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BSc in Applied Chemistry

Quantification and Characterization of the phenolic compounds of kenaf leaves

Dissertation for the Master Degree in Technology and Food Security

Orientador: António Segura Carretero, Professor, CIDAF-UGR
Co-orientador: Ana Luísa Fernando, Assistent Professor, FCT-UNL

Júri:

Presidente: Prof. Doutor Nuno Carlos Lapa dos Santos Nunes
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Astract:

In the food industry is necessary to bring new ways to improve the taste and quality of food and add health benefits for the consumer, some herbs and plants are a good natural source of antioxidants having the ability to protect the human system from many chronicle diseases, such as Parkinson and inflammatory processes. Combining these two worlds there is a way to acquire a new product.

To know if the plant chosen is suitable to be a future add to a certain food it’s necessary to study the components of the plant and determinate the quantitative and qualitative composition of the metabolite group of interest, in this case phenolic compounds.

The plant of interest was *Hibiscus cannabinus* L. (Kenaf), from Malvaceae family that it’s known for its fibres but it is also used as an infusion or condimental herb and in traditional medicine as an antidote to poisoning with chemicals and venomous mushrooms, it’s known in Asia and Africa but still barely approached by European countries.

In order to separate and identify the phenolic compounds a liquid extraction with a polar solvent was the first step, it was chosen MeOH/H_2O and MeOH (80% w/w; 100%).

Was determinate the antioxidant capacity and the quantification of the phenolic content of the plant samples, for Everglades 41 the DPPH value of Ec50 was 27,33, The ORAC value was 1273,7 (µmol Trolox/mg sample) and the Folin-Ciocalteu 26,40 EAG (mg)/Sample (g)). For the Tainung 2 type was 43,00, 307.54 and 18,67, respectively.

The final step was the identification of the compounds by liquid chromatography followed by mass spectrometry with time of fly analyzer and quadrupole time of fly (HPLC-ESI-MS), were obtained 47 compounds for Everglades leaf and 37 for Tainung 2.

Key-Words: food quality, phenolic compounds, liquid extraction, antioxidant capacity, HPLC-ESI-MS, Kenaf.

Resumo:

Na indústria alimentar é necessário investigar novas formas de melhorar o sabor e qualidade dos alimentos, de preferência trazendo seus benefícios para a saúde do consumidor. Neste sentido, algumas ervas e plantas são fontes naturais de antioxidantes, apresentando capacidade de conferir protecção ao corpo humano de algumas doenças crónicas, tal como Parkinson e processos inflamatórios. A combinação destes dois mundos é um caminho para o desenvolvimento de um novo produto.

Para saber se a planta em questão é adequada para ser uma futura adição a um certo alimento (ou algum dos seus componentes) é necessário estudar os componentes da planta e determinar a composição quantitativa e qualitativa do grupo metabólico de interesse, neste caso os compostos fenólicos.

A planta de interesse foi a *Hibiscus cannabinus* L. (Kenaf), da família Malvaceae conhecida pelo teor de fibra de qualidade (os caules) mas também utilizada como chá ou condimento e na medicina tradicional como um antibiótico contra o envenenamento com químicos e cogumelos venenosos; é conhecida na Ásia e Africa mas ainda pouco estudada nos países Europeus.

De modo a separar e identificar os compostos fenólicos o primeiro passo utilizado foi a extracção líquida com um solvente polar, foi escolhido o MeOH/H_2O e MeOH (80% v/v; 100% v/v).

Foi determinada a capacidade antioxidante e a quantificados os compostos fenólicos das amostras, para a Everglades 41 o valor de Ec50 (método DPPH) foi de 27,33, o valor de ORAC de 1273,7 (μmol Trolox/mg amostra) e o de Folin-Ciocalteu 26,40 EAG (mg)/amostra (g)). Para a Tainung 2 foram 43,00, 307,54 e 18,67, respectivamente.

O passo final foi a identificação dos compostos por cromatografia líquida seguida de espetrometria de massa com analizador de tempo de voo e quadrupólo tempo de voo (HPLC-ESI-MS), foram obtidos 47 compostos nas folhas Everglades 41 e 37 em Tainung 2.

Key-Words: qualidade alimentar, compostos fenólicos, extracção líquida, capacidade antioxidante, HPLC-ESI-MS, Kenaf.

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Abbreviations

FOSHU	*FO*od for *S*pecified *H*ealth *U*ses
ROS	Reactive Oxygen Species
AAPH	2,2'azobis(2-amidinopropane)dihydrochloride
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate
ORAC	Oxygen Radical Absorbance Capacity
MeOH	Methanol
LC	Liquid Chromatography
HPLC	High Pressure Liquid Chromatography
UV	Ultra-Violet
MS	Mass Spectrometry
ESI	Electrospray

Chapter 1

1. Introduction

Food industry has always aimed to present new ideas and chased new techniques to improve the brands already on the market. In this scenario, functional food is an opportunity to innovate and one of the most interesting areas of study within this industry.

In Europe the functional foods are a topic of discrepancy, while in some Northern countries they have been questioned by the public, in Finland the consumers have accepted them with a more open mind (Annunziata, 2011). This study also reported that in general the consumers don’t have a clear idea of what are functional foods yet, so there is a need to clarify the audience that will purchase these new products.

The term of functional food arises in 1984 coined by Japanese scientists when studied the correlation between nutrition, sensory satisfaction, fortification and modulation of physiological systems. Some years later, in 1991 the *FO*od for *S*pecified *H*ealth *U*ses (FOSHU) was approved, where the rules for approval of a specific health-related food category were established (Siró *et al.,* 2008).

While the studies on this new type were explored, other countries started to define the term ‘’Functional Foods’’ and it began to appear more as a concept than an actual food category. According to the EU definition cited in Siró *et al* 2008 “a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule just as normal food form”.

In Portugal the functional food theme is not yet being discussed by the population although the typical Portuguese food has always been a point of interest for both tourism and their own people, following the Mediterranean type (FAO.org[1]).

The Mediterranean diet is characterized by a nutritional consisting in olive oil, cereals, fresh or dried fruits and vegetables, a moderate amount of fish, dairy and meat, and many condiments and spices with a touch of infusions and wine, depending on the country. Also in 2010, UNESCO inscribed the Mediterranean diet on the Representative List of the Intangible Cultural Heritage of Humanity.

[1] http://www.fao.org/docrep/016/i3004e/i3004e04.pdf. 02-05-2013, Granada, ES

1.1. Antioxidant Activity

Every day the human body takes oxygen that produces, along with other biological reactions such as mitochondrial respiratory chain and inflammatory condition (Fernandez-Panchon *et al.*, 2008), free radicals commonly named ROS (reactive oxygen species) including superoxide ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$) and other peroxides. Nevertheless, ROS encompasses not only oxygen radicals but also other non-radicals oxidizing agents (Buonocore *et al.*, 2010).

Free radicals are extremely reactive species with tendency to interact with other species and form new radicals that are not beneficial for human organism. They are capable to begin a chain of reactions that involve a number of steps, in which each step forms a free radical that is the trigger for the next one. There are three phases in this process, namely initiation (first step), propagation (second step) and termination (third step) (Buonocore *et al.*, 2010). During the first step, alkyl radicals are formed from reaction with oxygen molecules that propagate hydroperoxide and peroxide radicals in the second step, and finally, the third step encloses this process by the association of two radicals in a stable adduct form (Brand-Williams *et al.*, 1995).

Some studies (Fernandez-Panchon *et al*., 2008) have associated free radical activity with the development of certain degenerative diseases like cancer and coronary heart disease but it shouldn't be forgotten that ROS have also a beneficial side, as shown in the Table 1.1.

ROS are neutralized by antioxidants, naturally occurring or added to food such as certain vitamins (A, E, C), chlorophylls, carotenoids and phenolic compounds (Bianchi, 1999). Synthetic antioxidants could be considered not safe because of the toxicity associated with them so there is an aim of searching for natural antioxidants that could be added to food in order to produce a healthier and safer product. A variety of them have been already identified and isolated from fruits like acai berry, gogi berry, green tea (Finley, 2011) and vegetables.

Table 1.1: Beneficial vs. Harmful effects of ROS.

ROS

Beneficial effects	**Regulatory mechanisms:** NADPH oxidase (NOX) enzymes required for the differentiation and activation of myofibroblasts.
	Intracellular signaling: Transmission of biochemical signals from cell surface receptor-ligand
	Host defence against invading microbes: ROS-generating NOX enzymes
Harmful effects	Lipid peroxidation within cell membranes
	Oxidative damage to proteins
	Activation of pro-cell death factors

Equilibrium is needed in human organism between antioxidants and ROS, since this balance is destroyed and the antioxidant defenses become depleted, oxidative stress could occur and the damages would be inevitable. However, overtake of antioxidants also could be harmful because all natural compounds may cause toxicity when taken in excess. For example, a study (Finley, 2011) reported that caspase-3 and JNK, the cell death proteins, were activated by green tea epigallocatechin-3-gallate when taken in high doses so in order to promote the beneficial effects of these compounds an attention to the physiological conditions and the dosage used is needed.

1.1.1. Where to Find Antioxidants and Their Role

Fruits and vegetables are a good natural source of antioxidants which have the ability to protect the human system from many chronicle diseases, as above discussed. The antioxidants are also important in the food industry since oxidation of lipids cause off-flavours (Brand-Williams et al., 1995) and some chemicals that could not be healthy so the antioxidants will contribute to delay this process and maintain for a longer time the quality and good aspect of food.

Due to the toxicity and carcinogenicity of some synthetic antioxidants (Akter et al., 2010), there is a need from the food sector for new natural antioxidants such as plant-derived polyphenols. There is a rage of methods that allow the quantification of the antioxidant capacity of a certain sample, especially based on spectrophotometric measures.

Some of them measure the ability of the antioxidant to break the chain reaction of lipid peroxidation. In these cases, the lag phase is observed during the consumption of the antioxidant and then, a more rapid peroxidation occurs which could be detected by oxygen uptake or chemiluminescence (Bisby et al., 2008).

The oxidizing peroxyl radical source is usually an ‘’azoinitiator’’ as reported in Bisby et al., 2008, 2,2’azobis(2-amidinopropane)dihydrochloride (AAPH) which generates a carbon-centered radical by thermolysis and reacts with oxygen giving a reactive peroxyl radical, as shown in figure 1.1.

$$\text{AAPH} + 2O_2 \rightarrow 2\text{ROO}\bullet$$

Figure 1.1: Formation of the reactive peroxyl radical (Adapted Bisby et al., 2008).

In other methods, the reducing power of the antioxidant is established by the ability to reduce a colored radical such as 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and the antioxidant capacity is given by the measurement of the absorbance.

Alternatively, peroxyl radicals could be reduced by the antioxidant in competition with an indicator and so simple competition kinetics may be used to evaluate the rate constant for reaction of peroxyl radical with the antioxidant such as the case of the oxygen radical absorbance capacity assay (ORAC) (Bisby et al., 2008).

1.1.2. DPPH assay

The DPPH assay is based on the reduction of the radical DPPH• by receiving a hydrogen from the antioxidant species, the DPPH• is a stable radical which has an unpaired valence electron at one atom of Nitrogen Bridge, the molecular structure of this reagent is shown in figure 1.2. It is a simple and practical method so it has been used in plenty of laboratories for this kind of measurements.

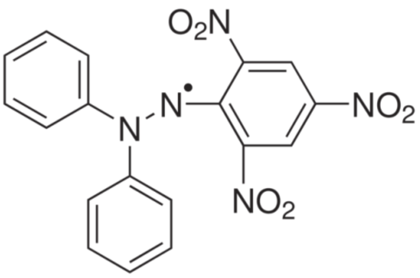


Figure 1.2: Molecular structure of DPPH• (Adapted from Sigma-Aldrich[2]).

In its radical form the DPPH absorbs at 515 nm (Brand-Williams et al., 1995) showing a violet color. When the reduction occurs by another radical or an antioxidant, the absorption disappears according to the figure 1.3 (Brand-Williams et al., 1995). Therefore, this method will give an overlook of the antioxidant power of the sample in question.

$$\text{DPPH}\bullet + \text{AH} \longrightarrow \text{DPPH-H} + \text{A}\bullet$$

$$\text{DPPH}\bullet + \text{R}\bullet \longrightarrow \text{DPPH-R}$$

Figure 1.3: Reduction of the DPPH radical (Adapted from Brand-Williams et al., 1995).

This method has been reported in some studies to determine the antioxidant activity in food and vegetable matrices (Brand-Williams et al., 1995; Herrero et al., 2011) and particularly, Mohd-Esa et al., 2010 studied samples of the genus *Hibiscus* L., species sabdariffa by this method.

[2]http://www.sigmaaldrich.com/catalog/product/aldrich/d9132?lang=es&region=ES, 26-04-2013, Granada, ES.

There are a number of different ways to apply this method with variations on the DPPH concentration or incubation time. In 2009, Sharma presented a study about the applications of the method and some differences on the protocols that resulted in variations in the results, especially in the Ec50 (the amount of sample needed to reduce the initial concentration of DPPH by 50%), and an extra difficulty in comparing data. This study also presents a standard procedure based in the use of a DPPH concentration of 50 µM in methanol, ethanol or buffered under dark or dim light within the sensitivity range of spectrophotometry (Sharma, 2009).

1.1.3. ORAC assay

Another widely used method to determine the antioxidant capacity is the ORAC assay which compares the antioxidant activity of the matrix with an analogue of vitamin E, commonly known as Trolox (Figure 1.4).

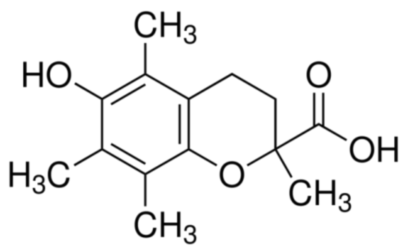


Figure 1.4: Molecular structure of Trolox, an analogue to vitamin E (Adapted from Sigma-Aldrich).

This assay has been reported as successful in the determination of antioxidant activity in fruits, beverages and plasma as well as more complex samples, so it also has been applied in cosmetic industry and nutraceuticals (Stockham et al., 2011).

In this assay, the β-phycoerythrin was introduced as an indicator for the estimation of antioxidants but nowadays the fluorescein is used as a more reliable indicator. The decrease of its fluorescence indicates the lag phase where there is a competition between indicator and antioxidant to react with peroxyl radical. On this method, it is possible to evaluate if the antioxidant is effective and the total antioxidant capacity of a plant extract by measurement of the area under the curve of fluorescence intensity versus time (Bisby et al., 2008).

For better understanding of the ORAC assay, the figure 1.5 shows the major reactions which include the reaction between the peroxyl radicals (ROO•) with fluorescein (FH) and the antioxidant (AH).

$$FH + ROO\bullet \rightarrow F\bullet + ROOH$$

$$F\bullet + F\bullet \rightarrow Products$$

$$AH + ROO\bullet \rightarrow A\bullet + ROOH$$

$$A\bullet + A\bullet \rightarrow Products$$

Figure 1.5: Main reactions of the ORAC assay (Adapted from Bisby et al., 2008).

1.1.4. Folin-Ciocalteu assay

A method widely used to quantify the total phenolic content in plant products is the Folin–Ciocalteu method. It is based on the reaction of phenols with a colorimetric reagent composed of phosphomolybdate and phosphotungstate, exemplified in figure 1.6. This method has been widely used for this kind of determination in food since it is simple and standardized, and the reagent is commercially available (Ramirez-Sanchez et al., 2010; Magalhães et al., 2010).

$$H_3PW_{12}O_{40} + H_3Mo_{12}O_{40} + \text{Phenolic compound} \longrightarrow W_3O_{23} + Mo_3O_{23}$$

Figure 1.6: Reaction of the Folin-Ciocalteu reagent with a phenolic compound.

The former products present a blue coloration absorbing UV-vis radiation in wavelength range of 700-760 nm (Cicco et al., 2009) so it is an easy method to apply, it has a simple interpretation and it may be used in any laboratory.

Nevertheless, this assay is limited by its lack of specificity, since other products of oxidation could interfere causing over estimation of the polyphenol content within the sample. Substances as sugars, aromatic amines, sulphur dioxide, organic acids and bases can react with the Folin–Ciocalteu reagent (Ramirez-Sanchez et al., 2010).

This method has been used in the study of a wide range of plant samples, including species belonging to the genus *Hibiscus* (Mohd-Esa et al., 2010).

1.2. Phenolic compounds

Phenolic compounds or polyphenols are extremely diverse, indeed, there are more than 8000 phenolic structures known according to Bravo, 1998 and they constitute one of the widest groups in plant metabolism. According to same authors, phenolic compounds can be classified by the number of phenol rings and their linkage, ranging from simple phenols to more complex structures. In figure 1.7 there are represented the principle families.

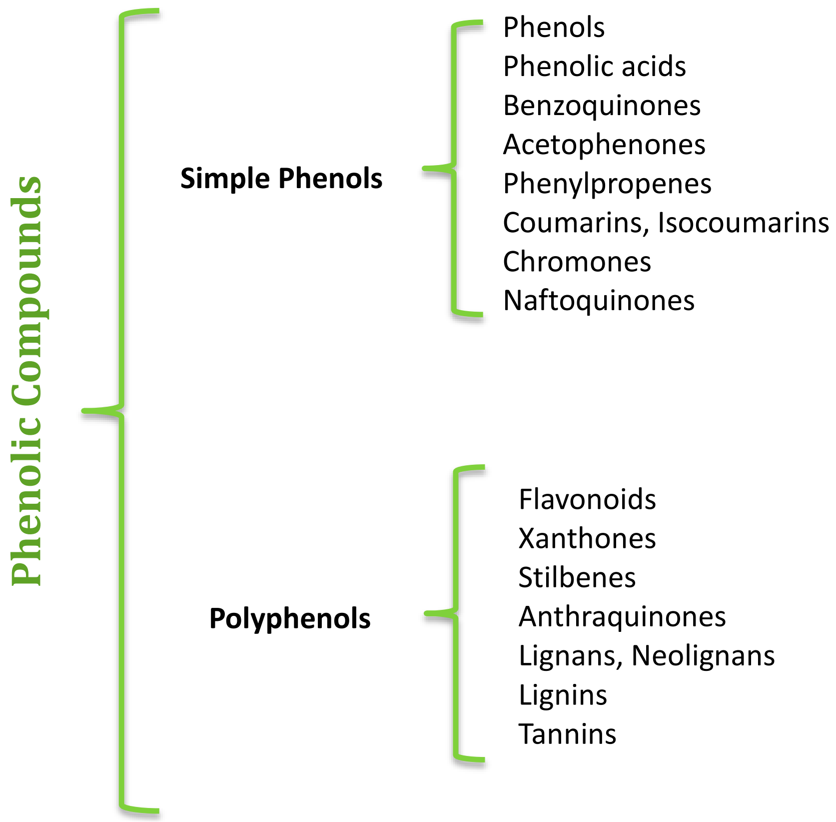


Figure 1.7: Principal families of phenolic compounds (adapted from Bravo, 1998).

Phenolic compounds also exhibit multiple forms, being divided into subclasses, within the families of phenolic compounds the flavonoids are one of the most important and one of the widest distributed in nature; they are distributed in subclasses as shown in figure 1.8.

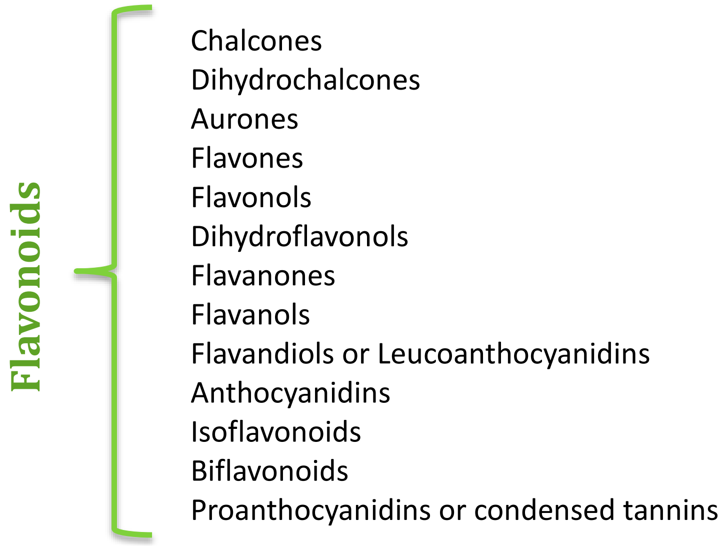


Figure 1.8: Subclasses of the flavonoids (Adapted from Bravo, 1998).

They primarily occur in conjugated form with one or more sugar rings linked to hydroxyl group or directly to an aromatic carbon, being glucose the most common although galactoses or xyloses are also found. Other common substitutes of phenolic rings are carboxyl, amines, lipids and other phenols (Bravo, 1998).

Despite phenols and hydroxybenzoic acids (Figure 1.8) have the simplest structures in phenolic compounds; they have been mentioned in many studies related with plant taxonomy, growth or reproduction (Bravo, 1998; Escarpa, 2001). They contribute to the color and taste of fruits and vegetables and they also are responsible for the lack of color when these are processed because of the formation of complexes with metal ions and proteins (Belitz et al., 2009).

Phenolic compounds are not homogeneously distributed in the plants. The insoluble phenols can be found in the cell walls where contribute to their strength while the soluble phenols are usually located within the plant cells vacuoles. In all cases, they play a regulatory role in the growth and morphogenesis of the plant and take part in the stress response to pathogens attack, (Naczk, 2006).

1.2.1. Interest in Phenolic Compounds

As said before phenolic compounds are antioxidants, which is especially important once they could act as anti-inflammatory and anti-carcinogenic.

Inflammation is a response of the organism to exposure to infectious agents, physical injury or antigenic stimuli however if the process is not efficient it becomes chronic, it becomes pathological. Some studies (Conforti et al., 2009; Soberón et al., 2010) showed that certain phenolic compounds are potent radical scavengers which moderately inhibited COX-2 activity, as an indication of anti-inflammatory potential. COX-2 is a key enzyme in prostaglandin biosynthesis and hyaluronidase, an enzymatic activity increased during chronic inflammation and it has been studied for anti-inflammatory aims (Soberón et al., 2010).

Cancer is a group of diseases associated with an altered control of the cell cycle, the imbalance in the control of cell proliferation is a primary characteristic of the cancer cells, so any molecule capable of inhibiting the proliferation of cancer cells may be useful as potential chemo preventive agent.

In order to study a solution for this problem Ren et al., 2003 correlated the activity of flavonoids and carcinogenic disease. Ornithine decarboxylase, a rate-limiting enzyme in polyamine biosynthesis related to cell proliferation in several tissues could be inhibited by flavonoids causing a decrease in polyamine and inhibition of DNA/protein synthesis. Furthermore, flavonoids are also effective at inhibiting signal transduction enzymes, for example, protein tyrosine kinase, protein kinase C and phosphoinositide 3-kinases which are involved in the regulation of cell proliferation.

Also Birt et al., 2001 and Yao et al., 2004 correlated the consumption of flavonols with the combat to cancer cells.

There have been found a relationship between their antioxidant activity and the chemical structure depend on the number and position of the hydroxyl group and on the pH, for phenolic acids the antioxidant capacity will be as higher as the hydroxyl group is free (Fernandez-Panchon et al., 2008), while in flavonoids this capacity will increase with the increase of hydroxyl groups and a decrease in glycosylation (Yao et al., 2004).

1.3. Characterization of phenolic compounds

To investigate the bioavailability and biochemical effect of a particularly plant of interest, there is the need to know the quantitative and qualitative composition of the metabolite group that occurs on that plant. These types of studies have become a growing field and a powerful tool in food science.

1.3.1. Metabolomics

Metabolomics is a group of integrated sciences that has the aim of identifying and quantifying the intercellular or extracellular metabolites. It could be considered as the final step in the study of the ‘’omic’’ sciences, beginning with the genomics (study of the evolution and functioning of the genes), transcriptomics (the study of the genes expression), proteomics (study of the structure and function of proteins) and finally, metabolomics as represented in figure 1.9.

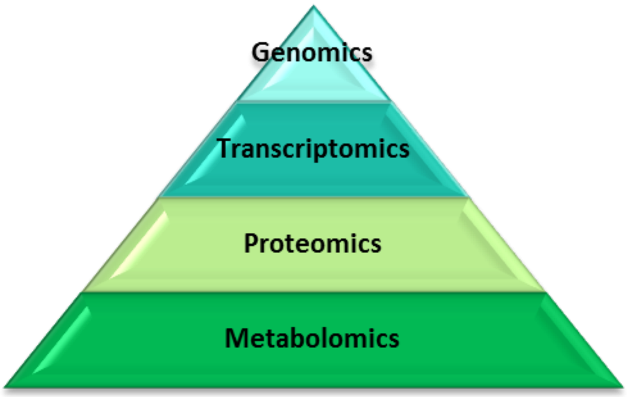


Figure 1.9: Diagram of ‘’omic’' sciences

There are different analytical strategies within metabolomics.

- ✓ Target analysis: Centered in one or a small group of metabolites of interest.
- ✓ Metabolite profiling: Focus on identifying and quantifying a certain number of related metabolites that could belong to the same family, for example phenolic compounds, or participate in the same metabolic pathway.
- ✓ Metabolomics: Analysis in which all the metabolites of a system are identified and quantified, revealing the metabolome of the system.

- ✓ Metabolite fingerprinting: Based on a qualitative screening to classify the samples according to their origin or biological relevance.

In order to obtain the required information about the metabolites of a system, the right techniques should be applied. The technique must be rapid and simple, comprehensive and give as much information as possible. Furthermore, the applied method must be standardized to enable the comparison of new samples with data from literature.

Anyway the first important step to a good characterization is the treatment of the sample.

1.3.2. Vegetable samples treatment

A considerable amount of methods have been proposed for the extraction of phenolic compounds from vegetable samples, the procedure of choice has a direct relationship with the matrix in question and the complexity of these compounds. Generally, the extraction process should be as mild as possible to maintain the integrity of the components in question.

The first step is to dry the sample by heat or lyophilization and freeze them with liquid nitrogen for example so it can be stored and then analyzed when suitable. When that moment comes, phenolic compounds could be extracted from the matrix by several different systems as exemplified in figure 1.10.

In general, phenolic compounds are weak organic acids with pKa range between 8 and 12 (Tura, 2002; Harnly et al., 2007), they can go from hydrophilic to hydrophobic and are usually readily extracted into aqueous alcohol, solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, DMSO have been used for the extraction (Harnly et al., 2007; Naczk, 2006).

Taamalli et al., 2012 studied a conventional method for extraction of bioactive compounds from raw materials, such as olive leaves, combining a mixture of methanol and water and maintaining it during 24h in the dark at room temperature.

It is important to remain in mind that to extract bioactive compounds from a plant is typically used a solvent extraction so the yield and antioxidant activity will depend strongly on the solvents.

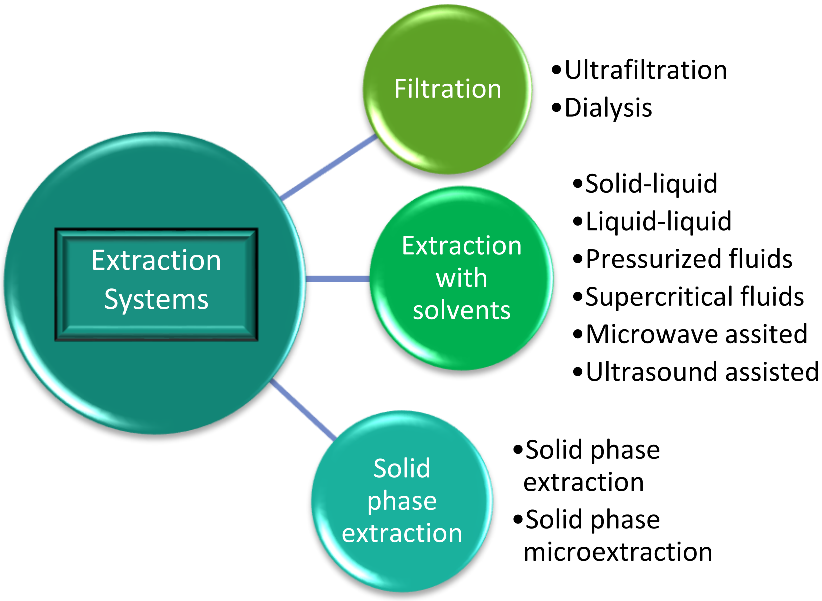


Figure 1.10: Extraction systems.

1.3.3. Characterization and quantification of phenolic compounds

The separation and identification of phenolic compounds is the next step to take after the extraction. In order to do that, analytical chemistry has some techniques based on the differences between the physical-chemical properties of the compounds which form the complex samples, including liquid chromatography.

1.3.3.1. Liquid chromatography

Chromatography is a vast area but, in a simple way, it could be separated in two distinct branches, gas chromatography (GC) and liquid chromatography (LC). GC provides better and faster separations and very good resolution, despite these advantages, many samples can't be handled by GC because they are insufficiently volatile and cannot pass through the column, or they are thermally unstable and decompose under the conditions of separation.

Snyder 1979 wrote that approximately only 20% of known organic compounds can be satisfactorily separated by GC, without prior chemical modification of the sample, whereas LC is ideally suited for the separation of macromolecules and ionic species of biomedical interest, labile natural products and a wide variety of other high molecular-weight and/or less stable compounds.

LC is a technique based on the separation of the compounds of a sample due to their different distribution between a liquid mobile phase and a stationary phase placed in a column. The stationary phase can be a porous solid or a thin layer of substance bound to a solid support, contained within a metallic tube that usually leads to the chromatographic column. The mobile phase is a solvent or a mixture of them which may present different pH values by addition of acids, bases or buffer solutions.

To separate its components, the sample is injected in the LC equipment and forced by a high pressure created by the pump to flow through the chromatographic column. In the column the components of the sample are separated according to their affinities by the mobile or the stationary phases in such a way that those components with higher affinity by the mobile phase will elute before than other components with higher affinity by the stationary phase (Snyder 1979).

When the analytes reach the end of the column, the detector produces a signal that is as intense and durable as the nature and quantity of the analyte. That signal is processed by a computer and recorded by the form of a chromatogram.

The LC equipment has the basics components which are shown in figure 1.11: the pump, the injector, the column, the oven, the detector and the recorder (computer).

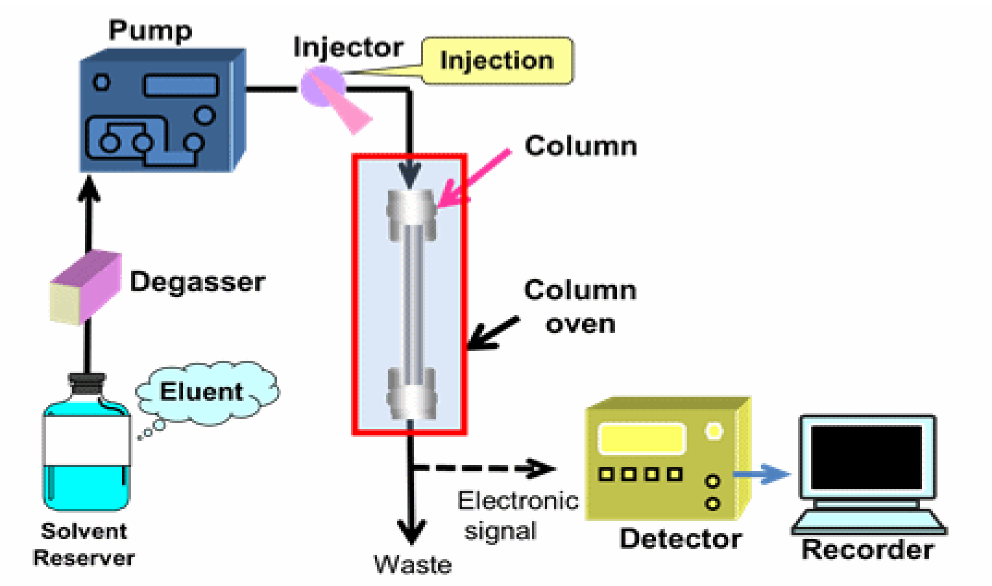


Figure 1.11: Schematic representation of a LC system.

The characteristics of the column are decisive for the type of LC applied, when the internal diameter of the column is between 1,5 - 4,5 mm and its length is between 3 – 30 cm it is a high pressure liquid chromatography (HPLC), in this case the flow of the mobile phase can be between 0,2 - 2,5 ml/min. Inside this category there is another section, when the size of the stationary phase particles are beneath 2 µm and its length is between 3 – 15 cm it's called RRLC (rapid resolution liquid chromatography) or UPLC (ultra pressure liquid chromatography), in this case the flow can be between 0,2 - 5 ml/min.

LC technique can be described in four different processes according to the nature of the stationary phase, the liquid-liquid (or partition), the liquid-solid (or adsorption), the ion-exchange and the size-exclusion (or gel) chromatography (Snyder, 1979).

When a liquid-liquid chromatography, also called partition chromatography (Snyder, 1979), is applied both phases are liquid but different in composition, once they must be immiscible, and the components of the sample distribute between these two phases. It is possible to differentiate two modes, normal-phase chromatography which the stationary phase is polar and the mobile phase non-polar, so the polar molecules stay stronger attached to the column and the elution starts with the non-polar compounds.

However, in reverse-phase chromatography the opposite happens since the stationary phase is non-polar and the mobile phase polar in consequence the non-polar molecules stay in the column more time.

In the liquid-solid chromatography, or adsorption chromatography (Snyder, 1979), the column packing is solid and the liquid phase transports the molecules of interest and the retention of these molecules occurs by adsorption over the solid phase surface.

Ion-exchange chromatography is characterized by the stationary phase containing fixed ionic groups such as SO-3, along with counter-ions of opposite charge.

Finally, in size-exclusion chromatography the stationary phase is a porous matrix and the size of the pore is determined so certain sized molecules could not pass into that matrix so they are excluded while the smaller molecules penetrate into the pores lasting longer than the larger molecules (Snyder, 1979).

Over the years, innumerous authors reported liquid chromatography as the technique of choice to analyze phenolic compounds (Harnly, 2007, Kalili, 2011, Taamalli, 2012), normally by reverse-phase because of their polar nature. The most common columns in the separation of phenolic compounds are those with modified silica with hydrocarbons chains such as C8 (n-octyl) or C18 (n-octadecyl), what changes between the numerous columns available in the market is the length, diameter and pore size (Harnly, 2007; Kalili, 2011).

The mobile phases are usually made by different mixtures of solvents like acetonitrile, water and methanol containing some modifiers such as acetic, formic or trifluoroacetic acids and salts as ammonium phosphate (Robards, 1999). The elution begin with the most polar compounds followed by a decrease of polarity, then the elution order can be synthetized as phenolic acids first followed by cinnamic acids and then flavonoids (Robards, 1999) although the overlap of some components could be practically impossible to avoid once that there are numerous components within these families.

1.3.3.2. Detectors

A number of detection systems might be combined with a LC instrument, ultraviolet-visible molecular absorption spectrophotometry (UV-Vis) and mass spectrometry (MS) are the most widely used to detect phenolic compounds.

1.3.3.2.1. UV-Vis Spectrophotometry

One detection system typically combined with HPLC is the UV-Vis spectrophotometry that is based on the interaction between the UV-Vis radiation and the analytes giving rise to a phenomenon of absorption of specific wavelengths of the radiation by the compounds.

UV-Vis absorption detectors may be of a fixed wavelength, a variable wavelength or a number of photosensitive diodes place side by side, known as "diode array detector" (DAD). In general, these detectors have a light source, a monochromator (prism), a sample holder and a detector of radiation, as represented in figure 1.12.

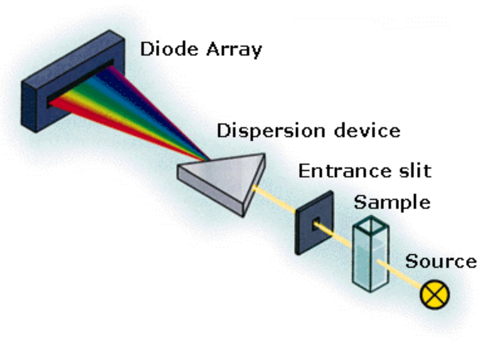


Figure 1.12: Schematic representation of a UV-Vis detector.

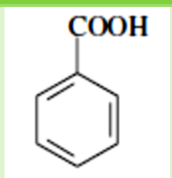
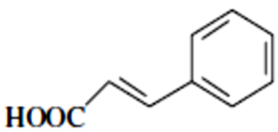
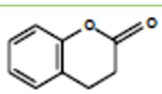
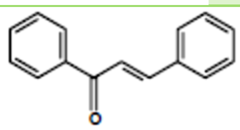
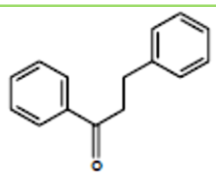
The light source, the most important part, consists in a lamp of deuterium filament (190-330 nm) and a lamp of tungsten filament (330-800 nm) so together they produce a light beam that runs the full range of the spectrum (Verma M. et al., 2011).

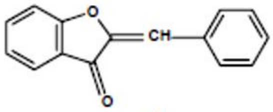
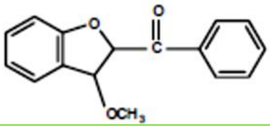
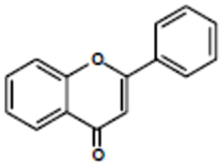
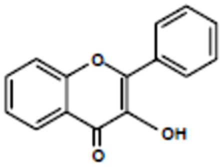
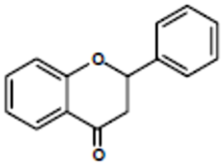
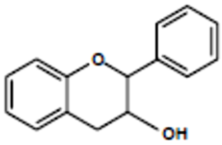
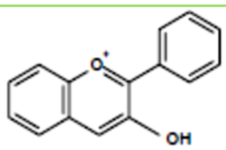
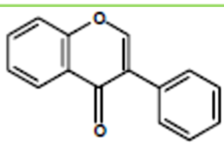
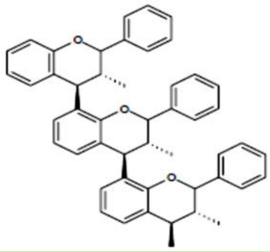
Phenolic compounds have multiple conjugated bonds that convert them into chromophores so they will present absorption bands in the UV and even in the visible (Skoog et al., 1998) as is the case with some flavonols and anthocyanins.

This detector is very useful since a huge range of molecules absorb radiation in this area of the electromagnetic spectrum but this fact has also become an inconvenient because of its lack of specificity.

UV-Vis does not provide structural information and by itself does not enable the unambiguous identification of compounds if standards are not available. In complex samples, compounds without commercially available standards are usually much more numerous than those with them. However, this technique provides useful information of the family which the analytes belong to once that each family has characteristic absorption bands, as explained in table 1.2.

Table 1.2: Characteristic absorption bands of some families of phenolic compounds.

Compound	Structure	UV Bands (nm)
Phenolic acids		270-280
Cinnamic acids		305-325
Coumarins		220-230 310-350
Chalcones		220-270 340-390
Dihydrochalcones		~ 220 ~ 280

Aurones	 	240-270 340-370
Flavones		250-270 330-350
Flavonols		250-270 350-380
Flavanones		270-295
Flavanols		270-280
Anthocyanidins		240-280 450-560
Isoflavones		245-270 300-340
Proanthocyanidins		~280

1.3.3.2.2. MS

Mass Spectrometry can be used as a detector combined with LC. The major advantage of this technique is its selectivity and its capability to give structural information about the analytes. This coupling (HPLC-MS) also presents the advantage of providing a second dimension of separation once that the compounds are separated according to their retention time by the HPLC, another separation takes place according to the mass to charge ratio (m/z).

MS is a technique in which the ions are separated in the vacuum by some kind of force according to their m/z. In general MS equipment has four essential parts, as seen in figure 1.13.

- ✓ Sample inlet system
- ✓ Ionization source
- ✓ Mass analyzer
- ✓ Detector

The sample can be introduced from a connection with a previous separation system, such as HPLC, or directly by infusion, heating or desorption.

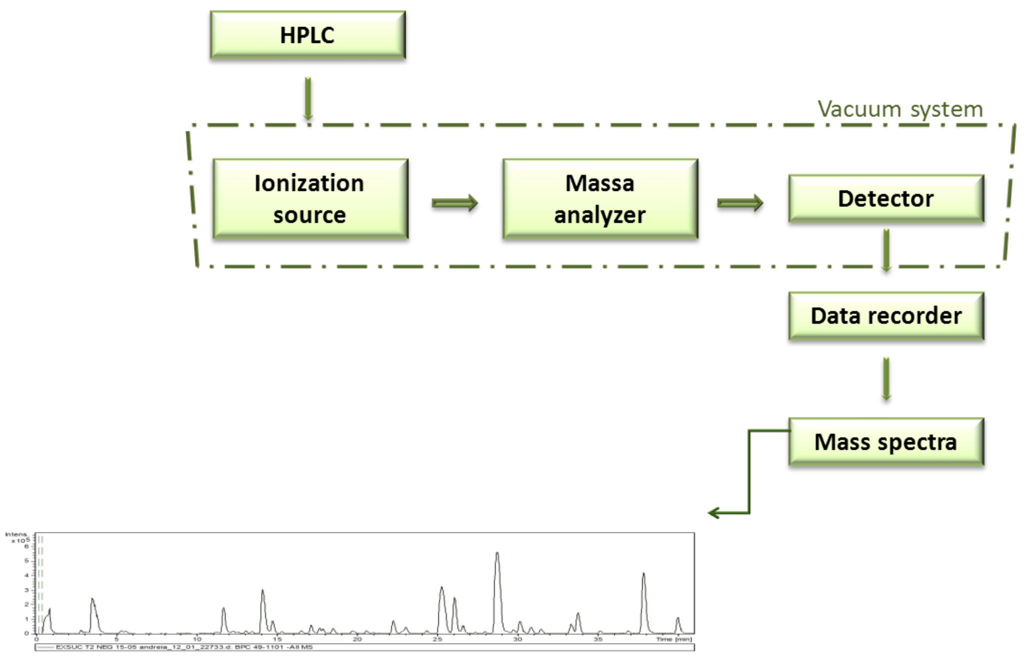


Figure 1.13: Schematic representation of a MS equipment coupled to a HPLC.

In the ion source the sample is ionized and then, the formed ions are transported by electric fields to the mass analyzer. The ionization of the sample could occur by several phenomena, among them the most important are:

- ✓ Chemical ionization (CI)
- ✓ Electrons impact (EI)
- ✓ Electrospray (ESI)
- ✓ Fast Atom Bombardment (FAB)
- ✓ Matrix Assisted Laser Desorption Ionization (MALDI)

When they come to the mass analyzer, the ions are separated according to their mass/charge and the analyzers could be:

- ✓ Electric or magnetic sector (could be together or not)
- ✓ Quadrupole (Q) or triple quadrupole (QqQ)
- ✓ Time of flight (TOF)
- ✓ Ion trap (IT)
- ✓ Quadrupole-time of flight (Q-TOF)

Mass spectrometry is the most popular technique in the field of metabolomics and LC-DAD-ESI-MS starts to be used for screening of botanical metabolites. Harnly, 2007 reported a LC-DAD-ESI-MS method for the identification of glycosylated flavonoids and other phenolic compounds and its versatility to identify 78 compounds, eight subclasses, in fruits, beans and herbs.

1.3.3.2.3. ESI

In the process of electrospray formation, the sample dissolved in a volatile solvent passes through a spray needle and high voltage is applied. The generated electric field produces oxidation-reduction reactions in a way that the sample comes out as electrically charged droplets (figure 1.14). This nebulization is assisted by the nebulizer gas flow, commonly N_2 (Bruker Daltonics, 2008).

The solvent evaporates from the droplets formed (desolvation) and those will increase the density of their electric charge. In consequence, the droplets shrink and the ions on the surface are forced to approach to each other due to electrostatic field applied between the capillary exit and the entrance to the mass spectrometer (Bruker Daltonics, 2008).

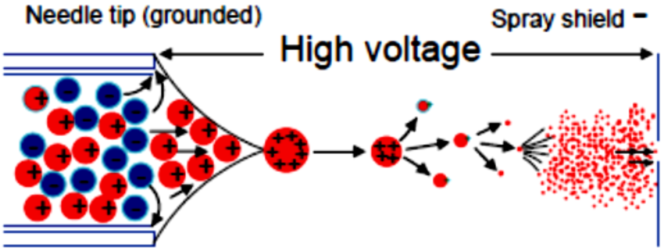


Figure 1.14: Representation of the formation of electrospray (Adapted from Bruker Daltonics, 2008).

At one point, the forces of repulsion become higher than the surface tension that keeps drops united in spherical form and the droplets are broken. Because of Coulombic repulsion forces, the surface tension of the droplets increases and they "explode" ("Coulomb explosion"), forming a series of charged droplets that continue suffering processes of evaporation and subsequent explosions.

Finally, charged ions are formed and pass to gas phase with one or more charge and they are attracted towards the entrance of the mass spectrometer in consequence of the voltage applied. In the ionization process, ions mono-or multicharged could be formed allowing the detection of compounds with high molecular weights in mass analyzers which work with limited range of m/z ratio.

The ionization could be in negative or in positive mode, forming $[M\text{-}nH]^{n-}$ by deprotonation or $[M\text{+}nH]^{n+}$ by protonation, respectively.

1.3.3.2.4. Q-TOF

The mass analyzers allow the separation of the previously formed ions in basis of different phenomena depending on the type of analyzer with different resolution degrees, providing information on their molecular mass.

One of these analyzers is the Quadrupole-Time-Of-Flight (Q-TOF) which has been seen as a powerful and robust instrument with unique capabilities since it provides data which enable positive identification by matching characteristics of the samples with standards or provisional identification based on structural information for the compound subunits (Harnly, 2007). The rapid acceptance of these instruments is mainly due to the combination of high sensitivity and mass accuracy for precursor and product ions and also to the simplicity of operation for those familiar with LC/MS analysis on quadrupole (Chernushevichet al., 2001).

This analyzer separates the ions according to different speeds in the tube of flight according to their m/z ratio. The ions are extracted from the ionization chamber and accelerated towards the flight tube by an electrostatic field which gives them a high kinetic energy. The ions with higher m/z will "fly" at a slower rate than ions with lower m/z so the resolution between ions of different m/z is as better as the tube length is longer and as lower is the dispersion of the initial kinetic energy between ions. The follow figure exemplifies the Q-TOF equipment (Figure 1.15):

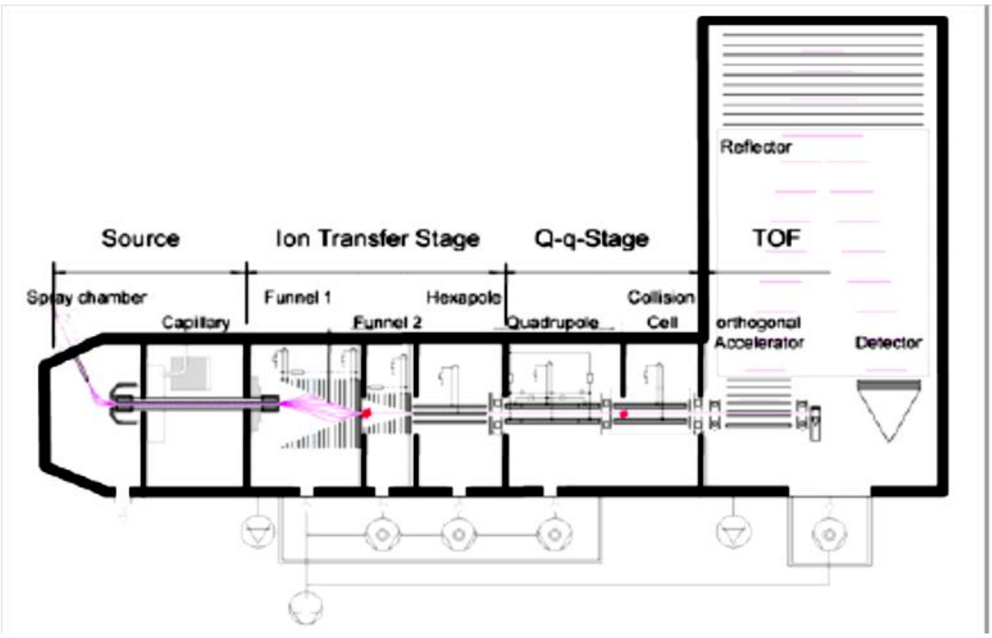


Figure 1.15: Schematic representation of a Q-TOF (Bruker Daltonics, 2008).

The sample gets in the nebulizing camera and there happens the formation of the spray. The formed ions pass through the unit of desolvation, which separates the areas with atmospheric pressure from the first area of vacuum and has a heater of the drying gas and a glass capillary.

Then the ions pass to the area of transmission of optical transfer that is composed by three parts in vacuum and are separated by funnels (concentric rings stacked in form of funnel) that prevent the loss of ions during the transmission ionic, increasing the intensity.

The ions are transferred by the hexopole to the more interesting part of the equipment, the quadrupole where it’s possible to select certain ions to fragment in a collision chamber, with the help of a collision gas normally N_2, so the fragmentation pattern would be better and, consequently, the final mass spectra improved.

After collision chamber the ions pass to the area of high vacuum while the lenses focus and direct them. After that, the ions reach the area of orthogonal acceleration that accelerates the ions in the inside of the fight tube, through an intermittent electric field (Bruker Daltonics, 2008).

According to their masses the ions are introduced to a greater or lesser extent in the reflector, behind it there are areas of tension which repel the ions that come, as expected the smaller ions repel easier. This reflector enables the correction of the initial dispersion of kinetic energies of the ions and simultaneously, it doubles the effective length of the tube of flight since the ions have to go through it twice until reaching the detector.

Finally, the detector converts an ion signal into an electrical signal and then it is transmitted to a digitizer, which card is mounted in the computer. Modern detectors used in QTOF mass spectrometers are designed as micro channel plate detectors; a microchannel plate assembly is a solid core with millions of small pores that are internally coated with a semiconductive layer. These pores are called microchannels and each of them works as an electron multiplier independently from the others (Bruker Daltonics, 2008).

1.4. Kenaf

1.4.1. The origins of Kenaf

The Malvaceae family, native from east-central Africa (Tao et al. 2011) is known for its high levels of cellulose within the stalk. The genus *Hibiscus* is typically used as an infusion or condimental herb but it can also be used in traditional medicine as antidote to poisoning with chemicals and venomous mushrooms (Maganha et al. 2010). This genus is very extensive with about 200 species, annual and perennial; the United States department of Agriculture (USDA) presented the taxonomic status of the species as shown in table 1.3.

Some investigations of the genus *Hibiscus* indicated the presence of useful biological activities such as antidiabetic, anti-inflammatory, antioxidant and antimutagenic (Maganha et al. 2010, Foyet et al. 2011) so the interest in this kind for plants started to grow in order to explore new ways to leverage the beneficial capabilities of the plants.

Table 1.3: Taxonomic status of Kenaf (Adapted from plants.usda.gov[3]).

Kingdom	***Plantae* – Plants**
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida*
Subclass	*Dilleniidae*
Order	*Malvales*
Family	*Malvaceae* – Mallow family
Genus	*Hibiscus* L. – Rosemallow
Species	*Hibiscus cannabinus* L. – Kenaf

One member of this genus is *Hibiscus cannabinus* L. (Figure 1.16) or Kenaf, the most common name, that is an annual dicotyledonous herbaceous plant (Maganha *et al.* 2010) similar to cotton (*Gossypium hirsutum* L.) and okra (*Abelmoschu sesculentus* L.) (Webber III, 1999), well known in Asia and Africa while in Europe the production is not yet extensive and it is used for the extraction of fibers.

Figure 1.16: Kenaf (omafra.gov.on.ca[4]).

[3]http://plants.usda.gov/java/profile?symbol=HICA5, 19-06-2013, Granada, ES.
[4]http://www.omafra.gov.on.ca/CropOp/en/indus_misc/fibre/ken.html, 25-06-2013, Granada, ES.

1.4.2. Uses of Kenaf

Despite its use as a spice in some Asian countries, in Europe it appears in middle 90's to be planted in lands not used for food stocks and to produced fiber, although the whole plant could be used as a profitable product, as shown in table 1.4.

Table 1.4: Utilities of different parts of Kenaf (Adapted from FAO[1]).

Main use	Detailed use	Used part
Material	Fibres	Bark
Food & beverage		Seeds
Material	Lipids/oil & fats	Seeds
Food additive	Condiment/seasoning	Leaves
Fuels	Fuelwood	Entire plant
Animal food (feed)		Seeds
Material	Dye/tannin	Bark
Fuels	Petroleum substitutes/alcohol	Seeds

It has been used for double panels, absorbent materials, strings, bags, wires, plastic biocomposites, materials for thermochemical processes like combustion, gasification and pyrolysis for energy production (ecocrop.fao.org[3]).

As said, this family is very rich in fibers and Kenaf is not an exception. Its fiber is similar to others from vegetable steams like hemp or flax, the average fiber length is about 2.5 mm and its composition is formed by cellulose (58 to 63%), hemicelluloses (21 to 24%) and lignin (12 to 14%). Compared with the hemp, the presence of lignin may represent a negative factor in the elasticity of the fiber what makes it less appropriated for textiles (Barbosa, 2010).

However, Kenaf is not used in its full potentials. Since the food sector is not paying much attention to Kenaf's leaves, the competition for water or land with food planting is not on the equation. As a result, Kenaf is very resistant and in semi-arid conditions like those found in Mediterranean region, it reaches significant levels of production only with a range of 250 to 400 mm of water, much less than those required for traditional crops such as maize, sugar beets or alfalfa (Alexopoulou et al., 2009). Therefore, it could be used as an important alternative crop in lands with poor or moderate water availability. Furthermore, it needs relatively low nitrogen levels, 50 to 100 kg N ha-1 (Alexopoulou et al., 2009).

Taking all this into account, kenaf could be cultivated in rotation system what is important for lands with just one type of cultivation such as cotton or cereals, helping the agriculture market as well.

1.4.3. Production of Kenaf

In order to have an idea of the Kenaf's production all over the world, in the year 2010/11 FAO reported the major countries that produce this plant (table 1.5) and it is possible to see that Asia appears as the big source of Kenaf with India holding almost 54% of the production, followed by Bangladesh with approximately 42% while others like Europe and America all together reach only 4%.

Table 1.5: Overview of Kenaf's production. Adapted from FAO, 2012.

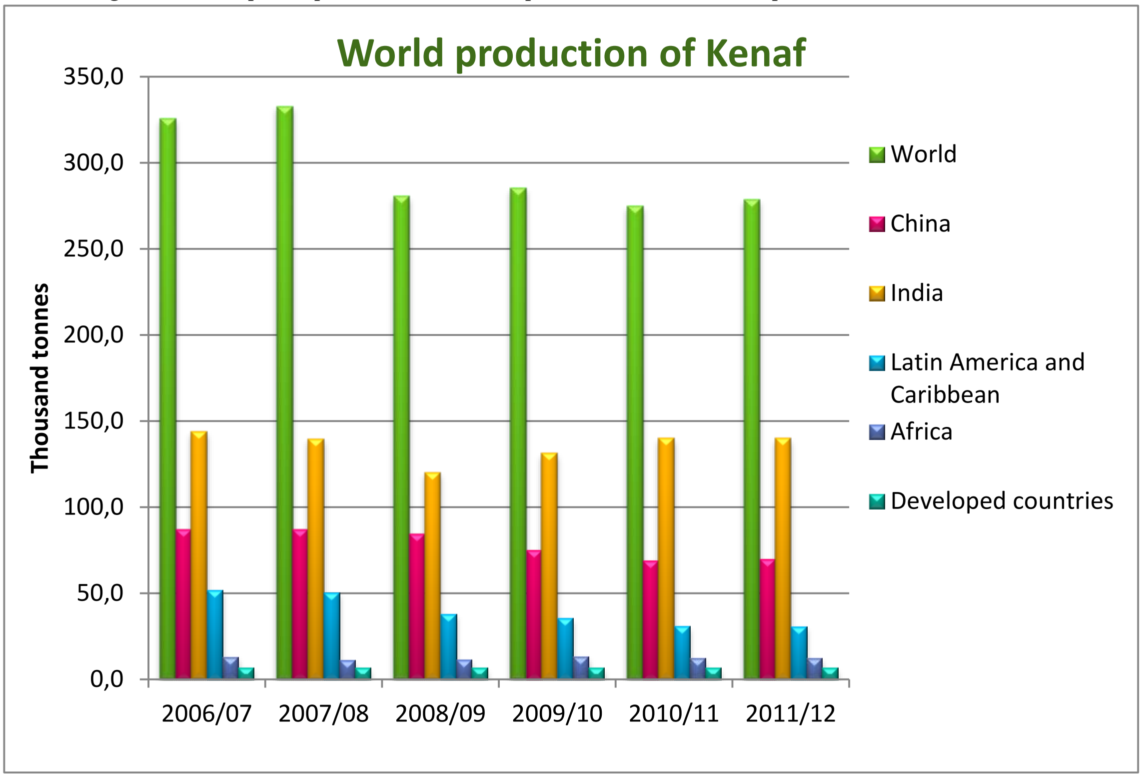
	Bangladesh	India	Myanmar	Nepal	Thailand	Others	Total
Production (Thousand tonnes)	1404,5	1800,0	7,8	14,4	1,7	133,2	3361,6

In the last years, the production of Kenaf has decreased regardless its potential, as seen in figure 1.17. India and China represent the biggest producers followed by Latin America and Caribbean with Brazil and Cuba in the head of the production with 46% and 21%, respectively (FAO, 2012). As seen before, the developed countries do not show an interest in the production of this plant, presenting the lowest production, however with studies related to the potential benefits of Kenaf, an increase would be possible.

Some years ago, Alexopoulou et al., 2009 studied this plant to assess the viability of the sowing in Europe so several Kenaf trials were established in some southern countries like Greece, Italy, France, Spain and Portugal, all Mediterranean regions. The yield capacity was observed as well as the factors that could interfere. The best results were obtained in Spain and the worst in Portugal and the yielding capacity of the variety Tainung 2 was slightly higher than Everglades 41.

In Portugal, Fernando et al., 2008 studied the adaptation of Kenaf in the region of Setubal, in the south of Lisbon and near the Atlantic Ocean. The idea was to evaluate if this plant could be used as an alternative in land use and if it could provide diversified opportunities for farmers and biological materials for the bio-based industries. According to them, the production of Kenaf could be achieved with 50% PET (potential evapotranspiration) irrigation level so it will contribute to a less depletion of the groundwater resources and it could be helpful to a new sustainable circuit between agriculture and bio industry.

Figure 1.17: Graphic representation of world production of Kenaf. Adapted from FAO, 2012 .



1.4.4. Kenaf Leaf

Kenaf produces simple leaves with jagged edges, which are positioned on the main stem (stalk) and along the branches. The shape of the sheet is determined by the variety of the plant which can be divided or whole. Varieties with divided leaves have three, five or seven lobes per sheet. Everglades-71, Tainung-1 and Tainung-2 are examples of varieties with leaf divided. The varieties of whole leaf are slightly lobed and heart-shaped, some examples are Everglades-41, Guatemala-4 and Guatemala-45 (Barbosa, 2010), shown in Figure 1.18.

Figure 1.18: Photograph of two types of Kenaf leaf: Everglades 41 and Tainung 2.

The good properties of Kenaf leaves already had started to hold attention. A study (Lee *et al.* 2007) shown that this plant could be able to modulate macrophage-mediated responses which could be good for therapeutic uses. There are some references to the presence of 10 components like ethyl alcohol, isobutyl alcohol, limonene, phellandrene, R-terpenyl acetate, citral, and other unidentified components in kenaf from Egypt (Maganha et al, 2010). Bindhu and Umadevi, 2013 also reported that Kenaf it is rich in calcium, iron, nitrogen, phosphorus and vitamin C.

1.5. Objective

The objective of this work was to identify and quantify the phenolic compounds on Kenaf's leaves, comparing variety Everglades 41 with Tainung 2. In order to accomplish this, a study of the best extraction system took place and posterior study of the antioxidant capacity of the leaves by DPPH, ORAC and Folin–Ciocalteu assays.

The identification of the phenolic compounds in the leaves was carried out using advanced analytical techniques such as liquid chromatography coupled to electrospray ionization mass spectrometry using quadrupole time-of-flight analyzers (HPLC-ESI-MS (Q-TOF)).

Chapter 2

2. Materials and Methods

All the extracts and measurements were done in triplicate.

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. The standards utilized, fluorescein, trolox, Folin–Ciocalteau reagent, AAPH and DPPH were from Sigma-Aldrich (Steinheim, Germany); methanol used for the extraction of the phenolic compounds was from Panreac (Barcelona, Spain); acetonitrile from Lab-Scan (Dublin, Ireland), and acetic acid from Sigma–Aldrich (Steinheim, Germany). Distilled water with a resistance of 18.2 M was deionized in a Milli-Q system (Bedford, MA, USA).

2.2. Plant samples

Kenaf (two varieties, Everglades 41 and Tainung 2) was planted in May and harvested in September 2005 at FCT-UNL, Portugal, before flowering. The leaves were then dried at 70ºC and stored in the dark at room temperature.

2.3. Plant extracts

Four different extraction protocols were tested. Two of them based on conventional solid-liquid extraction (maceration) and other two based on ultrasound-assisted extraction using MeOH or a mixture MeOH/H_2O (80:20) as extracting solvents. 500 mg of Kenaf's leaves, weighed by an analytical scale Mettler Toledo AB204-s/fact, were milled in 15 mL of MeOH or MeOH/H_2O (80:20), depending on the protocol, by an electric mill, IKA®T18basic ultra-turrax. Then, there were applied the two different methods of extraction of phenolic compounds: the overnight maceration and the ultrasound-assisted extraction.

2.3.1. Overnight maceration

The mixture of sample and 15 ml of MeOH or MeOH/H_2O (80:20) was maintained 12 h in the dark at room temperature in a stir plate (Agimatic-N) (Adapted from Taamalli, 2012). The extracts were dried in a rotary evaporator (Büchi R-200) at 40 ºC and then dissolved in 1 or 2ml of MeOH, depending on the oil obtained. These solutions were filtered through a 0.45 µm syringe filter and saved at −20 ºC to avoid any possible degradation until analysis.

2.3.2. Ultrasound-assisted extraction

To extract the phenolic compounds by ultrasound an adaption of the method used by Agbor, 2005 was applied. The mixture of 15 ml of MeOH or MeOH/H_2O (80:20) and sample was maintained for 2 h in the ultrasound bath (Branson 3510) at room temperature. Then, samples were centrifuged for 15 min at 4000 rpm using a centrifuge (Labofuge 200, Heraeussepatech) to remove solids. The supernatants were dried in a rotary evaporator at 40 ºC and then dissolved in 1 or 2 ml of MeOH, depending on the oil obtained. These solutions were filtered through a 0.45 µm syringe filter and saved at −20 ºC to avoid any possible degradation until analysis.

The pellet obtained after centrifugation was then utilized for 3 more cycles of extraction in the same conditions and the liquid phases saved every time in a different tube.

2.4. Antioxidant assays

2.4.1. DPPH

The antioxidant capacity of Kenaf leaves extracts was determined by the DPPH radical scavenging method, based on a procedure described by (Brand-Williams et al. 1995). Briefly, a solution was prepared dissolving 19.7 mg of DPPH in 100 mL of MeOH to obtain the stock solution at 500 µM. This stock solution was further diluted 1:10 with MeOH. Both solutions were stored at 4 ºC in dark bottles until use.

Different concentrations of extracts were tested (10 to 120 ppm) in such a way that 20 µL of these extracts solutions were added to 980 µL of DPPH diluted solution to complete the final reaction medium

(1 ml). After 4 h at room temperature in the dark, 200 µl of the mixture was transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (BioTek). DPPH–methanol solution was used as a reference sample and the DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or Ec50.

2.4.2. ORAC

Other method applied to determine the antioxidant capacity was the ORAC assay, based on the method described in Huang et al., 2002. A solution of fluorescein was prepared at a concentration of 56 nM and kept at least 30 min at 37ºC before use. Trolox solution was also prepared at a concentration of 1mM to make the calibration curve in a range from 1 to 13.5 µM. The extracts were tested in a range of concentrations from 0.1 to 50 ppm. All solutions were diluted in a buffer solution consisting of NaH_2PO_4 0,2M + Na_2HPO_4 0,2M (20:80, v/v) at pH 7.4 and kept at 4ºC.

The ratios of different mixtures were added to the well of the microplate as shown in figure 2.1, firstly without the radical AAPH which was added after 15 min at 37ºC in the microplate spectrophotometer reader and then, fluorescence was read at 520 nm (excitation wavelength at 485 nm) during 3 h which comprises 200 cycles with 8 s of orbital agitation.

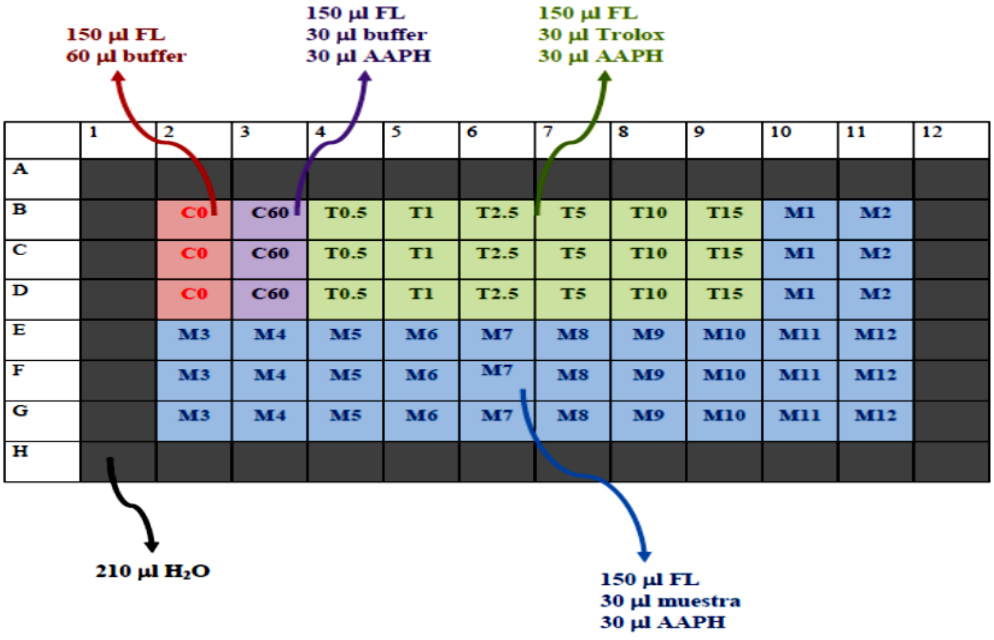


Figure 2.1: Schematic illustration of the microplate in the ORAC assay.

2.4.3. Folin-Ciocalteu

The total phenolic content was estimated in the extracts as gallic acid equivalents (GAE), expressed as mg gallic acid/g Sample according to the Folin–Ciocalteau method, based on a procedure described by Herrero et al. (2011). 10 µL of sample (3000 ppm) and 600 µL of H_2O were mixed and 50 µL of Folin–Ciocalteau reagent was added, and after 10 min 150 µL of Na_2CO_3 20% (w/v) and 19 µL of H_2O were added. After 2h of incubation at room temperature in the dark, 200 µL were transferred into a well of the microplate and the absorbance was measured at 760 nm in a microplate spectrophotometer and compared to the gallic acid calibration curve (from 25 to 2000 ppm) elaborated in the same way, substituting the sample by gallic acid.

2.5. HPLC-ESI-QTOF-MS analyses

HPLC analyses were made using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, an autosampler, a binary pump and a Diode Array Detector (DAD). The column used for the chromatographic separation was a Zorbax Eclipse Plus C18 (1.8 µm, 150 mm×4.6 mm) (Agilent Technologies, Palo Alto, CA, USA).

The conditions consisted of a gradient elution using aqueous acetic acid 0.5% (v/v) as mobile phase A and MeOH as phase B at a flow rate of 0.5 ml/min. The following gradient was applied: 0-7 min, 5-30% B; 7-65 min, 30-95% B; 65-67 min, 95-5% B and 67-75 min, 95-5% B. With 5 µLof sample injected.

HPLC system was coupled to microTOF-Q II (Bruker Daltonik, Bremen, Germany) equipped with an ESI interface operating in negative ion mode, considering a mass range of 50–1100 m/z and using a capillary voltage of +4000 V; dry gas temperature, 210 ºC; dry gas flow, 8.0 l/min; nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. The flow delivered into the MS detector from HPLC was split using a flow splitter 0.5 for stable electrospray ionization and reproducible results.

Moreover, automatic MS/MS experiments were performed adjusting the collision energy values as follows: m/z 100, 20 eV; m/z500, 25 eV; m/z 1000, 30 eV; and using nitrogen as the collision gas. For the necessary mass accuracy to identify compounds, external instrument calibration was used, for this, the calibrant used was sodium acetate clusters consisting of 5 mM sodium hydroxide and water: 2-propanol 1:1 (v/v) with 0.2% of acetic acid. This calibrant solution was injected in the instrument at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA). The accuracy of the mass data for the molecular ions was controlled by Data Analysis 4.0. Software (Bruker Daltonik) that offers a list of possible elemental formulas by using the GenerateMolecularFormulaTMEditor. The

Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

Chapter 3

3. Results and Discussion

3.1. Extraction method

To determine the best extraction methodology of phenolic compounds from kenaf leaf the two methods were tested, three ultrasound-assisted extractions and the overnight maceration, both cases with 100% and 80% of methanol. The respective concentrations of the obtained extracts were in Appendix 6.1, the percentage of extraction in each case was evaluated based on the ratio between the concentration of each extraction (mg extract/ml dissolution) and the summation of all extractions (Table 3.1).

Table 3.1: Percentage of extraction in each experiment, 1st, 2nd and 3rd ultrasound-assisted extraction.

	100% MeOH			**80% MeOH**		
%	Ultrasound Extraction			Ultrasound Extraction		
	1st	2nd	3rd	1st	2nd	3rd
Tainung 2	88,00	9,00	3,00	67,50	27,30	5,20
Everglades 41	77,40	13,80	8,80	79,70	15,50	4,80

As seen in both cases (Tainung 2 and Everglades 41) the percentage of extraction was higher in the first one, extracting more than 50% of mass, followed by the second and the third, giving reason to believe that probably a major quantity of the main components were obtained in the first extraction, anyway the second and third extraction weren’t a waste.

In the Tainung 2 case the first extraction showed a better result with 100% MeOH than with 80% while in the second and third extraction the experiments with 80% MeOH showed better results. In the Everglades case the first and second extractions showed similar behavior with 100% and 80% of MeOH while in the third extraction the experiment with 100% was better than with 80%.

Another fact that was to take into account, the raw material was very fibrous, it wasn’t possible to be sure that was homogenous. If in this point of sample collection the fiber was taken in more quantity than the soft part of the leave then the percentage of extraction wouldn’t be so efficient.

In order to seen which method of extraction was better to obtain the phenolic compounds, the following step was to search for its antioxidant capacity, with the data provided by Folin–Ciocalteau and DPPH assay, showed in table 3.2 were all the results were referent to the initial sample of Kenaf leaf.

Table 3.2: Results of Folin-Ciocalteau (EAG (mg)/Sample (g)) and DPPH (Ec50) assay for Everglades 41 and Tainung 2, both ultrasound-assisted and maceration systems.

Everglades 41								
		Extraction 100% MeOH		Maceration 100% MeOH		Extraction 80% MeOH		Maceration 80% MeOH
	1	2	3		1	2	3	
Folin–Ciocalteau	13,67	3,06	0,74	15,08	19,26	3,40	0,43	43,56
DPPH Ec50		41,68		45,12		53,60		63,11
Tainung 2								
		Extraction 100% MeOH		Maceration 100% MeOH		Extraction 80% MeOH		Maceration 80% MeOH
	1	2	3		1	2	3	
Folin–Ciocalteau	9,64	0,86	0,37	10,17	19,17	1,98	0,49	8,69
DPPH Ec50		37,69		38,96		51,62		37,62

In terms of the DPPH assay, the Ec50 was used to compare the antioxidant activity of the samples. Following the analysis done by Brand-Williams et al., 1995, a lower value of Ec50 indicate a small quantity of sample needed to neutralize the radical DPPH, according to that a sample with high antioxidant capacity will present a lower Ec50 value. The time of reaction was also studied once that according to Alves et. al, 2010 this a parameter taking into consideration, was observed that after 1h of reaction this wasn't complete, longer times of incubation under room temperature and in the dark were tested and established that after 4h the reaction was completed and it was possible to make a more accurate interpretation of the data.

Focusing of table 3.2 the extractions at 100% MeOH presented a lower Ec50 than the extractions at 80% in both leaf cases, which indicate that with a solution only of methanol and this type of extraction the efficiency of the reaction with the radical DPPH was better.

When the macerations were observed we can distinguish two ways, comparing only the Everglades case, the Ec50 of the macerations was higher with 100% MeOH and with 80% comparing with the extractions. While in the Tainung 2 case the maceration at 100% MeOH showed a value of Ec50 very close to the extraction also with 100% MeOH, in the maceration with 80% MeOH it showed a lower value than the extraction.

On the other hand comparing Tainung 2 and Everglades 41, the macerations of the Tainung 2 leave showed better results than the Everglades 41 leave, especially in the maceration with 80% MeOH in which Everglades 41 presented an Ec50 of 63, the highest value. Analysing just the results of the DPPH assay we could say that Tainung 2 as a better antioxidant capacity than Everglades 41. In order to better understand how the total phenolic content of the Kenaf leaves, the results from the Folin–Ciocalteu method will be analyzed.

In each extraction the results were steady, the first extraction showed always better results followed by the second extraction and then the third. In the Everglades 41 case, comparing the Folin–Ciocalteu between the 100% extractions and the 80%, the first extraction at 80% showed a better result and the second and third ones appeared to be very close, nevertheless the difference in values between the first extraction to the second and third was high, which indicated that the majority of phenolic compounds were extracted in the first extraction and a small amount in the second and third.

In relation to the Tainung 2 leafs the behavior of the extractions appeared to be similar to the Everglades 41 case, except in the first extraction at 100% MeOH that it showed a lower value.

Comparing the Folin–Ciocalteu results of the macerations, the values didn't show similarities and the maceration at 80% of MeOH in the Everglades 41 case appeared to be very high in relation to the others, this result was also contradictory to the result of DPPH, as seen this maceration as a very high value of Ec50 which pointed to a low antioxidant capacity so it wasn't probable such a high value of phenolic content.

The Folin-Ciocalteu results aren't very trustworthy once that, as explained in the section 1.1.4, other substances like sugars, aromatic amines, sulphur dioxide, organic acids and bases can react with the Folin–Ciocalteu reagent (Ramirez-Sanchez et al., 2010). So it was useful to give an idea of the amount of phenolic compounds, nevertheless it can't be said with all sure that those values translate correctly the phenolic content of the samples.

The lake of stability in the results could be explained by the method itself once that the maceration takes place during 12 h at room temperature in a stir plate which could lead to an uncontrolled temperature or was possible that the stir wasn't homogeneous, another possibility to take in count was the time of the experiment that could be too much and some products of degradation could appeared and alter the results of antioxidant activity and phenolic contents.

Taken all the experiments in account, in order to be more certain of the results and because they appeared to be more trustworthy, the extractions at 100% MeOH were chosen as a better method for the quantification of phenolic compounds, and as seen the three extraction were still extracting compounds, because of that the next step was to combine the three of them, collecting the product of each one to the same falcon tube, then dried in a rotary evaporator and analyze the product of the three extractions together (The respective concentrations of the obtained extracts were in Appendix 6.2). The results were exposed in table 3.3 and were denominated by successive extraction.

Table 3.3: Results of Folin-Ciocalteu (EAG (mg)/Sample (g)), DPPH (Ec50) and ORAC (μmol Trolox/mg sample) assay for Everglades 41 and Tainung 2, successive extractions (complete data in appendix 6.3).

Everglades 41	Sucessive Extraction
Folin–Ciocalteau	26,40
DPPH Ec50	27,33
ORAC	1273,7

Tainung 2	Sucessive Extraction
Folin–Ciocalteau	18,67
DPPH Ec50	43,00
ORAC	307,54

In relation to the other methods tested, overall the Everglades 41 type showed better results than the Tainung 2, especially when it comes to DPPH and ORAC values. Giving a closer look to the data provided by the Folin-Ciocalteu assay the Everglades 41 showed a better result, so quantitatively this type appeared richer that Tainung 2 and for this it made sense that the Ec50 of the Everglades type showed a lower result once that if it has more content in phenolic compounds it will have a better antioxidant capacity.

In order to better understand the antioxidant capacity of the sample, the ORAC assay was performed, in this assay, according to Huang et al., 2002 the antioxidant capacity is translated by the equivalents of Trolox, expressed in μmol, per unit of mass of sample, in this case it was presented in milligrams of leaf.

The previous results on table 3.3 were confirmed; in this method the Everglades 41 case also has a superior result than Tainung 2, so if it has a higher ORAC result then it has a higher quantity of equivalents of Trolox implying a better antioxidant capacity of the sample.

In their study, Alves et. al, 2010 pointed out that the compounds that presented a higher number of OH substitutions show better activity in this assay, also referred the importance of the kaempferol and quercetin once that between compounds with similar structures the antioxidant activity will be proportional to the number of OH substitutions. So considering this theory, it was expected that the samples were rich in substituted molecules with OH and the presence of some kaempferol and quercetin derivatives.

3.2. HPLC-ESI-QTOF-MS analyses of Kenaf leaf

In order to identify the phenolic compounds in Kenaf the following HPLC/ESI-QTOF-MS chromatogram was obtained and interpreted.

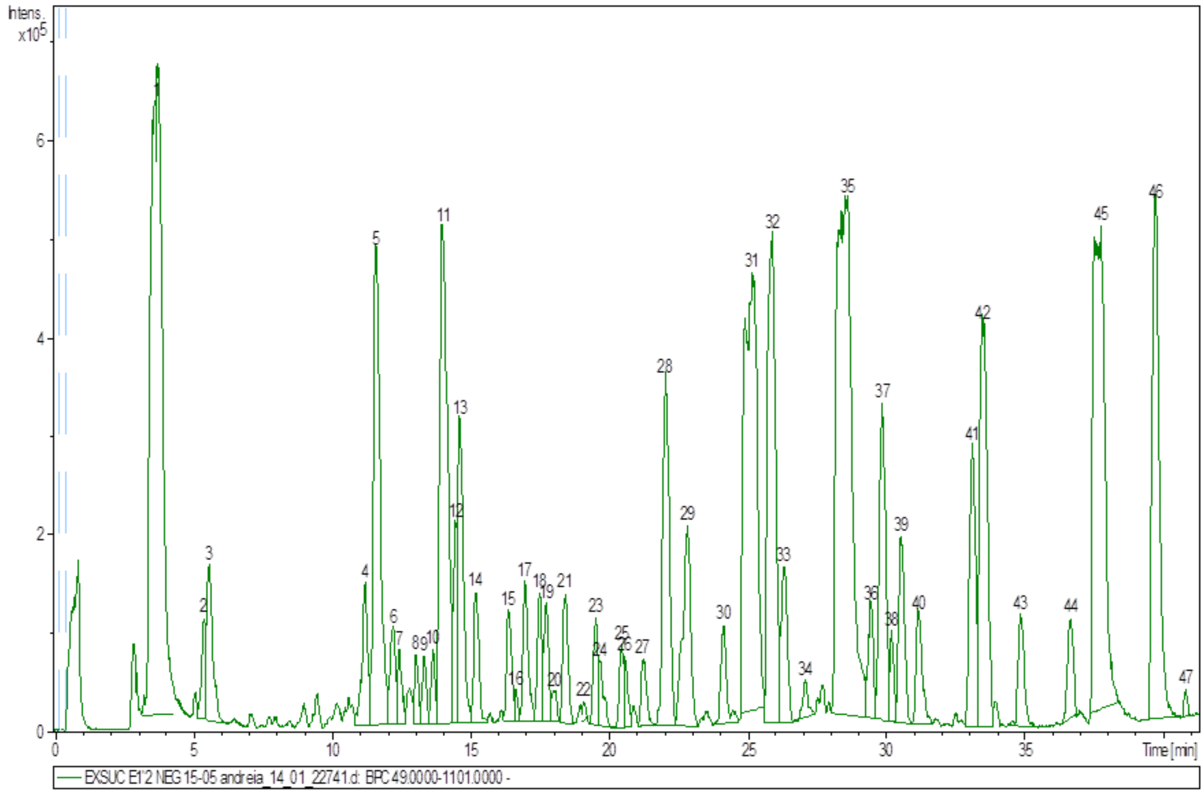


Figure 3.1: Base peak chromatogram (50–1100 m/z) of Everglades 41, in which the peaks are identified with numbers (1-47) according to the order of elution.

The interpretation of the MS and MS/MS spectra of each peak (figure 3.1) in comparison with those found in the literature, SciFinder data-base for chemical compounds and mass spectra data-bases such as Metlin, Metfrag and MassBank, was the main tool for identification of phenolic compounds.

The following table 3.4 shows the list of compounds identified in Everglades 41 type of leave, along with their retention time (RT) (min), the experimental mass of each compound, molecular formula, accurate mass of each molecular formula, the error (ppm) between both masses, the MS/MS ions used for the identification and proposed compounds. It also contains the mSigma value that is a numerical value that indicates how similar the theoretical and measured isotopic patterns are, in such a way that a low mSigma value indicates that the measured isotopic pattern of the peak is very similar to the theoretical isotopic pattern for the proposed molecular formula. The tolerance in the mSigma value is usually established at 50, although it is influenced by co-eluting analytes or matrix compounds, so it may be higher in some cases. In this table there is also the correspondent peak, for each compound, in the type Tainung 2 or the absence of that compound.

Table 3.4: List of compounds of Everglades 41 and its presence/absence in type Tainung 2.

Beginning with the analyses of the peaks in the table 3.4, peaks 1 and 2 were proposed to be derivatives from citric acid. Peak 1 was proposed to be hydroxycitric acid also reported in *Hibiscus sabdariffa*

Peak	RT (min)	m/z experimental	Molecular formula	m/z calculated	Abslute Error (ppm)	mSigma value	MS/MS Fragments (%intensity)	Proposed compound	Tainung 2 - Peaks
1	3.7	207.0140	$C_6H_8O_8$	207.0146	2.5	4.5	127,0022 (100) 115,0035(41.3) 133,0124(28.2) 189,0026(10.9)	**Hydroxycitric acid**	2
2	5.4	221,0294	$C_7H_{10}O_8$	221,0303	4.1	2.0	127,0033(100) 103,0026(17.8) 189,0036(10.4)	**Methoxycitric acid**	3
4	11.2	353,0892	$C_{16}H_{18}O_9$	353,0878	2.7	23.2	191,0561(100) 179,0367(28.1) 135,0447(18.5)	**Chlorogenic acid isomer 1**	5
5	11.6	353,0904	$C_{16}H_{18}O_9$	353,0878	7.2	13.7	191,0562(100), 179,0351(71.7) 135,0453(30.3)	**Chlorogenic acid**	11
11	14.0	353,0876	$C_{16}H_{18}O_9$	353,0878	0.3	1.8	191,0563(100)	**Chlorogenic acid isomer 2**	
13	14.6	353,0889	$C_{16}H_{18}O_9$	353,0878	3.0	2.5	173,0455(100) 191,0563(94.7) 179,0355(81.6)1 35,0452(41.6)	**Chlorogenic acid isomer 3: Cryptochlorogenic acid (4-caffeoylquinic acid)**	12

15	16.4	353,0868	$C_{16}H_{18}O_9$	353,0878	2.7	3.3	191 (100)	**Chlorogenic acid isomer 4**	14
17	17.0	337,0915	$C_{16}H_{18}O_8$	337,0929	4.2	18.6	191,0561(100) 163,0397(12.6) 173,0041(11.0)	**Coumaroylquinic acid**	15
19	17.7	367,1023	$C_{17}H_{20}O_9$	367,1035	3.2	5.8	191,0558(100) 173,0454(17.6) 193,0498(17.2)	**Feruloylquinic acid**	17
21	18.4	463,2176	$C_{21}H_{36}O_{11}$	463,2185	1.9	4.3	179,0553(100) 113,0236(59.9) 403,1946(52.2) 161,0416(49.4) 119,0342(38.2) 101,0235(36.1) 223,1339(32.5) 149.0445(24) 131,0339(18.3) 143,0363(16.4)	**[butoxy-hydroxy-methyl-trihydroxy-(hydroxymethyl)oxan]-oxy-hexahydro-cyclopenta[c]pyran-yl] acetate.**	18
23	19.5	593,1504	$C_{27}H_{30}O_{15}$	593,1512	1.4	5.0	285,0410(100) 447,0936(64.5) 431,0981(18.9) 286,0426(16.6)	**Kaempferol-glucoside-rhamnoside**	19
27	21.2	609.1433	$C_{27}H_{30}O_{16}$	609.1461	4.6	8.3	462,0798(50.5) 463,0873(26.6) 317,0277(4.5)	**hexahydroxyflavone-rhamnoside**	
28	22.0	609,1449	$C_{27}H_{30}O_{16}$	609,1461	1.9	1.5	446,0845(49,7) 447,0909(43.3) 463,0872(13.5) 301,0347(5.7)	**Quercetin-glucopyranosyl-rhamnopyranoside**	22
30	24.1	593.1491	$C_{27}H_{30}O_{15}$	593.1512	3.5	13.6	447,0931(100) 430,0905(50.8) 431,0936(28.7) 285.0400(8.7)	**Kaempferol-galactoside-rhamnoside isomer**	24
31	24.9	593, 1514	$C_{27}H_{30}O_{15}$	593, 1512	0.6	14.1	431,0971(80.1) 430,0898(64.7) 447,0920(45.9) 285,0410(21.9)	**Kaempferol-galactoside-rhamnoside isomer 2**	25
32	25.8	563,1429	$C_{26}H_{28}O_{14}$	563,1406	4.0	14.9	430,0898(100) 431,0963(76.3) 285,0401(22.1) 417,0812(11.8)	**Kaempferol-rhamnoside-xyloside**	26
33	26.3	463,0875	$C_{21}H_{20}O_{12}$	463,0882	1.6	5.1	300,0274(100) 301,0339(50.8)	**Isoquercitrin**	27
35	28.5	577,1604	$C_{27}H_{30}O_{14}$	577,1563	7.1	39.7	431,0970(100) 430,0895(42.1) 285,0402(33.7)	**Kaempferitrin**	29

36	29.4	447,0935	$C_{21}H_{20}O_{11}$	447,0933	0.5	7.6	300,0278(100) 301,0338(78.6)	**Quercitrin**	30
37	29.8	447,0942	$C_{21}H_{20}O_{11}$	447,0933	2.0	5.1	284,0328(100) 285,0392(43.3)	**Kaempferol-glucoside**	31
39	30.5	417,0822	$C_{20}H_{18}O_{10}$	417,0827	1.1	3.5	284,0330(100) 285,0383(33.7) 255,0305(5.9)	**Kaempferol-arabinoside**	32
40	31.1	417,0820	$C_{20}H_{18}O_{10}$	417,0827	1.7	6.0	284,0331(100) 285,0387(33.5)	**Kaempferol - arabinoside isomer**	33
41	33.0	447,0938	$C_{21}H_{20}O_{11}$	447,0933	1.1	7.2	301,0344(100) 300,0276(37.2) 151,0034(22.5)	**Quercitrin isomer**	34
42	33.4	431,1010	$C_{21}H_{20}O_{10}$	431,0984	5.7	15.7	285,0397(100) 284,0325(89,5) 255,0297(4,5) 257,0425(1.3)	**Kaempferol-rhamnoside**	35
43	34.8	301,0356	$C_{15}H_{10}O_7$	301,0354	0.9	4.2	151,0041(100) 179,9973(46.2) 273,0405(15.8) 121,0276(15.1)	**Qurcetin**	36
44	36.6	593,1306	$C_{30}H_{26}O_{13}$	593,1301	0.8	19.6	285,0408(86.5) 284,0330(44.3) 447,0925(5.6)	**Tiliroside**	37
45	37.4	431,1022	$C_{21}H_{20}O_{10}$	431,0984	8.8	34.9	285,0403(100) 257,0455(13.6) 151,0037(10.2)	**Kaempferol-7-O-rhamnoside**	38
46	39.6	285,0418	$C_{15}H_{10}O_6$	285,0405	4.8	4.4	285,0401(100)	**Kaempferol**	39

(Fernandez-Arroyo, 2011) and peak 2, methoxycitric acid. Both compounds presented the fragment 189 m/z corresponding to citric acid after the loss of H_2O 18 m/z and MeOH 32 m/z, respectively.

Several caffeoylquinic acids have been detected in Kenaf leaves. Peak 5 with m/z 353 was identified as chlorogenic acid (5-caffeoylquinic acid) by comparison with authentic standard, while peaks 4, 11, 13 and 15 were identified as its isomers due to their fragmentation pattern. As described in Vallverdú-Queralt et al. 2010, it was possible to differentiate three of them once that on one hand, chlorogenic and neochlorogenic acids gave a fragment at m/z 191 $[M\text{-}H\text{-}162]^-$ that corresponds to quinic acid and on the other hand cryptochlorogenic acid showed other fragment at m/z 173 $[\text{quinic-}H\text{-}H_2O]^-$ as base peak. Also chlorogenic and neochlorogenic acids could be distinguished by the intensity of the fragment at 179 that was quite intense in neochlorogenic acids but with a relative intensity lower than 5% in chlorogenic acid.

So with all these facts in count the peak 4 was established as neochlorogenic acid (3-caffeoylquinic acid) and the peak 13 as cryptochlorogenic acid (4-caffeoylquinic acid). The peaks 11 and 15 were other isomers with a fragmentation pattern more similar to chlorogenic and neochlorogenic acids than cryptochlorogenic acid but it wasn't defined were the substitution occurs.

Peak 28 at m/z 609 was identified as a quercetin-glucopyranosyl-rhamnopyranoside. There were two possible losses, hexose or rhamnose so they correspond to the fragments observed at m/z 447 $[M-H-162]^-$ and 463 $[M-H-146]^-$, as reported in Kerhoas et al., 2006, there is observed also a fragment at m/z 301 corresponding to the quercitin nucleus after the cleavage of the two sugars.

Peak 27 also with m/z 609, presented the molecular formula $C_{27}H_{30}O_{16}$ like peak 28 and fragment at m/z 463 $[M-H-146]^-$, nevertheless it presented a fragment at m/z 317 instead of 301 which indicates that the substitution is a rhamnose but the flavonoid core is not quercetin. The proposed molecular formula of this core was $C_{15}H_9O_8$, so this compound was proposed to be a hexahydroxyflavone-rhamnoside.

Kaempferol derivatives are major constituents of Everglades 41, such in a way that elution after 20 min shown many derivatives and with strong intensity. Kaempferol (figure 3.2) is a multiply-substituted flavonoid aglycone with different possibilities of substitution.

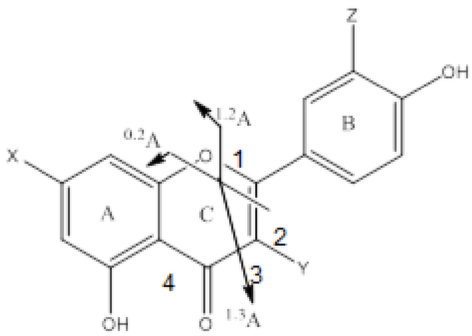


Figure 3.2: Basic structure of kaempferol, principal fragmentation routes and sites of substitution (X, Y, and Z).

Peaks 23, 30 and 31 were isomers with m/z 593 and their fragmentation patterns were very similar with differences between the relative intensities of the peaks, so all were proposed to be isomers of kaempferol-galactoside-rhamnoside.

So analyzing the fragments one by one, the fragment at m/z 431 was proposed to be the core kaempferol with a galactoside substitution after losing the rahmnoside moiety and the fragment at m/z 430 the some substituted kaempferol but deprotonated, while the fragment at m/z 447 appears also to be the kaempferol with the galactoside but the fragmentation of the rahmnose occurred differently, given origin to a fragment with higher m/z ratio. The fragmentation also allowed identifying kaempferol core at m/z 285.

Different stereochemistry of the kaempferol-galactoside-rhamnoside molecule could influence different fragmentation patterns, to distinguish the conformation of the molecules other analysis were necessary like NMR, anyway based on the information given by the utilized method it was possible to compare the different isomers.

The peak 23 showed, with higher relative intensity, the fragments at m/z 285 and 447 (100% and 64.5%, respectively) and with lower relative intensity the fragment at m/z 431 (18.9%). However, in the case of peak 31 the fragment at m/z 431 showed to be the main fragment (80.1%) while the ion at m/z 447 presented lower intensity (45.9%), in opposite to peak 30 that showed the fragment 447 as base peak and the 431 with 28.7%.

So according to figure 3.3, the kaempferol-galactoside-rhamnoside of peaks 23 and 30 could break preferably according to pathway b while the compound of peak 31 according to pathway a.

Moving to the peaks 35 and 46, they were identified as kaempferitrin (m/z 577) and kaempferol (m/z 285), respectively. In relation to kaempferol it was identified by comparison with its standard. Kaempferitrin showed a fragment at m/z 431 corresponding to the loss of a desoxyhexose molecule (Kerhoas et al., 2006) and another fragment at m/z 285 originated by the loss of the two desoxyhexose moieties and remaining the kaempferol core. These two phenolic compounds have been already reported as constituents of kenaf's leaves by HPLC-FTIR analyses in Rho et al., 2010.

Peaks 33 and 36 were identified as isoquercitrin and quercitrin, respectively, by comparison with their standards.

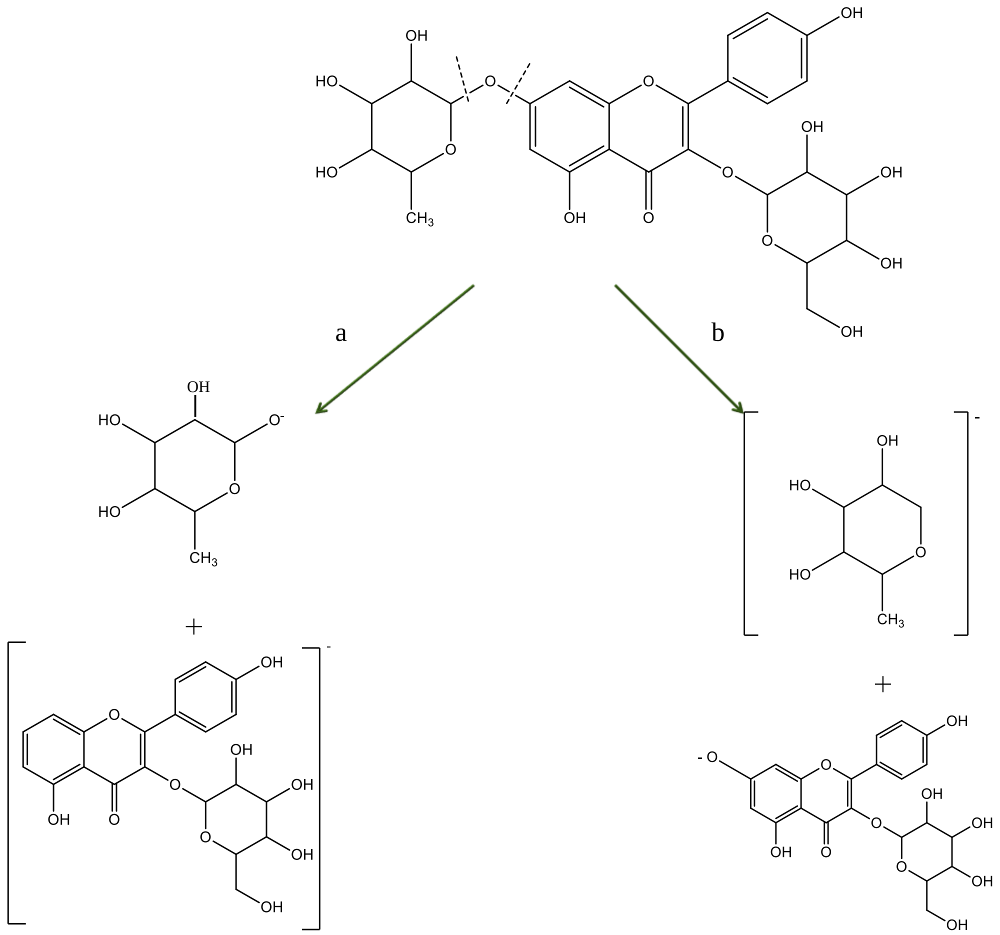


Figure 3.3: Fragmentation of kaempferol-galactoside-rhamnoside, pathway a gives origin to fragment 430 m/z while pathway b originates the fragment 447 m/z.

Peaks 37 and 41 presented the same molecular formula, nevertheless, the fragmentation pattern showed some differences, the peak 41, identified as a quercitrin isomer (quercetin-rhamnoside) produced the ion with m/z 301 (100% of relative probability) that corresponds to the core flavonoid quercetin deprotonated, supported by some studies like Sánchez-Rabaneda et al., 2003; Kammerer et al., 2004. The ion at m/z 300, $[M\text{-}H\text{-}147]^-$ mainly formed by the loss of the rhamnoside radical from the deprotonated core quercetin.

For other side, peak 37 was identified as kaempferol-glucoside, based on the similarity of fragmentation studied in Kammerer et al., 2004. This molecule showed the fragment 284 with 100% of relative intensity suggesting the loss of the glycoside radical $[M\text{-}H\text{-}163]^-$ from the deprotonated core of kaempferol, while the ion with m/z 285 corresponds to a depronated kaempferol. This compound was identified in the genus *Hibiscus* (*Hibiscus sabdariffa*) by Peng et al., 2011.

In relation to peak 43, data bases pointed to be quercetin. The study of fragmentation done by Febre et. al, 2001 showed that the fragmentation pattern could be very complex but one of the ways the C ring suffers a break, 1,3A by figure 3.2, gives origin to the fragment at m/z 151 (base peak of the MS/MS spectrum). This study also pointed out a pattern in which the bonds 1 and 2 are broken (figure 3.2) and a retrocyclization originates fragments at m/z 179 and 121, exemplified in figure 3.4.

Quercetin was also identified in the seedcake of Kenaf by Mariod et al., 2012 what reinforces the possibility of its presence also in the leaf.

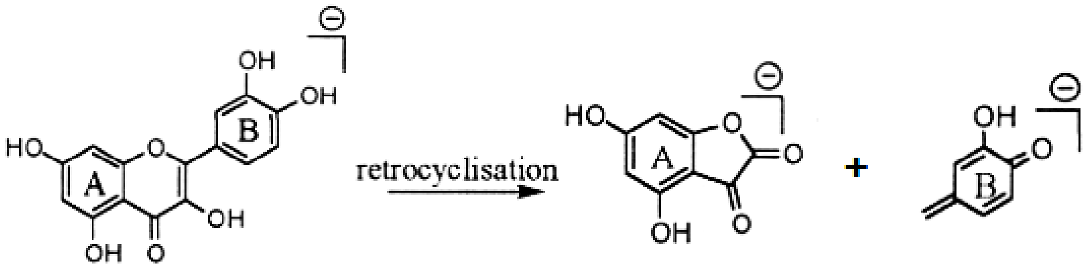


Figure 3.4: Example of the retrocyclisation that occurred in quercetin originating fragments at m/z 179 and 121, adapted from Febre et. al, 2001.

Peak 45 with m/z 431 and molecular formula of $C_{21}H_{20}O_{10}$ was primarily proposed to be a kaempferol-rahmnoside with the tipycal loss of the rhamnoside substitution $[M-H-146]^-$ giving origin to the fragment at m/z 285 (kaempferol core) with 100% of relative intensity.

Lu et al., 2009 studies of fragmentation of kaempferol-7-O-rhamnoside showed that this deprotonated molecule suffers conventional heterolytic cleavage to a large scale yielding abundant 285 m/z fragment; it was also observed a loss of $[M-H-174]^-$ that the same study assigned to the loss of CO by the aglycone to yield an ion at m/z 257. The subsequent cleave of the C ring as exemplified in figure 3.2, $^{1,3}A$, originated the fragment at m/z 151.

With the same molecular formula and m/z ratio appears the peak 42, nevertheless the fragment with m/z 257 showed very low relative intensity, suggesting that the position of the rhamnoside substitution is different. Also the fragment at m/z 255 appears with low intensity, this could be due to the loss of $COH^\bullet$ by the ion at m/z 284, product of a homolytic cleavage (Lu *et al.*, 2009).

Another kaempferol derivative was peak 32, supported by data-base MetFrag, it was proposed to be a kaempferol-rhamnoside-xyloside with m/z 563. It showed fragments at m/z 431 with proposed molecular formula of $[C_{21}H_{19}O_{10}]^-$ and 430, $[C_{21}H_{19}O_{10} - H]^-$, what points to a kaempferol with a rhamnose substitution. This was supported by the fragment corresponding to the kaempferol core, at m/z 285 and the loss of the rhamnoside substitution $[M-H-146]^-$ giving origin to the fragment at m/z 417.

Peaks 39 and 40 presented the same molecular formula $C_{20}H_{18}O_{10}$, proposed as kaempferol-arabinose and its isomer, respectively. As already observed in kaempferol derivatives, the fragment at m/z 285 corresponded to the kaempferol core and the fragment at m/z 284 to the deprotonated kaempferol. The fragment at m/z 255, which presented the minor relative intensity, correspond to the loss of the arabinose and the opening of the C ring, break $^{1,3}A$, as represented in figure 3.5.

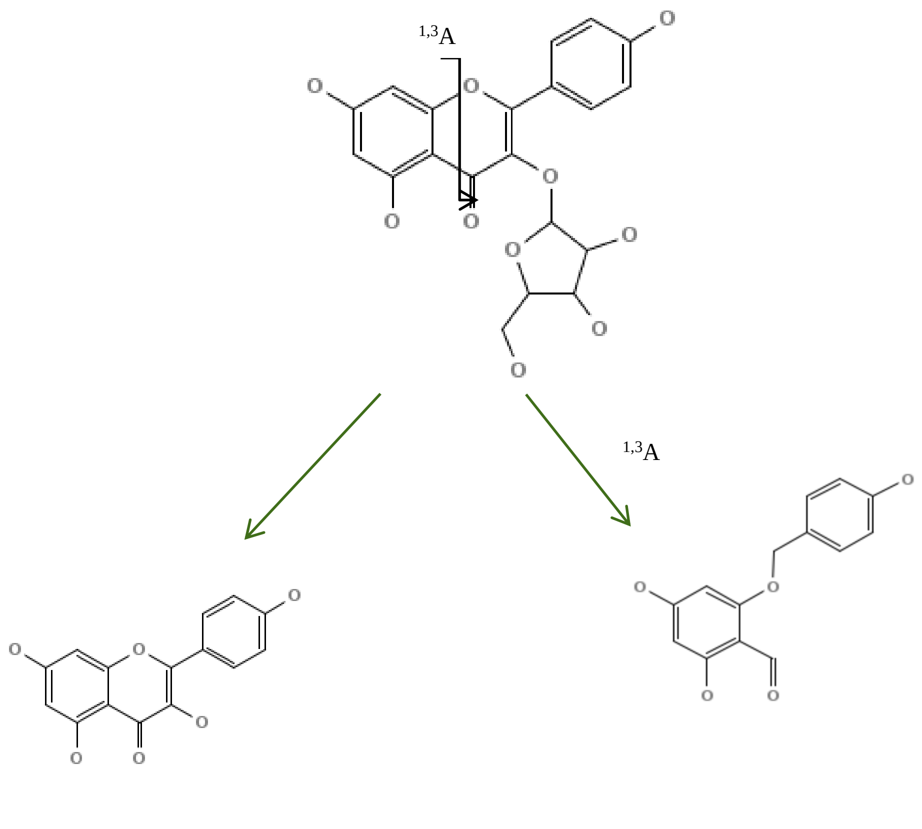


Figure 3.5: Representation of peak 39/40 and its fragments, at left the one correspondent to the m/z 285 and at right the one correspondent to m/z 255.

Moving to peak 44, there was a possibility of this being another kaempferol derivative because of the fragments at m/z 285 and 284, nevertheless, the fragment at 447 m/z matched with the fragmentation pattern, proposed by data-bases, of tiliroside. Also this compound was already identified in *Hibiscus* *sabdariffa* (Peng et. al, 2011) and *mutabilis* (Shanker et. al, 2007) so it is possible that another species of *Hibiscus*, such as *Hibiscus cannabinus*, also has this compound.

In relation to the minor peaks appearing between minutes 15 and 22, it was possible to propose some compounds. Peak 17 was proposed to be coumaroylquinic acid since the base peak of MS/MS spectrum was a fragment at m/z 191, which presented the molecular formula $[C_7H_{11}O_6]^-$ corresponding to quinic acid. The other fragment related to this molecule presented a relative intensity of 12.6% and a molecular formula $[C_9H_7O_3]^-$ and m/z 163 corresponding to the coumaric acid. The last fragment at m/z 173 could be explained by a loss of a water molecule from the quinic acid.

A similar compound was also identified in peak 19, with the fragment at m/z 191 as quinic acid and the fragment at m/z 173 the same molecule but with a loss of H_2O just like the previous case. The difference between both compounds appeared in the absence of the fragment at m/z 163 and the appearance of the fragment at m/z 193 with relative intensity of 17.2% and $C_{10}H_{10}O_4$ as molecular formula, like ferulic acid, these facts gave reason to propose this compound as feruloylquinic acid.

The peak 21 was proposed to be [butoxy-hydroxy-methyl-trihydroxy-(hydroxymethyl)oxan]-oxy-hexahydro-cyclopenta[c]pyran-yl] acetate (figure 3.6), not yet proposed to be a component of the *Hibiscus* genus. The fragments obtained by Data Analysis were found in this compound with the exception of the fragment at 223 m/z.

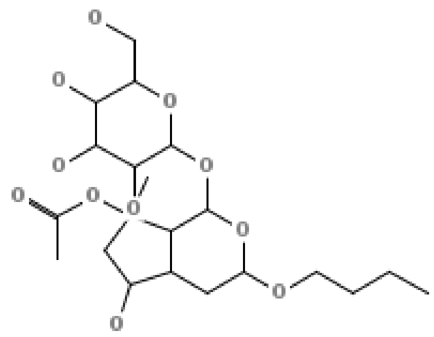


Figure 3.6: Proposed compound for peak 21, IUPAC name: [butoxy-hydroxy-methyl-trihydroxy-(hydroxymethyl)oxan]-oxy-hexahydro-cyclopenta[c]pyran-yl] acetate.

After analyzing the Everglades 41 list of compounds, it was demanding to compare with the Tainung 2 and one thing that was ease to observe was that the Tainung 2 chromatogram has fewer compounds than the Everglades one, while the Everglades 41 chromatogram has 47 peaks, the Tainung 2 has 37, a total difference of 10 compounds. It was also possible to see that between the 12 and 14 min and 16 and 22 min there were more compounds in the Everglades 41 chromatogram than in the Tainung 2, presented in the following figure (figure 3.7).

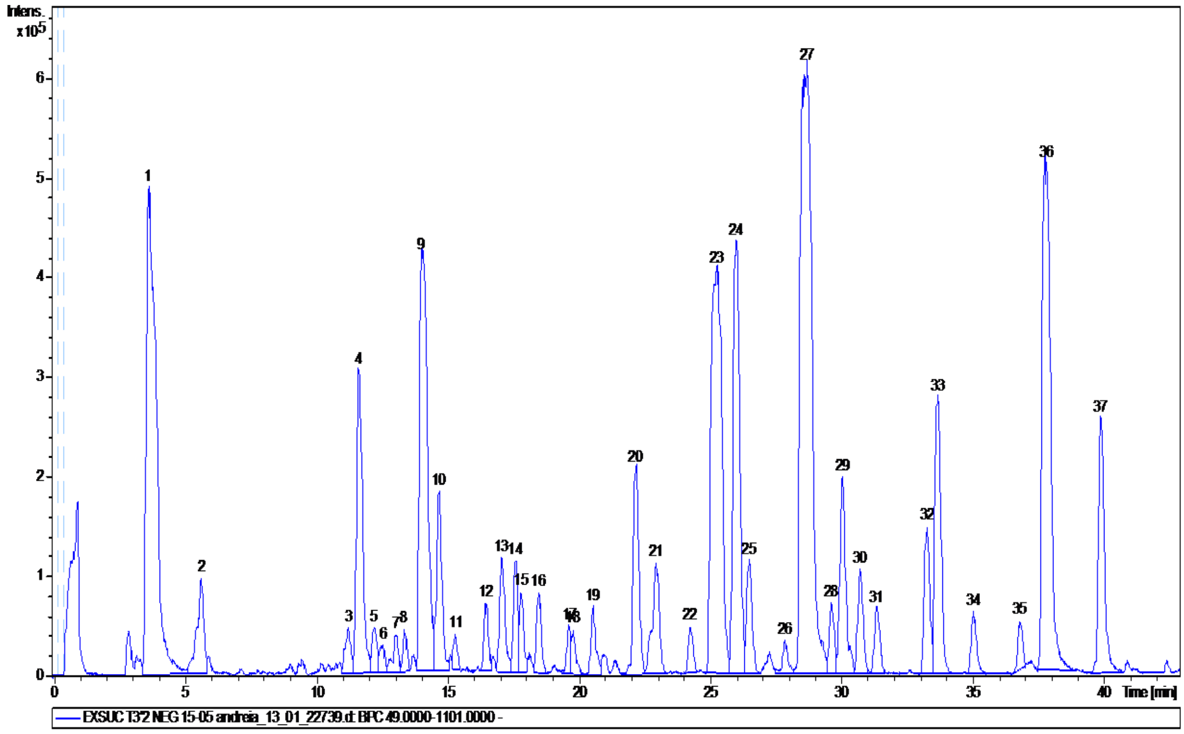


Figure 3.7: Base peak chromatogram (50–1100 m/z) of Tainung 2, in which the peaks are identified with numbers (1-37) according to the order of elution.

Nevertheless it was possible to find in the list of compounds of Tainung 2 (table 3.4), the majority of compounds found in the Everglades 41 list, with a few exceptions like the isomer 2 of chlorogenic acid (peak 11 of Everglades 41 chromatogram) that wasn't possible to find in the Tainung 2 one.

The peaks from the Everglades 41 chromatogram are also slightly more intense than the Tainung 2, but the of proportion of the intensity inside each chromatogram is similar, for example, peaks 30 and 31 of Everglades 41 chromatogram showed a similar difference of intensity as it's correspondents in the Tainung 2 chromatogram, the peaks 22 and 23. As the major difference between the two chromatograms, in the Tainung 2 one the peak 27 proposed as kaempferitrin is more intense than in the Everglades 41 (peak 35).

Thinking about the results obtained before, of the antioxidant activity and the total phenolic content, which were better results for Everglades 41 leaf, it made sense that in the Everglades 41 case the chromatogram appeared with more compounds and with peaks more intense (with the kaempferitrin exception).

Chapter 4

4. Conclusion

Considering the objectives for this work, that were to identify and quantify the phenolic compounds on Kenaf's leaves, comparing variety Everglades 41 with Tainung 2, and for that different extraction systems were tested, the antioxidant assays were carried out and then the identification of the phenolic compounds in the leaves, using advanced analytical techniques such as liquid chromatography coupled to electrospray ionization mass spectrometry using quadrupole time-of-flight analyzers (HPLC-ESI-MS (Q-TOF)). It can be stated that the objectives were accomplished.

The extraction system evaluated to be the best was the ultrasound-assisted extraction with 100% MeOH, the results of Folin–Ciocalteau, DPPH and ORAC pointed to Everglades 41 type to have more antioxidant potential and more phenolic contents.

After analyzing the HPLC/ESI-QTOF-MS chromatogram of Everglades 41 and Tainung 2 leaf, the one from Everglades 41 appeared to be richer in phenolic compounds, presenting 47 compounds against 37 from Tainung 2. Despite the fact that not all the compounds were identified, it was possible to propose 28 compounds for the Everglades 41 type and 26 of those compounds were also proposed to be in the Tainung 2 type.

Some of the compounds found in the Kenaf leaf were related before with the *Hibiscus* genus, nevertheless this study was a step forward for the comprehension of the phenolic compounds of Kenaf leaf.

It is important to reinforce the idea that this study was made also with the purpose to search for a candidate of a future add to food. Once that Kenaf is a source of antioxidants it could be seen as a possible candidate to grow in European countries and contribute with its phenolic compounds to a healthier food.

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References

- ✓ Agbor, G. A., Oben, J. E., Ngogang, J. Y., Xinxing, C., Vinson, J. A., Antioxidant capacity of some herbs/spices from Cameroon: A comparative study of two methods, *J. Agric. Food Chem.* **53**, 2005, 6819-6824.
- ✓ Akter, M. S., Ahmed, M., Eun, J., Solvent effects on antioxidant properties of persimmon (*Diospyros kaki* L. cv. Daebong) seeds, *International Journal of Food and Technology* **45** 2010, 2248-2264.
- ✓ Alexopoulou, E. Cosentino, S.L., Danalatos, N., Venturi, G., Fernando, A.L., Tenorio, J.L., Cadoux, S., Veccheit, M., Kipriotis, E. (2009) Biomass Yields of kenaf in Europe: Effect of site, year and cultural practices on yields. In: De Santi, G.F., Dallemand, J.F. Ossenbrink, H., Grassi, A., Helm, P. (eds) *Proceedings of the 17th European Biomass Conference and Exhibition, From Research to Industry and Markets*. 29 June - 3 July 2009, Hamburg, Germany, ETA-Renewable Energies and WIP-Renewable Energies, pp 138-141.
- ✓ Alves, C.Q., David, J. M., David, J. P., Bahia, M. V., Aguiar, R. M., Métodos para determinação de atividade antioxidante *in vitro* em substratos orgânicos, *Quim. Nova*. 2010; 33(10), 2202-2210.
- ✓ Annunziata, A., Vecchio, R., Functional foods development in the European market: A consumer perspective, *Journal of functional foods* **3**, 2011, 223-228.
- ✓ Barbosa, B. M. G., *Utilização de água residuais tratadas na irrigação de Kenaf (Hibiscus cannabinus* L.) – *efeito do ião amónio*, tese de doutoramento FCT-Unl, 2010.
- ✓ Belitz, H. D., Grosch, W., Schieberle, P., Food Chemistry 4th Edition revised and extended edition, *Springer*, 2009.
- ✓ Bianchi, M., Antunes, L., Radicais livres e os principais antioxidants da dieta, *Revista de nutrição*,**12**, 1999, 123-130.
- ✓ Bindhu, M. R., Umadevi, M., Synthesis of monodispersed silver nanoparticles using *Hibiscus cannabinus* leaf extract and its antimicrobial activity, *Spectrochimica acta part A: Molecular and biomolecular spectroscopy* **101**, 2013, 184-190.
- ✓ Bisby, R. H., Brooke, R., Navaratnam, S., Effect of antioxidant oxidation potential in the oxygen radical absorpition caparity (ORAC) assay, *Food Chemistry **108*** 2008, 1002-1007.
- ✓ Brand-Williams, W., Cuvelier, M. E., Berset, C., Use of a free radical method to evaluate antioxidant activity, *Lebensm-wiss u-technol* **28** 1995, 25-30.
- ✓ Bravo, L., Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance, *Nutrition reviews*, **56**, 1998, 317-333.
- ✓ Bruker Daltonics, Manual U. micrOTOF-Q II. 2008;0 (October).
- ✓ Buonocore, G., Perrone, S., Tataranno, M. L., Oxygen toxicity: chemistry and biology of reactive oxygen species, *Seminars in fatal & neonatal medicine* **15**, 2010, 186-190.

- ✓ Cicco, N., Lanorte, M. T., Paraggio, M., Viggiano, M., Lattanzio, V., A reproducible, rapid and inexpensive Folin-Ciocalteu micro-method in determining phenolics of plant methanol extracts, *Microchemical journal* **91**, 2009, 107-110.
- ✓ Escarpa, A., Gonzalez, M. C., An overview of analytical chemistry of phenolic compounds in foods, *Critical reviews in analytics chemistry* **31:2**, 2001, 57-139.
- ✓ Fabre, N., Rustan, I., de Hoffmann, E., & Quetin-Leclercq, J. (2001). Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *Journal of the American Society for Mass Spectrometry*, *12*(6), 707–15.
- ✓ FAO – Yute, Kenaf, Sisal, Abaca, Coir and Allied Fibres, STATISTICS, September 2012.
- ✓ Fernandez-Panchon, M. S., Villano, D., Troncoso, A. M., Garcia-Parrilla, M. C., Antioxidant activity of phenolic compounds: From *in vitro* results to *in vivo* evidence, *Critical reviews in food science and nutrition* **48**, 2008, 649-671.
- ✓ Fernando A, Morais J, Mendes B, Oliveira JFS (2008) Is kenaf an effective land-use alternative for the energy and industrial sectors in Portugal? Proceedings of the International Conference: Bioenergy: Challenges and Opportunities 237-242.
- ✓ Finley, J. W., Kong A. N., Hintze, K. J., Jeffery, E. H., Ji, L. L., Lei, X. G., Antioxidants in foods: State of science importante to the food industry, *j. agri. Food chem.* **59**, 2011, 6837-6846.
- ✓ Foyet, H. S., Hritcu, L., Ciobica, A., Stefan, M., Kamtchouing, P., Cojocaru, D., Methanolic extract of *Hibiscus asper* leaves improves spatial memory deficits in the 6-hydroxydopamine-lesion rodent model of Parkinson's disease, *Journal of ethnopharmacology* **133**, 2011, 773-779.
- ✓ Harnly, J. M., Bhagwat, S., Lin, L., Profiling methods for the determination of phenolic compounds in food and dietary supplements, *Anal bioanal chem* **389**, 2007, 47-61.
- ✓ Herrero, M., Temirzoda, T. N., Segura-Carretero, A., Quirantes, R., Plaza, M., Ibanez, E., New possibilities for the valorization of olive oil by-products, *Journal of chromatography A* ***1218***, 2011, 7511-7520.
- ✓ Kalili, K. M., Villiers, A., Recent developments in the HPLC separation of phenolic compounds, *J. Sep. Sci.* **34**, 2011, 854-876.
- ✓ Kammerer, D., Claus, Carle, A. R., Schieber, A., Polyphenol Screening of Pomace from Red and White Grape Varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS *J. Agric. Food Chem.*, **2004**, *52* (14), pp 4360–4367.
- ✓ Kerhoas, L., Aouak, D., Cingöz, A., Routaboul, J.-M., Lepiniec, L., Einhorn, J., & Birlirakis, N. (2006). Structural characterization of the major flavonoid glycosides from Arabidopsis thaliana seeds. *Journal of agricultural and food chemistry*, *54* (18), 6603–12.
- ✓ Lee, Y. G., Byeon, S. E., Kim, J. Y., Lee, J. Y., Rhee, M., H., Hong, S., Wu, J. C., Lee, H. S., Kim, M., J., Cho, D. H., Cho, J. Y., Immunomodulatory effect of *Hibiscus cannabinus* extract on macrophage functions, *Journal of ethnopharmacology* **113**, 2007, 62-71.

- ✓ Lu, L., Song, F. R., Tsao, R., Jin, Y. R., Liu, Z. Q., Liu, S. Y., Studes on the homolytic and heterolytic cleavage of kaempferol and kaempferide glycosides using electrospray ionization tandem mass spectrometry, *Rapid communiations in mass spectrometry*, 2010, 24, 169-172.
- ✓ Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., Lima, J. L. F.C., Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity, *Talanta* **83**, 2010, 441-447.
- ✓ Maganha, E. G., Halmenschlager, R. C., Rosa, R. M., Henriques, J. A. P., Ramos, A. L. L. P., Saffi, J., Pharmacological evidences for the extracts and secondary metabolites from plants of the genus *Hibiscus*, *Food chemistry* **1**, 2010, 1-10.
- ✓ Medina-remo A, Ja O, Vallverdu A, Lamuela-ravento RM, Andre C. Improved characterization of tomato polyphenols using liquid chromatography / electrospray ionization linear ion trap quadrupole Orbitrap mass spectrometry and liquid chromatography / electrospray ionization tandem mass spectrometry. 2010:2986–2992.
- ✓ Mohd-Esa, N., Hern, F. S., Ismail, A., Yee, C. L., Antioxidant activity in different parts of roselle (*Hibiscus* sabdariffa L.) extracts and potential exploitation of the seeds, *Food Chemistry* ***122***, 2010, 1055-1060.
- ✓ Mariod, A. A., Ibrahim, R. M., Ismail, M., & Ismail, N. (2012). Antioxidant activity of phenolic extracts from kenaf (*Hibiscus cannabinus*) seedcake. *Grasas y Aceites*, *63* (2), 167–174.
- ✓ Naczk, M., Shahidi, F., Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis, *Journal of pharmaceutical and biomedical analysis* **41**, 2006, 1523-1542.
- ✓ Peng, C.-H., Chyau, C.-C., Chan, K.-C., Chan, T.-H., Wang, C.-J., & Huang, C.-N. (2011). *Hibiscus* sabdariffa polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. *Journal of agricultural and food chemistry*, *59*(18), 9901–9.
- ✓ Ramirez-Sanchez, I., Maya, L., Ceballos, G., Villarreal, F., Fluorescent detection of (−)-epicatechin in microsamples from cacao seeds and cocoa products: Comparison with Folin–Ciocalteu method, *Journal of food composition analysis* **23**, 2010, 790-793.
- ✓ Rho, H. S., Ahn, S. M., Lee, B. C., Kim, M. K., Ghimeray, S. K., Jin, C. W., Cho, D. H., Changes in flavonoid content and tyrosinase inhibitory activity in kenaf leaf extract after far-infrared treatmet, *bioorganic & Medical chemistry letters* **20**, 2010, 7534-7536.
- ✓ Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., Glover, W., Phenolic compounds and their role in oxidative processes in fruits, *Food chemistry* **66**, 1999, 401-436.
- ✓ Sánchez-Rabaneda, F., Jáuregui, O., Casals, I., Andrés-Lacueva, C., Izquierdo-Pulido, M., & Lamuela-Raventós, R. M. (2003). Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (Theobroma cacao). *Journal of mass spectrometry : JMS*, *38* (1), 35–42.

- Shanker, R., Vankar, P. S., Dyeing cotton, wool and silk with *Hibiscus* mutabilis (Gulzuba)., *Dyes and Pigments*, 2007, 74(2), 464-469.
- Sharma, O. P., Bhat, T. K., DPPH antioxidant assay revisited, *Food Chemistry* ***113***, 2009, 1202-1205.
- Siró, I., Kápolna, E., Lugasi, A., Functional food, product development, marketing and consumer acceptance – A review, *Appetite* **51**, 2008, 456-467.
- Skoog, A. Douglas, Holler, F. James, Nieman, T. A., (1998) Chapter 13: Molecular Spectroscopy, Thomson Learning (Ed), Principles of Instrumental Analysis 5th Edition (pp. 300 – 325). UK: Brookes Cole.
- Snyder, L. R., Kirkland, J. J., Introduction to modern liquid chromatography, *John Wiley & Sons, Inc.* **2nd ed.**, 1979, USA.
- Stockham, K., Paimin, R., Orbell, J. D., Adorno, P., Buddhadasa, S., Modes of handling Oxygen Radical Absorbance Capacity (ORAC) data and reporting values in product labelling, *Journal of food composition and analysis* **24**, 2011, 686-61.
- Taamalli, A., Arráez-Tomán, D., Barrajón-Catalán, E., Ruiz-Torres, V., Pérez-Sánchez, A., Herrero, M., Ibañez, E., Micol, V., Zarrouk, M., Segura-Carretero, A., Fernández-Gutiérrez, A., Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells, *Food and chemical toxicology* **50**, 2012, 1817-1825.
- Tao, C., Jian-min, Q., Jian-tang, X., Pin-pin, C., Ai-fen, T., Fu-cheng, C., Wei, C., Optimization of two-dimensional gel electrophoresis for Kenaf leaf proteins, *Agriculture* sciences *in China* **12**, 2011, 1842-1850.
- Tura, D., Robards, K., Sample handling strategies for the determination of biophenols in food and plants, *Journal of chromatography A* **975**, 2002, 71-93.
- Verma, M., Nilapwar, S. M., Nardelli, M., Westerhoff, H. V. (2011) Chapter four – Absorption Spectroscopy, Methods in Systems Biology, Volume 500, pag. 59-75.
- Webber III, C. L., Whitworth, J., Dole, J., Kenaf (*Hibiscus cannabinus* L.) core as a containerized growth medium component, *Industrial crops and products* **10**, 1999, 97-105.

Appendix

Appendix 6.1: Data of the ultrasound-assisted extractions, divided into Tainung 2 and Everglades 41, with 100% and 80% of MeOH, which includes the mass of the flask, flask with the dry residue after the evaporation, volume of the MeOH used to reconstitute the dry residue, concentration of the dry residue expressed in mg/ml. Each extraction was done in triplicate (1, 2, 3).

Tainung 2

1st Extraction		mflask (mg)	m(flask + dry residue) (mg)	m(dry residue) (mg)	Vol. MeOH (mL)	C=m/v (mg/ml)
	1	47521,0	47582,2	61,2	2	30,6
100%	2	48187,6	48252,8	65,2	2	32,6
MeOH	3	48180,2	48258,7	78,5	2	39,25
	1	48179,2	48295	115,8	2	57,9
80%	2	47522,2	47672,3	150,1	2	75,05
MeOH	3	46415,3	46522,4	107,1	2	53,55
2nd Extraction						
	1	48207,5	48207,8	0,3	2	0,15
100%	2	46431,3	46440,8	9,5	2	4,75
MeOH	3	47521,9	47535,6	13,7	2	6,85
	1	55517,9	55656,2	138,3	2	69,15
80%	2	46415,5	46442,3	26,8	2	13,4
MeOH	3	46415,2	46436,1	20,9	2	10,45
3rd Extraction						
	1	48185,1	48185,5	0,4	1	0,4
100%	2	47525,2	47525,8	0,6	2	0,3
MeOH	3	46416,2	46423	6,8	2	3,4
	1	46417	46422,6	5,6	1	5,6
80%	2	46415,4	46426,4	11	2	5,5
MeOH	3	48179	48182,7	3,7	1	3,7

Everglades 41

1st Extraction		mflask (mg)	m(flask + dry residue) (mg)	m(dry residue) (mg)	Vol. MeOH (mL)	C=m/v (mg/ml)
	1	48179,3	48275,8	96,5	2	48,25
100	2	48179,2	48281,5	102,3	2	51,15
MeOH	3	48178,8	48268,5	89,7	2	44,85
	1	48177,8	48310,6	132,8	2	66,4
80	2	46414,5	46535,6	121,1	2	60,55
MeOH	3	47525,2	47642,5	117,3	2	58,65
2nd Extraction						
	1	48179,8	48198,3	18,5	2	9,25
100	2	48179,8	48198,2	18,4	2	9,2
MeOH	3	46417	46431,4	14,4	2	7,2
	1	47521,8	47547,2	25,4	2	12,7
80	2	46415,7	46447,3	31,6	2	15,8
MeOH	3	48179,4	48194,8	15,4	2	7,7
3rd Extraction						
	1	48179,3	48183,6	4,3	1	4,3
100	2	47522,5	47525,8	3,3	1	3,3
MeOH	3	47520,7	47529,4	8,7	1	8,7
	1	48184	48185,1	1,1	1	1,1
80	2	46420,6	46422	1,4	1	1,4
MeOH	3	47522,4	47530,9	8,5	1	8,5

Appendix 6.2: Data of the successive extractions, divided into Tainung 2 and Everglades 41, with 100% MeOH, which includes the mass of the flask, flask with the dry residue after the evaporation, volume of the MeOH used to reconstitute the dry residue, concentration of the dry residue expressed in mg/ml. Each extraction was done in triplicate (1, 2, 3)

Tainung 2

		mflask (mg)	m(flask + dry residue) (mg)	m(dry residue) (mg)	Vol. MeOH (mL)	C=m/v (mg/ml)
	1	48181,2	48239,0	57,8	2	28,9
100%	2	48180,2	48240,0	59,8	2	29,9
MeOH	3	48185,5	48233,7	48,2	2	24,1

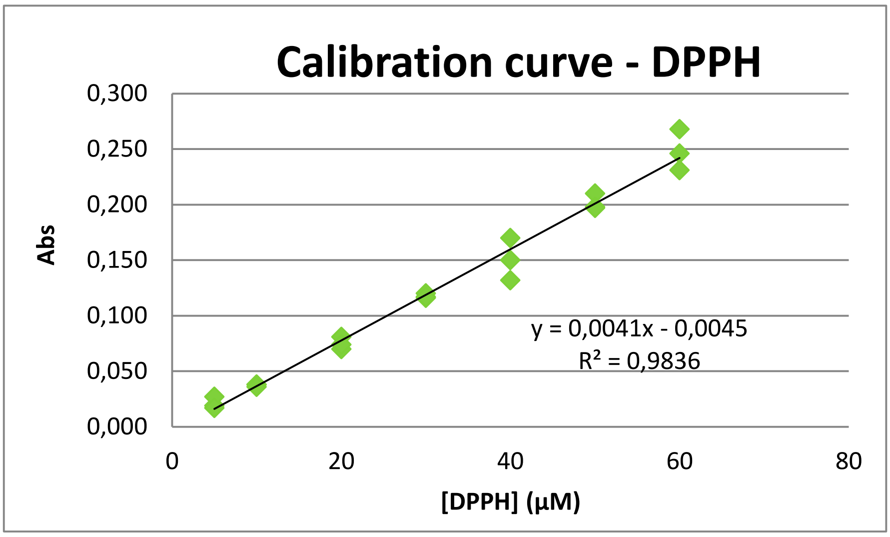
Everglades 41

		mflask (mg)	m(flask + dry residue) (mg)	m(dry residue) (mg)	Vol. MeOH (mL)	C=m/v (mg/ml)
	1	46415,8	46478,7	62,9	2	31,45
100%	2	46417,6	46484,1	66,5	2	33,25
MeOH	3	46419,9	46482,2	62,3	2	31,15

Appendix 6.3:

DPPH: Calibration curve done with the concentration of the standards and its absorbance at 516 nm. The equation was then utilized to calculate the concentration of DPPH.

Well ID	Conc (µmol)	516 nm
STD1	5	0,05600
	5	0,06500
	5	0,05500
STD2	10	0,07600
	10	0,07500
	10	0,07400
STD3	20	0,11900
	20	0,10700
	20	0,11200
STD4	30	0,15800
	30	0,15400
	30	0,15500
STD5	40	0,20700
	40	0,18700
	40	0,17000
STD6	50	0,24700
	50	0,23500
	50	0,23400
STD7	60	0,30600
	60	0,26900
	60	0,28300

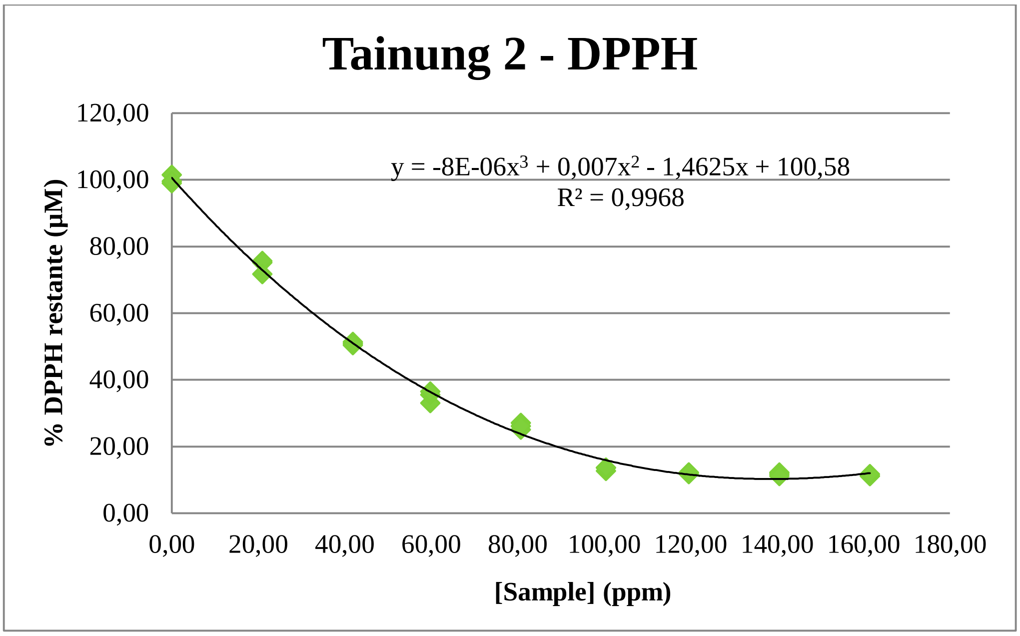


Tainung 2

[DPPH] (µM)	% DPPH (µM)	[Sample] (ppm)
36,95122	75,18610	20,9300
37,19512	75,68238	20,9300
35,24390	71,71216	20,9300
24,75610	50,37221	41,8600
25,24390	51,36476	41,8600
25,00000	50,86849	41,8600
16,21951	33,00248	59,8000
17,43902	35,48387	59,8000
17,92683	36,47643	59,8000
12,31707	25,06203	80,7300
13,29268	27,04715	80,7300
12,80488	26,05459	80,7300
6,21951	12,65509	100,4640
6,70732	13,64764	100,4640
6,70732	13,64764	100,4640
5,73171	11,66253	119,6000
5,97561	12,15881	119,6000
5,97561	12,15881	119,6000
5,97561	12,15881	140,5300
5,73171	11,66253	140,5300
5,48780	11,16625	140,5300
5,48780	11,16625	161,4600
5,48780	11,16625	161,4600
5,73171	11,66253	161,4600
49,87805	101,48883	0,0000
48,65854	99,00744	0,0000
48,90244	99,50372	0,0000

To calculate the remaining DPPH in solution:

% DPPH = ([DPPH] samples/ Average [DPPH] ctr) X 100



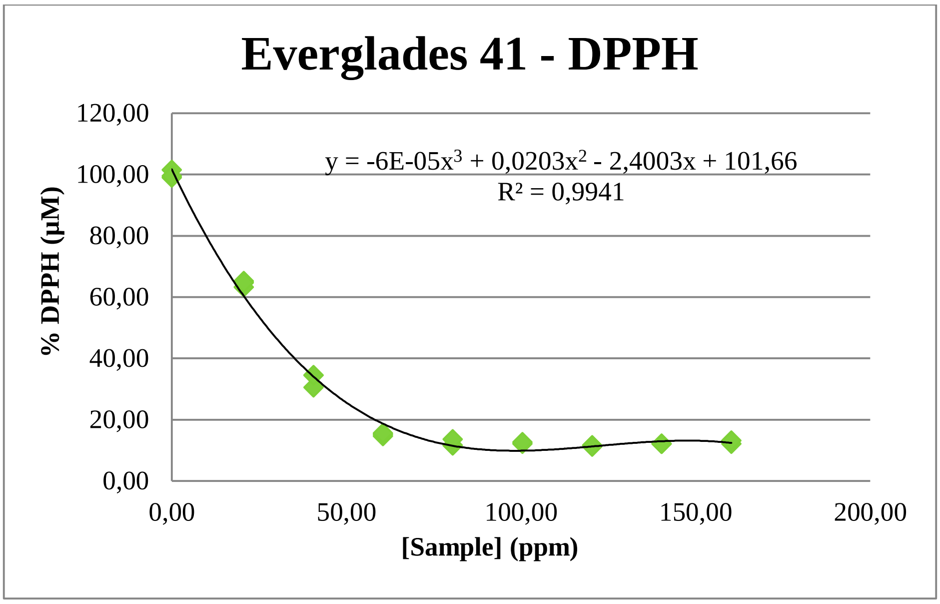
Everglades 41

[DPPH] (µM)	% DPPH (µM)	[Sample] (ppm)
32,07317	65,26055	20,61500
31,09756	63,27543	20,61500
31,82927	64,76427	20,61500
15,00000	30,52109	40,56500
16,95122	34,49132	40,56500
16,95122	34,49132	40,56500
7,43902	15,13648	60,51500
7,19512	14,64020	60,51500
7,68293	15,63275	60,51500
5,73171	11,66253	80,46500
5,73171	11,66253	80,46500
6,70732	13,64764	80,46500
20,36585	41,43921	100,41500
5,97561	12,15881	100,41500

6,21951	12,65509	100,41500
5,73171	11,66253	120,36500
5,73171	11,66253	120,36500
5,48780	11,16625	120,36500
5,97561	12,15881	140,31500
5,97561	12,15881	140,31500
5,97561	12,15881	140,31500
5,97561	12,15881	160,26500
28,17073	57,32010	160,26500
6,46341	13,15136	160,26500
49,87805	101,48883	0,00000
48,65854	99,00744	0,00000
48,90244	99,50372	0,00000

The red values weren't utilized to do the calibration curve. To calculate the remaining DPPH in solution:

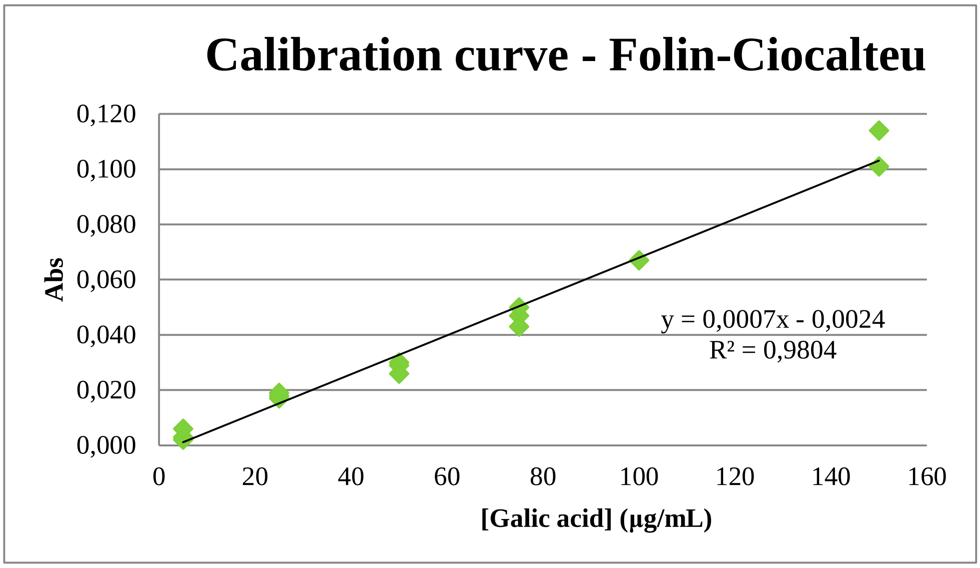
% DPPH = ([DPPH] samples/ Average [DPPH] ctr) X 100



Resolving both equations in order to X, the Ec50 was obtained.

Folin-Ciocalteu: The red values weren't utilized to do the calibration curve. Calibration curve done, to Tainung 2 and Everglades 41, with the concentration of the standards of Galic acid and the absorbance at 760 nm. The equation was then utilized to calculate the concentration of equivalents of galic acid (EAG).

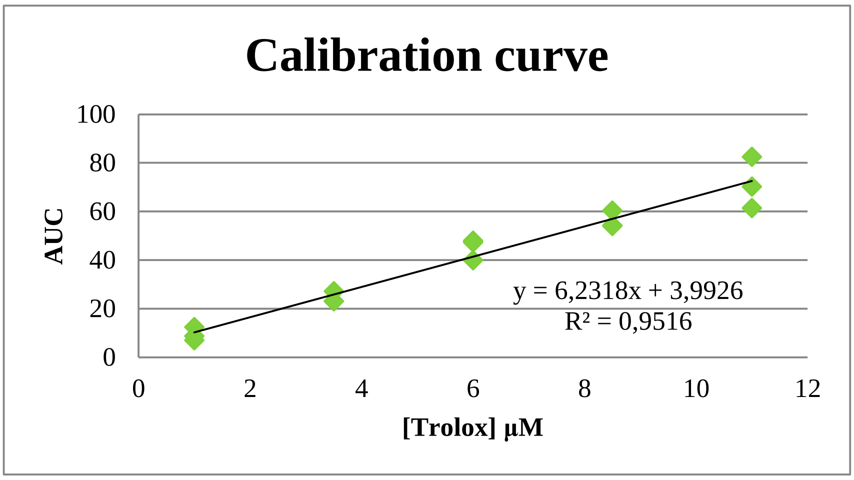
Well ID	[Galic acid] (µg/mL)	Abs 760 nm
STD1	5	0,0030
	5	0,0020
	5	0,0060
STD2	25	0,0180
	25	0,0170
	25	0,0190
STD3	50	0,0300
	50	0,0290
	50	0,0260
STD4	75	0,0470
	75	0,0500
	75	0,0430
STD5	100	0,0840
	100	0,0750
	100	0,0670
STD6	125	0,1030
	125	0,0950
	125	0,1090
STD7	150	0,1010
	150	0,1140
	150	0,0970



Sucessive extractions 100%MeOH	EAG (mg)/Extract(g)	m (dry residue) (g)	m simple (g)	EAG (mg)/Sample (mg)	Average	Standard derivation	Average of the 3 exp.
T1	74,61	0,0578	0,2022	21,33			
	77,21	0,0578	0,2022	22,07	20,6685	1,8227	
	65,10	0,0578	0,2022	18,61			
T2	55,39	0,0598	0,2120	15,63			
	55,39	0,0598	0,2120	15,63	16,4505	1,4298	18,67
	64,17	0,0598	0,2120	18,10			
T3	74,99	0,0482	0,2134	16,94			
	81,47	0,0482	0,2134	18,40	18,8906	2,2369	
	94,44	0,0482	0,2134	21,33			
E1	85,65	0,0629	0,2035	26,47			
	83,27	0,0629	0,2035	25,74	25,6552	0,8629	
	80,09	0,0629	0,2035	24,75			
E2	82,51	0,0665	0,2016	27,22			
	89,61	0,0665	0,2016	29,56	29,7898	2,6946	26,40
	98,81	0,0665	0,2016	32,59			
E3	87,28	0,0623	0,2141	25,40			
	85,67	0,0623	0,2141	24,93	23,7623	2,4382	
	72,03	0,0623	0,2141	20,96			

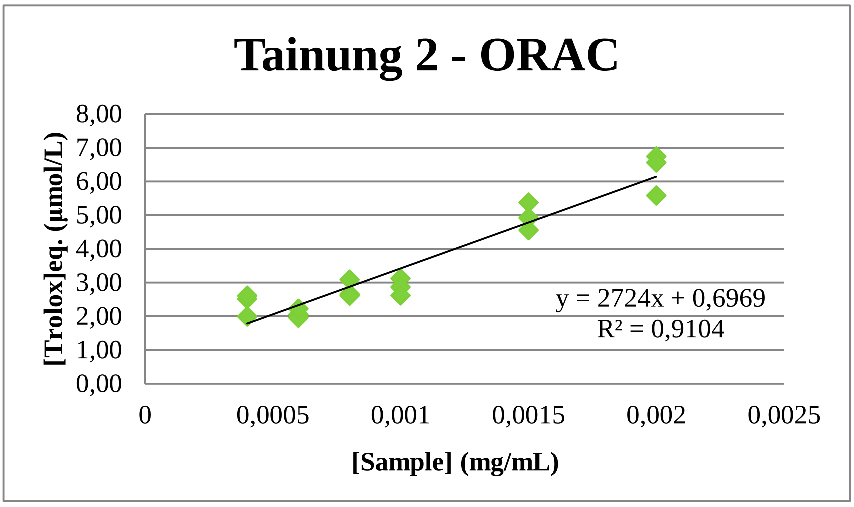
ORAC: The calibration curve was done, to Tainung 2 and Everglades 41, with the concentration of the standards of trolox and the area under the curve, given by the reading equipment, with the equation was possible to calculate the equivalents of trolox in the sample.

Well ID	Conc/Dil	AUC neta
STD1	1	12,542
	1	7,133
	1	8,724
STD2	3,5	27,267
	3,5	23,165
	3,5	23,159
STD3	6	40,033
	6	48,094
	6	47,345
STD4	8,5	60,367
	8,5	54,395
	8,5	54,043
STD5	11	61,504
	11	82,611
	11	70,37
STD6	13,5	71,529
	13,5	119,315
	13,5	97,094



Tainung 2

[Trolox]eq. (µmol/L)	[Extract] (mg/mL)
2,5175	0,0004
1,9907	0,0004
2,6061	0,0004
2,0532	0,0006
1,9513	0,0006
2,2184	0,0006
3,0860	0,0008
2,6598	0,0008
2,6131	0,0008
3,1271	0,001
2,8623	0,001
2,6239	0,001
4,5512	0,0015
5,3717	0,0015
4,9157	0,0015
5,5826	0,002
6,7376	0,002
6,5604	0,002



In order to find the equivalents of trolox in the leaf, the following equations were needed.

m=[Trolox]/[extract] (µmol/mg)

m=2724 µmol trolox - 1mg extract

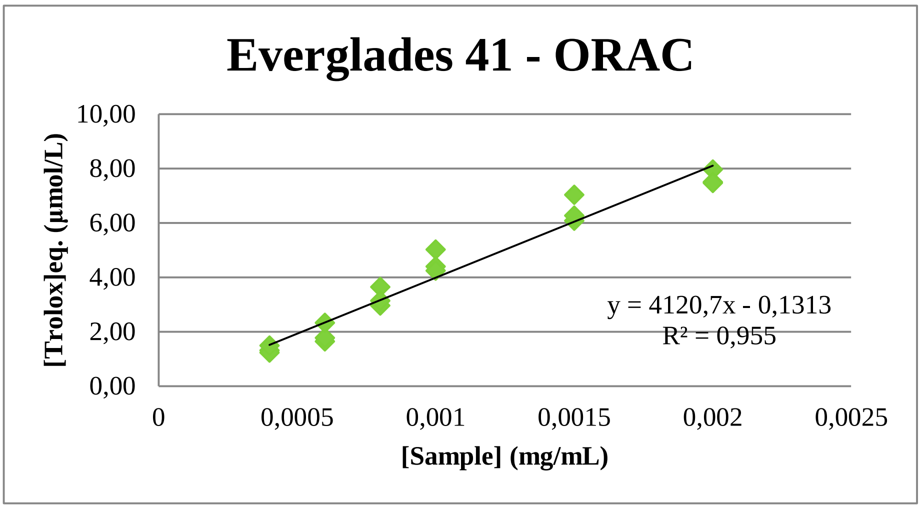
213,4mg original sample - 24,1 mg extract
1mg original sample - 0,1129 mg extract

0,1129 mg extract - 1mg origial sample
1mg extract - 8,8574 mg sample

2724µmol trolox - 8,8574 mg sample
307,54 µmol trolox - 1mg sample

Everglades 41:

[Trolox]eq. (µmol/L)	[Sample] (mg/mL)
1,3122	0,0004
1,2300	0,0004
1,4886	0,0004
1,6463	0,0006
1,7774	0,0006
2,3309	0,0006
2,9599	0,0008
3,6460	0,0008
3,1390	0,0008
5,0184	0,001
4,3965	0,001
4,2428	0,001
7,0372	0,0015
6,0781	0,0015
6,2745	0,0015
7,9637	0,002
7,5088	0,002
7,4679	0,002



The calculi were done the same way as Tainung 2.

Everglades 41: 1273,7 umol trolox/ mg sample