} oil) and linoleic (230 g kg^{-1} oil), with significant differences among the accessions. Most of the lipid traits significantly correlated with climatic traits. In particular, oleic acid negatively correlated with year potential evapotranspiration. These relations could emerge from a genotypic adaptation to the environment. The seed of *C. humilis* showed high content of lipids, which implies an importance of the species for feeding wild life during winter, and a high degree of unsaturation. Considering the importance of the unsaturated FAs in human and animal nutrition, the present results suggest that European fan palm could be introduced in breeding programmes and its seed extract used as FA in diet supplementation. Further studies are needed to identify the content of anti-nutritional or nutraceutical compounds.

Keywords: *European fan palm, dwarf fan palm, seed lipid, unsaturated fatty acid, plant biogeography*

Introduction

Seeds accumulate lipids, mainly triacylglycerols, thanks to an extension of the membrane-lipid and common biosynthetic pathway. Seed lipids and fatty acid (FA) composition diverges among *taxa* (Voelker & Kinney 2001) and have been related to plant biogeographic pattern because they support the early growth of the plantlet and thus can depend on environmental conditions. In addition, its saturated FA/unsaturated FA (SaFA:UnFA) ratio plays a role in plant adaptation to the environment because it stimulates the germination rate and plant growth at low temperatures (Linder 2000). Seed FA have been used to infer systematic and phylogenetic relationships at different taxonomic ranks such as *family* (Hohn & Meinschein 1976; Özcan 2008; Scialabba et al. 2010), *genus* (Graham et al. 1981; Lamarque et al. 2000) and *species* (Dodd and Rafii 1995; Sabzalian et al. 2008).

European fan palm (*Chamaerops humilis* L., *Arecaceae*) is the only native palm in western Europe where it plays an important ecological role in the Thermomediterranean vegetation belt, both in xerophitic shrub communities and degraded ecosystems (Muñoz & Alcántara 2013). However, its occurrence in the Mediterranean is progressively declining due to agricultural land reclamation, human activities and animal predation, and this led most of the Italian regions to envisage its protection in the Special Area of Conservation (SAC) defined in the European Union's Habitats Directive (92/43/EEC). In addition, infestation of European fan palm by the red palm weevil *Rhynchophorus ferrugineus* has recently been reported (Giovino et al. 2012a).

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Despite its importance, little information is available on the diversity of the native European fan palm populations. Aim of the present work was to characterize nine natural European fan palm populations collected in eight different SACs from Sicily for their seed lipid content and FA composition and to study its relation with the climatic characters of the collection sites.

Material and methods

Germplasm collection and characterization of the collection localities

A collection of natural populations of European fan palm (*Chamaerops humilis*) was performed on the basis of its distribution in Sicily (Table I). No seed was found in three populations from very arid

Table I. Identification, climatic characteristics and growing-season (1 September 2010–23 September 2011) weather data of the nine collection sites and Villa Cattolica (Bagheria, PA, Italy) in which the European fan palm seeds were collected.

Collection site Accession code	Capo Gallo CG	Capo Rama CR	Kolymbetra Ko	Monte Cofano MC	Marsala Ms	Raffarosso RR	Vendicari V	Zingaro (pop. 1) Z_1	Zingaro (pop. 2) Z_2	Villa Cattolica (control specimen) VC
Latitude N	38°13′	38°08′	37°17′	38°06	37°53	37°16′	36°48′	38°06′	38°06′	38°05′
Longitude E	13°18′	13°04′	13°35′	12°40′	12°31′	14°23′	15°06′	12°48′	12°48′	13°30′
Growing season (September 2010–September 2011)										
Rainfall (*R*)										
Year	651	710	532	727	606	753	820	716	716	642
Fall	246	277	208	277	261	253	290	268	268	193
Winter	218	253	238	288	233	257	374	269	269	222
Spring	65	74	52	104	72	139	125	107	107	98
Summer	59	24	5	19	12	13	6	18	18	33
Rainy days	66	81	46	82	73	65	67	83	83	64
ETp	1152	1036	1292	949	1125	1267	1111	1050	1050	1125
Mean temperature										
Fall	18.5	17.0	17.5	15.2	17.7	17.3	17.4	17.4	17.4	17.4
Winter	26.3	24.5	25.0	23.7	25.9	26.0	25.3	25.2	25.2	25.2
Spring	17.8	16.5	17.2	14.3	16.9	16.5	16.4	17.3	17.3	16.4
Summer	12.0	10.5	11.4	8.9	11.3	9.1	10.3	11.3	11.3	10.6
GDD	3478	2968	3138	3143	2418	3076	3026	3015	3015	2998
RH										
Min	41.3	46.8	47.9	51.5	56.1	46.2	45.7	48.2	48.2	42.6
Max	81.1	98.3	83.8	94.0	94.2	97.3	98.6	88.8	88.8	83.3
Climatic indexes										
AI	0.56	0.69	0.41	0.77	0.54	0.59	0.74	0.68	0.68	0.57
Ir	34.4	40.8	29.5	46.1	33.2	42.9	46.4	39.5	39.5	36.4
Ia	22.5	25.9	19.0	28.2	21.4	27.3	29.6	25.4	25.4	23.2
Im	−43.5	−31.5	−58.8	−23.4	−46.1	−40.6	−26.2	−31.8	−31.8	−42.9
R/GDD	0.19	0.24	0.17	0.19	0.30	0.24	0.27	0.24	0.24	0.21
(*R* − ETp)/GDD	−0.14	−0.11	−0.24	−0.17	−0.09	−0.17	−0.10	−0.11	−0.11	−0.16
Long-term average (30 years)										
Rainfall (*R*)										
Year	683	683	516	491	678	484	452	491	491	n.a.
Fall	238	238	173	178	220	168	190	178	178	n.a.
Winter	272	272	211	192	269	189	172	192	192	n.a.
Spring	147	147	117	109	162	108	67	109	109	n.a.
Summer	26	26	15	12	27	20	23	12	12	n.a.
Rainy days	72	72	59	61	77	58	42	61	61	n.a.
ETp	954	954	912	970	881	865	924	970	970	n.a.
Mean temperature										
Fall	19.9	19.9	21.5	21.2	18.5	18.0	19.9	21.2	21.2	n.a.
Winter	11.8	11.8	12.1	13.3	10.9	9.1	12.5	13.3	13.3	n.a.
Spring	15.1	15.1	14.5	15.4	14.5	13.0	14.6	15.4	15.4	n.a.
Summer	25.6	25.6	24.7	25.5	24.5	25.2	25.2	25.5	25.5	n.a.
Climatic index										
AI	0.72	0.72	0.57	0.51	0.77	0.56	0.49	0.51	0.51	n.a.
Ir	37.1	37.1	29.0	26.0	39.0	29.0	25.0	26.0	26.0	n.a.
Ia	24.0	24.0	18.0	17.0	25.0	18.0	16.0	17.0	17.0	n.a.
Im	−28.4	−28.4	−43.0	−49.0	−23.0	−44.0	−51.0	−49.0	−49.0	n.a.

Note: Climatic indexes on a long-term and growing-season basis are provided. See text for climatic indexes formula.

environments, probably due to wild animal predation, and so these populations were not included in the present analyses. Seeds were collected in late summer (19–23 September) 2011, and a total of nine populations and a single cultivated control tree growing in the historical Villa Cattolica of Bagheria (Palermo, Italy) were sampled. In the Zingaro collection sites, two putative populations, differing for morphological traits, were found, thus they were taken separately and named as Zingaro 1 and Zingaro 2. For each population sampled, the main morphometric descriptors were recorded and a bulk of ripe dates was harvested from at least 10 randomly chosen plants. For each collection site, the main long-term climatic parameters were gathered from the data recorded by the closest weather station with at least 30 years of consistent data as displayed in Cartabellotta et al. (1998). The climatic data of the last growing season (1 September 2010–23 September 2011) were provided by the Informative Agro-Meteorological Sicilian Service (SIAS 2012). Long-term climatic data considered were rainfall (R); mean maximum, mean minimum and mean average temperatures (T) on a per-season basis; number of rainy days per year; and mean maximum, mean average and mean minimum of potential evapotranspiration (ETp) on a per-year basis. The growing-season weather data considered were R; mean average temperatures, minimum and maximum relative humidity (RH); and growing-degree days (GDD at $t_0 = 10$°C) and ETp on a per-season basis.

With both climatic and weather data, the following indexes were calculated: FAO–UNEP aridity index (UNEP 1992) as R/ETp (AI); Lang index (Ir), as R/T (Kira 1976); De Martonne (1927) index (Ia), as $R/(T + 10)$; and Thornthwaite (1948) index (Im), as $(R \times \text{ETp})/\text{ETp} \times 100$. The Emberger (1955) index (Iq) was calculated on the basis of climatic data alone, $\text{Iq} = [R/(T_H^2 - T_C^2)] \times 100$, where T_H and T_C are the mean maximum temperatures of hottest and coldest months, respectively. Finally, by means of the weather data alone, two new indexes were calculated in order to take into account the amount of available rainfall per GDD. These indexes were calculated as follows: (1) the GDD/R ratio and (2) the $(R - \text{ETp})$/GDD ratio, where $(R - \text{ETp})$ was considered 0 if the actual difference was negative.

Seed lipids extraction and esterification

Dates were conserved at 4°C during transport and processed within 4 h of collection. Flesh was manually removed and the damaged seeds were discarded. Seeds were ground to 1 mm diameter with a cooled (10°C) laboratory mill (IKA A10 Analytical Mill) to avoid oxidation of the lipids. Ground seeds were dried under vacuum. Seed lipids were extracted by means of Soxhlet (Büchi, Italy) using *n*-hexane (HPLC-grade; Sigma-Aldrich, Milan, Italy) as solvent. Briefly, 15 g of dried ground seeds were placed into a cellulose cone and extracted with 400 ml solvent for 24 h at 50°C. The soxhlet was kept in the dark to avoid lipids photooxidation. After extraction, the solvent was removed via a rotary vacuum distillator at 50°C. The residual fatty fraction was weighed and lipid content determined. Lipids were collected by successive addition of 2, 2 and 1 ml of *n*-hexane and stored at −20°C for further analyses.

Fatty acid methyl esters (FAMEs) were obtained by trans-esterification mixing 2000 µl of oil in *n*-hexane (average oil content: 8%) and adding 950 µl of sodium methoxide 2 M in methanol and 50 µl of 1-Brome Tetradecane (Sigma Aldrich) as an internal standard. The mixture was kept at 72°C for 1 h. After esterification, vials were kept at 4°C until complete separation of the methanol/hexane phases. The top layer was diluted 10-folds with *n*-hexane and saved at −20°C until analysis.

Gas-chromatographic mass spectrometric analyses and peak identification

Three biological replicates were adopted for each accession. The analyses were performed in duplicate using a gas chromatographer (GC) Agilent (6890 series) coupled with a single quadrupole (SQ-5973 inert). Helium was the carrier gas. Injector was set in split mode (split ratio 1:100) at 250°C. Then, 1 µl of FAMEs in *n*-hexane was injected into a WAX column (Varian, VF WAXms 30 m × 0.25 mm × 0.25 µm). Oven temperature was held at 50°C for 2 min, then increased at a rate of 4°C min^{-1} to 220°C and held at 220°C for 15 min. The transfer line was held at 290°C.

Ion source was at electronic ionization (230°C and 70 eV). The quadrupole was kept at 150°C. Acquisition was made as total ion current (TIC). Acquisition ranges were from 40 to 300 atomic mass units (amu) until 25 min run time and from 40 to 400 amu from 25 min to the end of the run.

A chemical structure was assigned to each peak by comparison of each retention time with a standard FAMEs mix (Supelco 37 Component) and confirmed comparing the mass spectra with those in the NIST 2002 database. The baseline area (accounting for less than 0.1‰ of the total area) was removed and the peak areas corrected on the basis of the internal standard area.

Calculation and statistical analysis

The relative percentage of every single FA, the UnFA:SaFA ratio and the oleic-to-linoleic ratio (O:L) were computed.

All variables corresponding to proportions were arcsine-transformed to better fit with the Gaussian law distribution. All data were subjected to analysis of variance (ANOVA procedure; SAS, Institute, Inc. 2002) according to the experimental design, and treatment means were compared using least significant differences (LSD) at 5% probability level.

A principal component analysis (PCA) was performed (PRINCOMP procedure; SAS Institute, Inc. 2002) using the data on oil percentage, single FA percentages and UnFA:SaFa ratio to establish the importance of different traits in explaining multivariate polymorphisms. Previously to the PCA, mean values per trait were standardized to a mean equal to 0 and standard deviation (SD) equal to 1. Correlations among variables were calculated, and whereas two or more variables were highly correlated ($r > 0.70$), one of these was discarded from the analysis to avoid weighting distortion as suggested by Pengelly and Maass (2001). Only those principal components (PCs) with eigenvalue > 1 were retained for cluster analysis (CA, Kaiser 1960; CLUSTER procedure, SAS Institute, Inc. 2002), adopting Euclidean distance as a measure of dissimilarity and average method as clustering algorithm. The number of clusters was estimated by means of pseudo F and t^2 statistics (Milligan & Cooper 1985).

Relationships between the variables and the climatic or weather traits of the collection sites were calculated.

Results

The oil content of the 10 European fan palm (*Chamaerops humilis*) accessions collected in Sicily ranged from 5.05% to 6.07% of the seed weight.

The average FA composition of the seed oil is shown in Table II. Eleven FAMEs were identified by the GC/MS analyses. The most represented FAs were oleic (C18:1), linoleic (C18:2), lauric (C12:0), palmitic (C16:0) and myristic (C14:0), accounting for more than 98% of the total FAs. However, seed content in the most represented FAs significantly differed among the accessions, especially oleic and lauric.

O:L ratio was on average $2.08 \pm 0.18\,g\,g^{-1}$ (mean ± SD), with wide variations among the accessions ($P < 0.001$, LSD = 0.104; Figure 1).

On average, UnFA:SaFA ratio was $2.10 \pm 0.57\,g\,g^{-1}$, but highly significant differences for this trait were observed among the accessions sampled ($P < 0.001$, LSD = 0.357; Figure 2). In particular, Capo Rama population showed a UnFA:SaFA percentages of 53%, 86%, 91% and 141% higher than Marsala population, the average of the third cluster's, Kolymbetra and Capo Gallo populations.

A significant correlation was observed between some of the observed traits and some climatic variables (Table III). In particular, oleic acid negatively correlated with maximum year ETp and positively with summer and total rainfall and most of the climatic indexes. Opposite results were observed for lauric acid, which negatively correlated with spring maximum temperature. Seed lipid content correlated with the climatic indexes, especially with the Emberger index.

Some significant correlations were observed between the measured traits and the weather characters (Table IV), but none on a fall/winter basis. In particular, the O:L ratio negatively correlated with Spring + Summer 2011 rainfall. The UnFA:SaFA ratio positively correlated with Summer 2011 Maximum RH and *R*/GDD ratio. No correlation was observed between the seed content in oleic acid and the weather indexes. Lauric acid content negatively correlated with both Summer

Table II. Seed lipid FA composition (mg kg^{-1} DW) of the 10 *Chamaerops humilis* accessions.

	Hexanoic C6:0	Caprilic C8:0	Capric C10:0	Undecanoic C11:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Heptadecanoic C17:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2
Mean	1.4	0.7	1.1	0.1	125.8	47.3	106.3	0.0	9.9	477.5	229.6
SD	1.0	0.9	1.3	0.1	23.9	6.6	12.6	0.1	5.0	22.8	16.6
Range	2.7	2.8	4.3	0.4	75.5	23.3	42.7	0.3	17.0	77.4	51.6
Capo Gallo	2.4	0.0	0.3	0.1	76.4	49.1	139.5	0.3	11.4	477.3	243.2
Capo Rama	2.9	0.3	0.6	0.4	110.8	36.9	98.5	0.0	6.4	497.9	245.3
Monte Cofano	0.4	0.6	1.3	0.0	142.8	47.3	104.1	0.0	10.5	478.3	214.7
Villa Cattolica	1.1	0.4	0.6	0.0	122.8	49.2	111.3	0.0	8.5	458.8	247.3
Zingaro 1	0.8	1.8	1.0	0.1	138.5	46.8	97.0	0.0	8.5	473.3	232.2
Zingaro 2	2.3	0.5	0.9	0.2	149.4	53.9	103.7	0.0	7.0	456.8	225.3
Marsala	0.2	0.2	0.5	0.0	107.0	39.2	99.7	0.0	6.8	530.0	216.5
Kolymbetra	0.3	2.8	4.7	0.0	151.9	60.2	108.3	0.2	23.5	452.6	195.7
Raffarosso	1.4	0.7	0.8	0.3	143.6	45.5	96.7	0.0	7.6	468.9	234.4
Vendicari	2.4	0.2	0.5	0.2	115.4	45.4	104.7	0.0	8.4	481.7	241.2
P > F	*0.50*	*0.10*	*0.38*	**0.05**	**<0.001**	**<0.001**	**<0.001**	*0.54*	**0.001**	**<0.001**	**<0.0001**
$LSD_{0.05}$	*ns*	*ns*	*ns*	**0.4**	**26.1**	**13.1**	**7.4**	*ns*	**10.7**	**42.1**	**16.9**

Note: Variables in italic are non significant at $P > 0.05$, variables in bold are significant at $P \leq 0.05$.

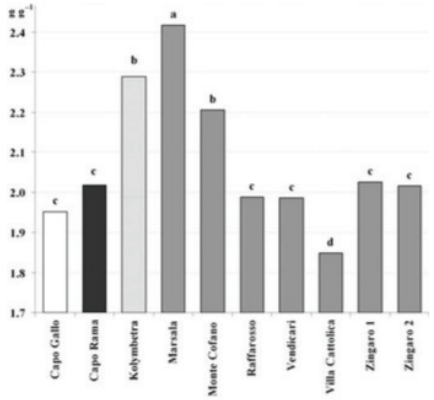

Figure 1. O:L ratio ($g\,g^{-1}$) in the seed lipid of the 10 *Chamaerops humilis* accessions. Bars with same letters are not significantly different at $P < 0.05$. $LSD_{0.05}$ is 0.1043 and corresponds to the unit of measure in *y*-axis. Bars with the same colour are in the same group as defined by cluster analysis.

2011 rainfall and the weather indexes. Opposite results were found for palmitic acid content. Finally, stearic and linoleic acid content negatively and positively correlated, respectively, with Spring + Summer 2011 rainfall.

PCA was performed taking into account 8 of 14 traits, as described above. The first three PCs cumulatively explained 84% of the total variation (Figure 3). PC 1 mainly correlated with the content in octanoic, stearic and linoleic acids, PC 2 with UnFA:SaFA ratio, lauric and palmitic acids, and PC 3 with undecanoic, oleic and linoleic acids.

Figure 3 shows the distribution of the PC 1 versus PC 2 and the eigenvectors of the original variables. By means of the CA, five groups were identified (Figure 4). Cophenetic correlation coefficient between the matrix of distance and the dendrogram was 0.8979. Four clusters were singularly formed by accessions coming from different environments (Kolymbetra, Capo Gallo, Capo Rama, and Marsala), whereas the last cluster consisted of all the other natural populations and the control specimen from Villa Cattolica.

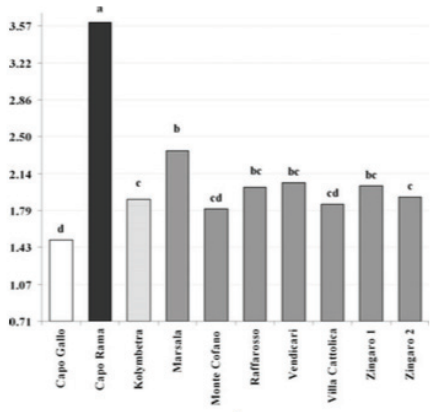

Figure 2. UnFA:SaFA ratio ($g\,g^{-1}$) in the seed lipid of the 10 *Chamaerops humilis* samples. Bars with same letters are not significantly different at $P < 0.05$. $LSD_{0.05}$ is 0.3573 and corresponds to the unit of measure in *y*-axis. Bars with the same colour are in the same group as defined by cluster analysis.

Discussion

Lipid content of European fan palm (*Chamaerops humilis*) seed was $54.8 \pm 3.2\,g\,kg^{-1}$ and correlated with climatic indexes. Similar seed lipid contents has been reported for other species in the same subfamily (*Coryphoideae*) such as *Sabal maritima*, *S. palmetto* and *Thrinax radiata* (Rodríguez-Leyes et al. 2007), whereas others such as *Phoenix dactylifera* (Besbes et al. 2004), *P. canariensis* (Nehdi et al. 2010) and *Washingtonia filifera* (Nehdi 2011) showed higher seed lipid content. The seed lipid content of the palms depends on several factors among which the subfamily. In the *Corypheae* and *Phoeniceae* palm tribes (both in the *Coryphoideae* subfamily), Litchfield (1970) observed that the seed lipid content of palms ranges from 30 to $140\,g\,kg^{-1}$. In a wide range of angiosperms, Levin (1974) has shown that the lipid content of the seed depends on specific ecogeographic adaptations and it is higher in those species evolved in resource-limited environment, such as arid environments. In addition, Westoby et al. (1992) suggested that in perennial plants that emerge under a seed canopy, seed storage compounds could be important in supporting plant growth before the complete establishment of the photosynthetic machinery, whereas in *Passiflora caerulea*, Mendiondo and Amela García (2009) showed that the seed lipid content plays a role in determining the physical dormancy, by increasing water impermeability, which is important in tropical climates. The European fan palm is among those palm species adapted to relatively cold and humid climates and usually grows in Mediterranean maquis with high radiation. This could explain its lower seed lipid content if compared with other palm species from the same subfamily.

The average contents in oleic (C 18:1) and linoleic (C 18:2) acids were 48% and 23%, respectively, with significant variations among the accessions. Besbes et al. (2004) found that the content in these acids varied between two different

Table III. Correlation coefficients between seed lipid traits and climatic variables.

	% Seed lipids	UnFA:SaFa	O:L	Hexanoic C6:0	Caprilic C8:0	Capric C10:0	Undecanoic C11:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Hepta-decanoic C17:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2
Mean rainfall (*R*)														
Fall	*0.64*	0.46	−0.04	0.45	−0.56	−0.44	0.24	**−0.90**	−0.56	0.47	0.39	−0.27	*0.63*	0.48
Winter	*0.62*	0.42	0.23	0.16	−0.32	−0.19	0.07	**−0.73**	−0.43	0.40	0.44	−0.07	*0.61*	0.17
Spring	0.54	0.33	0.39	−0.10	−0.19	−0.11	−0.06	−0.49	−0.35	0.25	0.32	−0.06	0.56	−0.04
Summer	0.55	0.42	−0.02	0.40	−0.57	−0.42	0.30	**−0.85**	*−0.62*	0.31	0.28	−0.26	**0.68**	0.49
Year	*0.64*	0.43	0.20	0.20	−0.39	−0.27	0.10	**−0.76**	−0.48	0.39	0.41	−0.14	*0.65*	0.23
Mean number of rainy days per year	0.52	0.31	0.35	−0.10	−0.22	−0.18	−0.06	−0.44	−0.36	0.22	0.26	−0.15	0.54	−0.02
Temperature (*T*, expressed in °C)														
Mean fall	−0.26	−0.22	−0.01	−0.09	0.53	0.50	−0.29	0.40	*0.62*	0.11	0.13	0.48	−0.58	−0.37
Mean winter	−0.02	−0.14	−0.05	0.04	0.16	0.10	−0.26	0.16	0.31	0.09	−0.03	0.11	−0.27	−0.13
Mean spring	0.21	0.03	−0.04	0.15	−0.06	−0.10	−0.18	−0.13	0.07	0.23	0.09	−0.05	−0.01	0.03
Mean summer	−0.30	0.03	**−0.81**	*0.62*	−0.27	−0.40	0.49	−0.08	−0.05	0.23	0.07	−0.34	−0.38	*0.62*
Max fall	−0.19	−0.10	−0.20	0.12	0.29	0.23	−0.11	0.19	0.42	0.22	0.19	0.25	−0.48	−0.10
Max winter	0.07	−0.08	0.00	0.07	0.17	0.16	−0.28	0.04	0.30	0.17	0.07	0.21	−0.20	−0.14
Max spring	0.58	0.39	−0.04	0.46	−0.38	−0.25	0.13	**−0.81**	−0.35	0.53	0.46	−0.06	0.46	0.36
Max summer	−0.13	0.20	−0.35	0.34	−0.20	−0.13	0.54	−0.28	−0.25	0.16	0.25	−0.07	0.02	0.41
Min fall	−0.29	−0.26	0.07	−0.18	*0.61*	*0.59*	−0.36	0.47	**0.68**	0.05	0.09	0.56	*−0.59*	−0.47
Min winter	−0.09	−0.18	−0.09	0.01	0.15	0.05	−0.24	0.25	0.30	0.02	−0.11	0.03	−0.31	−0.12
Min spring	0.06	−0.09	−0.03	0.02	0.05	−0.03	−0.24	0.10	0.19	0.09	−0.05	−0.03	−0.15	−0.08
Min summer	−0.15	−0.11	−0.40	0.26	−0.09	−0.23	0.03	0.13	0.12	0.07	−0.12	−0.22	−0.31	0.21
ETp														
Mean	−0.06	−0.02	−0.36	0.31	−0.03	−0.12	0.03	−0.01	0.15	0.23	0.09	−0.07	−0.29	0.19
Min	−0.08	−0.18	−0.08	0.01	0.16	0.07	−0.25	0.24	0.32	0.03	−0.10	0.06	−0.31	−0.13
Max	−0.53	−0.39	−0.11	−0.24	0.48	0.32	−0.24	**0.75**	0.58	−0.24	−0.26	0.20	**−0.69**	−0.35
Climatic indexes														
Lang index	*0.62*	0.42	0.26	0.12	−0.37	−0.24	0.10	**−0.73**	−0.50	0.33	0.37	−0.13	**0.68**	0.19
De Martonne index	*0.65*	0.43	0.23	0.15	−0.42	−0.29	0.10	**−0.75**	−0.53	0.34	0.36	−0.18	**0.70**	0.22
Emberger index	**0.70**	0.39	0.32	0.10	−0.33	−0.20	−0.06	**−0.72**	−0.41	0.38	0.37	−0.09	*0.66*	0.10
Thornthwaite index	*0.64*	0.41	0.31	0.08	−0.36	−0.22	0.06	**−0.72**	−0.50	0.31	0.35	−0.12	**0.70**	0.15
Aridity index	*0.64*	0.42	0.30	0.09	−0.37	−0.23	0.07	**−0.72**	−0.50	0.31	0.35	−0.13	**0.71**	0.16
Thorns														
Mean length	**0.70**	−0.03	0.52	−0.22	−0.38	−0.15	−0.45	−0.32	−0.16	0.29	0.15	−0.07	0.47	−0.27
Density	0.28	0.03	0.25	−0.17	−0.22	−0.14	−0.20	0.19	−0.07	−0.09	−0.25	−0.21	0.10	−0.23

Note: Values in italic are significant at $P < 0.1$, values in bold are significant at $P < 0.05$.

Table IV. Correlation coefficients between seed lipid traits and growing-season (1 September 2010–23 September 2011) weather data variables.

	% Seed lipids	UnFA:SaFa	O:L	Hexanoic C6:0	Caprilic C8:0	Capric C10:0	Undecanoic C11:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Hepta-decanoic C17:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2
Rainfall (*R*)														
Spring 2011	−0.42	−0.16	−0.45	0.14	−0.26	−0.44	0.29	0.36	−0.16	−0.43	*−0.60*	−0.55	−0.20	0.36
Summer 2011	0.10	−0.21	−0.49	0.38	−0.44	−0.41	−0.02	**−0.69**	−0.05	**0.82**	**0.64**	−0.14	−0.06	0.54
Spring + Summer 2011	−0.37	−0.28	**−0.73**	0.36	−0.51	**−0.68**	0.28	−0.03	−0.19	0.03	−0.24	**−0.63**	−0.24	**0.67**
Whole growing season	−0.14	0.11	−0.50	0.51	−0.47	*−0.60*	*0.55*	0.03	−0.39	−0.29	−0.46	**−0.64**	0.01	0.54
ETp														
Spring 2011	−0.13	−0.21	0.16	−0.20	0.32	0.45	−0.17	−0.10	0.31	0.26	0.47	0.54	−0.11	−0.22
Summer 2011	−0.17	−0.14	0.05	−0.11	0.13	0.21	0.08	−0.01	0.08	0.03	0.20	0.25	−0.04	−0.07
Spring + Summer 2011	−0.16	−0.17	0.09	−0.15	0.20	0.30	0.00	−0.04	0.16	0.11	0.30	0.36	−0.07	−0.13
Whole growing season	−0.26	−0.21	0.08	−0.16	0.32	0.41	−0.04	0.02	0.30	0.14	0.36	0.46	−0.18	−0.18
Mean Temperature (*T*, expressed in °C)														
Spring 2011	−0.14	−0.14	−0.19	0.28	−0.03	−0.08	0.05	−0.50	0.11	0.46	0.53	0.06	0.03	0.25
Summer 2011	−0.09	−0.29	−0.14	0.14	−0.19	−0.26	0.02	−0.45	−0.01	0.36	0.40	−0.12	0.13	0.25
Whole growing season	−0.15	−0.13	−0.13	0.26	0.04	−0.06	0.06	−0.42	0.14	0.39	0.49	0.05	0.03	0.18
Minimum relative humidity														
Spring 2011	0.09	0.14	0.33	−0.25	0.23	0.18	0.00	*0.60*	0.02	−0.51	−0.46	−0.01	−0.04	−0.43
Summer 2011	−0.08	0.07	0.13	0.00	0.33	0.18	−0.07	0.22	0.26	−0.05	0.02	0.09	−0.13	−0.25
Whole growing season	0.46	0.09	**0.74**	*−0.60*	0.14	0.19	−0.30	0.44	−0.13	−0.51	−0.43	0.02	0.35	**−0.66**
Maximum relative humidity														
Spring 2011	0.19	*0.56*	0.09	0.19	−0.29	−0.31	*0.59*	0.15	**−0.64**	**−0.69**	**−0.66**	−0.50	0.43	0.14
Summer 2011	0.20	**0.75**	−0.07	0.44	−0.40	−0.40	**0.71**	−0.10	**−0.74**	*−0.57*	*−0.59*	*−0.56*	0.49	0.38
Whole growing season	0.29	*0.57*	0.20	0.16	−0.29	−0.30	0.54	0.13	**−0.66**	**−0.69**	**−0.65**	−0.49	0.52	0.06
GDD														
Spring 2011	0.11	−0.08	−0.10	0.25	−0.11	−0.01	−0.06	**−0.69**	0.06	**0.64**	**0.68**	0.22	0.13	0.23
Summer 2011	−0.11	**−0.77**	0.05	−0.27	0.01	−0.02	−0.46	−0.13	0.34	0.36	0.36	0.13	−0.09	−0.11
Spring + Summer 2011	−0.03	*−0.60*	−0.01	−0.09	−0.04	−0.02	−0.37	−0.40	0.27	0.54	*0.56*	0.19	−0.01	0.02
Whole growing season	−0.14	−0.43	−0.10	0.10	0.04	0.02	−0.17	−0.42	0.29	*0.56*	**0.64**	0.21	−0.07	0.09
R/GDD														
Spring 2011	−0.30	−0.19	−0.26	−0.01	−0.15	−0.30	0.15	0.51	−0.07	−0.49	**−0.64**	−0.42	−0.22	0.13
Summer 2011	0.08	−0.22	−0.51	0.36	−0.45	−0.42	−0.05	**−0.65**	−0.04	**0.80**	*0.59*	−0.15	−0.08	0.54
Whole growing season	0.49	**0.68**	0.12	0.17	−0.30	−0.13	0.28	−0.13	*−0.56*	−0.27	−0.36	−0.20	0.41	0.13
(*R*-ETp)/GDD														
Spring 2011	−0.23	−0.30	−0.21	0.01	−0.08	−0.21	0.02	0.45	0.07	−0.34	−0.50	−0.28	−0.25	0.04
Summer 2011	0.21	−0.37	−0.25	0.35	−0.29	−0.22	−0.07	**−0.73**	0.09	**0.92**	**0.88**	0.11	0.00	0.29
Whole growing season	0.42	0.20	0.12	−0.13	−0.26	−0.10	−0.12	0.05	−0.29	−0.14	−0.32	−0.11	0.18	−0.03
Lang index														
Spring 2011	−0.35	−0.16	−0.39	0.08	−0.25	−0.39	0.24	0.39	−0.15	−0.45	*−0.62*	−0.49	−0.19	0.29
Summer 2011	0.10	−0.19	−0.50	0.38	−0.45	−0.42	−0.02	**−0.67**	−0.06	**0.80**	*0.61*	−0.15	−0.06	0.55
Whole growing season	0.02	0.06	−0.22	0.18	−0.34	−0.37	0.29	0.22	−0.32	−0.37	−0.53	−0.43	0.02	0.23
De Martonne index														
Spring 2011	−0.38	−0.16	−0.41	0.11	−0.25	−0.41	0.26	0.38	−0.16	−0.45	*−0.61*	−0.52	−0.20	0.32
Summer 2011	0.10	−0.20	−0.50	0.38	−0.45	−0.42	−0.02	**−0.68**	−0.06	**0.81**	*0.62*	−0.15	−0.06	0.54
Whole growing season	−0.03	0.09	−0.33	0.32	−0.40	−0.47	0.40	0.15	−0.36	−0.35	−0.53	−0.53	0.03	0.36

(*continued*)

TABLE IV – (*Continued*).

	% Seed lipids	UnFA:SaFa	O:L	Hexanoic C6:0	Caprilic C8:0	Capric C10:0	Undecanoic C11:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Hepta-decanoic C17:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2
Thornthwaite index														
Spring 2011	−0.32	−0.13	−0.38	0.15	−0.25	−0.43	0.27	0.38	−0.16	−0.46	*−0.63*	−0.54	−0.17	0.30
Summer 2011	0.08	−0.17	−0.51	0.40	−0.43	−0.43	−0.01	**−0.64**	−0.05	**0.77**	*0.57*	−0.18	−0.07	0.55
Whole growing season	0.33	0.24	−0.10	0.29	−0.53	*−0.55*	0.31	−0.06	−0.54	−0.29	−0.48	*−0.59*	0.33	0.30
Aridity index														
Spring 2011	−0.32	−0.13	−0.38	0.15	−0.25	−0.43	0.27	0.38	−0.16	−0.46	*−0.63*	−0.54	−0.17	0.30
Summer 2011	0.08	−0.17	−0.51	0.40	−0.43	−0.43	−0.01	**−0.64**	−0.05	**0.77**	*0.57*	−0.18	−0.07	0.55
Whole growing season	0.33	0.24	−0.10	0.29	−0.53	*−0.55*	0.31	−0.06	−0.54	−0.29	−0.48	*−0.59*	0.33	0.30

Note: Values in italic are significant at $P < 0.1$, values in bold are significant at $P < 0.05$.

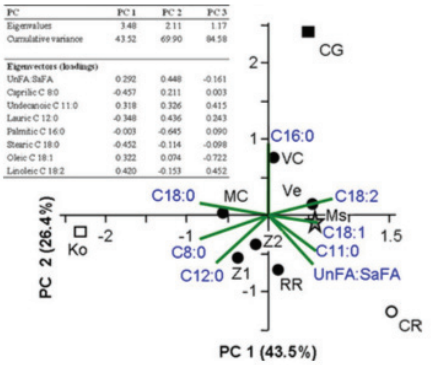

Figure 3. Biplot for first two PCs. Values in brackets represent the contribution of each variable to the explained variance. The lines intersecting at (0,0) represent the original variables. See Table I for accession acronyms and Table II for variables acronyms. The length of each vector is proportional to its contribution to the PCs and axes are in eigenvalues scale. Symbols correspond to the different groups as defined by cluster analysis. The table contains eigenvalues, cumulative variance of the first three PCs and correlation coefficients between the PCs and the selected characters.

genotypes of *P. dactylifera*, whereas Rodríguez-Leyes et al. (2007) found few differences between two species of *Sabal*, a genus related to *Chamaerops*. The oleic and linoleic contents in the seed lipids of other species differ among lines and show a high degree of hereditability (e.g. Kondra & Thomas 1975; Green 1986; Takagi & Rahman 1996). In *Brassica napus*,

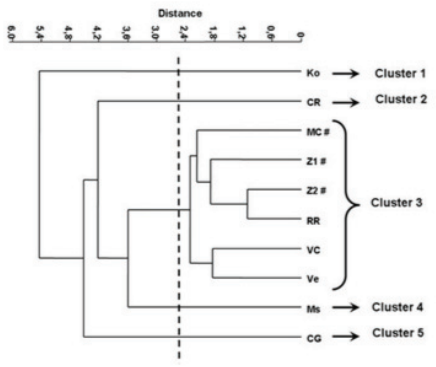

Figure 4. Dendrogram classification of *Chamaerops humilis* accessions clustered with average method (truncated at 5-group level, vertical dotted line) on the basis of the FA composition of the seed lipid. See Table I for acronyms of the accessions. Accessions followed by # come from very close environments.

Schierholt and Becker (2001) observed low environmental effects on the oleic acid seed content, mainly due to the control of this trait by a single main gene and argued that some other minor effects (e.g. genotype × environment and transcription of minor genes) would exert some secondary influence on the content in oleic acids. In our experiment, a high range of inter-population variation among the FAs was observed, which could imply a high variability for the genes encoding for the oleic and linoleic acids contents. In addition, this variability could be higher than what observed due to the dioecious nature of the species and thus to the lack of expression of regressive alleles encoding for the oleic and linoleic acids seed content.

Linolenic acid was not found in the European fan palm seed, whereas it was clearly displayed in the standard FAMEs reference (data not shown). Other palms show no or very little linolenic acid in the seed lipids (Nehdi et al. 2010; Nehdi 2011), whereas the same acid was found in the fruit lipids of similar palms (El Arem et al. 2011) of the *Corypheae* and *Phoeniceae* palm tribes. Linolenic acid is produced by desaturation of linoleic acid; however, desaturases are not present in all plant tissues (Ohlrogge & Jaworski 1997). In seeds, the synthesis of oleic acid takes place in proplastids, whereas its further desaturation to linoleic and linolenic acids directly occur in the cytosol (Browse & Somerville 1991). In the seed lipids of palms, the linolenic acid is usually low or absent (Opute 1979), thus it is possible that palms do not produce linoleic desaturases in the kernel.

The O:L ratio widely varied between the accessions. In other species, O:L ratio is associated with lipid stability (Branch et al. 1990; Murkovic & Pfannhauser 2000). In *Helianthus annuus*, Flagella et al. (2002) suggested that temperature and water availability could play a role on the activity of seed desaturases during the grain-filling period. In the present work, the O:L ratio depended more on linoleic ($R = -0.83$) rather than oleic acid content ($R = +0.50$) and was highly correlated with the climatic mean temperature during the grain-filling period ($R = -0.81$), but not with growing-season summer rainfall ($R = -0.49$ at $P > 0.05$), thus we hypothesize that a genotypic adaptation to the environment is responsible for the differences in this trait.

As for the O:L ratio, also the UnFA:SaFA ratio widely varied among the accessions and correlated with RH and *R*/GDD during the growing season, but not with the climatic traits. According to Linder's hypotheses (Linder 2000), the UnFA:SaFA ratio has an evolutionary meaning in increasing the germination rate. European fan palm is widespread in semi-arid Mediterranean environments and its seed can take several months to germinate due to a hard coat (Giovino et al. 2012b), which implies that the plantlet will stand during spring and summer characterized by high ETp and low rainfall, thus, in theory, a correlation should be found between climatic traits and UnFA:SaFA ratio. However, this trait also depends on the activity of the seed desaturases, which are temperature-dependent (Flagella et al. 2002). In the sites of collection from the present study, the grain-filling period occurs during a highly heat-stressing period and this can have some influence on the content in the most represented UnFAs and SaFAs. Indeed, we observed a strong correlation between the weather data and lauric and palmitic acids, the most represented SaFAs.

The clustering analysis performed on the basis of the FAs and UnFA:SaFA ratios grouped together accessions from both very close (Zingaro 1, Zingaro 2 and Monte Cofano) and far sites (Rafforosso and Vendicari) and the control specimen from Villa Cattolica. The seed FAs have been related to biogeographic distribution and climatic conditions during the germination phase (Linder 2000) and also have a systematic significance (Graham et al. 1981). However, some other pedo-climatic traits such as soil texture, canopy shading and competition between adult plants and plantlets could be important in determining the fitness of seeds with unpredicted relative proportions of saturated and unsaturated FAs. Anyway, four accessions coming from very different environments in terms of pedo-climatic traits (Kolymbetra, Capo Gallo, Capo Rama and Marsala) were clearly differentiated, and the correlation between the climatic variables and the FA traits (especially, the most represented UnFa and SaFA and the lipid content) were often significant.

In conclusion, seeds of the European fan palm show a high content of unsaturated FAs, such as oleic, when compared with other palm species. In *Serenoa repens*, a species closely related to *Chamaerops humilis*, the lipido-sterolic extract of the berry has proven to be efficient for the treatment of benign prostatic hyperplasia (Wilt et al. 2002). Considering the importance of the unsaturated FAs in human and animal nutrition and that wild animals enrich their diet during winter with seeds rich in lipids (Salmaso et al. 2009), the present results suggest that *Chamarops humilis* could be introduced in breeding programmes and its seed extracts used as FA in diet supplementation. Further studies are needed to highlight the genotypic differences of the European fan palm populations and the content in other lipids and sterols, as well as anti-nutritional compounds.

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