UNIVERSITY *MEDITERRANEA*

OF REGGIO CALABRIA

DEPARTMENT OF AGRARIA

PhD Course on:
"AGRICULTURAL, FOOD AND FORESTRY SCIENCES"

CICLO XXXII (2016-2019)

S.S.D AGR/15

PhD Thesis

RECOVERY OF FRACTIONS WITH BIOLOGICAL AND CHEMICAL ACTIVITY FROM CITRUS BY-PRODUCTS

PhD thesis of
Dr. Chiara Cariello

PhD Coordinator
Prof. Marco Poiana

Thesis Supervisor
Prof. Marco Poiana

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The thesis work was carried out in the food technology laboratory at the *Mediterranean* University of Reggio Calabria. The subject of study, namely the citrus by-products was financed by the Agrumaria Reggina company located in Reggio Calabria.

The aim of this PhD thesis research t is to study the recovery of compounds with high added value from orange and lemon by-products considering their possible use for “functional” food production. During these three years the research activities concerned the production and characterization of phenolic extracts from the by-products of the citrus company, the enrichment of different food matrices and the evaluation of functional properties of the products obtained.

The study of extraction processes of phenolic compounds from citrus by-products showed like the extract has an high content of polyphenols and better antioxidant activity that could be used to improve the stability of different model food systems.

The aim is also to develop a virtuous cycle of production, processing, consumption and re-use of the raw material by treating waste

RIASSUNTO

Il lavoro di tesi è stato svolto presso il laboratorio di tecnologia alimentare dell'Università Mediterranea di Reggio Calabria. L'oggetto di studio, ovvero i sottoprodotti agrumari, sono stati finanziati dall’azienda Agrumaria Reggina con sede a Reggio Calabria.

Lo scopo di questa di tesi di dottorato è stato quello di studiare il recupero di composti ad alto valore aggiunto da sottoprodotti di arance e limoni considerando il loro possibile utilizzo per la produzione di alimenti "funzionali". Durante questi tre anni le attività di ricerca hanno riguardato la produzione e la caratterizzazione di estratti fenolici ottenuti dai sottoprodotti

dell'azienda agrumicola, l'arricchimento di diverse matrici alimentari e la valutazione delle proprietà funzionali dei prodotti ottenuti.

Lo studio dei processi di estrazione dei composti fenolici dai sottoprodotti degli agrumi ha dimostrato come l'estratto abbia un alto contenuto di polifenoli e un'elevata attività antiossidante che potrebbe essere utilizzato per migliorare la stabilità dei diversi sistemi modello alimentari.

L'obiettivo è stato anche quello di sviluppare un ciclo virtuoso di produzione, lavorazione, consumo e riutilizzo della materia prima trattando i rifiuti.

KEYWORDS: Citrus byproducts, maceration extraction, ultrasound extraction, phenolic compounds, antioxidant activity, UHPLC-DAD.

1. INTRODUCTION

For many decades scientific research has turned its attention to the waste deriving from the processing of products in the agri-food industries.

The interest in these materials is due in order to minimize waste, to extract from these chemical compounds of considerable scientific impact, to promote their recycling or to reduce their disposal costs. Finding no place on the market, they would be disposed of and treated as waste materials, increasing pollution problems.

Also citrus industries produce high amounts of solid waste that become difficult to manage. The waste itself contain important components such as pectin and antioxidants that could exploit and generate earnings. Citrus is an interesting case of study about this possibility.

1.1 The history of citrus fruits

The citrus fruits belong to the Rutaceae family, subfamily of the Aurantioideae, their history has its roots in very ancient times, in fact several sources say that the origins of this family are located in Asia more than 4000 years ago. (*Zaragoza et al, 2012*).

Before its appearance in Europe, orange was certainly grown in China and botanists gave the name of Citrus sinensis.

The first citrus fruit known in the Middle East and in Europe was probably the cedar. The spread of citrus fruits in the Mediterranean areas of Europe, particularly of lemon and bitter orange, is due to the Arabs starting from the 10th century.

Sweet orange arrived in Europe in the 13th century probably through the two routes: the first was that of the Arabs who had discovered it during the travels of their caravans in the Far East and the other by the Genoese and Venetian navigators who brought it in Italy from their trips to China.

The last to arrive in the Mediterranean gardens was the mandarin, whose cultivation in Sicily began only in 1800.

If we pass from history to legend many have identified in the orange the mythical golden apple, sacred to Jupiter, of the garden of the Hesperides which gave immortality and which was stolen by Hercules during one of his twelve labors. The memory of this legend has remained linked to the botanical name of the fruit of citrus fruits, called esperidio. (*Lupo S, 1990*).

But even if in the 13th and 14th centuries in Sicily and Spain citrus fruits were intensively cultivated, they were still a precious commodity: just think that among the gifts sent to Spain to Queen Eleanor of England seven oranges are mentioned.

The use of lemon as a condiment and its use as a drink became widespread starting from the 16th century when its property of preventing and treating an avitaminosis, scurvy, very common in those period, was discovered. (*Calabrese, F. 2002*)

The first news concerning the extraction of essential oils from citrus fruits date back to the middle of the XVI century with references to extraction systems using distillation processes starting from chopped lemon and orange peel and also from bitter orange flowers to obtain the oil that was called Neroli, in homage to the wife of the Count of Nerola, to whom the merit goes for having introduced this excellent fragrance to the European nobility of the time.

The processes of industrial transformation of citrus fruits originated in Italy, to be precise in Sicily, with the manual extraction of the essential oils of lemon with the "sponge" method with the private peel of the pulp that was considered a secondary product. Only in a second time did the industrial exploitation of the juice begin.

1.2 The importance of citrus fruits in Italy

In recent decades citrus cultivation in Italy has experienced a remarkable evolution but also a substantial change. From 1975 to today, there has been a significant decrease in the export of fresh produce, a marked increase in domestic trade and above all in the quantities used by the industry. The processing industry has been the activity that has most supported citrus cultivation and that has developed and evolved the most.

The 2016 ISTAT data confirm that citrus surfaces in 2011 were around 170,000 hectares, with greater production of oranges, followed by mandarins, lemons and others. Among the regions, Sicily ranks first with almost 95.00 hectares cultivated, followed by Calabria with about 43.000 hectares, Puglia, Sardinia, Basilicata. (ISTAT, 2016).

In Italy, the citrus processing industry is characterized by the presence of few rational and modern companies, with a high technological level and good working capacity with plants for the production and extraction of essential oils and juices. The companies are located mainly in Sicily and Calabria, followed by some small companies in Campania, Lucania and Sardinia.

The data in the 2016/2017 ISTAT campaign show the product flows in Italy in thousands of tons.

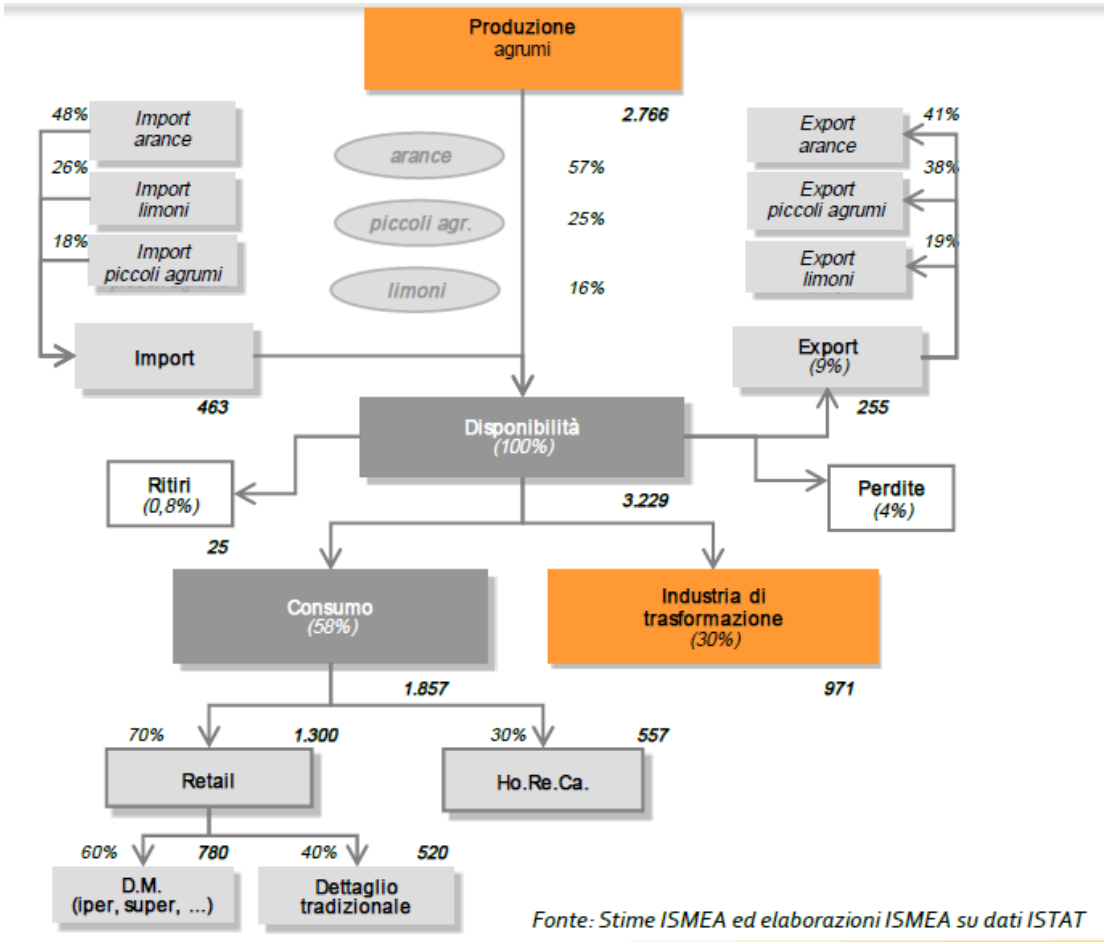

Figure 1- Istat data

1.3 Transformation process

After harvest the product is subjected to a fungicide treatment for later be placed in special rooms awaiting processing. Citrus fruits then are unloaded on the roller conveyors, this operation must be carried out with the utmost care since the product could be damaged due to the blows suffered and release essential oils and other components. The washing is carried out by mean of system placed in line or using large tanks where fruits are put into, with the first method being the most used. Washing is carried out by a series of brushes with the support of nozzles that supply water with added chlorine or sanitizing agents and detergents, at the end the rinsing with drinking water provides to remove the claim to observe the fruit under special absorbent sponges.

Apart the fresh fruit directly marketed, other products could be obtain from citrus by mean of different process applied, the most important are essential oils and juices. It must be noted that different citrus fruits bring to different products, with essential oils being always the most important one, while juice, albedo and others represent the secondary product, depending on the specie of citrus.

During the years, all the processes were greatly improved with the introduction of new machines allowing to obtain more valuable products. The most used extraction systems are the in-line (**Fig. 2**), which is the most suitable in the world, therefore simultaneous extraction of essential oils and juices. During the extraction, the two fractions do not come into contact at all, providing high quality products, with less pollution of the materials.

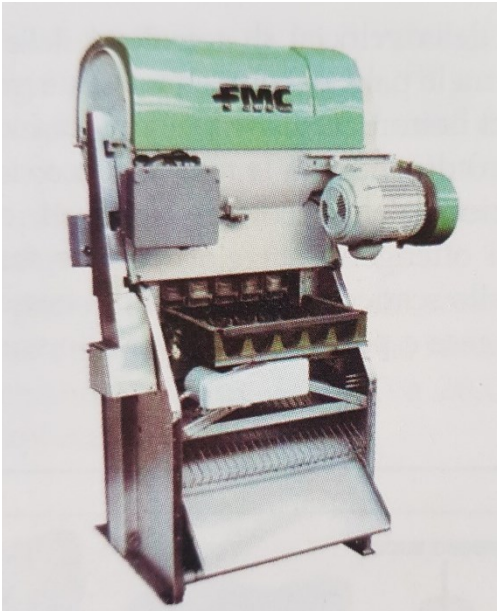

Figure 2- Juice and essence extractor FMC Technologies Italia SpA

(*Citrus trattato di Agrumicoltura Vacante e Calabrese 2009*)

The extraction of essential oils (**Figure 3**) is based on the alternating motion of two overlapping cups,

one fixed and the other mobile, the mobile one lowers on the fruit, lying inside of the fixed cup, exerting a first compression on the flavedo that causes the rupture of the utricles and leakage of essential oil, which is removed with water.

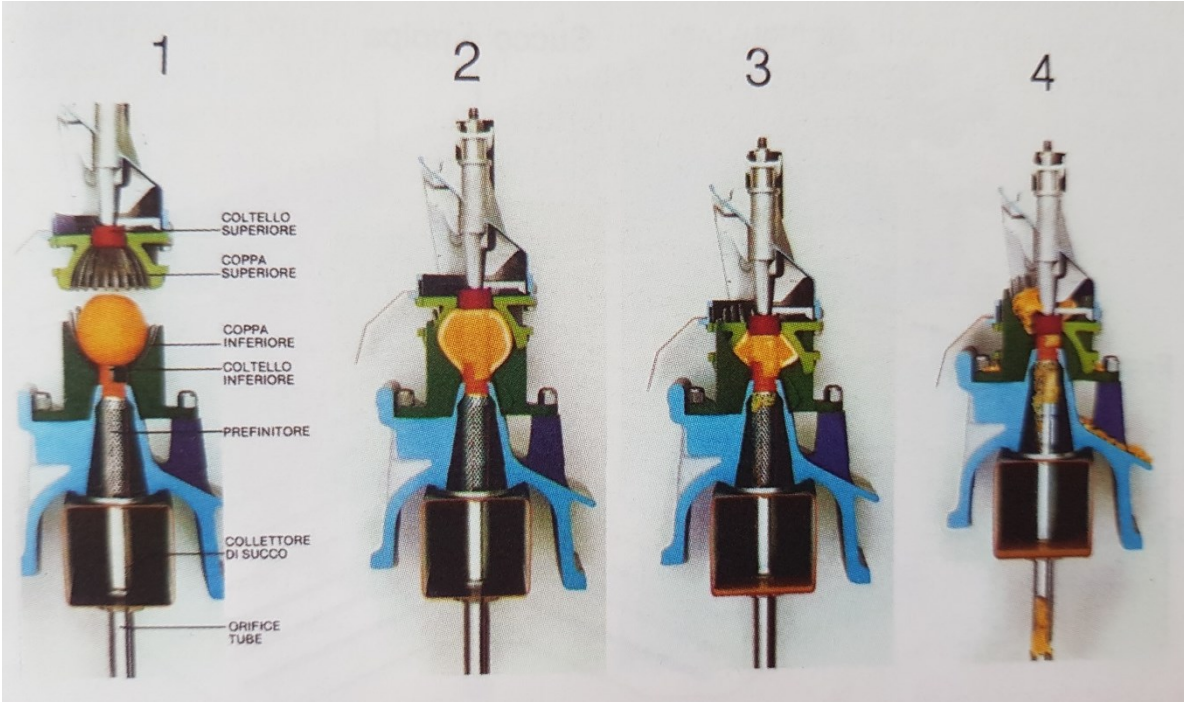

Figure 3- Representation of the different stages of juice extraction and essence with FMC extractors (*Citrus trattato di Agrumicoltura Vacante e Calabrese 2009*)

After extraction, the emulsion is separated by centrifuge. The obtained essential oil must be deterpenated,. This is carried out by mean of distillation or other technique. Monoterpenes could give some unwanted characteristic, mainly toxic, the removal of these components from the products improve the quality of the essence and bringing a better scent of the essence. Other high boiling components such as furocumarins, that could create concerogenic mutations to the skin if the products (essential oils) containing them are used as cosmetic ones. Also these are remove, by mean of distillation or low temperatures.

The extracted juices undergo to different operations such as pasteurization (**Figure 4**) and a possible concentration; although some juices are subjected to a preventive refining to

eliminate the content of suspended solids. This operation is used above all for the production of carbonated beverages, as opposed to juices where taste and freshness are of minor importance.

Once the juice is refined, it is pasteurized at 90-95 ° C for 15-60 seconds carefully check due to high susceptibility to temperatures of nutritional components and enzymatic and microbiological activity.

At the end of process concentration could occur, by this operation water is take away, and makes easier transport and store juices.

Figure 4- Pasteurization

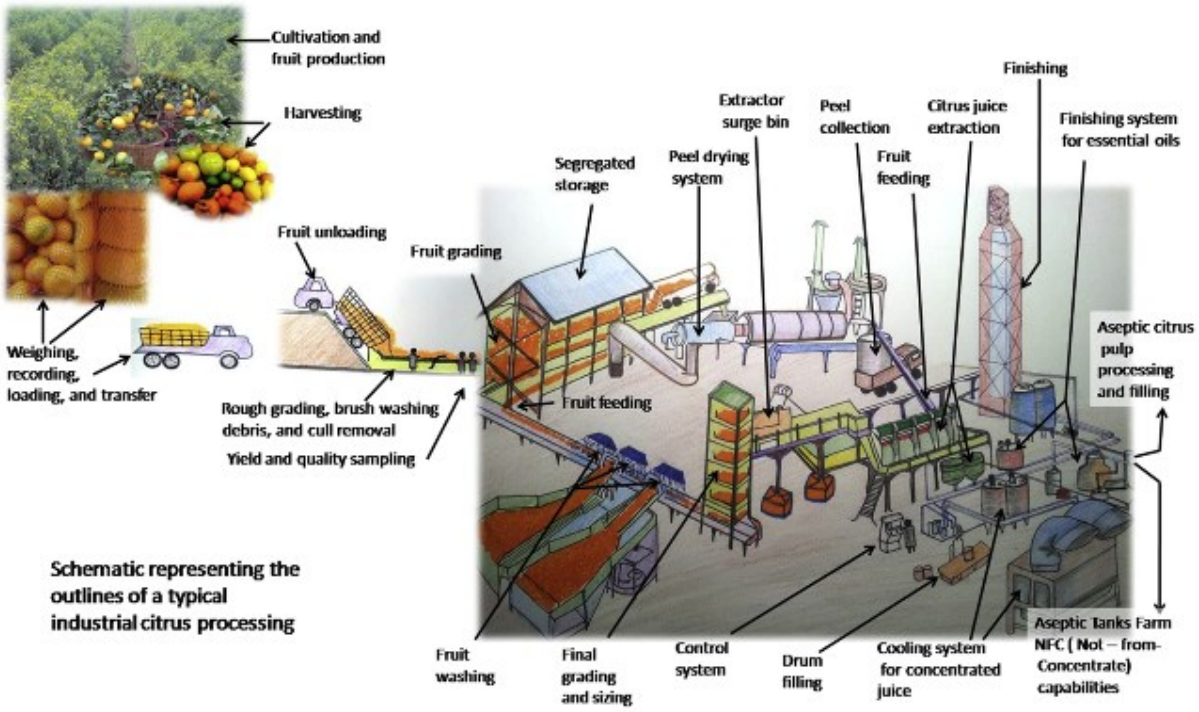

Figure 5 - Outline of citrus processing and product formation from citrus farm to the market *(Sharma, K et al 2017.*

1.4 Food waste

1.4.1 Definition of Recovery

There are some rules about the waste handling, the Directive 2008/98/EC says that:

In Article 3 of the Waste Framework Directive, the definition is inserted, among many others of "recovery" as "any operation whose main result is to allow the refuses to play a useful role by replacing other materials that would otherwise have been used to perform a particular function or to prepare them to perform this function, within the plant or in the economy in general "; definition that completes its understanding in Annex II, reporting "a non-exhaustive list of recovery operations", in particular for the thesis dealt with under item" R3: Recycling / recovery of organic substances not used as solvents (including composting operations and other biological transformations).

1.4.2 Definition of Waste or By-Product

For a better understanding of the problems related to the re-use of waste is necessary to define the definition of "by-product" and "waste". Little clarity, in fact, he expressed the Legislative Decree 152/2006 (art. 183, paragraph 1) passed by the current Legislative Decree 205/2010, which sets out the same Waste Framework Directive: “The decision [...] of the European Parliament and of the Council, [...] requests the extension or revision of waste legislation, in particular to clarify the distinction between what is rejection and what is not ... ".

The definition of "waste" is contained in article 3, as follows: "any substance or object which the holder discards or intends or is required to discard "; in particular, food waste, which is of interest to us here, is included in definition of "organic waste": "biodegradable waste from gardens and parks, waste food and cooking products from households, restaurants, catering services and retail outlets and similar waste produced by the food industry plants ".

At this point, it is good to point out that from Directive 2008/98 / EC they have exemption of "animal by-products, including processed products ..." of which once again, the present paper will not deal with it.

The definition of "by-product" is placed in a separate article (Article 5) with respect to the definitions (Article 3). Under the heading "by-products" the Directive reports how much follows:

1. A substance or object resulting from a production process whose primary purpose is not the production of that article may not be considered waste pursuant to Article 3, point 1, but by-product only if the following conditions are met:

a) it is certain that the substance or object will be further used;

b) the substance or object can be used directly without any further treatment other than normal practice industrial;

c) the substance or object is produced as an integral part of a production process;

d) further use is legal, the substance or object satisfies, for specific use, all relevant requirements concerning the products and the protection of health and the environment and will not lead to overall negative impacts on the environment or human health.

2. On the basis of the conditions set out in paragraph 1, they can measures should be taken to establish the criteria to be met so that specific substances or objects are considered as by-products

and non-waste in accordance with Article 3, point 1. Such measures, designed to amend non-essential elements of this Directive, supplementing it, they are adopted according to the regulatory procedure with control referred to in Article 39, paragraph 2.

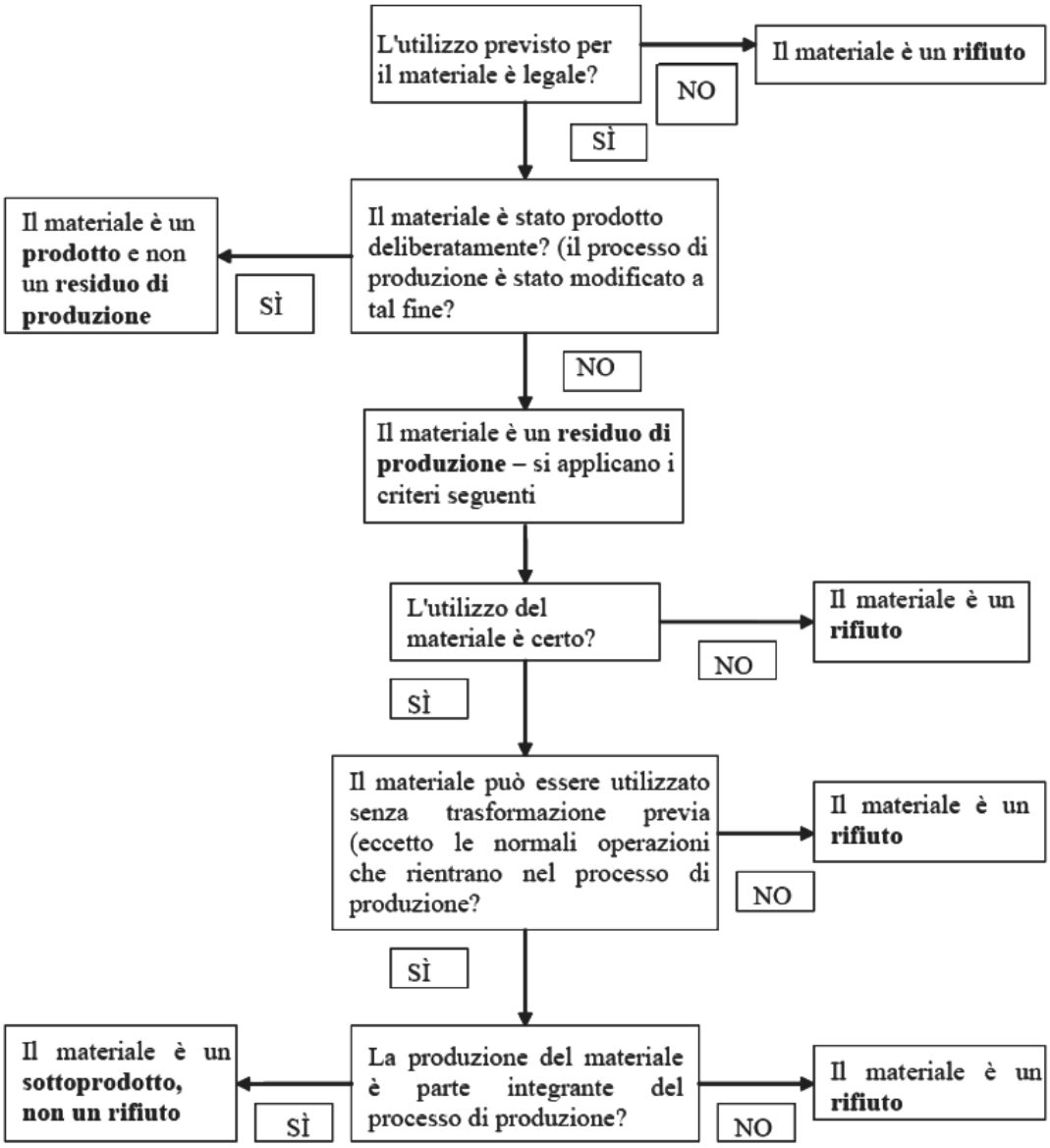

Figure 6- Decision diagram for the assignment of the term "by-product" waste according to the legislation. Source: http://www.compost.it/

2. STATE OF THE ART

2.1 Processes for the extraction of citrus wastes

The main products of citrus processing are the juice and the essential oils. The periods of production and industrial processing of citrus fruits are notably different.

Citrus are balls of juice sacs protected by a waxy skin called peel. (**Fig. 7**) The peel is made up of a thin outer layer called the flavedo and a thicker, fibrous inner layer called albedo. Oranges have characteristic coloured substances called carotenoids in the flavedo. Vesicles (small sacs or cavities) containing peel oil also present in the flavedo contribute to the fruits fresh aroma. The internal part, white and spongy, albedo, contains same substance that influence juice quality if they find their way into extracted juice. These substances include flavonoids, d-limonene, limonin and pectin.

Endocarp is known as the edible portion of the fruits. It consists of a central fibrous core, individual segments and an outer membrane. Segments contain juice vesicles, or juice sacs, that are held by a waxy substance. Seeds may be present within the segments.

In the juice vesicles are present juice, droplets of juice oil and lipids. Juice contains also sugars, acids, vitamins, minerals, pectins, coloured substances and other components. After juice is extracted, part of citrus are recovered as pulp.

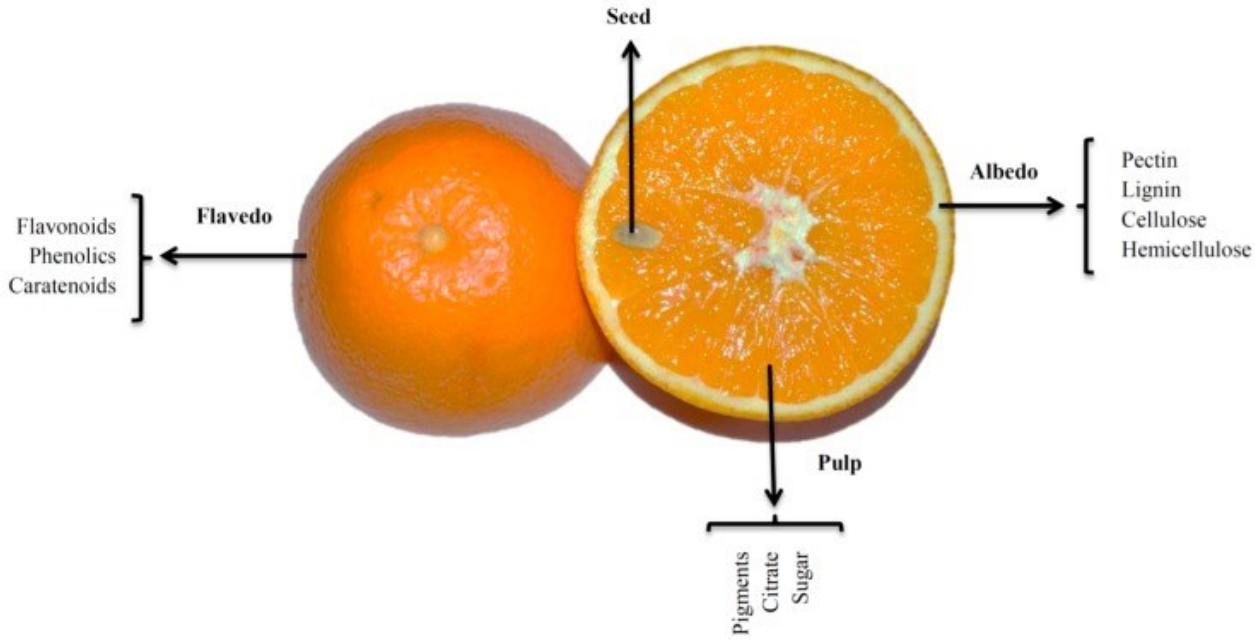

Figure 7 - Anatomy of a citrus fruit

The aim of the juice extraction process is to remove maximum amount of juice from fruit without including any peel. But there is a compromise between the possible juice yield and the product quality. The yield is 40-60% by weight depending on the fruit species, variety and local climate.

Citrus has two primary products: whole fruit and juice, while has got many secondary products, the so called by-products that help to maximize profits and minimize waste.

2.1.1 Citrus by-product and their possible uses

Citrus fruits are commonly processed into cloudy juice (*Lavaj et al 2012*), and approximately 45 to 60% of the weight of these fruits is discarded as waste, consisting of peel, seeds, membrane and juice vesicles. In addition, sugar and limonoids are typical value-added by products extracted for various industries, but polyphenols and particularly flavonoids are left in the peel, which is then dried, mixed with dried pulps and used for feed. Citrus by-products may increase the economic yield of the citrus processing industries. In a study, by Sharma et

al. it is observed the use of citrus peel as an economically valuable source of high-added value compounds as it contains a significant amounts of various carotenoids, flavonoids, sugar, dietary fiber, polyphenols, essential oils and ascorbic acid. Most important is the high quantity of sugars present in citrus waste, suitable for fermentation in bioethanol production and as a substrate for solid state fermentation. There were studied various methods of extraction for value-added products from citrus by-products and their potential utilization as a source of a number of functional compounds.

If up to more in the recent past, waste management has only been considered with a view to disposal, the goal pursued today is to reduce the quantity of the same and to promote, at the same time, profitable recovery and recycling activities. To composting, aimed at recovery and enhancement of organic materials, such as citrus by-product, which, otherwise, they would see the transfer as an alternative placement hypothesis to landfill.

Therefore the pulp can also be used for agronomic purposes, as a soil conditioner or as a basis for the production of compost; its use has also been proposed as fuel or as an organic matrix for the production of bioethanol e biogas.

High value added uses are being developed recently (such as production of fibers and other products for human consumption), which so far have however, very small quantities of product are affected.

2.1.2 Essential Oil

Essential oils are mainly located in otricoli (oil-bearing glands) arranged irregularly in the layers immediately below the fruit's subepidermal tissue rich in pigments and known as flavedo or epicarp. Moreover, the odorous components are not present exclusively in the citrus fruit rind, but are also found in the endocarp, in the flowers in the leaves and in the

young branches. Essential oils are mixtures of physically and chemically different substances, among which monoterpenes, hydrocarbons and sesquiterpenes predominate.

2.1.3 Dietary Fiber

Citrus fruits are excellent source of dietary fiber, soluble and insoluble. Soluble fiber includes pectin, gum, mucus and part of cellulose. Insoluble fiber includes cellulose, hemicellulose and lignin (Table 1). Lemon, among the citrus fruits, contain the higher amount of fiber (soluble and insoluble), 14 g/100 g. In general, citrus peel contains of 50-60 % cellulose and hemicellulose, that is a good raw material for their extraction. Citrus fiber can be considered as a biologically active compounds (BAC) due to the presence, in addition to the polysaccharides, of polyphenol like components used like inhibitors of lipid oxidation in meat products and improving oxidative stability for prolonging the shelf-life of meats.(*Sayago-Ayerdi et al 200*3). In addition citrus juice fiber has been used as a novel fat replacer in ice cream (*de Moraes Criztel et al, 2013).*

Dietary fiber has been known for over 2000 years in different forms. It is defined by the Association of Official Analytical Chemists (AOAC, 2000), such as the set of polysaccharides and vegetable waste materials that are resistant to hydrolysis (digestion) by human digestive enzymes, and they cannot be metabolized or absorbed. So dietary fiber is seen as a natural component of food (Cho et al., 1997). The fibers traditionally used in food technology come from cereals. However, the fibers of fruits and vegetables, although less studied, have greater nutritional qualities and technology. Recently, extraction studies have been conducted composition of the fibers of different origins. In the case of dietary fiber from fruits, they are gaining more importance than cereals because they have a more balanced ratio of total dietary fiber (TDF), and therefore they protect better the health of consumers (Borroto et al., 1995). The TDF content is also influenced by the extraction method. Generally, most fruit and

vegetables have values between 1% and 2.2% of TDF; the values are normally expressed as a percentage by weight of food (Mongeau et al., 2001). Dietary fiber intake in countries developed is lacking, and several studies on food guidelines recommend an increase consumption of fiber-rich products such as fruits and vegetables (Gibney etal., 2000). In this sense, the fiber obtained from the citrus peel constitutes a viable alternative to grain-based fiber. The process of obtaining dietary fiber from citrus peels is described in **Figure 8.**

The premix of hazel peels and fruits is homogenized and then pressed. It is adding water to this homogeneous mixture in a proportion varying from 1: 3 at 1:10; the whole is then subjected to an enzymatic and chemical treatment at controlled temperature, which determines the final characteristics of the fiber obtained. The treated mass is dried in a rotary dryer, and whatever turns out it can be fragmented into dry flakes. These flakes are ground and the resulting powder is sieved until it reaches the average size of the required particles (MPS). The type of fiber obtained depends on the source, the treatment phase with water and MPS. It is clear that the method used determines the quality of dietary fiber. The best source of dietary fiber is the peel of Navel oranges and lemons (Figuerola et al., 2005).

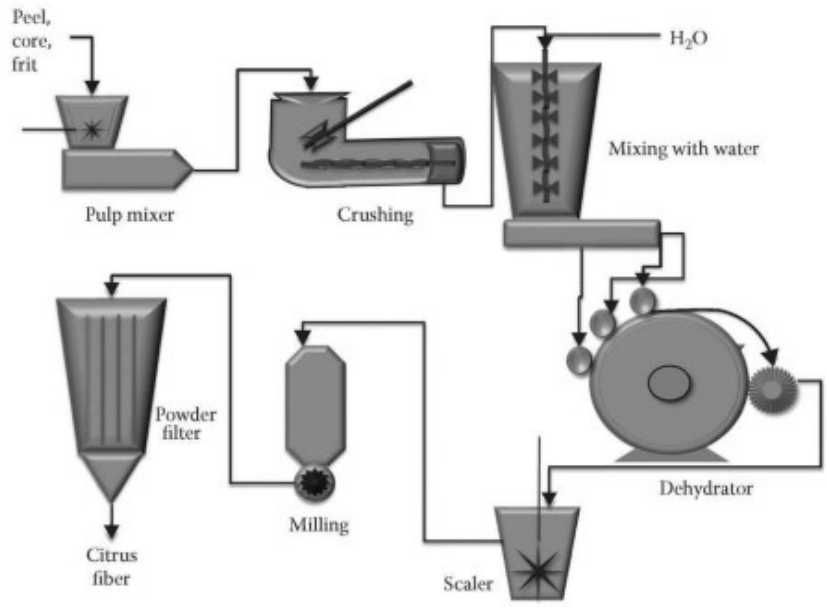

Figure 8-Citrus fiber production (Gibney et al 2000)

Table 1- Fiber composition (% dry weight) of lemon by-products. (*Marin et al, 2007)*

Citrus Waste	Pectin	Lignin	Cellulose	Hemicellulose
Lemon peels	13.00±1.06	7.56±0.54	23.06±2.11	8.09±0.81
Lemon pulp	22.53±1.95	7.55±0.66	36.22±3.24	11.05±1.09

2.1.4 Pectin and its extraction process

Pectin, represent a family of heterogeneous polysaccharides. It is a natural multifunctional ingredient which imparts textural and rheological properties to a wide range of food (Ngouemazong et al, 2015). In particular, lemon waste contains 12-23 % on DM of pectin. This is important because show a lot of opportunities in valorization of citrus by-product for pectin production. Pectin can be considered as a bioactive compound due to its function as a dietary fiber, and recent study reported possible anti-cancer activity.

As previously reported, pectin is a heteropolysaccharide composed of the union of several different monosaccharides, extracted from fruit from protopectin in it contained. Pectins form gelatinous colloids, abundant in the cell wall of fruit: they are hydrolysed with maturation by some enzymes such as pectase and pectinase. Pectin is used in industry food as a gelling agent, especially in the creation of jams. European market is an important consumer of pectin (600 t / year). The manufacturers who use citrus peels have found that their concentrate of pectin cannot be used in most applications due to its unpleasant smell. For this reason, they produced solid pectin from precipitation with alcohol or salts. The current situation in the sector is the result of this evolution towards the production of pectin powder. In recent years, American apple pectin producers have experienced an increase in costs compared to citrus pectin producers. The result was that the producers of citrus pectin dominate the market (The Copenhagen Pectin Factory, 1998). Currently, a key factor for pectin extraction is the washing

process and treatment of citrus peel first drying, as well as the drying process itself (Johnston, 2002).

These processes, together with coagulation and purification, constitute the points critics of the production process from an environmental point of view. The traditional production process is shown in **Figure 9.**

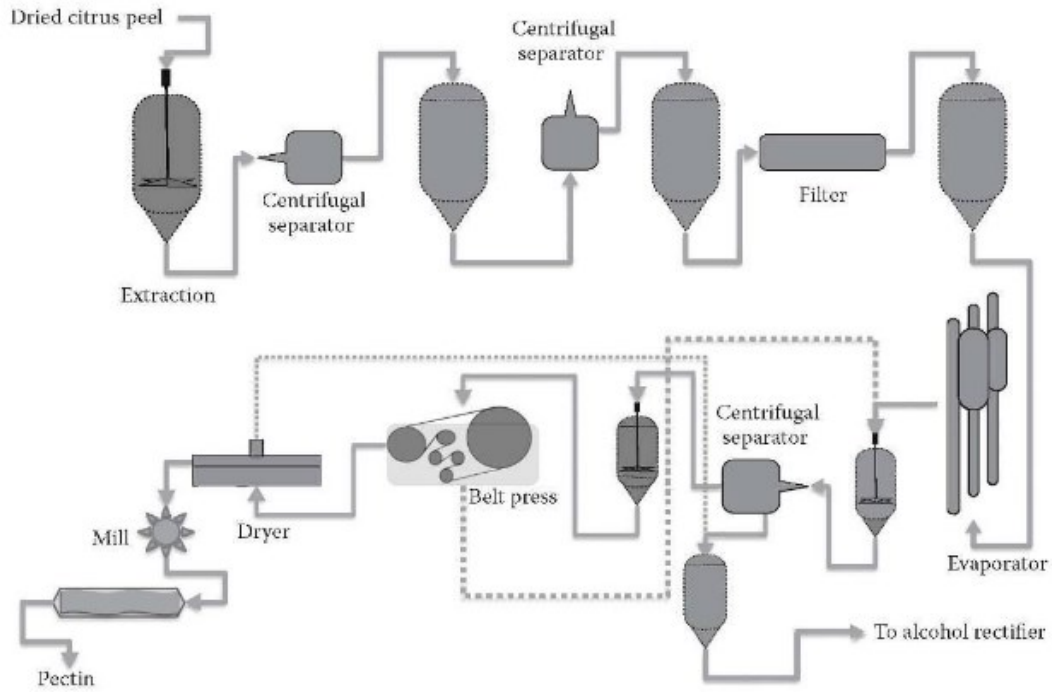

Figure 9 - Flow chart for industrial production of pectins (Transformación de Aditivos, 1998).

The processes can be grouped into phases common to all techniques production (Transformación de Aditivos, 1998) and shown in **Figure 9**. The industrial production of pectin occurs through the following stages: in the first stage, the citrus peels must be thoroughly washed to eliminate the greatest number of soluble impurities, as they could compromise the further purification process. The skins are then subjected to a drying process, which decreases the moisture content and allows the peel stabilization for storage, reducing transportation costs. These stages are usually performed in facilities close to the

manufacturing plants citrus juices. In traditional extraction, the peel must be subjected to the enzymatic inactivation process. The pectin extraction is carried out at high temperature and in an acid environment. Subsequently, a separation centrifuge allows to obtain a very fluid and turbid liquid, which is filtered to obtain a clarified and concentrated liquid. Ethanol is added to this concentrate, causing flocculation. Then, in a new trial of separation, the whole is pressed into a belt press, obtaining the pectin soaked in ethanol. This material is dried, grinding and refining, leaving a white powder that is pectin commercial. This production scheme has the following drawbacks: need for an intense treatment of the raw material that involves the generation of a large quantity of polluting waste, an extraction process in acid medium at high temperature, and a purification step in alcohol which it entails high installation and production costs and generates a great deal quantity of polluting slag. Therefore, alternative procedures are needed for stabilize the citrus peels without drying, using other processes extraction such as cavitation or ultrasound, to purify and dry the peel without ethanol flocculation. Future developments in industrial production of pectin will have such goals.

2.1.5 Limonene recovery from citrus processing

Limonene is a hydrocarbon, which takes its name from the lemon; the peel of lemon, like other citrus fruits, contains large quantities of this compound chemical, which is largely responsible for the characteristic smell. The main naturally occurring chemical compound of major interest in the industrial field and commodity is the D-limonene. Limonene is common in the production of products cosmetics; given its orange smell, D-limonene is used in industry food as a flavor enhancer; it is also used in botany as an insecticide. The use of limonene is increasing significantly as a solvent for cleaning surfaces and can also be considered a biofuel.

Currently, there are three ways to get limonene as a by-product of citrus fruits:

- recovery with an evaporator, from the residual heat that comes from the peel drying process;
- stripping on the essential oil emulsion, using extractors without centrifuges;
- distillation.

From a commercial point of view, it is interesting that the citrus juice industry creates a large amount of waste, used to obtain limonene. The low cost of limonene (2 - 7 € / kg), extracted from the residues, aroused great interest for the chemical industry. For these reasons, its market is expanding.

2.1.6 Citric Acid

The best raw material for the production of citric acid at an industrial level is made up of citrus juices, in particular those obtained from lemon, bergamot and lime. Citric acid is used in alcoholic beverages and in various food products, in the pharmaceutical industry, in the dry cleaners, etc.

The process for obtaining citric acid from citrus juices is the so-called Scheele process, named after the chemist who for the first time in 1784 isolated citric acid. It can be summarized in the following equations:

$$2\ C_6H_8O_7 + 3\ Ca(OH)_2 \Longrightarrow Ca_3(C_6H_5O_7)_2 + 6H_2O$$
$$Ca_3(C_6H_5O_7)_2 + 3H_2SO_4 \Longrightarrow 2\ C_6H_8O_7 + CaSO_4$$

Citric acid in the form of calcium salt precipitates and subsequently it undergoes to decomposition with sulfuric acid. The resulting citric solution is sufficiently pure to crystallize. In fact, in untreated citrus juices, crystallization is impossible due to the presence of impurities, such as sugars, pectin, inorganic salts. Currently the production of natural citric

acid is in decline, almost supplanted by citric fermentation with Aspergillus niger strains beetroot or sugar cane molasses.

2.1.7 Citrus wastes derived feed for animals

Citrus by-product can be dried or pelletized for animal feed. The dried citrus processing wastes contains 10% of moisture with 30-40% sugar, 15-25% pectin, 8-10% cellulose and 5-7% hemicellulose (*Grohman et al 1994*). Dried citrus pulps are added as supplements to the cereal diet to the cows, because are highly digestible and energetic compared to cereals. The energy from the feed is due to soluble carbohydrates and digestible fiber. Digestion of the feed takes long time for rumination and produce large quantity of saliva.

Dried citrus pulp can replace up to 20% of the concentrated cereal diet for dairy cattle (Assis et al 2004). Dried citrus is not recommended for pigs and poultry because of the fiber content and presence of limonin that is toxic to monogastrics (Gohl, 1982).

It has been reported that dried citrus pulp can be incorporated in diets of rabbits up to 20-30% levels (Hon et al. 2009). To increase the density of citrus pulp, it has to be sun dried and pelleted and should be ensiled (fermented). Ensiled citrus pulp having a pleasant odour, is mixed with grass, or cereal straw in order to increase dry matter content.

2.1.8 Agronomic use

Use as a soil conditioner. The agronomic use of citrus by-product represents a suitable cultivation practice to increase the content of organic substance in the soil, increasing fertility. The organic substance improves the resistance to the driving action of rain and water infiltration capacity of the soil, with consequent limitation of runoff and water erosion processes. Citrus by-products brings nutrients to the soil; **Table 2** shows the average content of some macroelements in their fresh state and dried in the sun.

Table 2- Characteristics of fresh and sun-dried citrus by-product (% V/W)

	Fresh Citrus by-products	Dried Citrus by-products
Organic C	50	39
Total N	1.3	1.5
C/N	38	26
P_2O_5	0.28	0.36
K_2O	1.1	1.5

The phytotoxic effects of the content of essential oils in citrus by-products (0.3%) suggest the administration in advance of the sowing operations in herbaceous crops and the administration without immediate incorporation in the soil in tree crops.

Today, it is considered of marginal interest the direct use of the roller as a conditioner also for the regulatory difficulties connected to the moisture content (50%) and the pH (> 6.0). The use of naturally dried citrus by-products could allow to reduce the incidence of transport and distribution costs per unit of dry weight, as well as the regulatory limits connected to the high humidity content of the by-product.

Compost Production (**Figure 10**). Citrus by-products like all other vegetable matrices can be used for the production of quality compost. The composting process allows to increase the degree of humidification and mineralization of the organic substance, increase the content of the macronutrients and reduce the humidity of the by-product. The critical elements of this process are the duration, yield and cost-effectiveness of production, but only if the citrus processing industry is close to the drying and composting site.

Figure 10- Compost Production

2.1.9 Energy production

Citrus by-product is also used as a fuel or organic matrix for the production of biogas or bioethanol. Dried citrus by-products are used for direct combustion in heat production plants for the drying of biomass, for the sole production of electricity or cogeneration or domestic pellet stoves. Its calorific value is less than 12-13 MJ kg^{-1} on dry weight. This energy potential is limited to the high humidity of the by-product in which its preliminary drying is necessary. The extraction of bioethanol from the citrus by-product consists in the hydrolysis of pectin, cellulose and hemicellulose in simple sugars; the subsequent alcoholic fermentation of simple sugars produces ethanol and carbon dioxide (Wilkins et al 2007).

What limits the process of extracting bioethanol from citrus by-products is the high content of essential oils, which hinder alcoholic fermentation due to their toxicity towards many organisms.

2.2 Citrus by-products a source of bioactive compounds

2.2.1 Polyphenols compounds in Citrus by-products

During the industrial processing of citrus, large quantities of agroindustrial wastes such as peels, stones, seeds and other residues are produced. The by-products contain valuable nutrients and biomass, which may be converted into value-added products. Currently, only a

fraction of total peel residue is utilized as beverage bases, marmalades, and candied peel. Peels have a higher concentration of phenolic compounds. However, this citrus part is the richest source of bioactive phenolic compounds, especially flavonoids, with comparatively higher content respect to the edible parts.

Polyphenols are natural antioxidants in plants, especially in fruits and vegetables, which have a vital role in human health thanks to their free radical scavenging activity (*Ross et al, 2009*).

The flavonoids present in citrus consist of flavones, isoflavones, flavonones, flavonols, and anthocyanidins (*Sreenath H, 1995*)

The flavonoids are distributed in the different parts of the citrus but in greater quantity they are in the solid parts: flavedo, albedo, radial septa, vesicular membranes.

For example, in the orange peel (albedo and flavedo), both glucosidic flavanones and polymethoxy-flavones are present. Being present mainly on the albedo, the total flavonoid content of the rind exceeds by more than ten times the juice content. Moreover, the totasl content varies during the harvesting period, sensibly rising with the maturation process. Among the various citrus fruits, one of the riches one in flavonoids is the bergamot, which grows almost exclusively in the Reggio Calabria province.

The content of some glucosidic flavanones in the fresh and dried Citrus by-products (peel, membrane and pulp) of the orange is shown in the **table 3.**

Table 3 - Flavonoids in the Citrus by-products fresh and dried (the citrus processing industry)

Citrus by-product	Total Flavonoid mg/Kg	Hesperidin mg/Kg	Narirutin mg/Kg	Didimin mg/Kg
Fresh	12500	8000	450	220
Dried	30300	15400	11000	750

The main flavonoids present in citrus species are Hesperidin, Naringin and Narirutin, are reported in **figure 11**. Their molecule have a heterocycle skeleton with different chemical groups substituted that give their activity.

Flavonoids are most important for scientific world thanks to health related properties, which are based in their antioxidant activity. Epidemiological studies have illustrated that heart disease are inversely related to flavonoid intake. Flavonoids prevent the oxidation of low-density lipoprotein reducing the risk for the development of atherosclerosis. They also have an antitumor and antidiabetic effect.

.

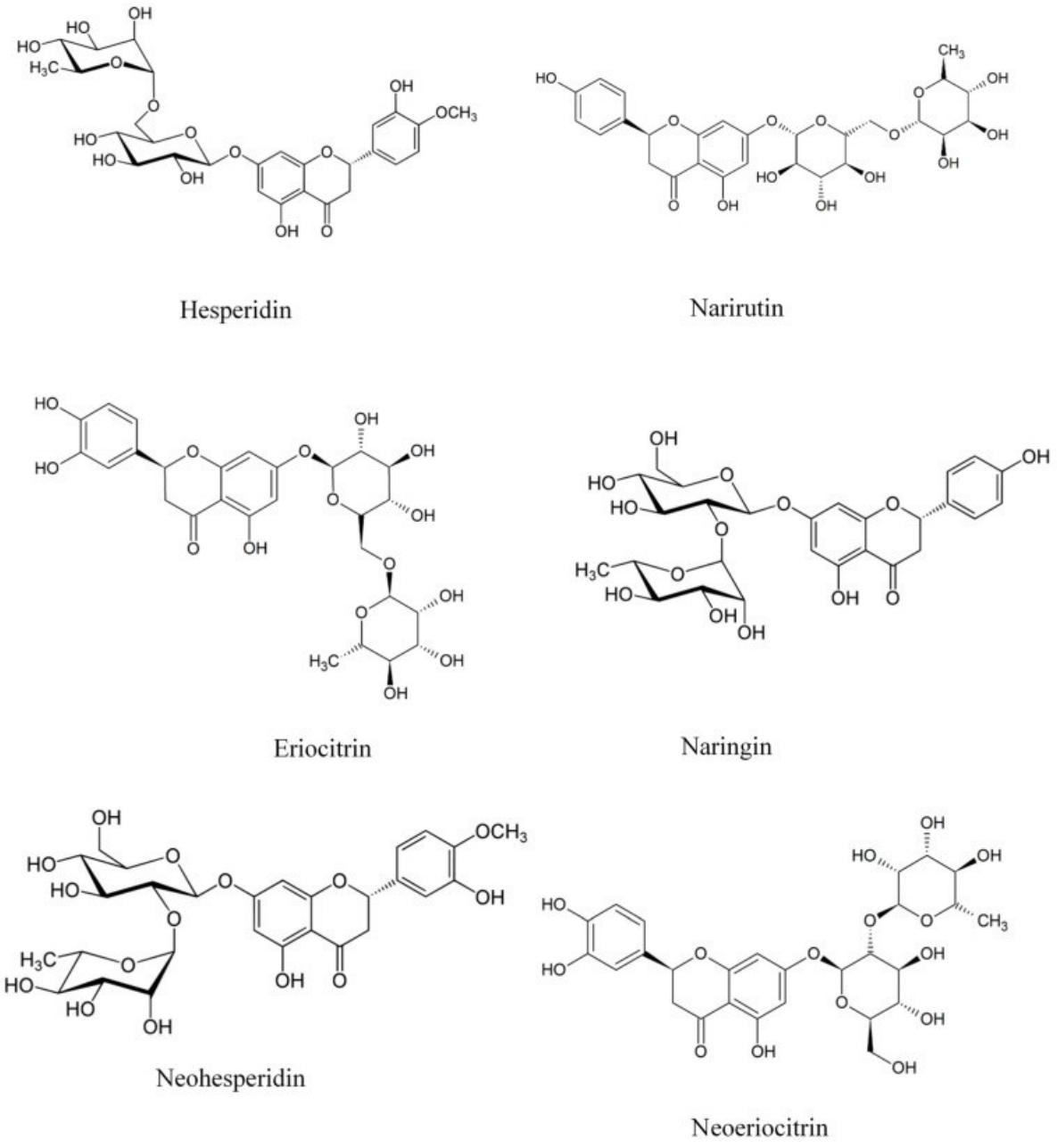

Figure 11 -The most flavonoids found in citrus species

2.2.2 Polyphenols

The most abundant and important antioxidants present in the fruits and vegetables of our diet are polyphenols, which can also represent 90% of the total antioxidant capacity assumed daily. According to Georgé et al., 2005 the daily intake of polyphenols is 10 times greater than vitamin C, 100 times of vitamin E and even 500 times of carotenoids. Phenol has the

structure of benzene, with an –OH group which gives it particular chemical properties (Georgé et al., 2005).

In table 2 is reported the classification of different phenol type compounds base on the cycle skeleton and its substituents. From table is possible show the complexity of these compounds from which derives their wide activities.

Table 4- Classes of the main bioactive phenolic compounds (Harborne, 1989).

Class	Skeleton
Simple phenols, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, (e.g., coumarins)	C_6-C_3
Naphthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbeni, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignani, neolignans	$(C_6$-$C_3)_6$
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Hydrolyzable tannins	$(C_6$-$C_3)_n$
Condensed tannins	$(C_6$-C_3-$C_6)_n$

In **figure 12** are reported the most important phenol compounds and their derivatives such as tannins, glycoside phenols and other polymers.

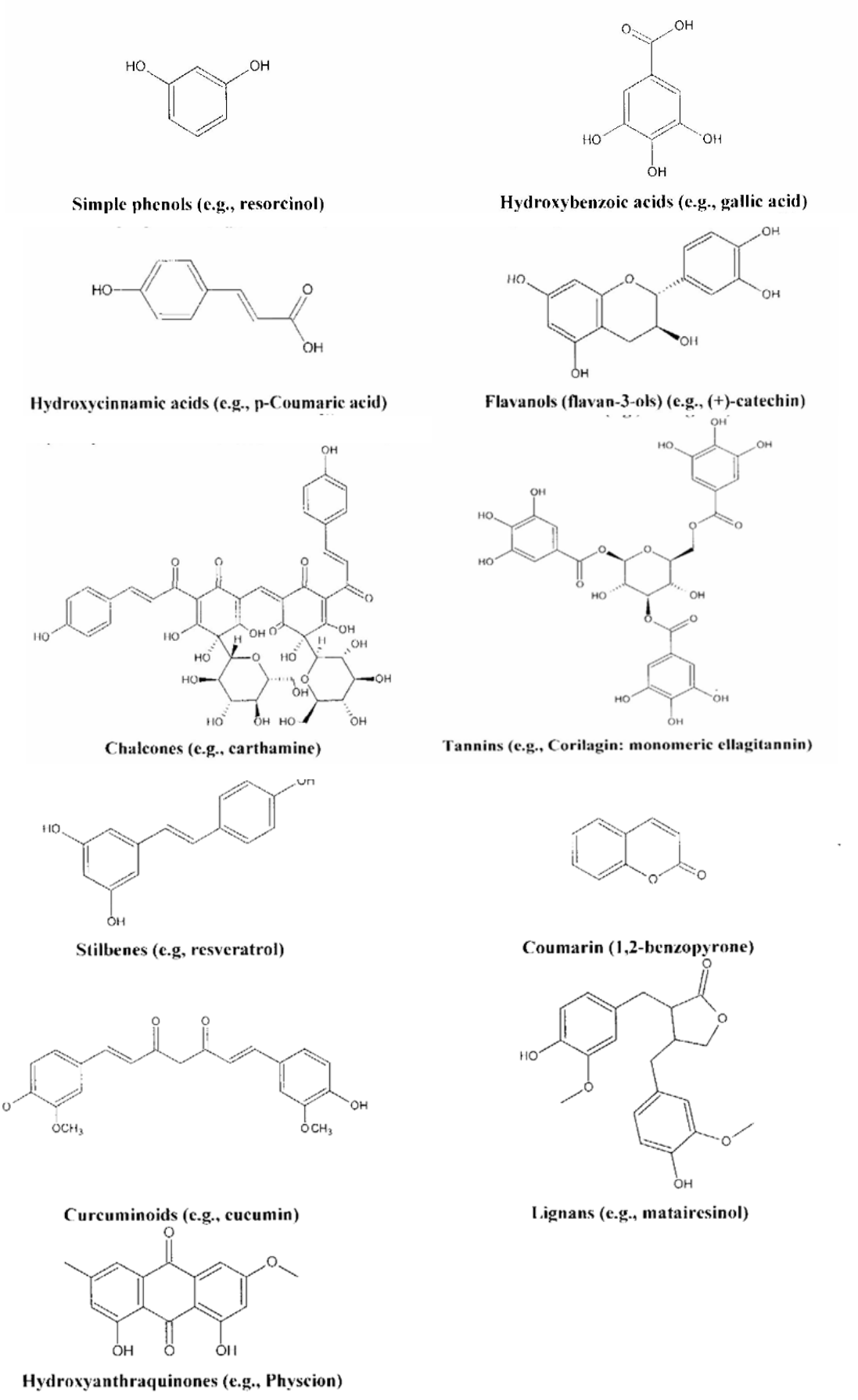

Figure 12 - Basic chemical structure of phenols in fruits and vegetables (*Apak et al., 2007*)

As previously reported, polyphenols are molecules with at least one of the hydroxyl group substituted on the carbon ring. They could be considered as derived from L-phenylalanine (*Petti and Scully, 2009; Naczk and Shahidi, 2006*), so a classification of the main phenolic compounds can be taking into account the carbon skeleton (Table 5 and Figure 9), while, based on their molecular weight, they can be distinguished in compounds with low, medium and high molecular weight (Table 3).

Table 5 - Classification of phenolic compounds based on molecular weight.

Phenolic class	Structure	Molecular weight
Simple phenols	C_6-C_1	Low
Hydroxybenzoic acids	C_6-C_1	
Hydroxycinnamic acids	C_6-C_3	
Flavonoids	C_6-C_3-C_6	Intermediate
Hydrolyzable tannins	$(C_6$-$C_3)_n$	High
Condensed tannins	$(C_6$-C_3-$C_6)_n$	

2.3 Health benefits and potential antioxidant of Citrus by-products

Citrus wastes derived phytochemicals and value added compounds are utilized in designing healthy foods, nutrient supplements, preservatives, flavoring agents in foods processing, health and power drinks. Citrus wastes are also utilized in cosmetic formulations for skin, hair and nails, antifungal and antibacterial lotions, soaps, toiletries and perfumes. Nutrition and food scientist are researching on functional foods and nutritional supplements that can reduce the risk of diet related disorders and diseases. Food containing antioxidants have proven to provide prospective effects against degenerative processes caused by oxidative stress (*Kaur and Kapoor, 2001*). Citrus fruits contain polyphenols, carotenoids, flavonoids, vitamins,

minerals and dietary fibers which help in curing obesity. Contain also other substances with anticancer and antioxidant activities (*Edwards-Jones 2004*).

2.3.1 Antitumor Effects

Flavonoids can inhibit carcinogenesis (*Fotsis et al 1997*). Some flavonoids are stated to be potent inhibitors of cell proliferation (*Caltagirone et al. 2000*). A large clinical study suggest the presence of an inverse association between flavonoid intake and the subsequent incidence of lung cancer. This effect was mainly ascribed as quercetin, which in a study provided >95% of the total flavonoid (*Puri M et al., 2012*). Quercetin and Apigenin inhibited melanoma growth and influenced the invasive and metastatic potential in mince (*Puri M et al., 2012*).. This finding may offer new insights about possible therapies for metastatic disease.

2.3.2 Anti-Atherosclerotic Effects

Flavonoids are important to have a major influence on the vascular system thanks to her oxidative properties. A study have pointed out that flavonoid intake protect coronary heart disease. Flavonoids if consumed regularly might reduce the risk of death from coronary heart disease in elderly men (*Hertog et al 1993*) . A Japanese study reported an inverse correlation between flavonoid intake a total plasma cholesterol concentration (*Arai et al. 2000*). Oxidative stress and vascular damage play an important role in dementia, and the intake of red wine prevents the development of dementia (Orgogozo et al. 1997).

2.3.3 Antidiabetic Effects

Flavonoids, in particular Quercetin, has an antidiabetic activity, Vessal et al (2003) reported that quercetin brings about the regeneration of pancreatic islets and probably increases insulin release in streptozotocin induced diabetic rats. Quercetin stimulated insulin, Hesperidin and Naringin both play crucial roles in controlling the progression of hyperglycemia.

2.4 Green Extraction

Consumer demand for substances don't derived from synthetic processes and more natural food has increased the research on the recovery of natural added value compounds from citrus waste. (*Marangoni, 2016*).

The importance of antioxidant mechanism is to understand the biological meaning of antioxidants, their possible uses, their production by organic synthesis or biotechnological methods.

One of the main reasons for the low levels of the citrus agroindustry residues utilization is the lack of effective and cost-effective extraction methods for compounds with the required quality. "Food-extractions" therefore have a potential to overcome such limitations and higher yields and energy savings. (Chemat et al, 2012). The so named "green" solvents are water in addition to renewable solvents like ethanol. (*Kerton et al, 2009*) This is important to note that controversy exist regarding how "green" ionic liquids are due to potential hazards to the environmental eco-system. (*Thuy Pham et al, 2010*).

2.5 ROS production and containment activities.

Free radicals are particularly reactive atoms that contain at least one electron unpaired in their outermost orbital. This chemical characteristic makes them unstable and pushes them - in search of balance - to bind with other molecules, stealing from them the electron necessary to equalize their electromagnetic charge. This process is called an oxidation-reduction reaction (redox), which consists in the exchange of electrons between a molecule that buys them (oxidant) and one that loses them (reducing).

Free radicals fall into the category of oxidizing chemical species (SCO), molecules capable of removing, under certain conditions, one or more electrons or hydrogen atoms from other chemical species. The best known are ROS, (Reactive Oxygen Species), reactive oxygen species whose production is the natural consequence of normal cellular biochemical reactions, especially of those that use oxygen to produce energy.

Among these are the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which can give rise to the hydroxyl radical ($OH \cdot^-$). The great reactivity of these species derives either from their radical nature (in the case of superoxide and the hydroxyl radical) or from the propensity to radical reactions in the presence of metals such as iron or copper (in the case of hydrogen peroxide).

But there are also free radicals other than reactive oxygen molecules and equally harmful.

Among these, the main ones are:

- RNS, or Reactive Nitrogen Species, in which the element whose atom directly participates in the oxidizing action is nitrogen;
- RCS, or Reactive Carbon Species (carbon content);
- RSS, or Reactive Sulfur Species (sulfur content).

2.6 Standardized analytical method for determining the antioxidant activity capacity

The antioxidant activity is an important function of many substances

There are a lot of known methods that assess the ability of scavenger against other Reactive Oxygen Species (ROS), other than peroxyl radical. There are several essays for the evaluation of the antioxidant power in which they are generated in situ the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2OR), Hydroxyl radical ($OH^{\bullet}$), Hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), The nitric radical oxide (NO) and the peroxynitrite ($ONOO^-$). In many of these tests by mean of the oxidation of a target compound, ROS state is monitored, and the extent of the inhibition caused by the presence of the antioxidant is measured. The determination is carried out mainly through measurements of absorbance, intensity of fluorescence or chemiluminescence, depending on the characteristics of the target molecule selected.

2.6.1 DPPH Assay

This assay uses the reduction of the 2,2-diphenyl-1-picrilhydrazyl radical (DPPH•) aside of antioxidant.

The reaction can be studied spectrophotometrically, measuring the disappearance of the peak typical of DPPH• at 515-528 nm. Recently a variant has been developed of the method based on the amperometric reduction of the DPPH• radical to a carbon electrode (*Milandovic et al. 2006*) The registered current measured is proportional to the concentration of DPPH•. The ability of oxygen scavengers measured by this method is influenced by pH and solvent. Moreover, a determining factor for the reaction between antioxidant and DPPH to take place is steric accessibility of the radical: some large antioxidants that react quickly with peroxy radicals, may not be quantified through this essay. Other disadvantages of the method are the fact that the radical DPPH• has no similarities with the reactive oxygen species; Moreover, it seems that some reactions of DPPH with polyphenols are balanced (*Huang et al. 2005*).

Despite the limitations, the DPPH• method is widely used as the radicals are stable, commercially available and do not need generate in situ (*Magalhaes et al. 2008*).

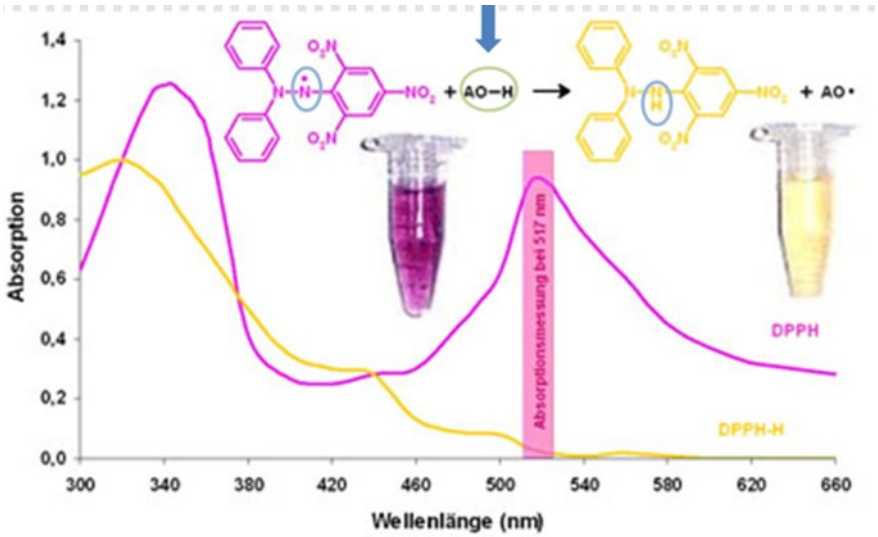

Figure 13 - Reaction mechanism of DPPH with an antioxidant (AO-H) (Boling et al 2014).

2.6.2 ABTS Assay

The ABTS method involves the formation of the 2,2'-azinobis- chromophore cation radical (3-ethylbenzotazolin-6-sulfonate) (ABTS• +), which presents absorption maxima at 414, 645, 734, and 815 nm, but the determination at 734 nm is preferred because minimizes interference due to other components and sample turbidity.

The original method is based on the generation of the ferrylmioglobin radical that reacts with ABTS to form the cation radical ABTS• +. In this first version of the method the sample must be introduced before the formation of the ABTS• +. The ABTS•+ just generated is immediately consumed by the sample, until antioxidant substances are present.(*Miller et al. 1993*). The order of reagent introduction has been much discussed, due to possible reactions between the antioxidant molecules of sample and the hydrogen peroxide which is used for the generation of ferrylmioglobin. It was therefore proposed a variant of the method in which the sample is introduced after the generation of radical ABTS• + and the difference in

concentration of ABTS is determined spectrophotometrically before and after the reaction with the antioxidant. Some modifications of the method were proposed and they involved the formation of the ABTS radical•+, the reaction time and the measurement wavelength used for spectophotometric measures. The ABTS • + can be for example generated using manganese dioxide, AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride) or potassium persulphate.

The results are generally expressed as Trolox equivalent antioxidant capacity (6-hydroxy - 2,5,7,8-tetramethylchroman - 2 - carboxylic),

The test is very simple and applicable for routine analysis. Scavenging with the ABTS method can be carried out at various pHs with the possibility of studying the effect of pH on the antioxidant action, and the solubility of ABTS• + both in water and organic solvents allows the analysis of lipophilic and hydrophilic antioxidants. The disadvantages are that the result depends not only on the antioxidant capacity of the sample, but also from the kinetic behavior of the antioxidant substances that it make up. The radical ABTS• + is not commercially available, but it must be prepared in situ (*Magalhaes et al. 2008*).

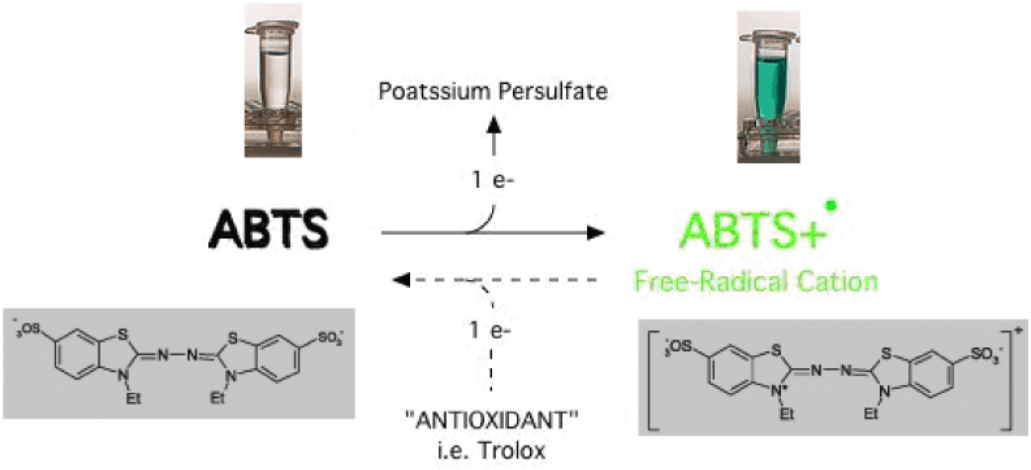

Figure 14 – ABTS chemical reaction (*Boling et al. 2014*)

2.6.3 ORAC Method

The ORAC (Oxygen Radical Absorbance Capacity) method is another of the most common methods for the determination of the capacity of scavenger against peroxyl radicals. The reaction is reported in **figure 15**. The principle of the essay is based on the measurement of the decrease in fluorescence intensity of a target molecule with fluorescent character (for example fluorescein), under a constant stream of peroxy radicals, generated by the thermal decomposition of an azo compound. The rate of spontaneous fluorescein decomposition is slowed by the presence of antioxidant chain-breaking type. The reaction is conducted for long periods (greater than 30 minutes) and the quantification of antioxidant power is carried out by measuring the difference between the subtended area to the curve that represents the oxidation of fluorescein in the absence and in the presence of antioxidant.

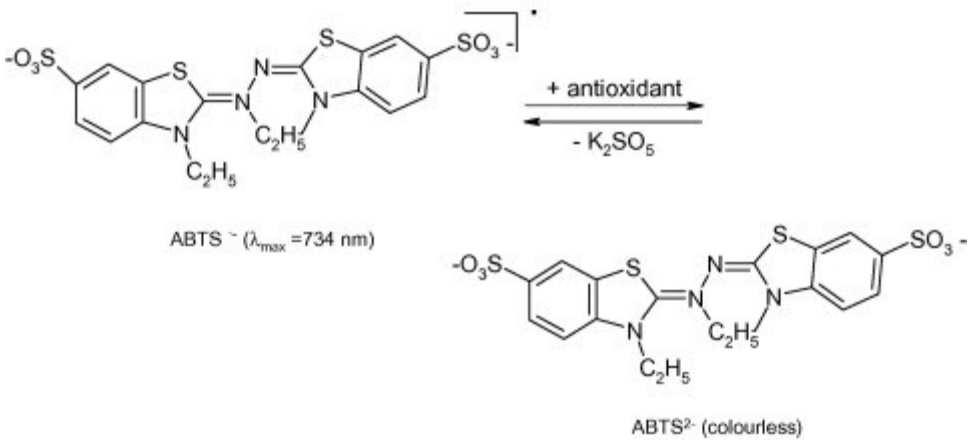

Figure 15 Reaction of the ABTS radical in the presence of the antioxidant compound during the ABTS assay (Zulueta et al 2009).

2.6.4 TRAP Assay

The TRAP assay (total radical antioxidant parameter) was developed for the determination of the antioxidant status of Human plasma. The Method is based on the measurement of time that the molecular oxygen uses to inhibit the plasma peroxidation reaction induce by peroxy radicals, generated by decomposition, have thermal of an azo-compound (**figure 16**).

The results (TRAP value) are expressed as µmol of ROO• trapped per liter of plasma (*Wayner et al 1985*).

In the original TRAP assay the oxygen electrode was used as a detector; in more recent versions of the method it is preferred to use β - phycoerythrin as fluorescent target and the ability of the plasma to be determined say or slow down the oxidation of the target, by fluorescence intensity measurements *(Magalhães Lm, et al 2008).*

The test is based on the measurement of O uptake during a controlled peroxidation reaction, promoted by the thermal decomposition of 2,2'-azobis-(2-amidopropane) (ABAP), which produces $ROO•_2$ at a constant rate . This starts with the addition of ABAP to human plasma; the parameter to be evaluated is the "delay time" of the absorption in plasma induced by the antioxidant compounds present in the medium. The delay time is measured from the O2 concentration data in plasma diluted in a buffer solution monitored with an electrode. In addition to ABAP, other free radical initiators have been used, such as the ABTS (Bartosz G, 1998), dichlorofluorescein diacetate, phycoerythrin, and luminol. One of the main disadvantages of the TRAP method is the possibility of an error in the detection of the end point caused by the instability of the O_2 electrode, because this point can take 2 h to reach. To minimize this problem, the electrochemical detection of O_2 can be performed with a chemiluminescent detection based on the use of luminol and horseradish peroxidase (Bastos et al, 2003).

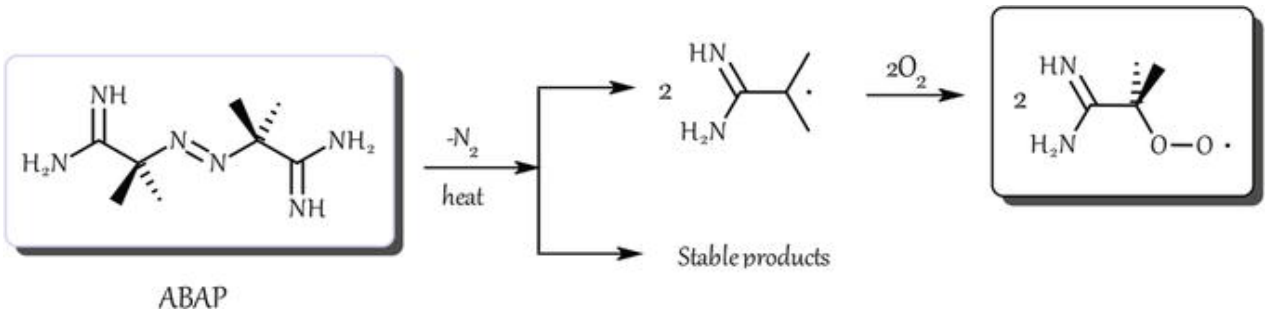

Figure 16- Thermal decomposition of 2,2′-azobis-(2-amidopropane) (ABAP) (Santos-Sánchez, Intech 2019)

2.6.5 FRAP Assay

The FRAP (Ferric Reducing Antioxidant Power) assay measures the ability of antioxidants to reduce iron (III) complex with 2,4,6-tripyridyls-triazine [Fe (III) - (TPTZ)2] 3+ to another complex of iron (II)[Fe (II) - (TPTZ)2]2+ , intensely colored in blue, in an acid environment. The reducing power is calculated by monitoring the increase in absorbance at 593 nm, comparing with that of one antioxidant standard solution (eg ascorbic acid). This essay is not widely used because of its many limitations: the value of reducing power might be affected by any substance that has the potential to reduce lower than that of the redox pair Fe (II) / Fe (III). On the other hand, there may be antioxidants that slowly reduce Fe (III) compared to analysis time (general 4 minutes) and therefore they come only partially quantified. Moreover, since the FRAP method measures the reducing capacity, antioxidants that act only from chain-breakers are not quantified. Finally the concomitant one production of Fe (II), a well-known pro-oxidant, can lead to the production of oxygen radicals additional (*Magalhães Lm et al, 2008*).

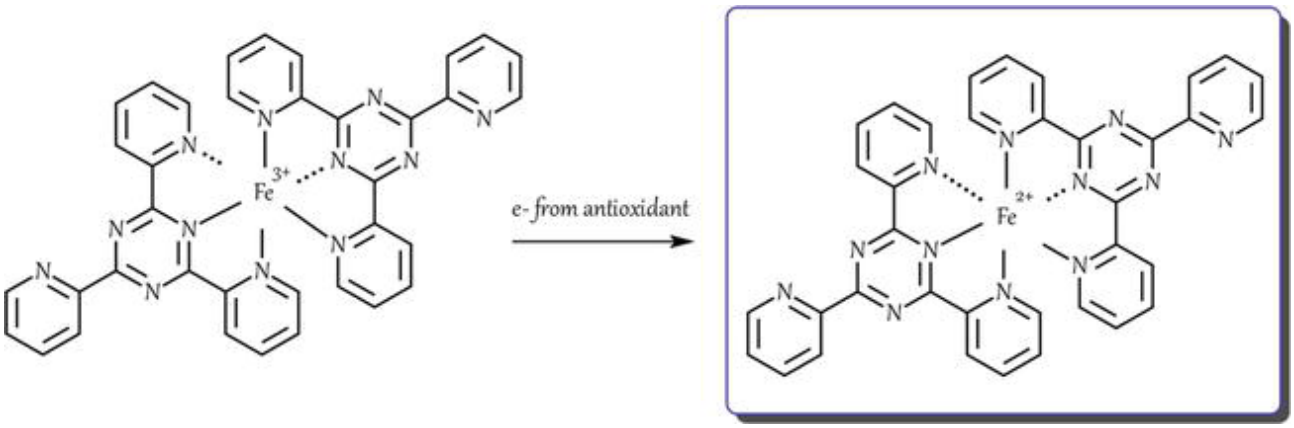

Figure 17- Reaction mechanism of FRAP in presence of an antioxidant. (Santos-Sánchez, 2019)**.**

3. MATERIALS AND METHODS

This project consists of three researches:

Research 1: The recovery of phenolic compounds from citrus by-product, produced from typical Calabria lemon was performed. The optimization of solvent extractions was applied in order to evaluate among these, which would allow to obtain extract with higher amount of phenolic compounds and a valuable level of antioxidant activity, in order to use as "functional" ingredients.

Research 2: The effect of citrus by-product phenolic extract was evaluated in a hydrophilic model food system. Different assays were performed in order to evaluate the suitable of the extract as natural antioxidant to improve the quality of model food.

Research 3: The addition of extracts was carried out on real food system, Natural Apple Juice. The aim of this experimentation was evaluated the addition of a phenolic extract in a complex matrix and its potential interactions with the other compounds contained in it.

3.1 Research 1: Selection and Characterization of phenolic extract from Citrus by-product.

The aim of this research was to obtain extracts containing interesting molecules with antioxidant activity from Citrus by-products typical of Calabria and Sicily. The extracts were obtained by means of extraction with different solvents and purification procedures, the best of them will be set up. Extraction, identification and evaluation of the components and its biological activity was studied. The goal of the research is the possible use of these natural extracts of food interest as "additives", alternative compounds to synthetic antioxidants.

3.1.1 Samples

Citrus by-product were obtained in the 2016 season from Lemons and Oranges coming from Sicily and Calabria. Their transformation took place at Agrumaria Reggina company (Reggio Calabria, Italy) and samples were analyzed at the laboratory of Food Technologies of the Mediterranean University of Reggio Calabria (Italy).

Initially, during the preliminary phase, citrus by-product of lemon and orange was analyzed (**Table 6**).

The starting materials were treated at a wet (or fresh) state and after drying. The best results were obtained from the dried lemon by-product.

Tabel 6 – List of samples Preliminary research

Citrus by-product	Denomination of Sample
Lemon Fresh	LF
Orange Fresh	OF
Lemon Dried	LD
Orange Dried	OD

3.1.2 Characterization of Citrus By- product

The sample of Citrus by- product was freeze-dried by a Lyophilizer (**Figure 18**) for 72 hours at T° of -90°C.

Freeze-drying, in fact, exploits the low temperature and the vacuum to sublimate frozen water.

and subsequently stored under vacuum conditions at room temperature for further analyses.

Figure 18- Lyophilizer

3.1.2.1 Colour analysis (Cie-Lab)

This analysis was performed using a device: a tristimulus colorimeter (Minolta CR-300) basing on the CieLab (L*, a*, b*) system.

This apparatus is used to determine the quantity of primary colors making up a light under examination, by comparison between this and a suitable mixture of primaries colors.

The system proposed by the C.I.E. (Commission Internationale de l'Eclairage) in 1931, defines color by three parameters and is based on the concept that the sensation of color caused by any color in a normal observer. The color can be reproduced by appropriately mixing three colored lights, having very spectral compositions different from each other and such that none of the three can be obtained by combining the other two. Sources with these characteristics are called primary ones.

If all the chromatic sensations can be produced by modulating three fundamental lights, it will be sufficient, starting from a white light source, to subtract from it different intensities of primary lights to obtain the same effect. To absorb more or less these three lights, three colors are used, called "primary colors". They are the only material colors capable of absorbing each one of the three primary lights (blue, green and red):

- YELLOW: controls (absorbs) the blue light;
- MAGENTA: controls (absorbs) green light;
- CYAN: controls (absorbs) the red light.

The system can be used with good results to establish color specifications and relative range.

There are different imitation functions to calculate the values of any color:

• Yxy color system;

• L * a * b * color system;

• L * C * h * color system.

The system used for this internship is: CIELAB L * a * b *.

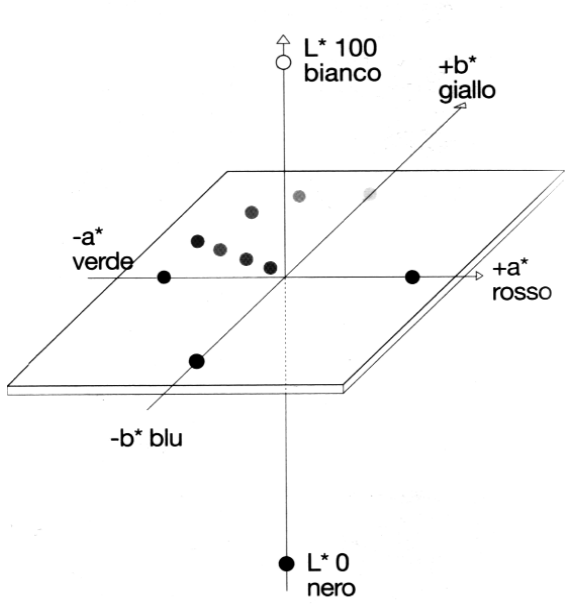

Figure 19 - CIELAB colorimetric system (CIE 1976 - L * a * b *)

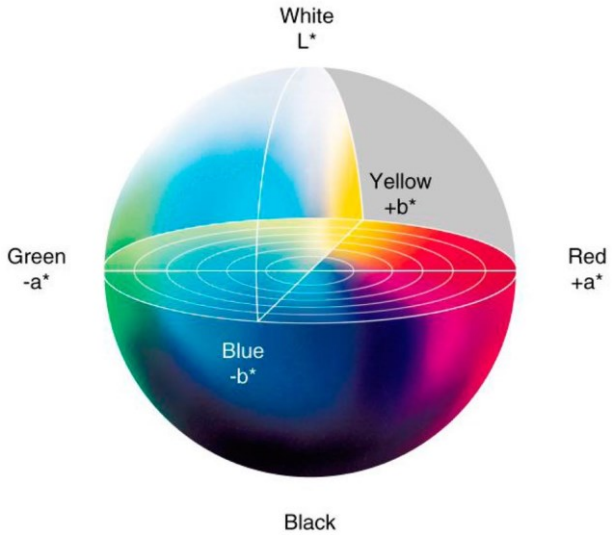

Figure 20 - CIELAB colorimetric system (CIE 1976 - L * a * b *)

The L* coordinate is clarity, which is solely a function of Y (X, Y, Z are the colorimetric functions of the surface, tristimulus function). The L* ranges from 0 to 100 values. However, the perfect black (Y = 0) assumes the value - 16, which is a system anomaly. In the diagram the clarity L* is represented by a vertical axis, with black at the bottom, white at the top and between the two extremes, all the shades of gray.

The a * and b * coordinates are perpendicular to each other and to the L* axis. They are the chromaticity coordinates. The a * and b * axes intersect at the neutral point, where (a *, b *) has the value (0,0). The limits of a and b are comprised approximately between + 80 and - 80. If one turns in a counter-clockwise direction, one starts from red (axis a * positive) goes to yellow (axis b * positive), to green (axis a * negative) to get to blue (b * negative axis) passing through all the intermediate shades.

Saturation is given by the distance between the point representing the color of coordinates (a *, b *) and the neutral point (crossing of the axes a * and b *), it is indicated with C.

The colour analysis was carried out using the Konica Minolta CM-700d tristimulus colorimeter (**Figure 21**).

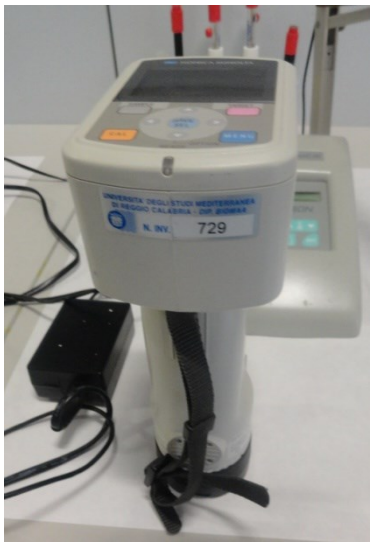

Figure 21 - Konica Minolta CM-700d tristimulus colorimeter.

3.1.2.2 pH and total soluble solids (°Brix)

The pH analysis was performed on different samples of extract of citrus by pH meter (Basic Model 20, Crison) (**Figure 22**), previously calibrated with standard solutions.

Figure 22 - pH meter

The Brix grades express the percentage value of total soluble solids (SST) expressed as sucrose and were determined by a digital refractometer PR-201α (Atago, Tokio, Japan) (**Figure 23**) and expressed as °Brix (sucrose percentage).. The determination is carried out at 20 ° C and from the value of the refractometric index the value of ° Brix was obtained by means of tabulated data.

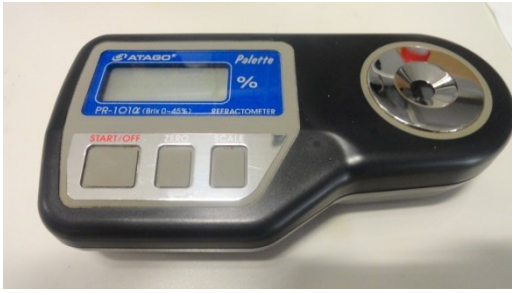

Figure 23 - Rifractometer

3.1.2.3 Acidity analysis

The total titratable acidity (TA) was assessed by titration with NaOH (0.05) and expressed as citric acid %. Take 2 mL of sample, add about 10 mL of distilled water and titrate with NaOH 0.05 using a color change by phenolphthalein (Acid/Base Indicator), 1% phenolphthalein in 95% ethyl alcohol.

3.1.2.4 Dry matter

A Thermobalance (**Figure 24**) was used to determine the dry matter (Sartorius MA37), this analyzer uses infrared rays that penetrate into sample without being hindered and heat directly. The analysis was performed in duplicate using 1 g of sample at 105 ° C until constant weight is reached. In this process, the sample was weighed before and after being heated and the difference between the two weighings was calculated. The results were expressed as a percentage of humidity.

Percentage content of the current mass in the test (dry matter)

W [%] = ma / ms * 100%.

Figure 24 - Thermobalance

3.1.3 Extraction of phenolic compounds

Maceration and Ultrasound Assisted Extraction (UAE) techniques were used for polyphenols extraction from orange and lemon by-products powders. (**Figure 25**).

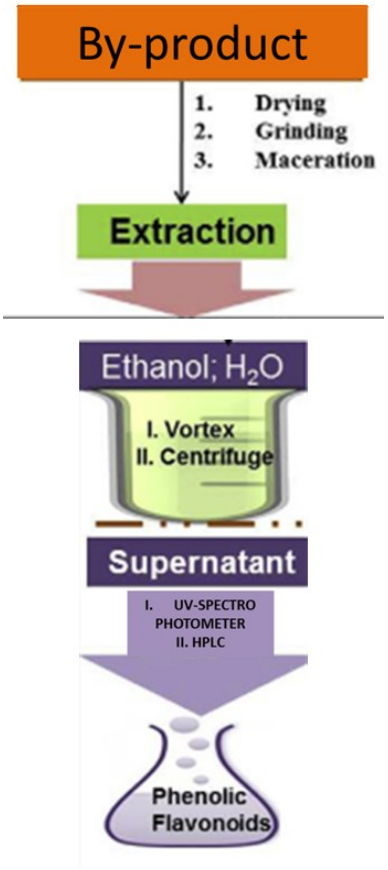

Figure 25 - Phases of the extraction process

3.1.3.1 Solvent extraction by maceration

Lemon peel powders were subjected to extraction as described by (*Elfalleh et al, 2012*) with modifications.

Preliminary studies were performed also to evaluate an optimal sample/solvent ratio (1:2, 1:5, 1:10) and extraction temperature (30°C, 40°C). After these studies, extraction was carried out using different solvents (ethanol, methanol, acetone and ethyl acetate) at two different

concentrations (50% of solvent and 50% of water; and 80% of solvent and 20% of water) with 1:10 sample/solvent ratio and extraction temperature of 40°C. (**Figure 26**).

The preliminary trials were carried out as follows, the extraction by maceration took place 1g of peel powder samples were extracted by specific solvent, concentration level, extraction temperature and sample/solvent ratio for 20 hours under agitation. The extracts were filtered through Whatman filter paper and centrifuged (Nuve NF 1200R) at 5000 rpm for 10 minutes. The supernatant was collected, and the solvent was evaporated with a rotary evaporator under vacuum (Heidolph VV200 equipped with Heidolph Waterbath WB 2000) at 35/40°C to obtain the extract, which was filtered using PTFE 0.45-µm syringe filter, then collected in amber glass bottles and stored at refrigeration temperature of 4°C temperature until subsequent analyses.

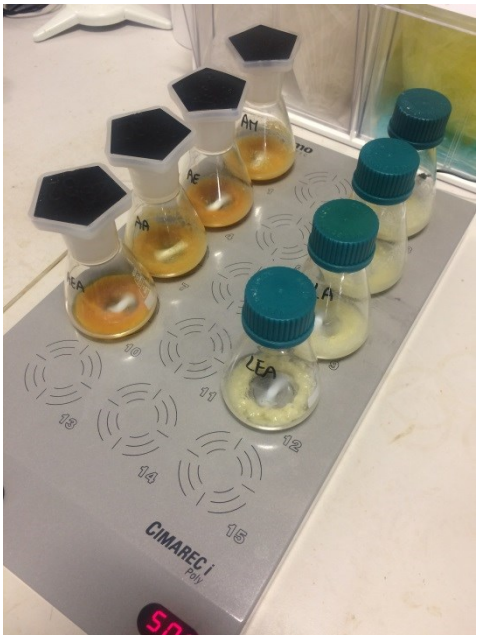

Figure 26- Orange and Lemon Samples with different solvents

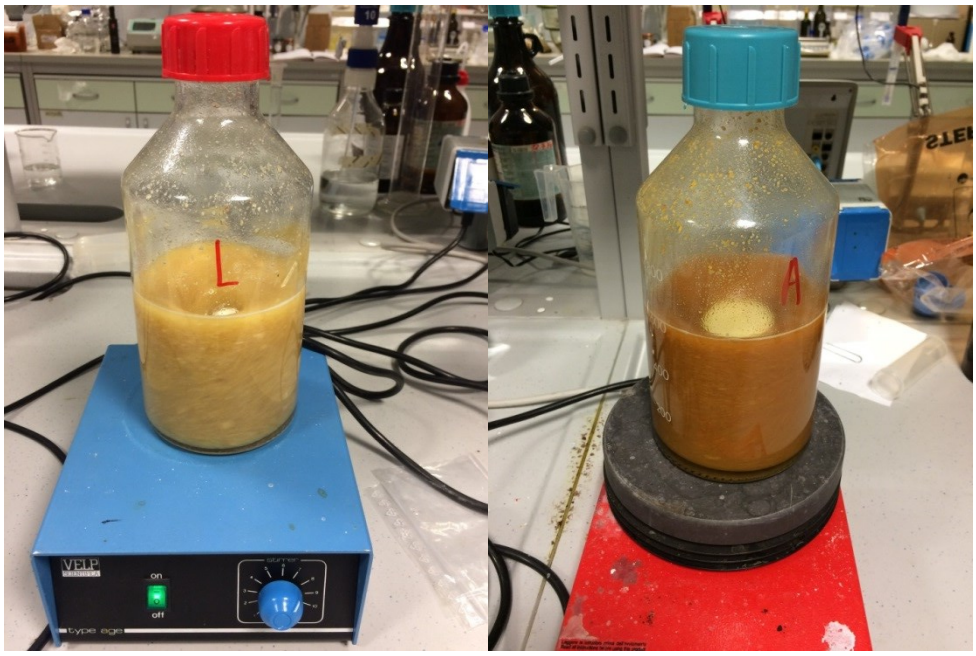

Figure 27 - Traditional solvent extraction - Maceration

3.1.3.2 Assisted ultrasound extraction

The extraction of bioactive compounds under ultrasound irradiation is one of the upcoming extraction techniques that can offer high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (*Chemat et al. 2008*).

The extraction of polyphenols from orange and lemon peel powders were conducted using the assisted ultrasound extraction (UAE) technique as described by (Bimakr et al, 2013) with slight modifications.

Preliminary studies were carried out to determine the optimal sample/solvent ratio (1:5,1:10), extraction temperature (30°C and 40°C) and extraction time (30, 60 minutes). From the preliminary trials an extraction procedure was set up.

After the preliminary studies, 10-g kinnow peel powder samples were extracted by solvents ethanol 80%, concentration levels set up by the preliminary trials: sample/solvent ratio, 1:10; extraction temperature, 40°C; extraction time, 60 minutes in a sonicator, **Figure 28**

(Transsonic 700; Elma, Wetzikon, Switzerland) at 40 kHz frequency. The extracts were filtered through Whatman filter paper; then they were centrifuged in a refrigerated apparatus (Nuve NF 1200R) at 5000 rpm for 10 minutes. the supernatant was collected, and the solvent was evaporated with a rotary evaporator (Heidolph VV200 equipped with Heidolph Waterbath WB 2000)) under vacuum at 40°C to obtain the extract.

Finally the residue was further filtered using PTFE 0.45-µm syringe filter (diameter 15mm), collected in amber glass bottle and stored at refrigeration at 4°C temperature and in a thermostat at 25°C, both kept in the dark for 50 days of storage. Samples were analyzed at T0, T7, T15, T30, T40, T50. All extractions were performed in duplicate.

Figure 28 - Assisted ultrasound extraction

3.1.4 Total phenol content determination with Folin Ciocalteau Method

The total polyphenol content of citrus by-product extracts was measured using the Folin-Ciocalteau method as described by (*Singleton et al 1999).* An aliquot of 500 µL of ethanolic extract was mixed with 2.5 mL of 10% Folin-Ciocalteau reagent dissolved in distilled water and 2.5 mL of 7.5% sodium carbonate. The blank contained 500 µL ethanol, 2.5 mL Folin-Ciocalteau reagent (10 times diluted), and 2.5 mL of 7.5% sodium carbonate. The samples were incubated at 25°C for 30 minutes for the development of a blue color. (**Figure 29**). The absorbance was measured at 765 nm with a UV-VIS Spectrophotometer Agilent 8453 (Agilent Technologies, Italy). A similar procedure was carried out for gallic acid standard solution, and the calibration curve was prepared from various concentrations of gallic acid (**Figure 30**). The total polyphenol content was expressed as mg gallic acid equivalent (GAE)/g extract.

Figure 29 - Total phenol content determination

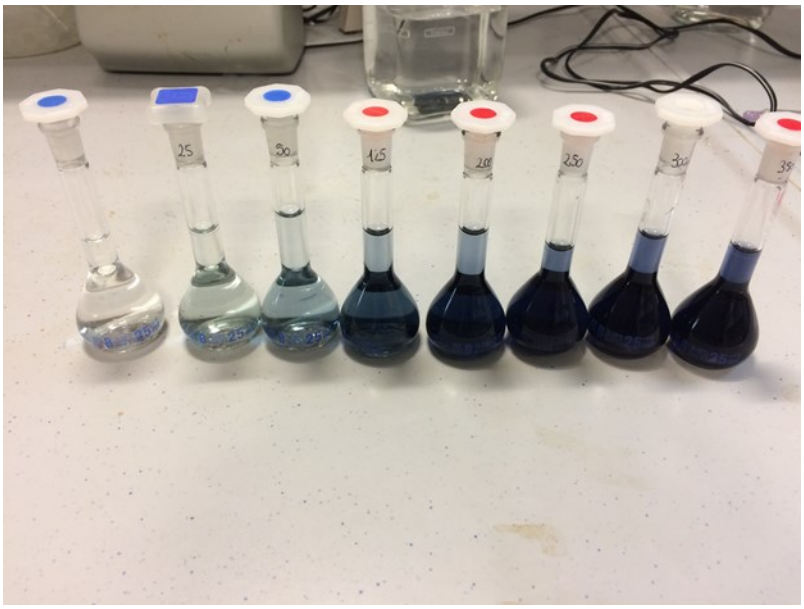

Figure 30 - calibration curve was from various concentrations of gallic acid.

3.1.5 Evaluation of antioxidant activity

The DPPH and ABTS assays were carried out on the extract obtained from the citrus by-product.

3.1.5.1 DPPH assay

In this study the antioxidant activity was determined using DPPH 2,2-diphenil-l-picrylhydrazil radical assay. The DPPH radical scavenging effect was determined as described by Brand-Williams et al. (1995) with some modification. A methanolic solution of DPPH $6*10^{-5}$ M was prepared and stored in dark at temperature of -18°C. 10 µl of citrus by-products extract were mixed with 2990 µl of DPPH for 15 minutes in a cuvette in the dark and at room temperature. The absorbance was determined at 515 nm using a spectrophotometer (Perkin-Elmer UV-Vis).

The results of citrus by-products were expressed as percentage of inhibition of ethanolic extract by applying the following formula:

$$\%\ Inibition_{\ 515nm} = 100 * \frac{(A_{t0} - A_{tf})}{A_{tf}}$$

Were A_{t0} is the value of absorbance of DPPH solution at the initial time while A_{tf} is the value of absorbance measured after 15 minutes. All measurements were performed in triplicate.

Trolox was used as a standard antioxidant and ethanolic extract activity was expressed in mM of Trolox equivalents.

3.1.5.2 ABTS assay

The radical scavenging capacity of the citrus by-products for the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate) radical cation was determined as described by *Re et al.(1999)* with some modifications. The solution of ABTS was produced by mixing 7 mM ABTS and 2.4 mM potassium persulphate ($K_2S_2O_8$) solution. The solution was placed at room temperature for 16 hours in the dark before use, in order to achieve a stable absorbance value. The mixture obtained was diluted with ethanol (1:80) to give a blue-green colour that showed an absorbance of 0.70 (±0.02) at 734 nm using the spectrophotometer (Perkin-Elmer UV-Vis). 2990 µl of ABTS was mixed with 10 µl of citrus by-products extract and the absorbance was measured after 6 minute.

The results were expressed as percentage of inhibition of ethanolic extract by applying the following formula:

$$\%\ Inibition_{\ 515nm} = 100 * \frac{(A_{t0} - A_{tf})}{A_{tf}}$$

Were A_{t0} is the value of absorbance of ABTS solution at the initial time while A_{tf} is the value of absorbance measured after 6 minutes. All measurements were performed in triplicate.

The antioxidant capacity of the samples is expressed in ÌM of equivalent Trolox.

3.1.6 Identification and Quantification of phenolic compounds by HPLC

Phenolic compounds in Citrus by-products were determined by HPLC/DAD. The analyses were performed on a Knauer (Asi Advanced Scientific Instruments, Berlin) system equipped with two different pumps Smartiline Pump 1000, a Rheodyne injection valve (20 µL), and a photodiode array detector UV/VIS equipped with a semi micro-cell. Processing data were carried out using Clarity Software (Chromatography Station for windows). Compounds were separated on a Phenomenex C18 column (250 mm x 4.6 mm, 5µm). The separation chromatographic elution of La Torre et al (2006) was used. The mobile phase was prepared with formic acid in water at pH=3 (solvent A) and formic acid in acetonitrile at pH=3 (solvent B). The gradient was: 0.01-20.00 min at 5% of solvent B; from 20.01 to 50.00 min, 5-40% B; 50.01-55.00 min, 40-95% B; 55.01-60.00 min 95% B isocratic. The column temperature was 30°C and the flow rate was 1.0 mL/min. Before the injection, samples were filtered through a 0.45 µm membrane filter, and the injection volume was 20 µL.

Peaks (**Figure 31**) were monitored at different wavelengths: 280, 254 and 365 nm. The identification and quantification was carried out by mean of the retention times compared with standards. Analyses were performed in triplicate.

The quantification of phenolic compounds, present in Natural Apple Juice, was performed by HPLC.

The major flavonoids present in apple juice and samples are Neohesperidine, Naringin, Narirutin Hesperidin, were evaluated by HPLC.

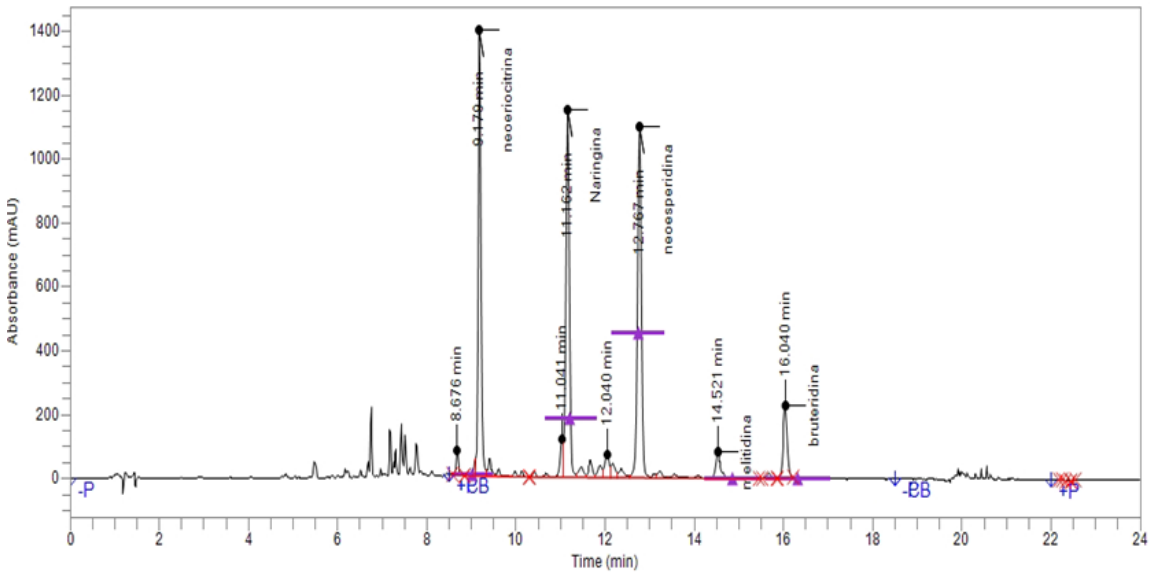

Figure 31 - Example of HPLC chromatogram of an unknown mixture of the five flavonoids considered in this study using the parameters described in this report.

3.1.7 Statistical analysis

Each value is the mean of three replicates. Analysis of variance (one-way ANOVA) was conducted using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL) and the Tukey's test was used to determine any significant difference among all treatments at $p < 0.05$.

3.2 Research 2: Addition to different model drink system of Citrus by-product extract

Addiction water was performed in the laboratory of Food Technologies of Mediterranean University of Reggio Calabria (Italy).

3.2.1 Samples preparation and identification

The samples monitored were prepare as follow, their identifications were reported in **Table 7.** For the preparation of the model system, the water was added with phenolic extract obtained from lemon by- product (WL5). The extract was dissolved at concentration of 5% of polyphenol ethanol extract (5mL of extract in 100mL of water). Samples were homogenized with a vortex until complete homogenization, observed as a clear solution. Moreover, an other types of samples were prepared, WLFC5, with 12 g of fructose + 1 g of citric acid with a concentration of lemon polyphenols ethanol extract at 5%.

Enriched water were stored in aseptically sterile glass bottles (30 ml of capacity) at 4 °C and at 25 °C. Samples (tree different replicate for each concentration and type) were analyzed on the 0^{th} day and on the 7^{th}, 15^{th}, 30^{th}, 40^{th}, 50^{th} day of storage.

Table 7 – List of sample Second Research

samples	
WL5-4°C	Water enriched with 5%of extract of Orange by-product- preserved at 4°C
WL5-25°C	Water enriched with 5%of extract of Orange by-product- preserved at 25°C
WLFC-4°C	Water enriched with 5%of extract of Lemon by-product + Fructose + Citric Acid - preserved at 4°C
WLFC-25°C	Water enriched with 5%of extract of Lemon by-product+ Fructose + Citric Acid - preserved at 25°C

3.2.2 Evaluation of antioxidant activity of model drink system

The antioxidant activity of the model drink system was evaluated by measuring the radical scavenging activity DPPH and ABTS assay.

DPPH

The solution was prepared as reported in Section 3.1.5.1. 10 µl of citrus by-products extract 2990 µl of DPPH were mixed to 2990 µl of DPPH for 15 minutes in a cuvette in the dark and a room temperature. The decrement of absorbance was determined at 515 nm using a spectrophotometer (Perkin-Elmer UV-Vis). The DPPH values expressed as % inhibition were calculated by applying the formula (section 3.1.5.1). All measurement were performed in triplicate.

ABTS

The solution was prepared as reported in Section 3.1.5.1. 2990 µl of ABTS was mixed with 10 µl of citrus by-products extract and the absorbance was measured after 6 minutes . The ABTS values expressed as % inhibition were calculated by applying the formula (section 3.1.5.2). All measurement were performed in triplicate.

3.2.3 Microbiological analysis

Samples were analyzed for microbiological count (1 mL/plate, triplicate) in a PCA (Plate Count Agar) for aerobic mesophilic bacteria count at 32 °C for up to 3 days, Dichloran Rose Bengal chloramphenicol Agar for the research of yeast and mould at 26 °C for five days before counting the colonies. Each test was done in triplicate (**Figure 32**).

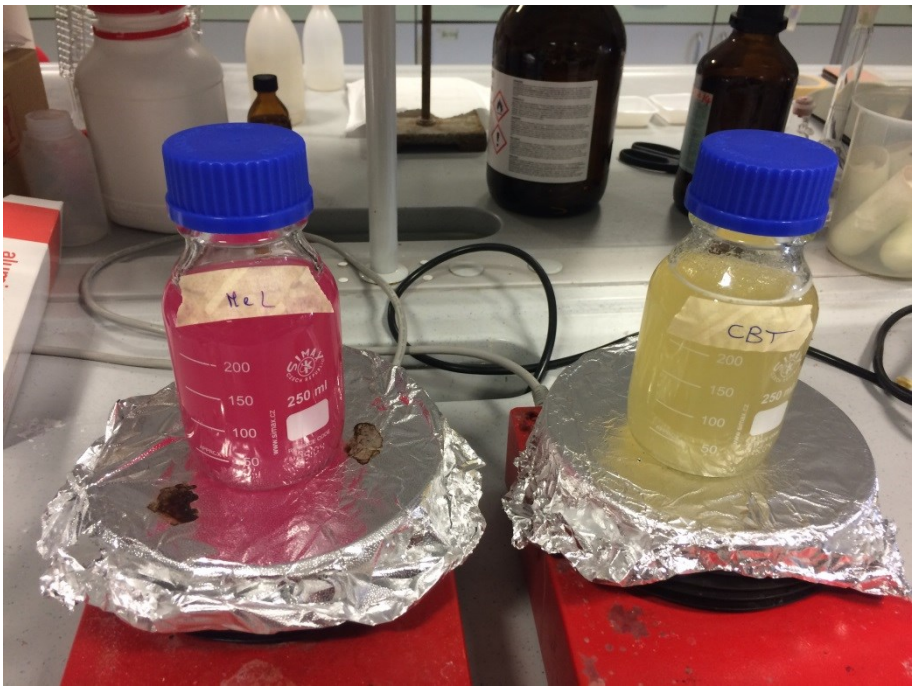

Figure 32 – Preparation of culture media

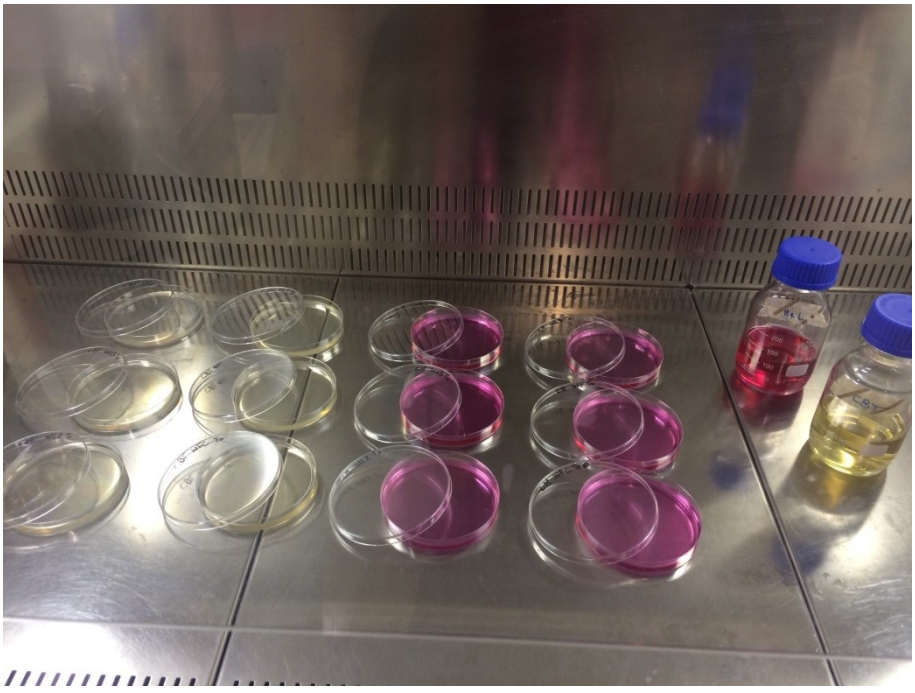

Figure 33 – PCA (Plate Count Agar) and Dichloran Rose Bengal chloramphenicol Agar

3.3 Research 3: Addition to different real drink system of commercial extract

3.3.1 Sample preparation and identification

The last research of the study was the addition of the extracts obtained and real food matrix evaluating the impact on formulation and stability of them (shelf life). The extract has been added to apple juice.

The apple juice and samples (obtained by adding extract to it) were characterized.

Production of Natural Apple Juice was performed in the food technology laboratory at the *Mediterranean* University of Reggio Calabria. The apple juice was extracted with a juice extractor for fruit and vegetables (Kenwood Estrattore JMP601SI PureJuice). Golden quality apples were used, and chemical analyzes were carried out on the juice as it is.

Quality parameters (Total soluble solids, pH, colorimetric analysis), total flavonoids, total phenolic compounds and antioxidant capacity (DPPH and ABTS) were reported in section 3.3.2 for method and in section 4.3. for the results.

The Apple Juice was enriched to 5% of ethanol extract of lemon by-product, 12,5 mL of extract was added in 250 mL of apple juice.

Apple Juice without extract added was also made following the same procedure and it was used as control and stored at 4°C (two independent replicate sample for each) until further analysis. All samples were analysed in triplicate at the production day, at 0^{th}, 1th, 2th,3th, 4^{th}, 7^{th} and 10^{th} day of storage.

Table 8 – List of sample Third Research

samples	
M	Natural Apple Juice
MC	Natural Apple Juice enriched with 5% of concentrate of Lemon By-product- 4°C

3.3.2 Evaluation of antioxidant activity of Apple Juice and Apple Juice enriched with extract

The antioxidant activity of the Apple Juice was evaluated by measuring the radical scavenging activity DPPH and ABTS assay.

DPPH

The solution was prepared as reported in Section 3.1.5.1. 10 µl of citrus by-products ethanol extract were mixed to 2990 µl of DPPH for 15 minutes in a cuvette in the dark and a room temperature. The decrement of absorbance was determined at 515 nm using a spectrophotometer (Perkin-Elmer UV-Vis). The DPPH values expressed as % inhibition were calculated by applying the formula (section 3.1.5.1). All measurement were performed in triplicate.

ABTS

The solution was prepared as reported in Section 3.1.5.2. 2990 µl of ABTS was mixed with 10 µl of citrus by-products ethanol extract and the absorbance was measured after 6 minutes . All measurement were performed in triplicate.

3.3.3 Microbiological analysis

Samples were analyzed for microbiological count (1 mL/plate, triplicate) in a PCA (Plate Count Agar) for aerobic mesophilic bacteria count at 32 °C for up to 3 days, Dichloran Rose Bengal chloramphenicol Agar for the research of yeast and mould at 26 °C for five days before counting the colonies. Each test was done in triplicate.

3.3.4 Sensory analysis of enriched juice

The sensory analysis was carried out by mean of a panel of 8 panelists participated, in order to evaluate the shelf life of the product and therefore its acceptability.

The sensorial evaluation was carried out using the QDA method, (Quantitative Descriptive Analysis) it consists of 2 steps. At first, descriptors for aroma, taste and aftertaste were collected and described. In the second step, these descriptors are proposed to the panelists for a more accurate definition and for the presentation of the reference standards (Apple Juice).

The profile obtained through QDA made it possible to quantify the characteristics of the product on a scale from 0 to 10 (where 0 indicates absence and 10 indicates presence at the highest level of perception) and sheet divided into four parts is drawn up:

- VISUAL SENSATION
- OLFACTORY SENSATION
- FLAVOR
- TEXTURE.

within these four areas descriptors are introduced.

Figure 34 reported the final data sheet and on it judges reported the values of each descriptor that were recorded as average on radially arranged scales. By joining all the midpoints together, a spider plot is obtained which represents the sensorial profile of the product (**Figure 35**).

This method was chosen because it is useful when a modification to a product is introduced (in this case, the lemon by-product ethanol extract has been added in apple juice) or for monitoring the profile during storage.

VISUAL SENSATION	from 0 to 10	OLFACTORY SENSATION	from 0 to 10
BRILLIANCE		FERMENTED	
COLOR UNIFORMITY		VEGETABLE	
TURBIDITY		CITRUS FRUITS	
FLAVOR	from 0 to 10	SWEET	
SWEET		MEDICINE	
BITTER		FRUITY	
ACID		ACID	
PERSISTENCE		**TEXTURE**	from 0 to 10
ASTRINGENCY		GRIT	
COTTO		STICKY	
SPICY		FLUIDITY	

Figure 34- Sensory Analysis Card

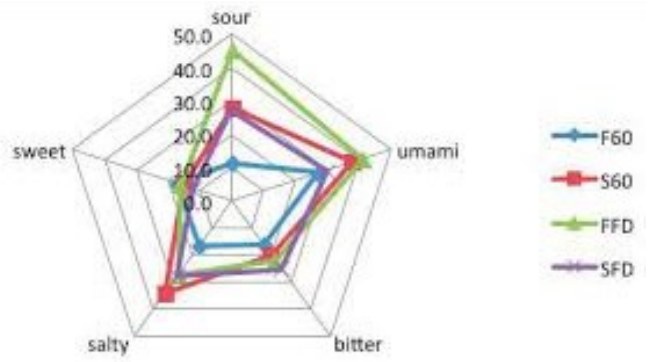

Figure 35- Example of spider plot.

4. RESULTS AND DISCUSSION

Research 1

4.1 Characterization of phenolic extract obtained from citrus by-products extracted by UAE.

The extraction yield of each sample was recorded in the range of 10.90 to 12.95 % on the dry basis of sample weight by using UAE method. This method was found to be the best.

The extracts were stored in amber glass bottle at two different temperatures: a refrigeration one of 4°C and at 25°C, both of the sets of samples were kept in the dark for 50 days.

The quality parameters, Total soluble solids (TSS) and pH of citrus by-products extract, are shown in **Table 9**.

TSS, expressed as °Brix, as well as the pH, of lemon by-products extracted by ultrasound method (LEU), remained constant during the 50 days of monitoring.

The TSS is an important parameter related to the quality characteristics of citrus by-products which comes from the quality of the fruit, due to the sugar to acid ratio.

The TSS defines the concentration of soluble solutes that in the extracts are probably represented by active molecules such as phenols and others.

The values obtained, show stability of the citrus juice processing waste extract in a hydrophilic matrix, also this tells us that such compounds can be high content in the discarded parts and not only in the fruits.

Table 9 – Chemical characteristics of citrus by-product ethanol extract

sample		°Brix		pH
LEU		MEDIA ± DS		MEDIA ± DS
	T0	30,40±0,00	T0	3,40±0,01
	T7	30,40±0,00	T7	3,39±0,00
	T15	30,40±0,00	T15	3,38±0,00

	T30	30,40±0,00	**T30**	3,38±0,00
	T40	31,00±0,00	**T40**	3,38±0,00
	T50	31,30±0,00	**T50**	3,39±0,01
Sign.		Ns		Ns

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Table 10 shows the color determination with the results expressed according to the CIE Lab scale (L *, a *, b *).

Color has to be considered as a special parameter that seems to be one of the first attributes of quality that a consumer perceives. The color parameters (L *, a *, b *) of lemon by-product has statistically significant differences, in particular the parameter *a (greenness/redness) increases during storage of 50 days, probably because the extract tends to brown due to the Maillard reaction between some of its components, and/or it undergoes to and oxidation state of sensible compounds contained in it.

Table 10 – Determination of colour of citrus by-product ethanol extract

sample		**L**		***a**		***b**
LEU		**MEDIA ± DS**		**MEDIA ± DS**		**MEDIA ± DS**
	T0	42,87±0,05^{ab}	**T0**	0,52±0,02^{c}	**T0**	4,67±0,02^{ab}
	T7	43,07±0,09^{a}	**T7**	0,53±0,02^{c}	**T7**	4,60±0,08^{bc}
	T15	42,82±0,10^{abc}	**T15**	0,58±0,02^{bc}	**T15**	4,65±0,03^{abc}
	T30	42,92±0,13^{a}	**T30**	0,60±0,02^{bc}	**T30**	4,39±0,14^{c}
	T40	42,60±0,09^{c}	**T40**	0,65±0,04^{ab}	**T40**	4,80±0,17^{ab}
	T50	42,64±0,23^{bc}	**T50**	0,72±0,08^{a}	**T50**	4,86±0,23^{c}
	Sign.	**	**Sign.**	**	**Sign.**	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Lemon and other citrus are considered healthy due to their high content of antioxidants, which help to reduce free radicals in the body. Antioxidants are secondary metabolites found naturally in plants as citrus fruits. An antioxidant can be defined like a substance that inhibits or prevents oxidation of a substrate.

As well as in fruits, interesting amounts of antioxidants are present in the waste.

The antioxidant activities were determined by free radical scavenging activity (DPPH and ABTS assay methods). For DPPH assay (**Table 11)** the samples analyzed have statistically significant differences, at time 0 samples have the highest value respectively of 56.08 % of

inhibition which over time decreases slightly until it reaches 43.84 % of inhibition at 50 days of storage. The relation between antioxidant activity and storage time is depicted by a regression coefficient, R^2=0,62.

Regarding ABTS assay (**Table 12**), it has statistically significant differences, but less highlighted than the DPPH. Some studies have suggest that phenolic compounds dominate the total antioxidant capacity of citrus by-products, in fact this compounds together with flavonoids have an important role due to their ability to scavenge free radicals. The antioxidant ability measured by mean of ABTS showed an unclear trend, this is demonstrated by the relation between antioxidant activity and storage time is depicted by a regression coefficient, R^2 = 0,04, due to the minimum level observed after 30 days of storage with a subsequent increase.

The decrease of antioxidant ability could be related to total phenol content. As reported in **table 13** these components, measured by Folin test showed a constant decrease during storage and this aspect could explain the same decrease of the antioxidant activity measured.

Table 11 - Antioxidant activity measured by DPPH assay in lemon by-product ultrasound extract.

sample		DPPH (% I)
LEU		**MEDIA ± DS**
	T0	56,08±0,00^{a}
	T7	53,98±0,00^{ab}
	T15	54,76±0,01^{ab}
	T30	41,54±0,00^{d}
	T40	48,79±0,02^{bc}
	T50	43,84±0,01^{cd}
	Sign.	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD^{a} test.

Table 12 - Antioxidant activity measured by ABTS assay in lemon by-product ultrasound extract.

sample		ABTS (% I)
LEU		**MEDIA ± DS**
	T0	20,79±1,63[ab]
	T7	21,95±0,02[ab]
	T15	23,36±0,08[a]
	T30	16,40±0,46[c]
	T40	19,01±1,09[bc]
	T50	21,97±0,42[ab]
	Sign.	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Total phenolic compounds were measured by Folin-Ciocalteu based on the absorbance values of extract solutions that reacted with a reagent that produce a complex, this is compared with standard solutions of gallic acid equivalents. In **Table 13** were reported the total phenols content measured along time and expressed as mg of gallic acid on kg of extract. The phenolic content of citrus peel processing waste extract appears to have statistically significant differences during the time, and polyphenols do not undergo to a severe deterioration during 50-day monitoring. The relation between poliphenols content and storage time is depicted by a regression coefficient, $R^2 = 0,79$.

Significative is the low level of these compounds at the Time 0, this is probably due to a dissolution and dispersion effect. In fact, considering the next observations it is a clear decreasing trend, statistically significant as reported in table 13.

Table 13 - Total polyphenols content in lemon by-product ultrasound extract.

Sample		Total Polyphenols (mg/kg GAE)
LEU		**MEDIA ± DS**
	T0	38140,84±9,25[e]
	T7	42721,42±47,24[a]
	T15	42451,45±17,04[b]

T30	41951,45±19,97[c]
T40	40641,87±22,40[d]
T50	36942,15±32,14[f]
Sign.	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

HPLC coupled with a UV-Vis (DAD) detector was employed to separate and to quantify phenolic compounds. (**Figures 36-37**)

In general, citrus presented a high content of phenolic acid. This one constitute a wide range of bioactive molecules. In particular Ferulic acid may be important in the prevention of Alzheimer's disease, cancer, diabetes, hypertension, atherosclerosis, inflammatory diseases and others illnesses. Ferulic acid is a polyphenol very abundant in nature especially in vegetables, citrus fruits, bran and corn. Several studies have shown its antioxidant action, due to the ability to sequester free radicals and induce the up-regulation of cytoprotective enzymes such as heme oxygenase-1, heat shock protein 70, kinases 1/2 regulated by extracellular and Akt signals.

Ferulic Acid, was the major phenolic components **Table 14** (33,62 mg/L). The results are in agreement with those reported by other authors.

The genus citrus is considered the major source of flavanone glycosides, and the most abundant components being narirutin, naringin and hesperidin and citrus waste contained significantly concentration of flavonoids.

A lot of study have shown that has antioxidant and antimicrobial activity, moreover, anti-inflammatory and antioxidant properties, naringin and hesperidin have been suggested in the literature to the potential blood cholesterol-lowering ingredients. Its activity has been pharmacologically evaluated in terms of chemoprevention of carcinogenesis.

Six flavonoids were quantified in the extracts: Erioitrin, Neoeriocitrin, Narirutin, Naringin and Hesperidin. The results are shown in **Table 14**. Narirutin was the major phenolic component (6.657,57 mg/L of product).

Table 14 - Chromatographic characterization of Citrus by-product ethanol extract.

Phenolic compounds	Concentration (mg/L of product)
Ferulic Acid	33,62

Erioitrin	2.692,74
Neoeriocitrin	230,70
Narirutin	6.657,57
Naringin	559,09
Hesperidin	1.767,47

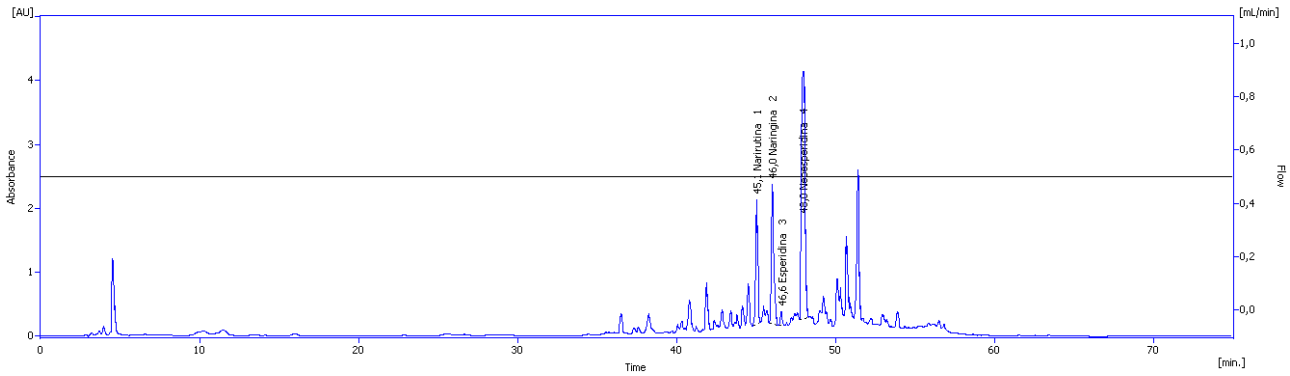

Figure 36 - Identification of phenolic acids and flavonoids in citrus by-product lemon extracts was determined by HPLC

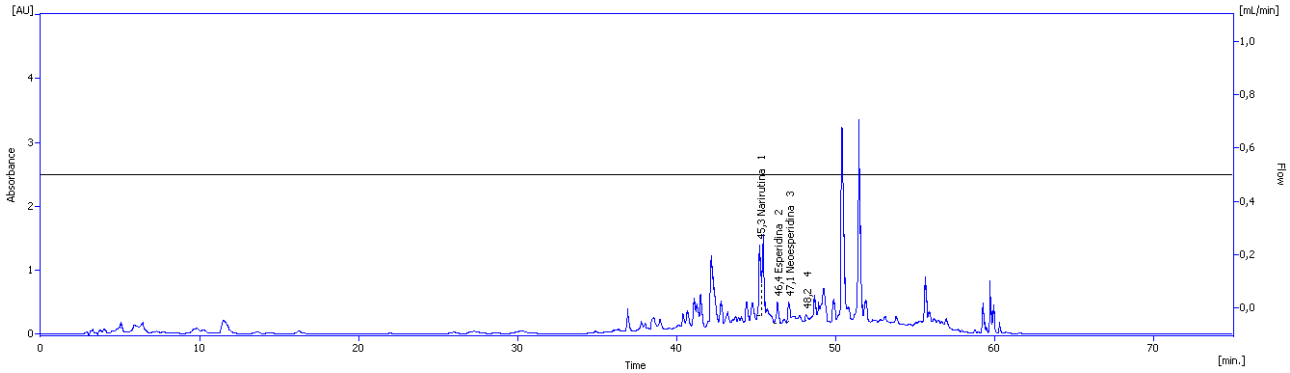

Figure 37 - Identification of phenolic acids and flavonoids in citrus by-product orange extracts was determined by HPLC

Research 2

4.2 Monitoring of extraction evolution in hydrophilic substrate

The extract obtained as previously reported was added to a hydrophilic system, the sample was stored at controlled temperatures (4 and 25 °C) and monitored .

Samples stored at 4 ° C were evaluated for 50 days, while the samples stored at 25 ° C had a shelf life of only 15 days, as it was evaluated according to the microbiological parameters. After 15 days the total bacterial load found positivity and consequently neither sensory analysis nor chemical parameters were carried out.

The analysis showed that interaction effects of storage time and temperature were significant ($P<0.01$) on pH and Total soluble solids (TSS). **Table 15** shows the low amount of sugar expressed in °Brix, furthermore this amount decreased during storage, most likely because a slight microbiological growth was observed at 40 days that could have consumed the little substrate present. This effect was for all the conditions, formulation (WL and WLF) and temperatures (4°C and 25°C).

The presence of citric acid added in the WLFC sample has maintained constant pH values over time more acidic than WL5.

Even if fructose and citric acid were added, the WLFC samples there was no clear difference compared to WL5, in fact there are slightly higher values of Bx ° and lower of the pH in WLFC.

Table 15 – Chemical characteristics of water enriched sample.

sample		°Brix		pH
		MEDIA ± DS		MEDIA ± DS
WL5 4°C	T0	2,00±0,40^{a}	T0	3,06±0,00^{a}
	T7	1,70±0,00^{b}	T7	3,05±0,01^{b}
	T15	1,77±0,46^{d}	T15	3,01±0,00^{e}
	T30	1,60±0,00^{c}	T30	3,04±0,00^{c}
	T40	1,70±0,00^{b}	T40	3,04±0,00^{c}
	T50	1,45±0,07^{d}	T50	3,02±0,00^{d}
Sign.		**		**
WLFC 4°C	T0	2,55±0,07^{a}	T0	2,57±0,00^{a}
	T7	2,30±0,00^{c}	T7	2,56±0,01^{ab}
	T15	2,20±0,52^{c}	T15	2,56±0,00^{ab}

	T30	2,40±0,00[b]	**T30**	2,55±0,01[ab]
	T40	2,60±0,00[a]	**T40**	2,53±0,00[c]
	T50	2,30±0,00[c]	**T50**	2,55±0,01[bc]
Sign.		**		**
WL5 25°C	**T0**	2,00±0,40[a]	**T0**	3,06±0,00[a]
	T7	1,40±0,00[b]	**T7**	3,06±0,00[a]
	T15	1,30±0,00[c]	**T15**	3,03±0,01[b]
Sign.		**		**
WLFC 25°C	**T0**	2,55±0,07[a]	**T0**	2,57±0,00[b]
	T7	2,30±0,00[b]	**T7**	2,59±0,00[a]
	T15	2,20±0,00[c]	**T15**	2,60±0,01[a]
Sign.		**		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Color has to be considered as a special parameter that seem to be one of the first attributes of quality that a consumer perceives (*Chen et al 2005*). For this reason, in the case of modify product, color is an important parameter, which needs to be taking into account during the choice of the drying method (*Ghanem et al 2015)*.

The hydrophilic sample enriched with 5% of the lemon by-products extract and stored at 4 ° C (WL5) there was no significant differences in the parameters L (clarity) and *b (yellowness-blueness) during storage. While the parameter *a (greenness/redness) was significant, in fact during the shelf life the sample tends to browning, like explain in a study *Ibarz et al 1999*. In fact process of food leads to change in color due to various mechanism, including the degradation of pigments, oxidation of ascorbic acid, and Maillard reaction. (Serkat et al 1976; Ghanem et al 2015; Aghajanzadeh et al 2016).

For this reason in the samples enriched with fructose and citric acid stored at 4 ° C and 25 °C (WLF) The samples enriched with fructose and stored at 4° showed significant differences of a*, which increase duering time. This effect was due to fructose and citric acid tend to change the color.

There is a significant difference for the a * parameter for sample enriched with 5% of the lemon by-products extract stored at 25 ° C WL but for WLFC There was no significant difference in hydrofilic.

Table 16 – Determination of color of water enriched sample

sample		L		*a		*b
		MEDIA ± DS		MEDIA ± DS		MEDIA ± DS
WL5 4°C	**T0**	43,07±0,76	**T0**	0,42±0,08^{a}	**T0**	3,57±0,69
	T7	43,10±0,22	**T7**	0,52±0,03^{a}	**T7**	3,87±0,04
	T15	43,09±0,10	**T15**	0,52±0,01^{a}	**T15**	3,81±0,01
	T30	43,13±0,14	**T30**	0,56±0,02^{a}	**T30**	3,71±0,02
	T40	42,62±0,06	**T40**	0,60±0,02^{a}	**T40**	3,87±0,03
	T50	43,16±0,22	**T50**	0,58±0,04^{b}	**T50**	3,90±0,02
Sign.		ns		**		Ns
WLFC 4°C	**T0**	43,03±0,67^{ab}	**T0**	0,49±0,12^{c}	**T0**	4,00±0,51
	T7	43,48±0,05^{a}	**T7**	0,47±0,01^{a}	**T7**	3,83±0,01
	T15	43,39±0,08^{a}	**T15**	0,51±0,01^{bc}	**T15**	3,81±0,01
	T30	43,01±0,31^{ab}	**T30**	0,57±0,05^{bc}	**T30**	3,71±0,02
	T40	42,62±0,11^{b}	**T40**	0,60±0,01^{b}	**T40**	3,92±0,05
	T50	42,98±0,19^{ab}	**T50**	0,59±0,03^{b}	**T50**	3,89±0,03
Sign.		**		**		Ns
WL5 25°C	**T0**	43,07±0,76	**T0**	0,42±0,08^{b}	**T0**	3,57±0,69
	T7	42,83±0,38	**T7**	0,54±0,05^{a}	**T7**	3,84±0,03
	T15	43,07±0,06	**T15**	0,51±0,01^{a}	**T15**	3,87±0,01
Sign.		ns		*		Ns
WLFC 25°C	**T0**	43,03±0,67	**T0**	0,49±0,12	**T0**	4,00±0,51
	T7	43,32±0,36	**T7**	0,52±0,04	**T7**	3,73±0,02
	T15	43,22±0,19	**T15**	0,51±0,01	**T15**	3,87±0,02
Sign.		ns		ns		Ns

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD^{a} test.

Table 17 showed the antioxidant activity of samples measured by DPPH assay. There was a decrease in antioxidant capacity during the 50 days of storage at 4°C and 25°C, both in sample WL5 and WLFC.

The drop of antioxidant activity at 25 °C was showed after 7 days of storage, so the increase of temperature appear dramatically and storage period reduced amount of antioxidant capacity.

Table 17 - Antioxidant activity measured by DPPH assay in water enriched sample

Sample		DPPH (% I)
		MEDIA ± DS
WL5 4°C	T0	33,69±0,00[a]
	T7	32,42±0,00[a]
	T15	23,14±0,02[b]
	T30	16,10±0,02[c]
	T40	14,46±0,01[c]
	T50	10,39±0,02[d]
Sign.		**
WLFC 4°C	T0	35,56±0,01[a]
	T7	19,32±0,01[b]
	T15	15,60±0,00b[c]
	T30	13,20±0,02[cd]
	T40	12,08±0,01[cd]
	T50	11,19±0,01[d]
Sign.		**
WL5 25°C	T0	33,69±0,00[a]
	T7	13,82±0,00[b]
	T15	14,92±0,01[b]
Sign.		**
WLFC 25°C	T0	35,56±0,01[a]
	T7	11,80±0,01[c]
	T15	19,57±0,00[b]
Sign.		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Table 18 showed the antioxidant activity of sample measured by ABTS assay. In the test with ABTS there was no significant differences in sample WL5 stored at 4°C, like other samples WLC at low temperature showed a slight decrease in the ABTS values during time. Sample WLFC 4°C showed a more significant decrease, in fact at the T0 has a value of 14.38 % of inhibition and at the end of storage has a value of 9.14 % of inhibition.

Table 18 - Antioxidant activity measured by ABTS assay in water enriched sample

Sample		ABTS (% I)
		MEDIA ± DS
WL5 4°C	**T0**	14,52±1,61
	T7	14,67±0,07
	T15	14,57±0,25
	T30	10,15±0,65
	T40	11,97±5,88
	T50	13,99±1,00
Sign.		Ns
WLFC 4°C	**T0**	14,38±1,29^{a}
	T7	11,11±0,04^{b}
	T15	10,45±0,49^{bc}
	T30	8,42±0,30^{c}
	T40	8,81±1,39^{bc}
	T50	9,14±0,59^{bc}
Sign.		**
WL5 25°C	**T0**	14,52±1,61^{a}
	T7	7,38±0,04^{b}
	T15	14,15±1,15^{a}
Sign.		**
WLFC 25°C	**T0**	14,38±1,29^{a}
	T7	10,97±0,29^{b}
	T15	10,65±1,38^{b}
		*

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD^{a} test.

Based on the absorbance values of extract solution that reacted with Folin-Ciocalteau reagent and compared with the standard solution of gallic acid equivalents, total poliphenols content is given in **Table 19**. All the samples showed the same range and trend, the values underwent a decrease during storage for 50 days and for all of them appeared statistically significant differences. In fact they have a relation between poliphenols content and storage time is

depicted by a regression coefficient, $R^2 = 0.95$, this explains a close relationship between the parameters.

Table 19 – Total polyphenols in water enriched sample

Sample		Polyphenols (mg/kg GAE)
		MEDIA ± DS
WL5 4°C	**T0**	2210,721±1,65[a]
	T7	2173,416±1,25[ab]
	T15	2122,888±30,48[b]
	T30	1977,847±48,93[c]
	T40	1963,912±17,59[c]
	T50	1924,265±22,38[c]
Sign.		**
WLFC 4°C	**T0**	2328,926±0,07[b]
	T7	2267,746±1,57[b]
	T15	2085,445±0,59[bc]
	T30	2578,283±207,71[a]
	T40	2076,997±42,84[c]
	T50	1998,255±62,94[c]
Sign.		**
WL5 25°C	**T0**	2210,721±1,65[a]
	T7	2066,208±2,15[b]
	T15	1927,41±33,86[c]
Sign.		**
WLFC 25°C	**T0**	2328,926±0,07[a]
	T7	2120,225±3,75[b]
	T15	2019,674±4,62[c]
Sign.		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

The main phenol contents of samples WL5 and WLFC preserved both at 4°C, are reported in **Table 20 and 21**.

The concentration of phenolic compounds it is similar in both samples.

The main phenolic compounds was Narirutin WL5 T0 (1031.75 mg/L) and WLFC T0 (995.18 mg/L) and also at T60 it is the highest quantities respect others.

Naringin in samples WL5 remain constant in fact there are no significant differences, like in samples WLFC Ferulic acid remain constant in time.

Erioitrin and Hesperidin undergo a decrease over time in both samples WL5 and WLFC.

Table 20 – Chromatographic characterization in water enriched sample

	Phenolic compounds WL5 4°C		Concentration (mg/L of product)			
	Ferulic Acid	**Erioitrin**	**Neoeriocitrin**	**Narirutin**	**Naringin**	**Hesperidin**
T7	1,49[b]	125,82[b]	14,43[a]	1031,75[c]	26,06	87,51[a]
T40	1,54[b]	137,49[a]	13,96[b]	1182,32[b]	26,32	70,21[b]
T60	1,62[a]	94,02[c]	13,19[c]	1210,66[a]	25,52	65,54[c]
Sign.	**	**	**	**	Ns	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Table 21 – Chromatographic characterization in water enriched sample.

	Phenolic compounds WLFC 4°C		Concentration (mg/L of product)			
	Ferulic Acid	**Erioitrin**	**Neoeriocitrin**	**Narirutin**	**Naringin**	**Hesperidin**
T7	1,54	121,91[a]	16,31[a]	995,18[c]	28,68[a]	99,25[a]
T40	1,55	91,03[b]	11,63[c]	1110,59[a]	25,32[b]	67,49[b]
T60	1,26	92,34[b]	11,90[b]	1048,70[b]	25,30[b]	67,49[b]
Sign.	Ns	**	**	**	**	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

4.2.1 Microbiological analysis

WL5 and WLFC they was tested for microbiological growth and samples did not show measurable mesophilic aerobic microorganism colonies (<1 cfu/mL) over time regardless the storage conditions.

Research 3

4.3 Effects of enrichment on stability of apple juice

By-products of citrus fruits processing as a source of functional compounds and their applications in foods and drinks is a promising field. in this case, the ethanolic extract of citrus by-products is used to add it to the apple juice

In table 23 are reported Brix, pH and Acidity of apple juice during storage time Total solid soluble expressed as °Brix, was constant during the 10 days. The apple juice sample added with the extract (MC) has 1°Bx higher compared to apple juice as it is, probably contained in the extract.

As regards the results of the TSS and the pH, the values remain similar but the zero standard deviation means that they are different. The Acidity analyzes, there are no differences, in fact the values remain constant.

The acidity of the samples is very important, as the right sugar / acid ratio allows to extend the shelf life and increase the quality of the sample (*Jayasena and Cameron, 2008*).

Table 23 – Chemical characteristics of apple juice and enriched apple juice

sample		Brix		pH		% acidità
		MEDIA ± DS		MEDIA ± DS		MEDIA ± DS
M 4°C	T0	10,00±0,00^{bc}	T0	3,81±0,01^{c}	T0	1,47±0,03
	T1	10,10±0,00^{abc}	T1	3,84±0,01^{b}	T1	1,55±0,03
	T2	9,95±0,07^{c}	T2	3,81±0,01^{c}	T2	1,44±0,00
	T3	10,20±0,00^{a}	T3	3,80±0,00^{c}	T3	1,47±0,03
	T4	10,15±0,07^{ab}	T4	3,86±0,00^{a}	T4	1,55±0,03
	T7	10,05±0,07^{abc}	T7	3,81±0,01^{c}	T7	1,55±0,03
	T10	10,10±0,00^{abc}	T10	3,80±0,00^{c}	T10	1,47±0,03
Sign.		*		**		Ns
MC 4°C	T0	11,80±0,00^{ab}	T0	3,55±0,02^{b}	T0	2,73±0,03^{a}
	T1	11,85±0,07^{ab}	T1	3,48±0,00^{c}	T1	2,78±0,03^{a}
	T2	11,75±0,07^{a}	T2	3,55±0,01^{b}	T2	2,73±0,03^{a}

	T3	11,80±0,00^{ab}	**T3**	3,58±0,01^{ab}	T3	2,71±0,00^{a}
	T4	11,95±0,07^{a}	**T4**	3,56±0,00^{b}	T4	2,71±0,00^{a}
	T7	11,90±0,00^{ab}	**T7**	3,60±0,00^{a}	T7	2,76±0,00^{a}
	T10	11,80±0,00^{ab}	**T10**	3,51±0,01^{c}	T10	2,60±0,03^{b}
Sign.		*		**		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

The table 24 showed the determination of color of apple juice and enriched apple juice, color has to be considered as a special parameter that seems to be one of the first attributes of quality that a consumer perceives (*Chen et al. 2005*).

The value *a (greenness/redness) of apple juice (M) at T0 (0.92) was darker than the (MC) (0.16), it could be a masking effect at T0, in fact, with the course of time a * increases.

Ultimately it seems that there is a slight improvement due to the addition of the extract after a few days but that this improvement did not last until the 10th day.

A shelf-life of 10 days has been estimated, as it is the limit where the flavor does not change.

Table 24 – Determination of color of apple juice and enriched apple juice

sample		**L**		***a**		***b**
		MEDIA ± DS		**MEDIA ± DS**		**MEDIA ± DS**
M 4°C	**T0**	44,85±0,35^{b}	**T0**	0,92±0,04^{c}	**T0**	5,84±0,14^{ab}
	T1	45,07±0,06^{ab}	**T1**	0,92±0,01^{c}	**T1**	5,59±0,04^{cd}
	T2	45,07±0,08^{ab}	**T2**	0,95±0,01^{bc}	**T2**	5,56±0,02^{d}
	T3	45,09±0,07^{ab}	**T3**	1,05±0,01^{a}	**T3**	5,72±0,03^{bc}
	T4	44,97±0,03^{ab}	**T4**	0,98±0,02^{b}	**T4**	5,71±0,12^{d}
	T7	45,19±0,03^{a}	**T7**	0,94±0,00^{c}	**T7**	5,83±0,02^{b}
	T10	45,17±0,12^{a}	**T10**	0,82±0,01^{d}	**T10**	5,95±0,01^{a}
Sign.		*		**		**
MC 4°C	**T0**	45,58±0,83	**T0**	0,16±0,05^{e}	**T0**	6,29±0,46^{a}
	T1	45,65±0,02	**T1**	0,59±0,01^{d}	**T1**	5,68±0,14^{d}
	T2	45,49±0,09	**T2**	0,80±0,01^{b}	**T2**	5,81±0,02^{cd}
	T3	45,63±0,04	**T3**	0,81±0,01^{b}	**T3**	5,88±0,02^{bcd}
	T4	45,83±0,01	**T4**	0,75±0,01^{c}	**T4**	5,92±0,05^{bcd}
	T7	45,55±0,05	**T7**	0,81±0,01^{b}	**T7**	5,96±0,02^{abc}
	T10	45,35±0,13	**T10**	1,12±0,02^{a}	**T10**	6,18±0,04^{ab}

Sign.	Ns	**	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Table 25 showed the changes in the antioxidant activity of apple juice (M) and apple juice enriched with citrus juice processing waste extract (MC) stored at 4°C with a shelf-life of 10 days.

There is a significant difference between the added apple juice (MC) compared to the natural one (M) and this is evidenced since the firs observation. The antioxidant activity of the sample (MC) at T10 (20.97) shows an even greater value than T0 (10.31), most likely as the extract it took time to distribute and mix with apple juice. This is proved by the higher value after 1 day storage that in MC reached the antioxidant of 31%. After then this parameter constantly decreased during time.

Similar results can be seen in the ABTS assay (Table 23), this confirms the trend of the antioxidant activity and the necessity of a complete homogenization of the added extract.

Table 25 - Antioxidant activity measured by DPPH assay in apple juice and enriched apple juice

Sample		DPPH (% I)
		MEDIA ± DS
M 4°C	**T0**	5,47±0,01
	T1	4,74±0,01
	T2	4,12±0,00
	T3	5,86±0,02
	T4	3,57±0,00
	T7	4,00±0,01
	T10	6,08±0,01
Sign.		ns
MC 4°C	**T0**	10,31±0,01^{c}
	T1	31,72±0,01^{a}
	T2	24,35±0,01^{ab}
	T3	25,66±0,01^{ab}
	T4	18,67±0,00^{bc}
	T7	27,22±0,01^{ab}
	T10	20,97±0,02^{abc}
Sign.		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Table 26 - Antioxidant activity measured by ABTS assay in apple juice and enriched apple juice

Sample		ABTS (% I)
		MEDIA ± DS
M 4°C	T0	10,21±0,88[a]
	T1	10,51±2,46[a]
	T2	6,42±2,31[ab]
	T3	6,59±0,86[ab]
	T4	9,83±0,11[a]
	T7	8,05±2,83[b]
	T10	5,52±0,37[ab]
Sign.		*
MC 4°C	T0	10,99±2,01[c]
	T1	31,67±5,41[ab]
	T2	30,37±3,06[ab]
	T3	34,75±0,56[a]
	T4	26,31±0,03[ab]
	T7	27,22±0,03[ab]
	T10	23,03±0,60[b]
Sign.		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

In comparison with the apple juice (M), apple juice with extract (MC) contained significantly higher concentration of total poliphenols, (M) at T0 showed 441.95 mg/L while MC) 13911.85 mg/LThe MC decreased quickly during storage, but the values remained always higher than M, simple apple juice.

Antioxidant activity showed a correlation with polyphenols in (MC), in fact after 10 days both values tend to decrease over time.

Table 27 – Total polyphenols in apple juice and enriched apple juice

Sample		Polyphenols
		MEDIA ± DS
M 4°C	T0	441,95±0,30[a]
	T1	355,23±2,70[c]
	T2	416,34±35,23[ab]
	T3	341,29±0,47[c]
	T4	366,21±0,05[bc]
	T7	348,93±1,04[c]
	T10	330,54±0,58[c]
Sign.		**
MC 4°C	T0	13911,85±75,97[a]
	T1	12609,23±23,18[c]
	T2	12169,08±197,23[de]
	T3	11984,16±7,79[e]
	T4	13354,68±22,40[b]
	T7	12492,56±30,58[cd]
	T10	10932,85±21,52[f]
Sign.		**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

Erioitrin, Narirutin and Hesperidin, and Narirutin were measured by HPLC. The main component was Narirutin at T0 amount 617,20 mg/L after 10 day of storage at 4°C was 282,76 mg/L(Table 28). The same situation occurs for hesperidin, which halves after 10 days.

In research 2 Narirutin had a different trend, in aqueous medium it didn't decrease, but remained rather constant. Instead, in the MC sample the value of Narirutin tends to decrease. This effect could be due to the apple components.

Table 28 – Chromatographic characterization of apple juice and enriched apple juice

	Ferulic Acid	Erioitrin	Narirutin	Hesperidin
T0	0,87[c]	92,06[a]	617,20[a]	30,71[a]
T1	0,85[c]	93,42[a]	528,68[c]	29,15[b]
T3	1,41[a]	86,92[c]	579,88[b]	27,81[c]
T7	1,17[b]	90,00[b]	582,10[b]	26,10[d]

T10	0,85[c]	87,45[c]	282,76[d]	16,68[e]
Sign.	**	**	**	**

Values are means ± SD. *Significance at P <0.05; **Significance at P <0.01; n.s. not significant. Data followers by different letters for each different solvent volume to sample ratio are significantly different by Tukey HSD[a] test.

4.3.1 Microbiological analysis

Ethanolic extracts of lemon by-product has high antimicrobial activity that can may be attributed to the presence of polyphenols. In fact, the sample MC showed that ethanolic extracts possessed maximum antimicrobial activity against Total bacterial count and mold and yeast test. Probably the concentration of ethanol as an inhibitor for microorganisms (Cowan 1999) and the use of this solvent is very important for the conservation of the samples.

4.3.2 Sensory analysis and acceptability of products

The samples were monitored and analyzed sensorially until there were no microbiological positives and until they proved to be safe for tasting. It can be stated that the samples up to the 10th day of storage at 4 ° C were found to be acceptable.

The organoleptic parameters were evaluated, above all sight, taste and smell.

The visual sensations (**Figure 38**) describe the decaying curve of the shelf-life and the parameters of brightness, color uniformity and turbidity are acceptable up to the 10th day of shelf-life, because most of the evaluations are positive with all that there is a decrease of the parameters of brightness and uniformity of the color and increase the turbidity.

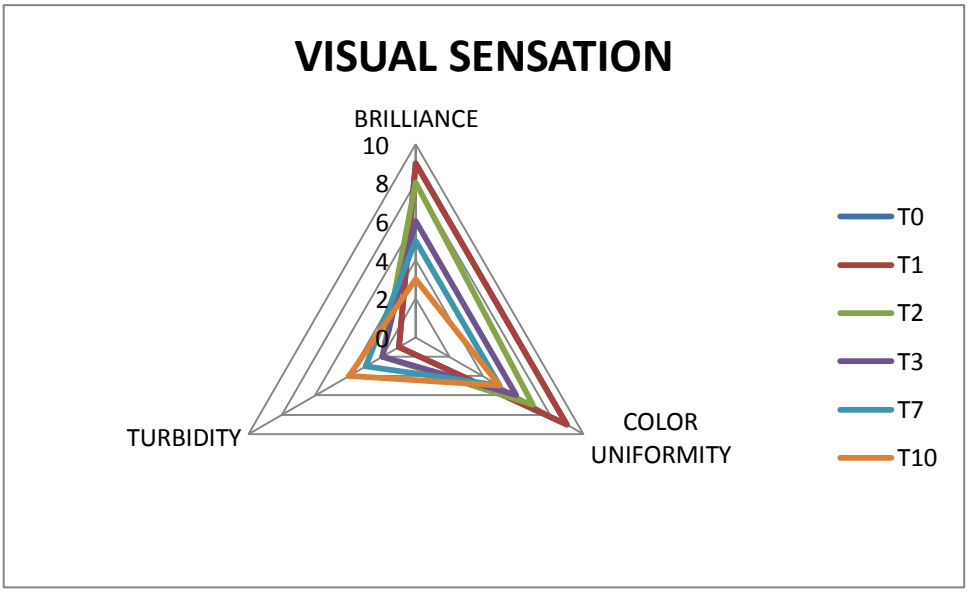

Figure 38- Visual Sensation of Apple Juice enriched with ethanolic extract of lemon by-product.

The olfactory sensation (**Figure 39**), at t0 they have a greater fruity, and sweet smell. At T10, on the other hand, the smell of fermented and acid begins to be perceived, this indicates a decrease in shelf-life.

The negative aspects that should be highlighted are the hints of acid and fermented, which are high at T10, probably due to the presence of sugars in the apple which start fermentation after 10 days.

The citrus smell, in this case lemon, does not influence the natural smell of apple juice.

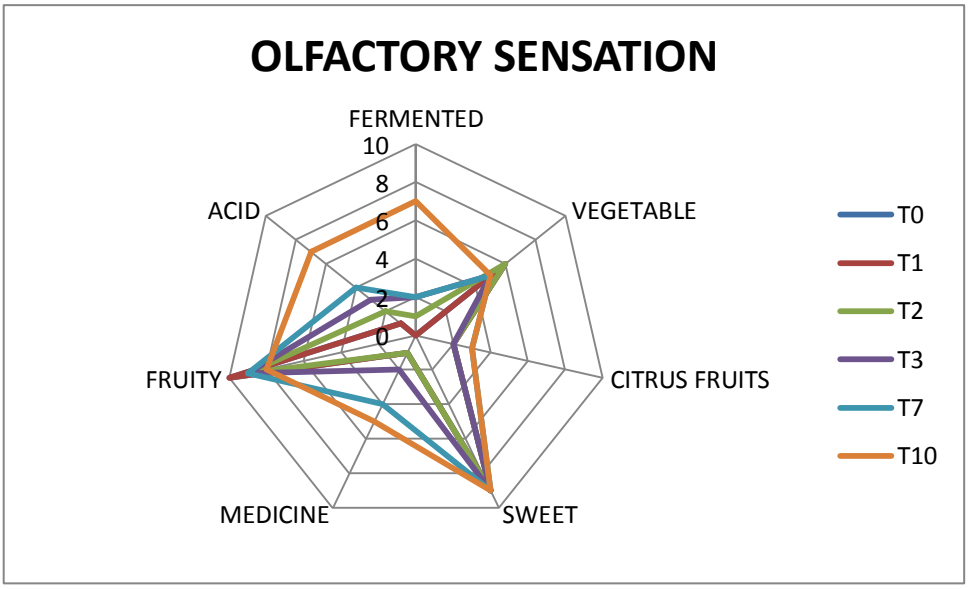

Figure 39- Olfactory Sensation of Apple Juice enriched with ethanolic extract of lemon by-product.

Flavor (**Figure 40**) it reflects the olfactory characteristics, in fact the sweet parameter is confirmed on tasting and the acid, astringency and spicy hints are found only slightly after 10 days.

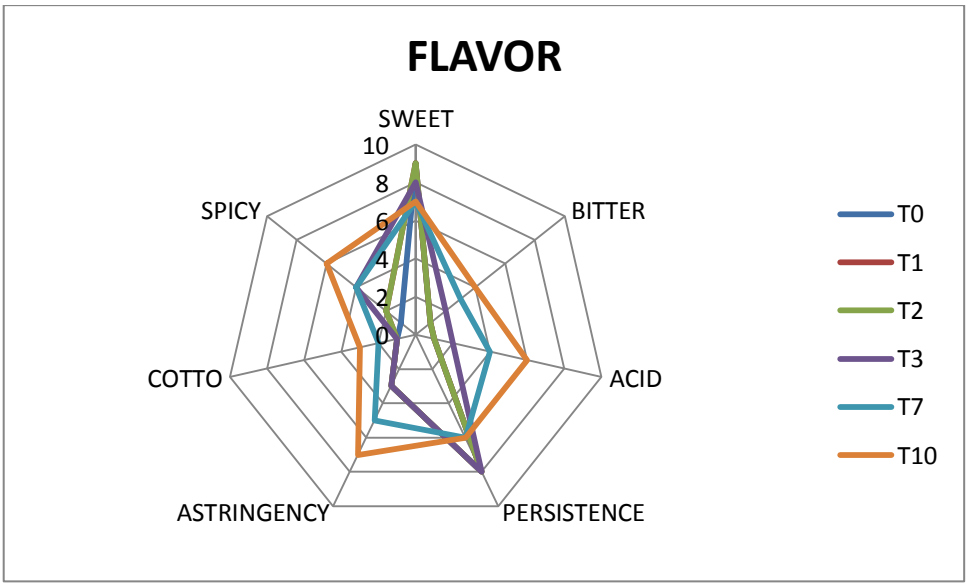

Figure 40- Flavor of Apple Juice enriched with ethanolic extract of lemon by-product.

As regards the texture (**Figure 41**) of the sample of apple juice added, it is fluid and sticky, the latter is due to the presence of sugar, and this also explains the sweetness parameter that persists throughout the shelf-life. moreover, it does not present a grain, therefore the sample is smooth and acceptable up to 10 days.

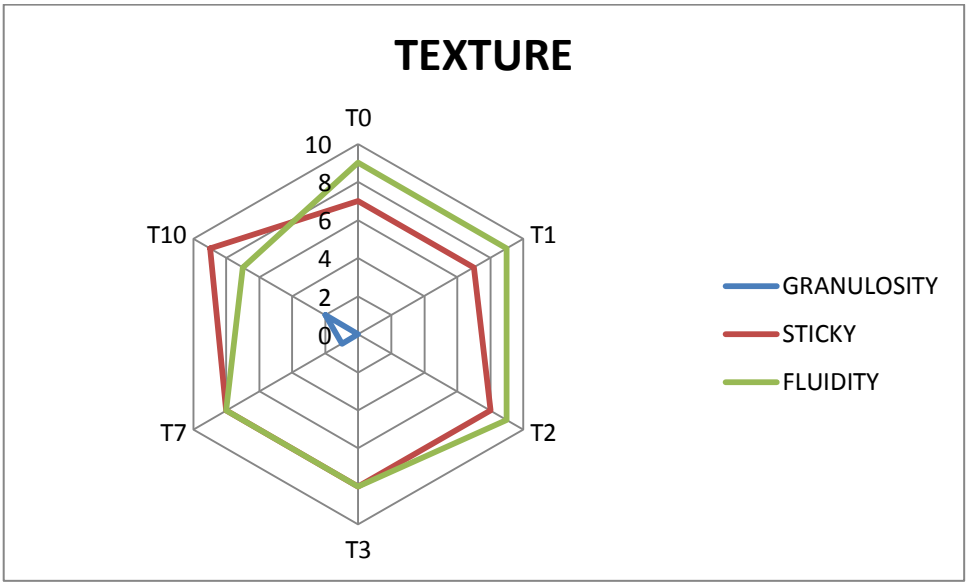

Figure 41- Texture of Apple Juice enriched with ethanolic extract of lemon by-product

5. CONCLUSIONS

This PhD research project has analyzed and evaluated the quality of the citrus by-product as a raw material for the production of interesting products that can have a good grip on the market.

The extraction technologies used, starting from a raw material destined, in most cases, to be used for zootechnical, agronomic or energy production, have allowed to obtain other destinations such as human nutrition.

Citrus by-products are considered to be economic and renewable resource for valuable compounds which can be used in pharmaceutical, nutraceutical, food and beverage, health drinks and cosmetic industries.

Moreover, certain requirements must be meet to reuse the waste economically and effectively.

In the light of the results obtained and the considerations we have made, we can consider the citrus by-product a very interesting raw material from the technological point of view that can be recycled and enhanced by exploiting the useful and beneficial components naturally present.

The results obtained, have identified possible transformations of this type of product able to reduce the volume of the by-product, but, above all, to exploit the useful compounds inside it.

Moreover, citrus by-product must be treated with food grade solvents as much as possible, as it is added to food.

It was concluded that lemon by-product is a potential source of phenolic compounds with antioxidant and antimicrobial properties and can be utilized as an ingredient for the preparation of functional food.

The UAE of phenolic antioxidants from lemon by-products with ethanol-water mixtures appeared effective in comparison to conventional procedure. The sonication power as the most influential factor in the UAE process followed by temperature and ethanol to water ratio.

UAE can be called an “environment-friendly” or “green” technique.

UAE extraction of polyphenols from food by-products by using food grade solvents has a strong potential of industrial development as an efficient and environment-friendly process for the preparation of extracts rich in natural antioxidants aimed at replacing synthetic antioxidant.

Conclusion Research 1

The best extraction method was found to be the ultrasound method, this statement comes from the observed antioxidant activity data. The procedure involved a ratio of 1:10, 1g of powder was added to 10 ml of water/ethanol solution (20:80) and subjected to ultrasound for 60 minutes at 40°C and 40 kHz.

This extract showed the better antioxidant activity performed by DPPH an ABTS assay. The extract obtained was characterized by the presence of compounds such as phenolic acid and flavonoids at high concentration (Ferulic Acid 33,62 mg/L; Erioitrin 2.692,74 mg/L; Narirutin 6.657,5 mg/L 7; Hesperidin 1.767,47 mg/L).

The high total polyphenol content and antioxidant activity of Citrus by-product extract together to an easier extraction method made this extract very attractive for addition to model food.

The stability of the extract is very important to the addition to a food matrix with the aim of a best preservation and an extension of the shelf life without any qualitative modifications of the matrix itself.

Conclusion Research 2

The addition of phenolic fraction to water was carried out to verify if citrus by-product ethanolic extract could be usei n formulation of functional enriched water.

Water enriched with 5% of ethanolic extract (WL5), and Water enriched with 5% of ethanolic extract, Fructose and Citric acid (WLFC5), stored at 4°C for 50 days and 25°C for 15 days and monitored for some qualitative indexes.

The best results were observed on WL5, as it has a greater shelf-life than 50 days, compared to WLFC5 which could have had a shelf-life of only 15 days for microbiological reasons.

WL5 has proved to be a natural preservative to avoid the microbiological growth and samples did not show measurable mesophilic aerobic microorganism colonies (<1 cfu/mL) over time regardless the storage conditions.

Therefore, citrus by-products may be considered an excellent source of poliphenols with potential health benefits when added foods are eat.

Conclusion Research 3

Phenolic extract characterized by the high amount of Erioitrin, Narirutin and Hesperidin, and Narirutin was used in order to evaluated the the possibility of adding it in a more complex matrix of water, in this case it has been added in apple juice. The study included the possibility of addition and its stability.

There is a noticeable difference between the control sample (Natural apple juice) and the added sample (Natural Apple Juice enriched with 5% of concentrate of Lemon By-product).

Both they have been preserved at 4°C and stored for only 10 days for microbiological reasons.

Ethanolic ectract has proved to be a natural preservative to avoid the microbiological growth and samples did not show measurable mesophilic aerobic microorganism colonies (<1 cfu/mL) over time regardless the storage conditions.

From the sensorial analysis it can affirmed that the addiction of lemon by-products ethanolic extract gave a slight hint by slightly modifying the taste of apple juice, but does not negatively alter it. The extyractc could be use as ingredient in multi-fruit juices or in stronger flavors, in order to camouflage the flavor.

6. FUTURE PRESPECTIVES

Observing the obtained results of this PhD thesis the next step will to be find an efficient purification of phenolic compounds useful for the food industry. The aim of this view is to obtain a concentrated ingredient that could easily use in food industry.

The exploitation of citrus by-products as a source of functional compounds and their application in food and beverages, pharmaceuticals, and cosmetic units is promising.

In addition to the routine analyses, has been essential to analyze the composition and amounts of bioactive compounds before adding them as drink ingredients.

This also depends upon the stability of the compounds during preservation shelf life after processing and during the storage time required for selected food or demanded by client.

ACKNOWLEDGEMENTS

It is my duty to dedicate this space of my work to the people who have contributed, with their tireless support, to the realization of the same.

First of all, a special thanks to my Tutor Prof. Marco Poiana, for his immense patience, for his infinite availability and timeliness to all my requests, for his indispensable advice, for the knowledge transmitted throughout the research and drafting of the paper.

Thanks to the laboratory of Food Science and Technology of the Mediterranean University of Reggio Calabria and its staff, which for three years has allowed me to carry out my research. In particular to Dr. Alessandra De Bruno who has always supported me with practical suggestions.

Thanks to the Agrumaria Reggina company and to all its Research and Development laboratory. Special thanks to Dr. Francesco Chirico who allowed me to complete my research, giving me trust.

Thanks to my parents who helped me to overcome the most difficult moments. Thanks for always being there especially in times of despair.

Thanks to my grandmother who allowed me to complete the writing of this paper.

Thanks to my family because it is always present. For me it's my everything.

Thanks to my husband for always believing in me, supporting me in good and bad luck.

Last but not least I thank my son, who, coming into the world, taught me the importance and beauty of the gift of Life, and I promise to convey to him what life has taught me.

REFERENCES

- Adom K. K., Sorrells M. E., Liu R. H. (2005). Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *Journal of Agricultural and Food Chemistry* (53): 2297-2306.

- Aires A., Carvalho, R., & Saavedra, M. J. (2016). Valorization of solid wastes industry processing: Extraction and optimization of polyphenols, tannins and its potential for adhesives, cosmetic and pharmaceutical industry. *Waste Management*, **48**: 457–464. (2016)

- Apak R., Güçlü K., Demirata B., Özyürek M., Çelik S. E., Bektaşoğlu B., Berker K. I., Özyurt D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules*, (**12)**: 1496-1547.

- Assis A, Campos J.M.S, FilhoS, Queiroz A.G, Lana R, Euclides R.F (2004). Citrus pulp in diets for milking cows. Intake of nutrients, milk production and composition. *Revista Brasilera de Zootecnia, 33, 242-250.*

- Bartosz G, Janaszewska A, Ertel D, Bartosz M. (1998). Simple determination of peroxyl radical-trapping capacity. *International Union of Biochemistry and Molecular Biology*.;**46**(3):519-528.

- Bastos EL, Romoff P, Eckert CR, Baader WJ. (2003). Evaluation of antiradical capacity by H2O2−hemin-induced luminol chemiluminescence. *Journal of Agricultural and Food Chemistry*.;51(25):7481-7488.

- Bimakr M, Abdul Rahman R, Taip FS, Mohd Adzahan N, Sarker ZI, Ganjloo A. Ultrasound-assisted extraction of valuable compounds from winter melon (Benincasa hispida) *seeds. Int Food Res J (2013);*20:331e8

- Brand-Williams W, Cuvelier M.E, Berset C. (1995). Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaftund Technologie,* **28**: 25-30.

- Cabras P., Martelli A. (2004) *Chimica degli alimenti*. Piccin Editore, Padova, Italia.

- Calabrese F. (2002) Origin and history. In: G. Dugo e A. Di Giacomo (eds.), Citrus. The genus citrus, Cap 1, Taylor and Francis Books Ltd, Londra.

- Caltagirone S, Rossi C, Poggi A, et al (2000) Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *International Journal of Cancer* 87, 595-600.

- Carratù B. Sanzini E. (2005). Sostanze biologicamente attive presenti negli alimenti di origine vegetale. Annuario Istituto Superiore della Sanità, (41): 7-16.

- Cautela D., Laratta B., Santelli F., Trifirò A., Servillo L., Castaldo D. (2008) Estimating Bergamot Juice adulteration of lemon juice by high-performance liquid chromatography (HPLC) analysis of flavanone glycosides. *Journal of Agricultural and Food Chemistry*. (56): 5407-5414.

- Chemat F., Vian M.A., Cravotto G. Green extraction of natural products: Concept and principles. Int. J. Mol. Sci. 2012;13:8615–8627. doi: 10.3390/ijms13078615.

- Choi IS, Cho EJ, Moon JH, Bae HJ (2015) Onion skin waste as a valorization resource for the by-products quercetin and biosugar. *Food Chemistry,* **188***:* 537-542.

- Di Donna L. De Luca G., Mazzotti F., Napoli A., Salerno R., Taverna D., Sindona G.

(2009) *Journal of Natural Product.* (72): 1352-1354.

- Dimitrios B. (2006). Sources of natural phenolic antioxidants. *Trends in Food Science & Technology*. (17): 505-512.

- Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives

- Elfalleh W, Hannachi H, Tlili N, Yahia Y, Nasri N, Ferchichi A. *Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower*. J Med Plant Res (2012); **6**:4724e30.

- Fernandez M de LA, Espino M, Gomez F JV, Silva MF (2018) Novel approaches mediated by tailor-made green solvents for the extraction of phenolic compounds from agro-food industrial by-products. Food Chemistry **239**: 671-678.

- Faller A. L. K. Fialho E. (2010). Polyphenol content and antioxidant capacity in organic and conventional plant foods. *Journal of Food Composition and Analysis*. (23): 561-568.

- Fotsis T, Pepper M.S, Aktas E, Breit S, Rasku S, Adlercreutz H, Vahala K, Montesano R, Schweigerer L. (1997). Flavonoids dietary-derived inhibitors of cell proliferation and in vitroangiogenesis. *Cancer Research*. 57. 2916-2921.

- Gabriele M, Frassinetti S, Caltavuturo L, Montenero L, Dinelli G, Longo V, Di Gioia D, Pucci L (2017) Citrus bergamia powder: Antioxidant, antimicrobial ant anti-inflammatory properties. *Journal of Functional Foods,* **31**: 255-265

- Gardana C., Nalin F., Simonetti P. (2008). Evaluation of flavonoids and furanocoumarins from Citrus bergamia (Bergamot) juice and identification of new compounds. *Molecules* (13): 2220-2228.

- Gattuso G., Barreca D., Caristi C., Gargiulli C., Leuzzi U. (2007). Distribution of flavonoids and furocoumarins in juices from cultivars of Citrus bergamia Risso. *Journal Food Chemistry* (55): 9921-9927.

- Gattuso G., Caristi C., Gargiulli C., Bellocco E., Toscano G., Leuzzi U. (2006) Flavonoid Glycosides in Bergamot Juice (Citrus bergamia Risso). *Journal of Agricultural and Food Chemistry*. (54): 3929-3935.

- Gawlik-Dziki U, Kaszuba K, Piwowarczyk K, Swieca M, Dziki D, Czyz J (2015) Onion skin- Raw material for the production of supplement that enhances the health-beneficial properties of wheat bread. *Food Research International,* **73**: 97-106

- Georgé S., Brat P., Alter P., Amiot M. J. (2005). Rapid determination of polyphenols and vitamin C in plant-derived products. *Journal of Agricultural and Food Chemistry,* (53): 1370-1373.

- Ghasemi K, Ghasemi Y, Ebrahimzedeh M.A. (2009) Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. Pak. J. Pharm. Sci., Vol.22, No.3, 277-281.

- G.L. La Torre, M. Saitta, F. Vilasi, T.M. Pellicanò, G. Dugo, Food. Chem. **94**, 640-650 (2006).

- Gohl B, (1982). Les aliments du betail sous les tropique. *Roma, Italy FAO. Division de Prodution et Santè Animale.*

- Grohman K, Baldwin E.A, Buslig B.S. (1994). Production of ethanol from enzymatically hydrolyzed orange peel by the yeast Saccharomyces cerevisiae. Applied biochemistry and biotechnology, 45 (46), 315-327.

- Halliwell B. Gutteridge J. M. C. (1990). Role of free radicals and catalytic metal ions in human disease: overview. *Methods in Enzymology*. (186)**:** 1-85.

- Halliwell B., Whiteman M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean?. *British Journal of Pharmacology* 142(2): 231–255.

- Harborne E. (1989). Phytochemicals in plant cell cultures. In: Cell culture and somatic cell genetics of plants serves. Constabel F. and Vasil I.K (Eds), Academic Press, San Diego.

- Hon F.M, Oluremi O.I.A, Anugwa F.O.I (2009). The effect of dried sweet orange (Citrus sinensis) fruit pulp meal on the growth performance of rabbit. Pakistan Journal of Nutrition, ,8, 1150-1155.

- Huang D, OU B, Prior R. *The chemistry behind antioxidant capacity assays.* Jo urnal of Agricultural and Food Chemistry; **53**, 6 (2005) 1841-1856.

- Heim K. E., Tagliaferro A. R., Bobilya D. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationship. *The Journal of Nutritional Biochemistry.* 13(10): 572-584.

- Hertog M.G, Feskens E.J, Hollman P.C, Katan M.B, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the zutphen elderly study. The Lancet (1993) 342, 1007-1011.

- Hervert-Hernandez D., Garcia O. P., Rosado J. L., Goni I. (2010). The contribution of fruits and vegetables to dietary intake of polyphenols and antioxidant capacity in a Mexican rural diet: Importance of fruit and vegetable intake. *Food Research International,* doi: 10.1016/j.foodres.2010.09.021

- Irkin R, Dogan Serap, Degirmenioglu N, Diken M.E, Guldas M. (2015). Phenolic content, antioxidant activities and stimulatory roles of citrus fruits on some lactic acid bacteria. *Arch. Biol. Sci., Belgrade*, **67**: 1313-1321.

- ISTAT (2016), Istituto Nazionale di Statistica.

- Jayasena V, Cameron I, (2008) °Brix/acid ratio as a predictor of consumer acceptability of Crimson Seedless table grapes. *Journal of Food Quality* 31(6):736 – 750.

- Katsampa P, Valsamedou E, Grigorakis S, Makris D (2015) A green ultrasound-assisted extraction process for the recovery of antioxidant polyphenols and pigments from onion solid wastes using Box-Behnken experimental design and kinetics. *Industrial Crops and Products,* **77**: 535-543.

- Kaur, C., Kapoor, H. C. (2001). Antioxidants in fruits and vegetables the millennium‟s health. *International Journal of Food Science and Technology.* (36):703-725.

- Kawaii S., Tomono, Katase Y., Ogawa E., Yano M. (1999) HL-60 differentiating

activity and flavonoid content of the readily extractable fraction prepared from Citrus juices. *Journal of Agricultural and Food Chemistry* (47): 128–135.

- Kertesz Z.I (1951). The pectic substances. *Interscience.* New York.

- Kerton F.M. In: Alternative Solvents for Green Chemistry. Kerton F.M., editor. RSC Publishing; Cambridge, UK: 2009.

- Khan M.K, Albert-Vian M, Fabiano-Tixier A.S, Dangles O,Chemat F. (2010) Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel. *Food Chemistry* **119**: 851-858.

- Klimczak I, Malecka M, Szlachta M, Gliszczynska-Swiglo A. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juice. *Journal of Food Composition and Analysis*, **20**: 313-322.

- Lombardo G. Malara D. Mollace V. (2009). Phytocomplex from bergamot fruti, process of manufacture and use as dietary supplement and in the pharmaceutical field International publication number: WO 2010/055490

- Lupo, S. (1990). Il giardino degli aranci in "Annales". Marsilio, Venezia.

- Magalhaes LM, Segundo MA, Reis S, Lima J. *Methodological aspects about in vitro*
evaluation of antioxidant properties. Analytica Chimica Acta; **613**, 1 (2008) 1-19.

- Mangiola C., Polimeni G., (1997). Un agrume molto colto. Il bergamotto, mito e storia. Editrice Culture.

- Magalhães Lm, Segundo Ma, Reis S, Lima Jlfc. *Methodological aspects about i n vitro evaluation of antioxidant properties.* Analytica Chimica Acta; 613, 1 (2008) 1-19.

- Mahato N, Sharma K, Sinha M, Cho M.H. (2018). Citrus waste derived nutra-/pharmaceuticals for health benefits: Current trends and future perspectives. *Journal of Functional Foods,* **40**: 307-316.

- Maugini E., Maleci Bini L., Mariotti Lippi M. (2006) Manuale di botanica farmaceutica. Piccin-Nuova Libraria.

- Milardovic
S, Ivekovic D, Grabaric B. A novel amperometric method for antioxidant activity determination using DPPH free radical. Bioelectrochemistry; **68**, 2 (2006) 175-180.

- Miller N, Rice Evans C, Davies M, Gopinathan V, Milner A. *A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant st atus in premature neonates.*Clinical Science (London); **84**, 4 (1993) 407-412.

- Morelli I., Flamini G., Pistelli L. (2005) Manuale dell'erborista. Biosintesi, estrazione e identificazione delle sostanze di origine vegetale. Tecniche Nuove.

- M'hiri N, Ghali R, Nasr B, Boudhrioua. (2018). Effect of different drying processes on functional properties of industrial lemon byproduct. *Process Safety and Environmental Protection,* **116**: 450-460.

- Mirabella N, Castellani V, Sala S (2014*)* Current options for the valorization of food manufacturing waste: a review*, Milan Journal of Cleaner Production,* **65**: 28-41.

- Nayak B, Dahmoune F, Moussi K, Remini H, Dairi S, Aoun O, Khodir M (2015) Comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from Citrus sinensis peels. *Food Chemistry,* **187**: 507-516.

- Naczk M. and Shahidi F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis.* (41): 1523-1542.

- Ninfali, P. Bacchiocca, M. (2003). Polyphenols and Antioxidant Capacity of Vegetables under Fresh and Frozen Conditions. *Journal of Agricultural and Food Chemistry.* (51): 2222-2226.

- Orgogozo J.M, Dartigues J.F, Lafont S, et al (1997). Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Review Neurology*. **153**: 185-192.

- Pagána J, Ibarza A, Llorca M, PagánaA, Barbosa-Cánovasc G.V. (2001). Extraction and characterization of pectin from stored peach pomace. *Food research International* **34**: 605-612.

- Pellegrino P, Mafrica R, Zappia R. (2015). In:Storie di bergamotto. Artemis s.r.l. Reggio Calabria, Italy.

- Petti S, Scully C. (2009). Polyphenols, oral health and disease: a review. Journal of Dentistry. (37): 413-23.

- Pilarski R, Zielinski H, Ciesiolka D, Gulewicz Antioxidant K. (2006). Activity of ethanolic and aqueous extracts of Uncaria tormentosa. *Journal of Ethnopharmacology*, **104**: 18-23.

- Podsedek A. (2007). Natural antioxidant and antioxidant capacity of Brassica vegetables: A review. *Food science and technology*. (40): 1-11

- Postorino E. Finotti E. Castaldo D. Pirrello A. (2002). La composizione chimica del "pastazzo" di bergamotto. *Essenze derivati agrumari*. (72): 15-19.

- Putnik P, Koracevic D.B, Jambrak A.R, Barba F.J, Cravotto G, Binello A, Lorenzo J.M, Shpigelman A. (2017). Innovative "Green" and Novel Strategies for the Extraction of Bioactive Added Value Compounds from Citrus Wastes. *Molecules* **22**: 680.

- Puri M, Lal Verma M, Mahale K (2012). Processing of citrus peel for the extraction of Flavonoids for biotechnological applications. *Biotechnological Approaches in Food Adulterans*, Taylor & Francis Group, USA View Project.

- Rafiq S, Kaul R, Sofi S.A, Bashir N, Nazir F, Nayak G.A. (2016). Citrus peel as a source of functional ingredient: A review. Journal of the Saudi Society of Agricoltural Sciences.

- Re R., Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medical*, **26**: 1231-1237.

- Robards K. (2003). Strategies for the determination of bioactive phenols in plants,

fruit and vegetables. *Journal of Chromatography A*. (1000)**:** 657–691.

- Ross K, Beta T, Arntfield S. *A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods.* Food Chem (2009); 113:336e44.

- Russo M, Bonaccorsi I, Inferrera V, Dugo P, Mondello L (2015*)* Underestimated sources of flavonoids, limonoids and dietary fiber: Availability in orange's by-products. *Journal of Functional Foods,* **12**: 150-157.

- Safdar M, Kausar T, Jabbar S, Mumtaz A, Ahad K, Saddozai A. (2017). Extraction and quantification of polyphenols from kinnow (Citrus reticulate L.) peel using ultrasound and maceration techniques. *Journal of Food and Drug Analysisis* **25**: 488-500.

- Saini A, Panesar P.S, Bera M.B, (2019). Comparative Study on the Extraction and Quantification of polyphenols from Citrus Peel using Maceration and Ultrasonic Tecnique. ISSN:2347-467X, Vol.07 No. 3, 678-685.

- Santos-Sánchez N.F, Salas-Coronado R, Villanueva-Cañongo C and Hernández-Carlos B. (2019). Antioxidant Compounds and Their Antioxidant Mechanism. *Institute of Agroindustry, Technological University of the Mixteca.*

- Huajuapan de León, Oaxaca, Mexico

- Saura-Calixto F., Serrano J., Goni I. (2007). Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chemistry.* (101): 492-501.

- Sharma K, Mahato N, Cho M.H. (2017). Converting citrus wastes into value-added products: Economic and environmently friendly approaches. *Nutrition* **34**: 29-46.

- Shi H., Noguchi N., Niki E. (2001). Introducing natural antioxidants. In: *Antioxidants in food. Pratical applications*. Pokorny J., Yanishlieva N., Gordon M. (Eds), CRC Press, New York.

- Shofinita D, Langrish TAG (2014) Spray drying of orange peel extracts: Yield, total phenolic content, and economic evaluation. *Journal of Food Engineering,* **139**: 31-42.

- Sindona G., Di Donna L., Dolce V. (2010). Natural molecole extracted from bergamot tissues, extraction process and pharmaceutical use. International application published under the patent cooperation treaty. International Publication Number: WO 2010/041290 A1

- Somogyi A., Rosta K., Pusztai P., Tulassay Z., Nagy G. (2007). Antioxidant measurements. *Clinical Physics and Physicological measurement*, (28): R41-R55.

- Sormoli ME, Langrish TAG (2016) Spray drying bioactive orange-peel extracts produced by Soxhlet extraction: Use of WPI, antioxidant activity and moisture sorption isotherms. *LWT-Food Science and Technology* **72**: 1-8.

- Sreenath H, Crandal P.G, Baker R. (1995) Utilizzation of Citrus By-Products and Wastes as Beverage Clouding Agents. Journal of Fermentation and Bioengineering, vol.80, No. 2, 190-194.

- Thuy Pham T.P., Cho C.-W., Yun Y.-S. (2010). *Environmental fate and toxicity of ionic liquids:* A review. Water Res.;44:352–372.

- Vessal M, Hemmati M, Vasei M. (2003). Antidiabetic effects of quercetin in streptozocin induced diabetic rats. *Comparative Biochemistry and Psycology- Part C toxicology and Pharmacology*. **135:** 357-364.

- Waladkhani A. R., Clemens M. R. (2001). Effect of dietary phytochemicals on cancer development. In: *Vegetables, Fruits, and Herbs in Health Promotion*. Watson R. R., Ph.D. (Eds), CRC Press.

- Wayner D, Burton G, Ingold K, Locke S. *Quantitative measurement of the total , peroxyl radicaltrapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins.* FEBS Letters; 18 7, 1 (1985) 33-37.

- Zhang Jiuxu. (2007). Flavonoids in Grapefruit and Commercial Grapefruit Juice: Concentration, Distribution and Potential Health Benefits. *Proc. Fla. State Hort. Soc.* **120**: 288-294.