INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO
MESTRADO EM ENGENHARIA QUÍMICA




Desenvolvimento e otimização de extração de antioxidantes de matrizes alimentares por QuEChERS

SARA MIGUEL DO VALE TEIXEIRA RAMOS
Outubro de 2016

POLITÉCNICO
DO PORTO

INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO

MESTRADO EM ENGENHARIA QUÍMICA

RAMO OPTIMIZAÇÃO ENERGÉTICA NA INDÚSTRIA QUÍMICA

Orientação:

Co-Orientação:

POLITÉCNICO
DO PORTO

Acknowledgments

For the development of this work, several people contributed, every single one in a different way. To these, I express my gratitude.

Firstly, I would like to thank to my mentors, Dr. Maria João Ramalhosa, Dr. Maria Fátima Barroso, Dr. Ana Carvalho and Dr. Manuela Moreira, for providing this subject enabling the execution of this work, the shared knowledge, the support given not only at work but also in my personal life. Without your support and understanding, this work would not be possible at all. To Dr. Cristina Delerue-Matos, for having me in GRAQ allowing the accomplishment of this thesis.

To my family, for the love and support given during my academic path.

To Carlos Rocha, for the love, understanding and support during this time. Without your support I could not achieve my goals, you are an essential part!

To my friend Gilda Reis, who accompanied me in these student years, for the support, the kind word and, above all, the friendship. With you I grow and became the professional I am today.

To my friends Ana Luísa Monteiro and Ana Gomes, for being always available when needed, for the amazing times and friendship.

To my friends João Noronha, Cristina Ferrão and Filipa Gonçalves, simply by always giving me strength to move on.

To all the others that I cannot mention, but somehow contributed to this happy ending journey, a warmful thank you.

ISEP, you provided me the best years of my life, for that I am eternally grateful.

Abstract

Nowadays there is a global trend towards the efficient utilization of natural resources, among others those that are found in food consumed by humans. This situation happens not only as a method to prevent pollution, but also as a practice to benefit from the compounds in several nourishments that can bring benefits to human health.

Taking that into consideration, this work intended to evaluate the antioxidant capacity and phenolic profile of apple species available in the Portuguese market, as apple is one of the most consumed fruit around the world. For this purpose, the extraction conditions of apple antioxidants by QuEChERS were developed and optimized. The results obtained were compared to a conventional extraction method so that it was possible to determine which parameters had the greatest influence in the extraction yield. The extraction efficiency was evaluated by the spectrophotometric methods, total phenolic content (TPC), total flavonoid content (TFC) and ferric reducing antioxidant power (FRAP), and the phenolic profile was assessed by high performance liquid chromatography with diode array detection (HPLC-DAD).

In comparison with the conventional extraction, the higher results were obtained with the QuEChERS extraction with salt S1, composed by magnesium sulphate and sodium acetate, which was able to promote the extraction and therefore the amount of extracted phenolic compounds. For the solvents tested, water was the extractor solvent that enabled to obtain the highest yield, either by conventional extraction (TPC – 154.4 ± 5.9 µg GAE/g; TFC – 98.7 ± 7.3 µg EE/g; FRAP – 71.7 ± 1.4 µg AAE/g) or by QuEChERS extraction (TPC – 505.5 ± 63.7 µg GAE/g; TFC – 881.6 ± 69.9 µg EE/g; FRAP – 25.7 ± 2.5 µg AAE/g).

Regarding the phenolic profile assessment, in the QuEChERS extract was possible to identify several acids, namely chlorogenic, caffeic, vanillic, *p*-coumaric acid and sinapic acids and only the flavonoid naringin. In the conventional extracts, the phenolic compounds identified were the same as for the QuEChERS extracts, although in the extract from acetonitrile solvent it was possible to quantify naringin and β-resorcyclic acid with the amounts of 0.64 ± 0.03 and 1.94 ± 0.09 mg/L, respectively.

Keywords: antioxidants, QuEChERS, apple, phenolics, flavonoids, spectrophotometric methods, HPLC-DAD

Resumo

Atualmente, existe uma tendência global para utilizar de forma eficiente os recursos naturais, entre outros os que são encontrados nos alimentos consumidos pela população. Tal situação sucede não só como um método de prevenção da poluição, mas também como um meio para beneficiar de compostos presentes nos vários alimentos que poderão trazer benefícios para a saúde humana.

Tendo isto em consideração, este trabalho teve como objetivo avaliar a capacidade antioxidante e o perfil fenólico de espécies de maçãs disponíveis no mercado português, sendo a maçã um dos alimentos mais consumidos por todo o mundo. Para o efeito foram desenvolvidas e otimizadas as condições de extração de antioxidantes da maçã por QuEChERS. Comparou-se os resultados obtidos com um método de extração convencional, de tal forma que fosse possível determinar quais os parâmetros que mais influenciam o rendimento da extração. A eficiência da extração foi avaliada através de métodos espetrofotométricos, teor de fenólicos totais (TPC), teor de flavonoides totais (TFC) e método de redução do ferro (FRAP), e o perfil fenólico foi avaliado por cromatografia de alta eficiência com deteção por um sistema de díodos (HPLC-DAD).

Em comparação com a extração convencional, os melhores resultados foram obtidos na extração por QuEChERS com o sal S1, composto por sulfato de magnésio e acetato de sódio, que por sua vez foi capaz de favorecer a extração e, consequentemente, a quantidade de compostos extraídos. Relativamente aos solventes estudados, a água foi o solvente de extração que permitiu obter o rendimento mais elevado, tanto pelo método convencional (TPC – 154,4 ± 5,9 µg GAE/g; TFC – 98,7 ± 7,3 µg EE/g; FRAP – 71,7 ± 1,4 µg AAE/g) como por QuEChERS (TPC – 505,5 ± 63,7 µg GAE/g; TFC – 881,6 ± 69,9 µg EE/g; FRAP – 25,7 ± 2,5 µg AAE/g).

Em relação à avaliação do perfil fenólico, na extração por QuEChERS foi possível identificar vários ácidos, nomeadamente ácido clorogénico, ácido cafeico, ácido vanílico, ácido *p*-cumárico e ácido sinápico, e apenas o flavonoide naringina. Na extração convencional os compostos fenólicos identificados foram os mesmos que na extração por QuEChERS, embora nos extratos com o solvente acetonitrilo tivesse sido possível quantificar a naringina e o ácido β-resorcílico com resultados de 0,64 ± 0,03 e 1,94 ± 0,09 mg/L, respetivamente.

Palavras-chave: antioxidantes, QuEChERS, maçã, fenólicos, flavonoides, métodos espectrofotométricos, HPLC-DAD

Index

Figure index

Table Index

List of abbreviations

AA	Antioxidant activity
ABTS	2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
DLLME	Dispersive liquid–liquid microextraction
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
FTC	Antioxidant activity in linoleic acid system with ferric thiocyanate reagent
GAE	Gallic acid equivalent
HPLC	High performance liquid chromatography
HPLC–UV	High performance liquid chromatography with ultraviolet detection
HPLC-DAD-MS/MS	High performance liquid chromatography with diode-array coupled to tandem mass spectrometry
MAE	Microwave assisted extraction
NTZ	Superoxide anion scavenging activity
ORAC	Oxygen radical absorbance capacity
PG	Propyl galatte
PLE	Pressurized liquid extraction
PSA	Primary-secondary amine
PTFE	Polytetrafluorethylene
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
QuEChERS-dSPE	Quick, Easy, Cheap, Effective, Rugged and Safe extraction with a clean-up dispersive solid phase extraction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
SFE	Supercritical fluid extraction
SLE	Solid liquid extraction
SMU-AEE	Simultaneous microwave/ultrasonic assisted enzymatic extraction
SWE	Subcritical water extraction
TA	Total Anthocyanins
TBHQ	Tertiary butylhydroquinone
TE	Trolox equivalent
TPC	Total Phenolic Content
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine
UAE	Ultrasound assisted extraction
UHPLC-PDA	Ultra-high performance liquid chromatography Photodiode Array

1. Introduction

Fruits, vegetables and other types of food contain several elements in their structure that present beneficial effects in human diet, if consumed in appropriate quantities. Its consumption is encouraged due to the reported multiple biological effects namely antioxidant, anti-inflammatory, anticarcinogenic, antimutagenic and antiproliferative activities (Silva *et al.* 2012). Among this food group, apple and its derivatives are one of the main products consumed all over the world (Dhillon *et al.* 2013).

Besides this, there is an increasing global trend towards the efficient utilization of natural resources. In fact, the direct disposal of agro industrial by-products in the environment represents a major cause for environmental pollution and an important loss of biomass that could be used for the production of different metabolites with added commercial value (Vendruscolo *et al.* 2007)(Penha *et al.* 2012). Consequently, sustainable food production and incorporation of added-value in by-products is a major issue in the agro and food processing industry.

This study intends to evaluate the antioxidant capacity and phenolic profile of different species of apples from the Portuguese market. Furthermore, the use of QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) during its extraction process was also evaluated, in comparison with the conventional extraction procedure, in order to ascertain its potential as a mode of enhancing process yield.

1.1. Antioxidants

The word antioxidant has many definitions, depending on which field it is being used. For example, the term antioxidant as used in the literature is often implicitly restricted to chain-breaking antioxidant inhibitors of lipid peroxidation (Sies 1996). However, nowadays it is known that antioxidants are also compounds that prevent cells membrane to damage as long as others molecules to oxidize. Therefore, a broader definition has been introduced, it has been defined as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that (Halliwell & Gutteridge 1995).

The main characteristic of an antioxidant is its ability to scavenge free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA (Deoxyribonucleic acid) and can initiate degenerative diseases (Figure 1). Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as

peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Prakash *et al.* 2011).

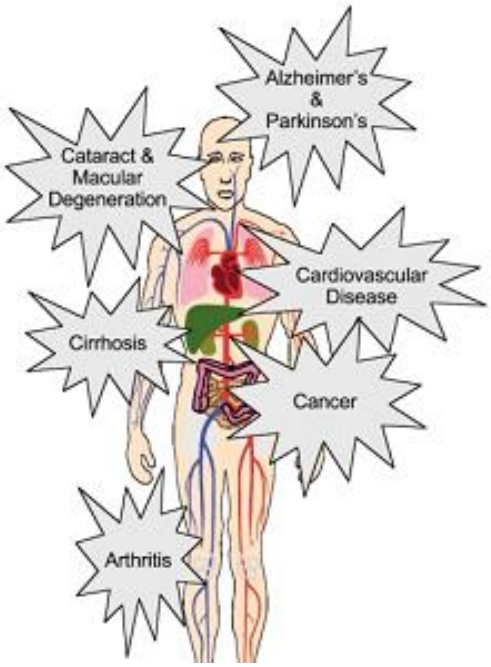


Figure 1 - Diseases caused by excess of ROS (reactive oxygen species) (adapted from Benzie 2003)

Free radical are species that have an unpaired pair of electrons that causes high instability and, consequently, high reactivity. These, in addition to a high reactivity, may start an initiation reaction and, as stated above, lead to degenerative diseases or others. The sources of free radicals can be endogenous and exogenous (Figure 2). Endogenous sources of free radicals are intracellular generated from auto-oxidation or inactivation of small molecules. Exogenous sources of free radicals are tobacco smoke, certain pollutants, organic solvents, anesthetics and pesticides (Rao *et al.* 2011). They derive from three elements: oxygen, nitrogen and sulfur, thus creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS). ROS include free radicals such as the superoxide anion ($O_2^{-\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide (NO), and other species like hydrogen peroxide (H_2O_2), singlet oxygen (O_2), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). RNS derive from NO by reacting with $O_2^{\bullet}$, and forming $ONOO^-$. RSS are easily formed by the reaction of ROS with thiols (Lü *et al.* 2010).

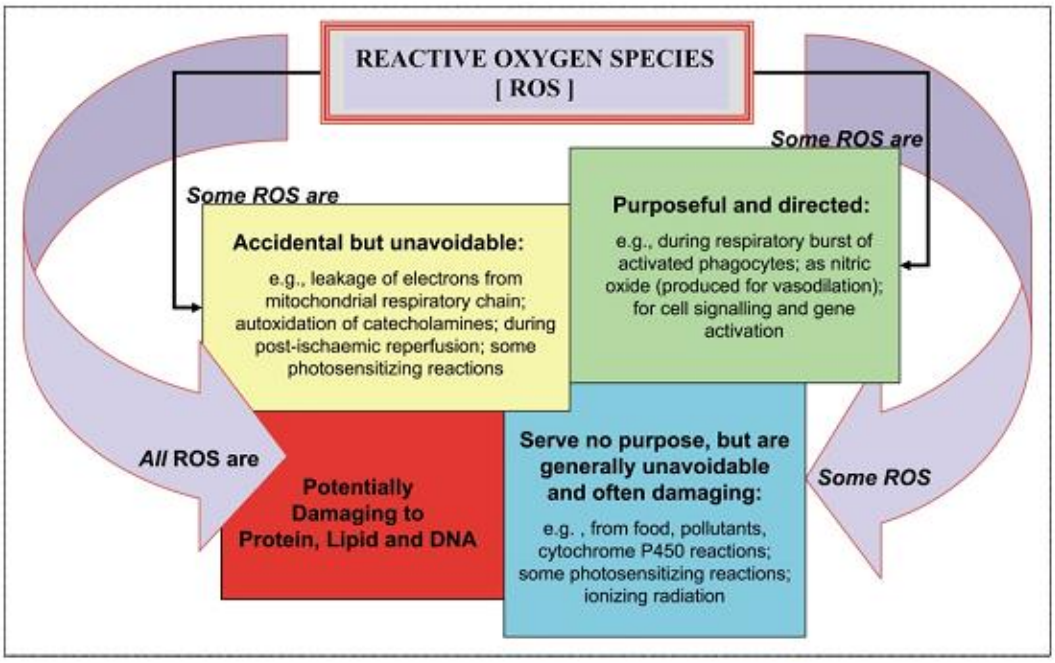


Figure 2 - Sources and characteristics of ROS (from Benzie 2003)

Essentially, the action of antioxidants against oxidative stress, as a result of the action of free radicals, involves several steps (Valko *et al.* 2007):

- preventive mechanisms;
- repair mechanisms;
- physical defenses;
- antioxidant defenses.

It is also important to note that, despite the action of antioxidants, oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses (Betteridge 2000), whereby it's not possible to dissociate this consequence from the action of these elements.

1.2. Classification of antioxidants

Usually, antioxidants contain at least one hydroxyl group. Nevertheless, among many authors, one option to categorize antioxidants is between synthetic antioxidants, natural antioxidants and other oxidation inhibitors (Durance 2002).

Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), Propyl galatte (PG) and Tertiary butylhydroquinone (TBHQ) are the most commonly used synthetic antioxidants in food industry. Their phenolic structure (Figure 3) can donate an electron to a free radical, thus stopping the oxidation mechanism (Ramalho & Jorge 2006).

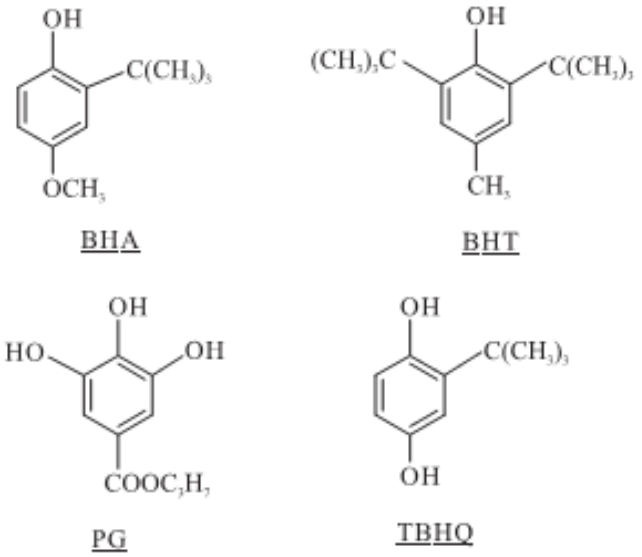


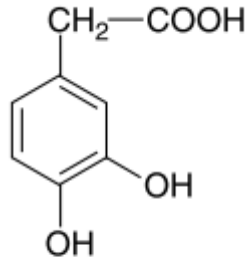
Figure 3 - Phenolic structure of synthetic antioxidants (Ramalho & Jorge 2006)

BHA is a more effective antioxidant in suppressing the oxidation of fats when compared to vegetable oils. BHT has similar properties to BHA and while the BHA is a synergist for propyl gallate, BHT does not have this property. BHA and BHT are synergistic with each other. PG is an ester of 3,4,5-Trihydroxybenzoic acid and has a large antioxidant activity. TBHQ is a white crystalline powder, slightly soluble in oils and fats and does not form a complex with iron and copper ions, such as gallate. TBHQ is also considered the best antioxidant for edible oils and, in conjunction with citric acid, has excellent synergy in vegetable oils (Ramalho & Jorge 2006).

Between the most commonly used natural antioxidants may be mentioned the tocopherols (vitamin E), the phenolic acids and carnosol for instance in rosemary (Ramalho & Jorge 2006). Nonetheless, according to the bibliography (Carocho & Ferreira 2013) natural antioxidants can be divided into two major systems, enzymatic system and non-enzymatic system. The enzymatic system is divided into primary enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, and secondary enzymes as glutathione reductase (Carocho & Ferreira 2013). In the non-enzymatic system, there are several components including co-factors, minerals, sulfur compounds, non-protein nitrogen compounds, vitamins and derivatives, carotenoids, flavonoids and phenolic compounds.

Vegetables, fruits and beverages are an important source of antioxidants, in fact it was demonstrated that oilseeds are also sources of natural antioxidants such as tocopherols. The best-known oxidation inhibitors are those present in olives, which are the fruits of virgin olive oil, that contain several antioxidants derived from hydroxytyrosol (Figure 4) (Durance 2002). In addition, sunflower seeds are rich in polyphenols as well as cottonseed contains gossypol, a polyphenolic compound with aldehydic groups possessing antioxidant properties. A variety

of cereals and grain vegetables contain several types of antioxidants such as phenolic compounds. Some substances belonging to this group act as cofactors of vitamin C, increasing its vitamin activity (Durance 2002).



Hydroxytyrosol

Figure 4 - Structure of natural antioxidants (Durance 2002)

1.3. Extraction Methods

Currently, there are several methods to extract antioxidants from matrices such as vegetables and fruits. This step is of utmost importance for the subsequent analysis. It is also important to note that the results obtained will strongly depend on the type of extraction, the matrix under extraction, the solvent chosen and operating conditions. However, the most effective extraction methods it's not described yet as many factors can be altered. Being so, a brief description of some used methods will be described.

Table 1 presents several types of antioxidants extraction, with different kinds of matrices, solvents and quantification methods. As it is possible to verify, there are several studies related with this type of antioxidants compounds which confirms the increasing global trend towards the utilization of natural resources, namely vegetables and fruits.

Table 1 - Antioxidant extraction by using several methodologies

Matrix	Compounds extracted/	Extraction tecnhique	Solvents	Quantification technique	Reference
Byproducts of Apple | Pear | Tomato | Artichoke	Antioxidant Capacity	SLE	Water | Methanol |Ethanol | Acetone | Hexane	TPC | DPPH | FTC | NTZ	(Peschel *et al.* 2006)
Fruit juice samples	BHA and BHT	DLLME	Hexanol | Octanol | Hexane | Ethyl acetate	HPLC	(Biparva *et al.* 2012)
Pomegranate seeds residue	Phenolic acids	SWE	Water | Methanol | Ethanol | Acetone	TPC | DPPH | ABTS | HPLC	(He *et al.* 2012)
Carrot | Tomato | Broccoli | Onion | Garlic | Green and red pepper | Beetroot	Protocatechuic acid | Catechin | Gentisic acid| Epicatechin | Vanillic acid | Syringic acid | Seringaldehyde| p-Coumaric acid | Ferulic acid | m-Coumaric acid | Rutin | Trans-esveratrol | o-Coumaric acid | Cinnamic acid | Kaempferol	QuEChERS-dSPE	Methanol | Water | Ethyl acetate | Acetonitrile	UHPLC-PDA	(Silva *et al.* 2012)
Phaleria macrocarpa	Phenolic | Flavonoid | Saponin | Alkaloid | Phytosterol | Tannin	SLE	Methanol	DPPH	(Andrean *et al.* 2014)
Red beets - beterraba	Betalains	MAE	Ethanol:water (1:1)	UV/Vis Spectrophotometer	(Cardoso-Ugarte *et al.* 2014)
Braeburn apple	Flavan-3-ol monomers |Phloridzin |Phlorogenic acid | Hyperoside | Isoquercitrin | Quercitrin | Ideain | Phenolic content	PLE	Acetone | Acetonitrile | Formic acid | Methanol	HPLC–DAD	(Franquin-Trinquier *et al.* 2014)

Golden apple peel	Catechin | Epicatechin |Chlorogenic acid | Phloridzin | Quercetin glycosides	SFE	Carbon dioxide | Ethanol | MeOH/Acetone	HPLC	(Massias *et al.* 2014)
Sugar beet molasses	Gallic acid | Vanillin | Hydroxybenzoic acid | Syringic acid cyanidin-3-O-rutinoside | Cyanidin-3-O-glucoside | Catechin | Delphinidin-3-O-rutinoside | Delphinidin-3-O-glucuronide | Ferulic acid	UAE	Ethanol	HPLC-DAD-MS/MS | TPC | AA| TA	(Chen *et al.* 2015)
Apple	Phenolic compounds	Enzyme extraction	Methanol/water (80:20)	DPPH | ABTS | FRAP	(Kim *et al.* 2016)
Wheat and rye bran	Antioxidant potential	PLE	Hexane | Acetone | Methanol:water (80:20%)	TPC | ABTS | DPPH | ORAC	(Povilaitis *et al.* 2015)
Red-fleshed apples	Phenolics |Flavonoids | Flavanols | Anthocyanins	SLE	Water | Formic acid | Acetonitrile	UV/Vis Spectrophotometer | HPLC	(Wang *et al.* 2015)
Nitraria tangutorun juice by-products	Phenols | Flavonoids | Anthocyanins	SMU-AEE	Methanol | Ethanol | Acetone	TPC | TA | Flavonoids | ABTS	(Wu *et al.* 2015)

Abbreviation definition: AA - Antioxidant activity; ABTS - 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; DLLME - Dispersive liquid–liquid microextraction; DPPH - 2,2-Diphenyl-1-picrylhydrazyl; FRAP - Ferric reducing antioxidant power; FTC - Antioxidant activity in linoleic acid system with ferric thiocyanate reagent; HPLC - High-performance liquid chromatography; HPLC–DAD - High-Performance Liquid Chromatography with Diode-Array Detection; HPLC-DAD-MS/MS - High-Performance Liquid Chromatography with Diode-Array Detection- Mass Spectrometer; MAE - Microwave assisted extraction; NTZ - Superoxide anion scavenging activity: ORAC - Oxygen radical absorbance capacity; PLE - Pressurized liquid extraction; QuEChERS-dSPE - Quick, Easy, Cheap, Effective, Rugged and Safe extraction with a clean-up dispersive solid phase extraction; SFE - Supercritical fluid extraction; SLE - Solid liquid extraction; SMU-AEE - Simultaneous microwave/ultrasonic assisted enzymatic extraction; SWE - Subcritical water extraction; TA - Total Anthocyanins; TPC - Total Phenolic Content; UAE - Ultrasound assisted extraction; UHPLC-PDA - Ultra High-performance liquid chromatography Photodiode Array.

1.3.1 Ultrasound assisted extraction (UAE)

Extraction enhancement by ultrasound has been attributed to the propagation of ultrasound pressure waves resulting in cavitation phenomena. Cavitation on the product surface causes impingement by micro-jets that result in surface peeling, erosion and particle breakdown. This effect provides exposure of new surfaces increasing mass transfer *(*Vilkhu *et al.* 2008), therefore the extraction is considered more efficient.

This technique can also be used as a complement, for example, if the matrix is dry, the ultrasound can facilitate hydration and increase the pores of the cellular walls. It may also be used to disintegrate the matrix, which will increase the area of the substrate subject to extraction. Both are options that increase the efficiency of extraction.

In 2015, Chen and co-authors carried out a study to extract polyphenolic compounds, antioxidants and anthocyanins from sugar beet molasses with acidic ethanol and ultrasonic treatment at 35 Hz and 450 W. The extract obtained had 17.36 mg GAE/100 mL in total phenolic content, 16.66 mg TE/g in antioxidant activity and total anthocyanins were 31.81 mg/100 g of the sugar beet molasses extract. Beside this, high performance liquid chromatography was also performed and ten compounds were determined (gallic acid, vanillin, hydroxybenzoic acid, syringic acid, cyanidin-3-O-rutinoside, cyanidin-3-O-glucoside, catechin, delphinidin-3-O-rutinoside, delphini- din-3-O-glucuronide and ferulic acid) (Chen *et al.* 2015).

1.3.2 Pressurized liquid extraction (PLE)

This technique is not more than one solvent extraction at a specific and controlled temperature and pressure. Initially it was mainly used in environmental pollutants in soil samples. However, given their “green” behavior when compared to other techniques, it began to be used in other matrices, such as food matrices.

As stated previously, PLE is a technique that involves extraction using liquid solvents at elevated temperature and pressure, which enhance the extraction performance as compared to those techniques carried out at near room temperature and atmospheric pressure. The merits of enabling the use of solvents at temperatures above their atmospheric boiling point is the enhanced solubility and mass transfer properties (Mustafa & Turner 2011).

For example, Polivaitis *et. al* (2015) developed a study to evaluate antioxidant potential of rye and wheat bran using different polarity solvents. The matrix (rye and wheat bran) was grounded in an ultra-centrifugal rotor mill and separated by different hole size sieves. Soxhlet

extraction was performed in an automated extractor as a standard technique using hexane and acetone, as solvents. PLE was executed in an accelerated solvent extraction apparatus, consecutively applying different polarity solvents, namely hexane, acetone and the mixture of methanol:water (80:20%). The extraction was performed at a pressure of 10.3 MPa and at a temperature of 80ºC. The highest extract yield was obtained from rye bran using methanol-water; particle size in most cases had a significant effect. Then the matrix was analyzed to evaluate antioxidant potential. Other case of study was achieved by Franquin-Trinquier *et al.* in 2014 to optimize antioxidant extraction of apple monomeric phenolics and to compare PLE and manual-liquid extraction. This author concluded that, in comparison with manual methods, PLE shows several advantages such as the increase of polyphenol concentrations and reduction of extraction time and organic solvent amounts (Franquin-Trinquier *et al.* 2014).

1.3.3 Subcritical water extraction (SWE)

According to Carabias-Martínez *et al.* (2005), a SWE is similar to PLE but using water as the extraction solvent. Temperatures between 100 and 374ºC and pressure high enough to maintain the liquid state are required. Unique properties of water are namely its disproportionately high boiling point for its mass, its high dielectric constant and high polarity. As the temperature rises, there is a marked and systematic decrease in permittivity, an increase in the diffusion rate and a decrease in the viscosity and surface tension. In consequence, more polar target materials with high solubility in water at ambient conditions are extracted most efficiently at lower temperatures, whereas moderately polar and non-polar targets require a less polar medium induced by elevated temperature (Asl & Khajenoori 2013).

As an example, He *et. al* in 2012, conducted a study of SWE from pomegranate seeds residues in order to determine total phenolic content (TPC) and antioxidant capacities of the extracts obtained. Water (at room temperature) was used and the extract was centrifuged at 3600 rpm for 10 min. The supernatants were evaporated to dry and dissolved in methanol, kept at −18 ◦C for subsequent analysis. The results showed that the optimum extraction time was 30 min, solid to water ratio was 1:40, and the highest TPC was obtained at 220ºC. High-performance liquid chromatography (HPLC) profiles revealed that nine compounds had antioxidant activity (He *et al.* 2012).

1.3.4 Microwave assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a process of using microwave energy to heat solvents in contact with a sample in order to partition analytes from the sample matrix into the solvent. The ability to rapidly heat the sample solvent mixture is inherent to MAE and is the main

advantage of this technique. By using closed vessels the extraction can be performed at elevated temperatures accelerating the mass transfer of target compounds from the sample matrix (Eskilsson & Björklund 2000).The main advantages are the rapid heating, which reduces the extraction time, the requirement of lower solvent volume and good reproducibility.

In 2014, Cardoso-Ugarte *et al.* did a study that intended, through this technique, to extract betalains from red beet. Several treatments with different combinations of time, power and duty cycle applied to the samples were studied. The combination of 400 W and 100% duty cycle for 90-120 seconds resulted in the highest amount of recovered betanines, whereas at 140-150 seconds the highest amount of betaxanthins was obtained. MAE extraction of betalains from red beets was performed using a Microwave Accelerated Reaction System. The colored extracts were kept in closed vials and analyzed in the same day. The authors concluded that betalain yields obtained by MAE were twice as high as those obtained during conventional extraction (Cardoso-Ugarte *et al.* 2014).

1.3.5 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a rapid, selective and convenient method for sample preparation prior to the analysis of compounds in natural product matrices. SFE is usually performed with pure or modified carbon dioxide, which facilitates off-line collection of extracts and on-line coupling with other analytical methods such as gas and supercritical fluid chromatography (Modey *et al.* 1996). Although the results are better with carbon dioxide, other fluid under appropriate conditions can be used, for example water, methanol, ethane, among others (Herrero *et al.* 2006).

The basic component required for performing SFE in the laboratory consist of (a) supply of CO_2 or some other potential fluid, (b) gas compressor or pump, (c) heated zone or oven, (d) extraction vessel or thimble, (e) outlet restrictor, and (f) extract accumulator or trap (Mustafa & Turner 2011). The basic hardware needed for a supercritical fluid extractor is shown in Figure 5.

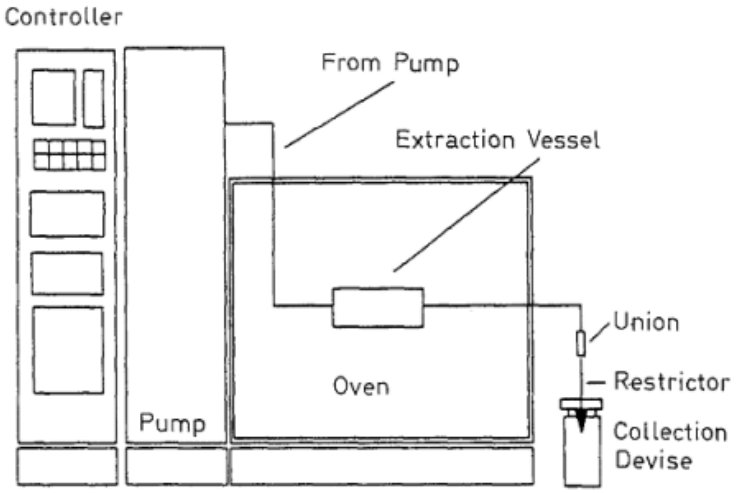


Figure 5 - General hardware needed for a supercritical fluid extractor (Hedrick *et al.* 1992)

An environmentally safe alternative, such as supercritical carbon dioxide, to organochlorine solvents which are widely used today in many government and industrial analytical laboratories for sample preparation is desirable (Hedrick *et al.* 1992). Supercritical fluids extraction (SFE) has immediate advantages over traditional extraction techniques: it is a flexible process, allows the elimination of polluting organic solvents and the expensive post-processing of the extracts for solvent elimination (Reverchon & De Marco 2006). The main disadvantage is the fact that it is a high investment when in laboratory scale but once the scale-up is completed, the cost is reduced.

In 2014, Massias *et al.* presented a work reflecting what has been stated about SFE. In this study, the aim was the recovery of phenolic compounds from apple peels using CO_2 and ethanol extraction. As for conventional extraction, nine phenolics were identified in SFE-extracts including the sugar based phloridzin and quercetin derivatives. Better results were then obtained, therefore proven the efficiency of SFE (Massias *et al.* 2014).

1.3.6 Solid liquid extraction (SLE)

Most industrial processes use solid-liquid separation in order to recover valuable solids or liquids. These options arise for reusing the fluid and / or solids for treating fluids before they are discarded. For example, in the bibliography, Peschel *et al.* in 2006, did a solid-liquid extraction in vegetables and fruits in which the raw materials were firstly extracted twice separately with water, methanol, ethanol, acetone and hexane, with a 10:1 solvent-raw material ratio, in closed vessels, by stirring at 25ºC for 4 h and being left to stand for another 4h (using a Soxhlet system for 6 h in the case of hexane). This process was finished with filtration and then analyzed, the main results were from apple 48.6 ± 0.9 mg gallic acid

equivalents/g dry extract, from pear 60.7 ± 0.9 mg GAE/g, from tomato 61.0 ± 3.0 mg GAE/g, golden rod 251.4 ± 7.0 mg GAE/g and artichoke 514.2 ± 14.9 mg GAE/g (Peschel *et al.* 2006).

1.3.7 Dispersive liquid–liquid microextraction (DLLME)

Dispersive liquid–liquid microextraction (DLLME) is a relatively new technique which, among others, requires low volume of solvent, has an environmental "friendly" character and a simple and economic technique. DLLME consists of two steps: (1) Injection of an appropriate mixture of extracting and disperser solvents into aqueous sample, containing the analytes. In this step, the extracting solvent is dispersed into the aqueous sample as very fine droplets and the analytes are enriched into it. Owing to the large surface area between the extracting solvent and the aqueous sample, equilibrium state is achieved quickly and the extraction is independent of time. (2) Centrifugation of cloudy solution. After centrifugation, analytes in the sedimented phase can be determined by analytical instruments (Rezaee *et al.* 2010).

This technique was used by Biparva *et. al* in 2012 for determination of synthetic antioxidants in fruit juice samples. Under the optimum conditions, the method yielded a linear calibration curve ranging from 10 to 2500 µg/L for BHA and 2 to 2500 µg/L for BHT, which was considered a satisfactory result (Biparva *et al.* 2012).

1.3.8 Ultrasound-assisted enzymatic extraction (UAEE)

Ultrasound-assisted enzymatic extraction (UAEE) is a technique that combines two methods that can be used isolated, as mentioned above in the case of ultrasound, for example.

Enzyme macromolecule-ultrasound interaction has a significant effect on the bioprocess efficiency. In addition to the effects of substrate fragmentation and micro-mixing, modification of protein tertiary structure by ultrasound is another influencing factor that enhances enzyme activity (Wu *et al.* 2014).

There are several studies assuming numerous combinations of parameters to use in both methods, combined or individually (Wu *et al.* 2014). The most effective method was achieved when the matrix was exposed to ultrasound and enzymes simultaneously. In this particular case, pumpkin pulp including the compound enzymes, was subjected to sonication with the ultrasonic power of 400 W for 20 min at 51.5°C, followed by compound enzyme inactivation. With this assay were obtained good extraction yields, the optimal yield was 4.33 ± 0.15%.

1.3.9 Simultaneous microwave/ultrasonic assisted enzymatic extraction (SMU-AEE)

This method combines three techniques previously mentioned. According to the authors (Wu *et al.* 2015), was the first time that an equipment was created on which an ultrasonic bath a microwave and an enzymatic digestion were attached for the extraction of antioxidant ingredients, from *Nitraria tangutorum* juice by-products (NJB). It was observed in this study that the antioxidant capacity was 27.62%–190.23% higher than those obtained by traditional extraction methods. The chemical composition assay suggested that the increase of antioxidant capacity in NJB extracts, by SMU-AEE, was achieved with the improvement of the extraction efficiency.

1.3.10 Quick, Easy, Cheap, Effective, Rugged and Safe extraction with a clean-up dispersive solid phase extraction (QuEChERS-dSPE)

The extraction by QuEChERS will be the main subject of the present study. This method emerged by the concern of Anastassiades *et al.* (2003) to find a viable technical and economic alternative for the determination of pesticide residues in fruits and vegetables. Prior to the development of this method, there's no research capable to stablish the minimum factors for a fast and easy extraction, maintaining high recoveries, covering a wide range of analytes, the selectivity and repeatability that a reliable procedure needs (Anastassiades *et al.* 2003). Being so, these authors performed a study to develop a simple, rapid, and inexpensive method that provides high-quality results, but minimizes the number of analytical steps, uses few reagents in small quantities and requires very little glassware. Later on, they made some amendments to the original procedure in order to improve the recoveries of certain pH-dependent pesticides and to expand the spectrum of commodities amenable to the method (Anastassiades *et al.* 2007).

QuEChERS extraction is divided into two stages, an initial single phase extraction with a solvent, followed by salting-out extraction/partitioning with salts and finally a dispersive solid phase extraction to clean the extract (due to possible interferences present as consequence of complex matrices) (Prestes *et al.* 2009).

Silva *et al.* (2012) conducted a study using this methodology which aimed to analyze the presence of low molecular weight polyphenols in eight vegetables (carrot, tomato, broccoli, onion, garlic, green and red pepper, and beetroot) along with the optimization of extraction solvents and sets of extraction salts. As main conclusion they determined that a mixture of acetonitrile and ethyl acetate (50/50) in presence of trisodium citrate dihydrate, disodium hydrogen citrate sesquihydrate, sodium chloride and magnesium sulphate as buffered salts on

the extraction/partitioning and magnesium sulphate, primary-secondary amine (PSA) and C_{18} as clean-up reagents, were the core chemicals to reach the aim of the study.

1.4 Extraction solvents

As many other factors, the choice of the extraction solvent must be done carefully. Among others, this decision should be meticulous and consider the following points:

- Selectivity;
- Extraction capacity of compounds with different polarities;
- Compatibility with various techniques;
- Low cost;
- Ease of handling;
- Environmental “friendly”;

Vilkhu *et al.* (2008) claims that, using conventional stirred extraction, ethanol was significantly less effective than ethyl acetate and butanone. However, applying ultrasound improved the performance of ethanol so much that it was comparable to butanone and ethyl acetate alone. Being so, ultra-sonication may reduce the dependence on a solvent and enable use of alternative solvents that may provide more attractive economic, environmental, health and safety benefits. For example, Chen *et al.* (2015) used ethanol as an extraction solvent and the technique chosen was UAE.
In 2014, Franquin-Trinquier and collaborators planned to extract phenolic compounds from Braeburn apples. They verified that pure methanol was the solvent with the best results, when using PLE, allowing the highest extraction yield, reduction of the extraction time, reduction of the extraction solvent amounts and the increase of the extracted phenolic compounds concentrations when in comparison with standard method.

Cardoso-Ugarte *et. al* (2014), used MAE and founded that by varying the energy applied to the matrix, different results were obtained, so, in this case the solvent wasn’t the main factor influencing the extraction efficiency.

One of the cases where it is actually determining the solvent used is SLE. Peschel *et al.* (2006), firstly, extracted the sample with water, methanol, ethanol, acetone and hexane, with a 10:1 solvent-raw material ratio. Then the plant material selected in the primary screening was separately extracted with ethanol/water (50:50) and acetone/water (80:20). The solvents were chosen because of their high sympathy for molecules containing hydroxyl groups and taking into account economic considerations imposed by the industrial context (Peschel *et al.* 2006).

Another example, studied by Biparva *et al.* (2012), where the choice of solvent is critical is the DLLME for determination of synthetic antioxidants in commercial fruit juice samples. This authors stated that in selection of extraction solvents, some properties must be considered such as (a) don't interfere with the peaks of analytes during direct injection into a chromatographic system, (b) lower density than water, (c) extraction capability of target compounds, (d) low solubility in water and (e) ability to form a stable two-phase system at the presence of a dispersive solvent. Considering these topics, five extraction solvents including hexane, ethyl acetate, hexanol, octanol and 2-Ethyl-1-hexanol were studied for the extraction of two target analytes. The conclusion was that 2-Ethyl-1-hexanol had high extraction efficiency. Despite this, in this method, it was also important to have attention to the volume used in order to perform an efficient extraction, because at excessively low or high volume the extraction efficiency decreases.

In a SMU-AEE, Wu *et al.* (2015) made some test to choose the best solvent (methanol, ethanol, acetone and their aqueous solutions) to extract antioxidant from *Nitraria tangutorun Bobr.* juice by-products (NJB). It was found that the antioxidant capacities of extracts varied significantly among each solvent, although the best result was obtained at 70% ethanol aqueous.

In the QuEChERS extraction made by Silva *et al.* in 2012, in order to get the highest extraction efficiency towards the target low molecular weight polyphenols (LMWPP), different partitioning solvents namely methanol (100%), water (100%), ethyl acetate (100%), acetonitrile (100%) and acetonitrile/ethyl acetate (50:50, v/v) were evaluated. By comparison, it was found that the solvent mixture used was the most efficient solvent for the extraction of target LMW-PPs from carrot samples. Conversely, water was found the solvent with the lowest extraction efficiency for the targeted compounds. Acetonitrile presented an advantage, being easily and effectively separated from the used sample by adding polar substances including buffered salts, namely sodium chloride and sulphate magnesium.

Although the selection of extraction solvent is a major concern also other factors can greatly influence the results. The choice of the parameters involved in extraction must be carefully chosen considering the compounds to be extracted.

2. Materials and methods

In order to evaluate the antioxidant capacity from the fruit extracted two types of extractions were performed (conventional and QuEChERS extraction) with different solvents. To accomplish the quantification of antioxidants in the samples it was necessary to perform an evaluation through methods previously studied and validated by Barroso and collaborators (2011), Benzie & Strain (1996) and Rubilar *et al.* (2007). The developed method was applied in Golden apples.

2.1. Samples and chemicals

2.1.1. Sample preparation

Golden apples were bought in a local grocery and in the supermarket. During sample preparation, apple peel was removed with a steel knife. The pulp was quickly grinded and stored at -20ºC for later procedure.

- **Purchase and sample preparation date: May 14th, 2015**

In Table 2 is listed the data of the Golden Delicious apple bought in a local grocery. These samples were prepared and used in all assays performed by conventional extraction.

Table 2 - Matrix data and treatment applied

Apple specie	Golden Delicious
Color	Green
Treatment applied	Processed into pulp
% Moisture pulp	91.4

- **Purchase and sample preparation date: June 2nd, 2015**

The QuEChERS trials were made with two types of Golden apple: the green golden apples were bought in the same local grocery as the ones used in conventional extraction, and the red Golden apples were bought in a supermarket.

The data of the batch analyzed, is described in Table 3:

Table 3 - Matrix data and treatment applied

Apple specie	Golden Delicious
Color	Green
Treatment applied	Processed into pulp
% Moisture pulp	95.2

- **Purchase and sample preparation date: June 2nd, 2016**

The information of the red apple batch, bought in a supermarket, is listed in Table 4:

Table 4 - Matrix data and treatment applied

Apple specie	Red Delicious
Color	Red
Treatment applied	Processed into pulp
% Moisture pulp	95.3

2.1.2. Chemicals

All the experimental work was performed at room temperature and all the solutions were made with deionized water. The extraction solvents used were methanol (VWR), deionized water, ethyl acetate (Merck), acetonitrile (Merck) and a blend of ethyl acetate with acetonitrile 50/50 (v/v).

To perform the quantification methods was necessary to prepare several solutions and special reagents in order to be able, through different reactions, to quantify the antioxidants. The reagents needed were:

- FRAP (ferric reducing antioxidant power)

  . Ascorbic acid (Merck);
  . Sodium acetate (Sigma-Aldrich);
  . Glacial acetic acid (VWR);
  . Commercial hydrochloric acid 37% (Sigma-Aldrich);
  . 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Fluka);
  . Iron(III) Chloride hexahydrate (Sigma-Aldrich).

With a blend of the sodium acetate with glacial acid acetic and deionized water was made an acetate buffer (pH = 3.6), to prevent pH variations. The commercial version of hydrochloric

acid at 37% was used to obtain a 40 mmol/L. The TPTZ reagent was made by mixing it with the solution of hydrochloric acid. At the end, FRAP reagent was obtained with the blend of the acetate buffer, the TPTZ reagent and the Iron chloride solution.

- TPC (total phenolic content)
  - Gallic acid (Sigma-Aldrich);
  - Folin-Ciocalteu reagent (Sigma-Aldrich) (1:1);
  - Sodium carbonate (Sigma-Aldrich);

- TFC (total flavonoid content)
  - Epicatechin (Fluka);
  - Sodium nitrite (Sigma-Aldrich);
  - Aluminum chloride (Merck);
  - Sodium hydroxide (Merck);

- HPLC
  - Formic acid (VWR);
  - Methanol (VWR).

2.2. Extraction procedure

To be able to quantify the antioxidant capacity from the samples it was necessary to perform the extraction. The obtained extracts were analyzed by spectrophotometric methods (FRAP, TFC and TPC) and by high performance liquid chromatography with ultraviolet detection (HPLC-UV).

2.2.1. Conventional extraction

For the conventional extraction, 10 grams of apple pulp was weighted and placed in a flask with 10 ml of the selected solvent. The flasks were placed in a shaker (Figure 6) (P Selecta – Rotabit) at room temperature for 30 minutes. Several extractions were made in order to use all the five solvents mentioned above in the point 2.1.2., the number of replicates for each solvent was three.

Figure 6 - Shaker (P Selecta Rotabit)

Afterwards, the flasks were removed from the shaker and the solutions obtained were then filtered in the dark with filter paper, in order to separate the liquid phase. The solutions were stored at -20ºC for later analyzes.

2.2.2. Extraction with QuEChERS

This extraction was performed in two steps. Firstly, the extraction itself, and then a clean-up step, to reduce possible interferences (Prestes *et al.* 2009).

In the first step, 7.5 grams of sample were weighted into a 50 mL PTFE tube, subjected to 5 minutes in a vortex and 5 minutes in an ultrasound bath, to prepare the matrix to the subsequent steps.

The extraction solvents mentioned in point 2.1.2 (and containing 1% of formic acid in case of red apples), were then added. The sample was subjected again to 5 minutes in vortex and 5 minutes in ultrasound to homogenize it and to ensure solvent interaction with the entire sample.

Then a salt was added. Two different salts were tested, composed by:

- Salt S1: Magnesium sulphate and sodium acetate (Agilent Bond Elut);
- Salt S2: Sodium chloride, magnesium sulphate, sodium citrate and sodium hydrogencitrate sesquihydrated (Agilent Bond Elut);

After the salt addition, the tube was briefly shaken to prevent the appearance of salt granules before 5 minutes in vortex. Then, the sample was centrifuged for 5 min at 5000 rpm (Thermo Scientific).

For the second step, the clean-up, a salt composed by PSA, C18EC and Magnesium sulphate (Agilent Bond Elut) was used. An aliquot of the supernatant, obtained in the prior step, was transferred to a clean-up tube and subjected to a vortex for 2 minutes. Then it was centrifuged for 2 minutes at 3000 rpm. The supernatant was stored at -20ºC for further analysis.

2.3. Total Phenolic Content – TPC

Total phenolic content was assessed by the Folin-Ciocalteau method according to *Barroso et al.* (2016). A standard curve with gallic acid (Appendix A), in the range of 10 to 200 µg/ml, was made by mixing it with deionized water, *Folin* reagent and, after 6 minutes resting without light, sodium carbonate. The same path was made for the blank and the samples.

The microplate was shaken with a medium velocity for 30 seconds and then, before the analysis at 765 nm, was left to rest for 90 minutes.

The microplate, before the 90 minutes are finished, should present a weak blue color, according the Figure 7.

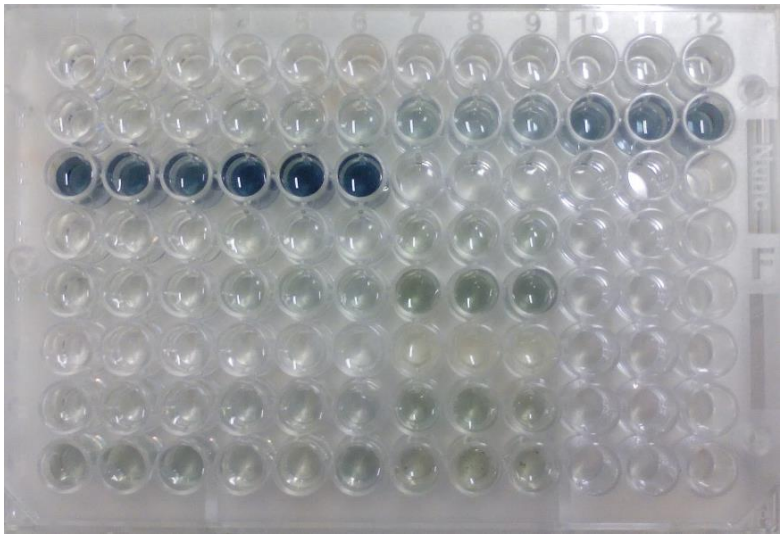


Figure 7 - Assay TPC, microplate before the analysis

After the analysis, and consequently the 90 minutes and the reaction complete, the microplate should present a strong blue color, as the Figure 8.

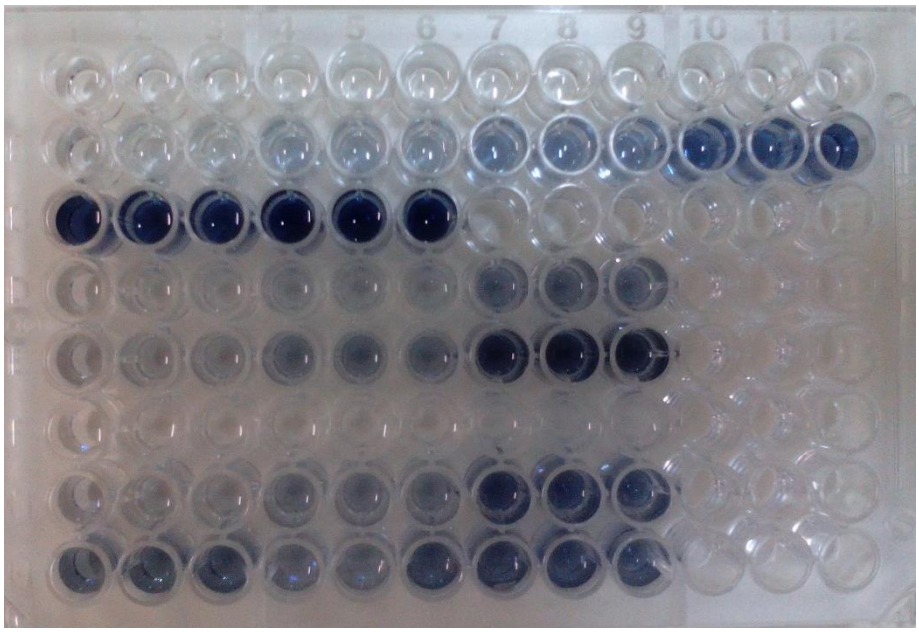


Figure 8 - Assay TPC, microplate after the analysis

2.4. Total flavonoids content – TFC

To measure the flavonoid content in the samples the assay was performed according to Barroso *et al.* (2011). The microplate was prepared and the blanks, the standard curve (Appendix B) (Epicatechin – concentration ranging between 15 to 300 µg/ml) and the samples, were set with the same steps, 100 µL of deionized water, 10 µL of sodium nitrite, 25 µL samples/blank/standard (t = 0 minutes), 15 µL of aluminum chloride (t = 5 minutes) and finally sodium hydroxide (t = 6 minutes).

The microplate was then slowly shaken for 30 seconds and the absorbance read at 510 nm. In TFC determination, the microplate should present a weak coral color and increase the strength, presenting at the end a strong color, as shown in the Figure 9.

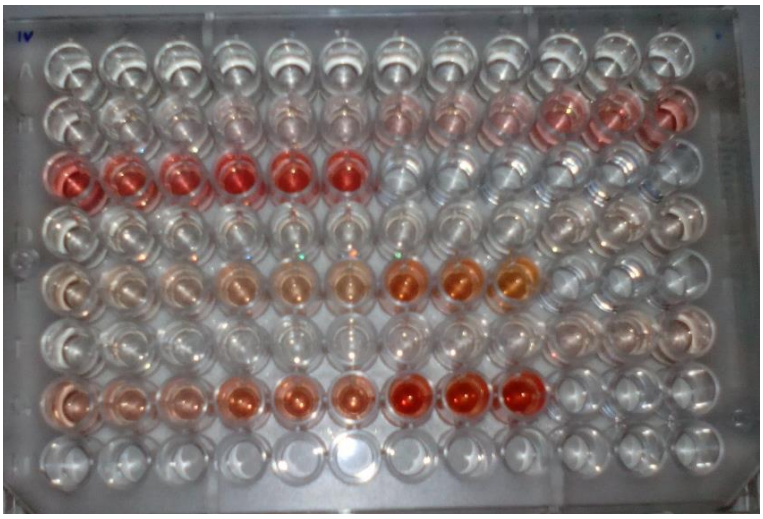


Figure 9 - TFC determination, microplate after analysis

2.5. Evaluation of antioxidant capacity – FRAP

To measure the “antioxidant power” the method chosen was the FRAP assay described by Benzie and Strain (1996) and modified by Barroso *et al*. (2016).

A standard curve, with a range between 5 and 100 µg/ml (Appendix C), was made with ascorbic acid, a strong antioxidant, and the FRAP reagent. In each well of the standard curve, 20 µL of standard and 180 µL of FRAP reagent, were distributed to react. The same procedure was made for the samples. The final aspect of a microplate prepared is shown in the Figure 10:

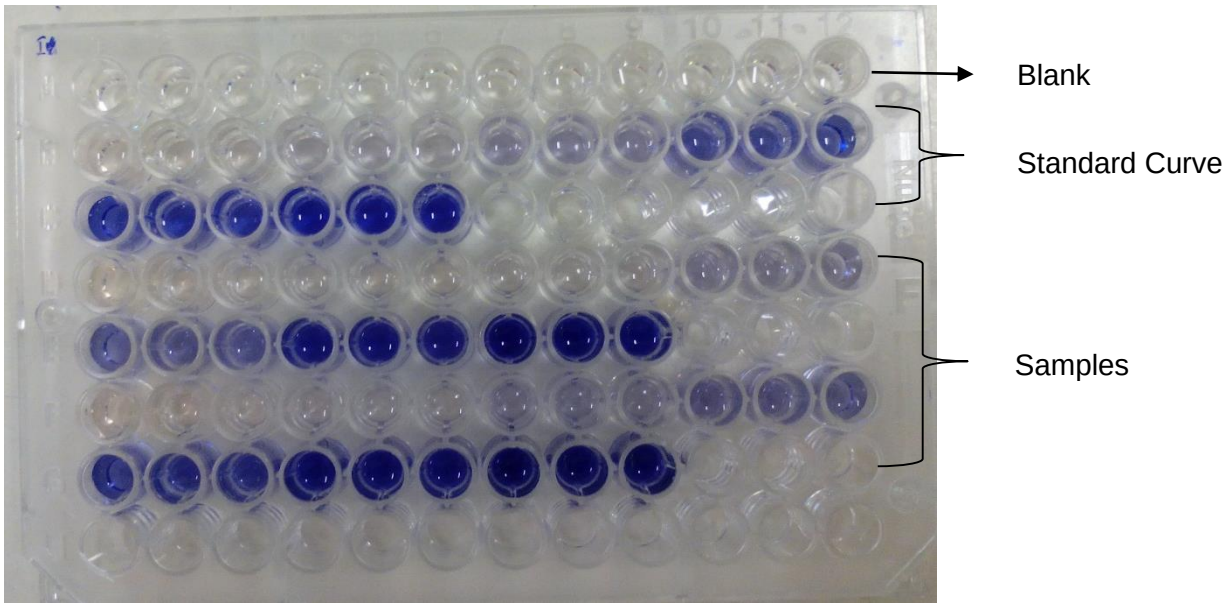


Figure 10 - Microplate ready to analysis (FRAP Assay)

After the plate is finished, is placed in the microplate reader (Biotek Synergy HT), slowly shaken for 30 seconds and the absorbance measured at 593 nm.

2.6. Characterization of phenolic profile by high performance liquid chromatography with ultraviolet detection

The phenolic composition of apple extracts was analyzed by the HPLC method described by Rubilar *et al.* (2007) with slight modifications. The chromatographic conditions for the HPLC assay were set as follows:

- Stationary phase: Gemini C_{18} column (250 mm x 4.6 mm, 5 µm) from Phenomenex.
- Mobile phase: methanol (A) and water (B) both with 0.1% formic acid. The flow rate was 1 $mL \cdot min^{-1}$, a gradient program was used as follows: 85% B in 0 min, from 85% to 70% B in 20 min, from 70% to 55% B in 20 min, from 55% to 50% B in 5 min, from 50% to 45% B in 5 min, from 45% to 30% B in 15 min, from 30% to 0% B in 10 min, followed by 100% A for 5 min and back to 85% B in 10 min and 10 min of reconditioning before the next injection.
- Injection volume: 20 µL.

Quantification of phenolic compounds was conducted at 280 nm for monomeric flavan-3-ols ((+)- catechin and (-)- epicatechin), hydroxibenzoic acids (gallic, vanillic, protocatechuic, syringic and β-resorcylic), naringin, naringenin and cinnamic acid. For the derivates of cinnamic acid (caffeic, chlorogenic, p-coumaric, ferulic and sinapic) at 320 nm and at 360 nm for rutin, quercetin and kaempferol.

2.7. Statistical analysis

Data statistical analysis was performed using a one-way analysis of variance (ANOVA) ($p<0.05$) of Microsoft Excel to assess differences between means.

3. Results and discussion

This chapter presents the results obtained, divided by the type of extraction technique used (conventional or QuEChERS). The solvents employed to carry out the extractions were water, methanol, ethyl acetate, acetonitrile and ethyl acetate/acetonitrile (EA/ACN) (50/50).

3.1. Conventional extraction

Table 5 shows the results of the spectrophotometric analysis (FRAP, TPC and TFC) obtained for the extracts from the conventional extraction procedure. Although the solvents ethyl acetate and EA/ACN were used exactly as the other (on the extraction assay), they are not mentioned or discussed here, due to the unfeasible spectrophotometric results (out of range).

Table 5 - FRAP, TPC and TFC values for the Golden Delicious Apple extracts using conventional extraction techniques

Solvent	TPC		TFC		FRAP	
	(µg GAE/g of sample)*	RSD (%)	(µg EE/g of sample)*	RSD (%)	(µg AAE/g of sample)*	RSD (%)
Methanol	72.0^{a} ± 6.7	9	18.8^{a} ± 0.7	4	40.0^{a} ± 3.1	8
Water	154.4^{b} ± 5.9	4	98.7^{b} ± 7.3	7	71.7^{b} ± 1.4	2
Acetonitrile	132.0^{c} ± 14.1	11	35.5^{c} ± 2.0	6	72.3^{b} ± 4.7	7

AAE – ascorbic acid equivalent; GAE – gallic acid equivalent; EE – epicatechin equivalent; RSD – relative standard deviation; *Values are means of 3 replicates; Values followed by different letters within each column are significantly different ($p < 0.05$)

As observed, water is the solvent that provides the higher results for the three methods tested, followed by acetonitrile and finally by methanol. Within each method, the type of solvent is a parameter that generates statistically different results, except for FRAP, where it is indifferent to use water or acetonitrile. Such conclusions are represented in Figure 11, which shows the performance and the comparison of each solvent, per analytical methodology (FRAP, TPC and TFC).

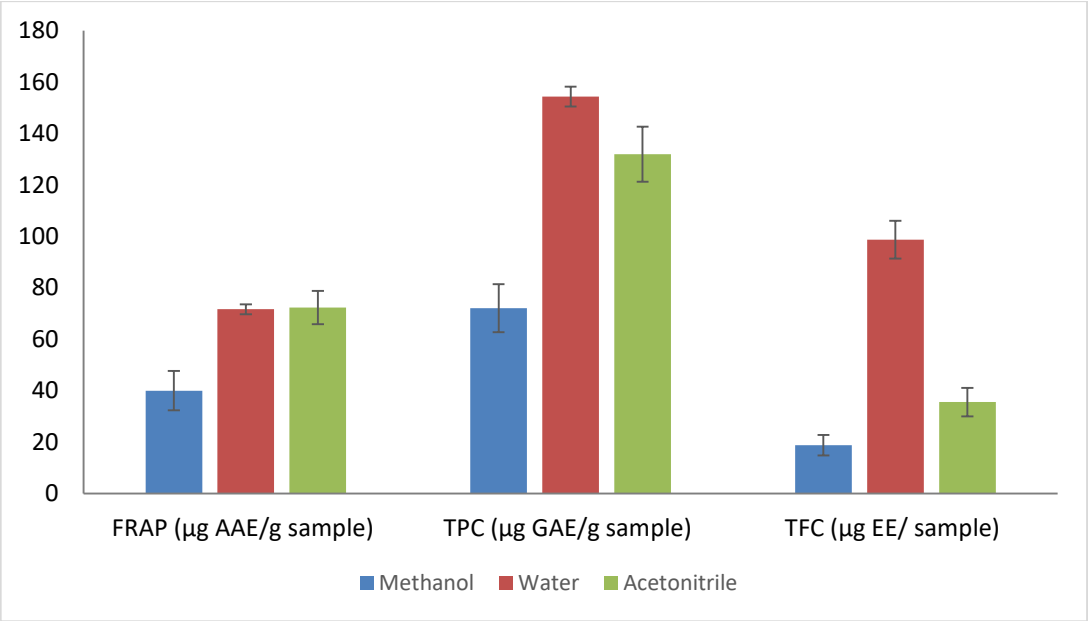


Figure 11 - Comparison between the solvent efficiency performance with the analytical methodology

This data complies with the information reported by Reis *et al*., 2012, where water extracted the highest amount of polyphenolic and flavonoid compounds reaching high TPC and TFC values. These results can be tentatively explained considering the polarity of the different solvents (Iqbal *et al*. 2012). Since water is the solvent which presents the highest polarity, followed by acetonitrile and methanol, it is not surprising that water is the solvent with higher extraction yield for phenolic compounds, as they are known to present polar structures (Iqbal *et al.* 2012).

Normally, as it is reported in the literature, the extraction with organic solvents is used in combination with water, in several proportions, which enhances the extraction of more compounds. For example, Vieira *et al.* (2011) used a mixture of acetone/water (80:20) to perform the extraction and TPC measurement in Golden Delicious specie achieving a TPC value of 128.33 ± 4.51 mg GAE/100 g of sample. In another study performed by Drogoudi *et al.* (2008), using methanol/water (80:20) as extractor, Golden Delicious species presented a TPC level of 3.7 ± 0.1 mg/g of apple. The TPC values presented in the reported study (extraction with 10 ml of solvent at 2000 rpm in a micro-dismembranator followed by centrifuge action), for several species of apples, were higher (0.1 - 11.9 mg/g of sample) than the TPC values obtained in the present study (72.0 – 154.4 µg/g of sample). Considering the literature mentioned, when solvents are mixed the extraction presents higher concentrations of TPC. However, it's important to consider the extraction conditions and the matrix under study, which, normally, affect the final contents achieved.

Concerning the TFC values, the obtained results are in accordance with the results of TPC mentioned before, since the flavonoids are a part of the polyphenols class and the variation within the solvents are consistent too. This values also complied with the work published by Alothman et al. in 2009 for tropical fruits. The TFC results obtained for pineapple, banana and guava using water as extract solvent were 3.34 ± 0.54, 13.7 ± 1.5 and 20.5 ± 4.3 mg of catechin equivalent / 100 g of fresh weight, respectively. Although the antioxidant standard used is not the same (however belongs to the same family) this study revealed that using water for the extractions, the TFC values obtained were higher than when it's used other solvents mainly methanol, ethanol, and acetone in different proportions. TFC results obtained when it was used 90% of methanol were 30 to 70% lower than when it was used only water. In fact, in this work, the differences onto TFC levels when it was used different solvents were 80% higher. This differences can be explained by the slight different in solvents proportion, by Alothman *et al.* (2009) it was used a ratio of 90:10 of methanol:water whereas in this study it was used 100% of methanol.

Regarding the antioxidant capacity evaluation (mean the FRAP methodology), the highest result obtained was when acetonitrile and water were used as extraction solvents, presenting FRAP values of 72.3 ± 4.7 and 71.7 ± 1.4 µg AAE/g, respectively. Although the solvent acetonitrile was not used in the study performed by Reis and collaborators (2012), the difference between the FRAP results obtained when it was used water and methanol as solvents complies with the published work, as with the methanol solvent a lowest FRAP value was observed. Being so, it can be said that the polar solvents used in extraction will enhance the redox reaction to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions.

Considering each method individually, the results obtained comply with the literature observed in general, nevertheless it is important to consider the variation between the solvent structures, and the antioxidants standards employed in the analytical procedures (Vieira *et al.* 2011).

In Figure 12 are presented the HPLC chromatograms at 280 nm of apple extracts obtained after CE with the solvents previously mentioned. The phenolic compounds contributing to the profile of the apple extracts were identified by the comparison of retention time and UV spectra with authentic standards, while the quantitative data was calculated from the calibration curves in the concentration range of 1 to 50 mg/L using a mixture of 18 standards listed in the section 2.6 from chapter materials and methods. Results were expressed as mg/100 g of sample.

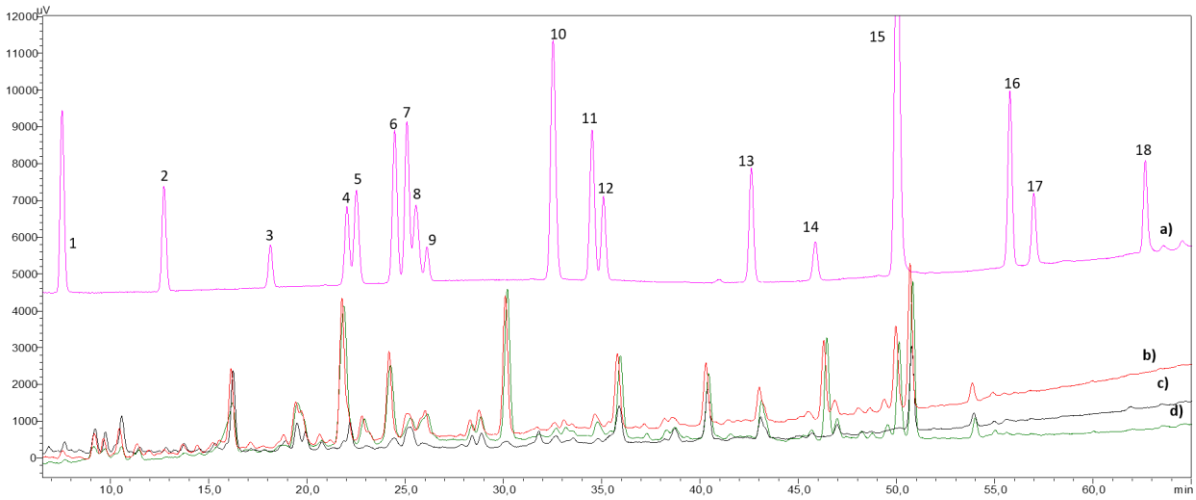


Figure 12 - HPLC chromatogram at 280 nm for a) polyphenols standard mixture of 5 mg/L, and apple extracts after CE with b) methanol, c) water and d) acetonitrile; (1) gallic acid, (2) protocatechuic acid, (3) (+)-catechin, (4) chlorogenic acid, (5) vanillic acid, (6) caffeic acid, (7) (-)-epicatechin, (8) syringic acid, (9) β-resorcylic acid, (10) p- coumaric acid, (11) ferulic acid, (12) sinapic acid, (13) naringin, (14) rutin, (15) cinnamic acid, (16) naringenin, (17) quercetin and (18) kaempfero

The phenolic compounds identified and quantified in apple extracts after CE are listed in Table 6.

Table 6 - Phenolic compounds identified in different extracts from apple after CE; results expressed as mg of compound/100 g dry weight, mean ± standard deviation, n=2, (LOQ, limit of quantification; LOD, limit of detection)

Sample	Compound	Concentration (mg/L)
Methanol	Chlorogenic acid	<LOQ
	Vanillic acid	<LOD
	Caffeic acid	<LOD
	β-resorcylic acid	<LOQ
	Naringin	<LOQ
Water	Chlorogenic acid	<LOD
	Vanillic acid	<LOD
	Caffeic acid	<LOD
Acetonitrile	Chlorogenic acid	<LOQ
	Vanillic acid	<LOD
	Caffeic acid	<LOQ
	β-resorcylic acid	1.94 ± 0.09
	Naringin	0.64 ± 0.03

In the HPLC analysis from the extracts of CE only for the solvent acetonitrile was possible to quantify to compounds the phenolic acid β-resorcylic and the flavonoid naringin. Other compounds, namely from the phenolic acid family were identified, but their quantification was not possible. Probably in a future work it can be made concentration step prior to the HPLC analysis in order to try to quantify a higher number of compounds. There were other peaks that were not identified, which could also be contributing to the phenolic composition from the apple extracts and in a future work it would be very useful if an HPLC analysis with mass spectrometry detection could be made.

3.2. QuEChERS extraction

QuEChERS extraction is a solid-liquid extraction with some modifications where salts are used to enhance the analytes extraction. To perform this extraction, two salts, S1 and S2, were chosen (Silva *et al.* 2012). Both salts have a compound in common, magnesium sulphate, which is used as a drying agent (to remove water from the organic phase), facilitates solvent partitioning and improves recovery of polar analytes (United Chemical Technologies 2016). Besides this, in salt S1 sodium acetate buffer is present, protecting base sensitive analytes from degradation, while salt S2 includes sodium chloride to limit polar interferences and several buffering citrate based reagents to preserve base sensitive analytes (Sigma-Aldrich 2016).

Golden Delicious - Salt S1

In Table 7 are presented the FRAP, TPC and TFC levels when it was used QuEChERS as extraction technique and using a sample batch bought and prepared in 2nd June 2015. For the QuEChERS extractions methanol, water and acetonitrile were used as solvents however only methanol and water were able to achieve results viable to analysis.

Table 7 - FRAP, TPC and TFC values for the Golden Delicious Apple extracts using QuEChERS as extraction technique

Solvent	TPC		TFC		FRAP	
	(µg GAE/g of sample)*	RSD (%)	(µg EE/g of sample)*	RSD (%)	(µg AAE/g of sample)*	RSD (%)
Methanol	128.6^{a} ± 5.0	4	507.6^{a} ± 34.0	7	29.0^{a} ± 2.9	10
Water	505.5^{b} ± 63.7	13	881.6^{b} ± 69.9	8	25.7^{a} ± 2.5	10

AAE – ascorbic acid equivalent; GAE – gallic acid equivalent; EE – epicatechin equivalent; RSD – relative standard deviation; *Values are means of 3 replicates; Values followed by different letters within each column are significantly different ($p < 0.05$)

Figure 13 show the FRAP, TPC and TFC values obtained when it was used as extraction solvent 100 % of methanol or 100 % of water and QuEChERS as extraction technique.

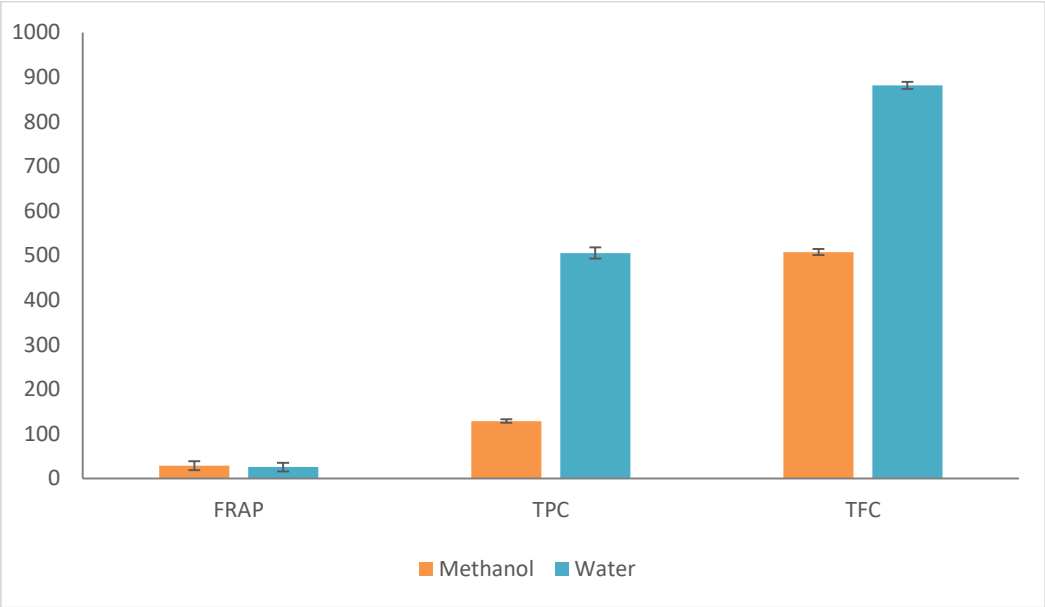


Figure 13 - Comparison between the solvent efficiency using QuEChERS (and Salt 1) and the analytical methodology

In these assays it was tested the salt S1 and two different solvents, methanol or water, moreover the influence of this salt onto the efficiency of the antioxidant extraction.

Following the results of CE, water is shown to be the extraction solvent with the ability to extract the highest amount of antioxidant compounds, proving that its polarity has a great influence on the extraction performed upgraded by the effect of magnesium sulphate from the salt with an improvement in the recovery of polar analytes.

TPC and TFC showed high values in the quantification, this proves that perhaps the constituents of the salts have a greater affinity to phenolic and flavonoid compounds which enhanced the extraction.

Regarding the FRAP values, the results obtained follow the prior ones achieved in CE. Once again, the only difference between the two extraction methods was the addition of salts. AA is a reducing agent however with the addition of salts, the reducing power may decrease (CE: 40.0 – 72.3 µg/g; QuEChERS: 25.7 – 29.0 µg/g) considering that the acidity is a major factor in this reaction kinetics (Fornaro & Coichev 1998) and the salts are composed mostly by buffers which can reduce the antioxidant capacity (FRAP).

In Figure 14 are presented the HPLC chromatograms at 280 nm of apple extracts obtained after QuEChERS extraction with water and methanol.

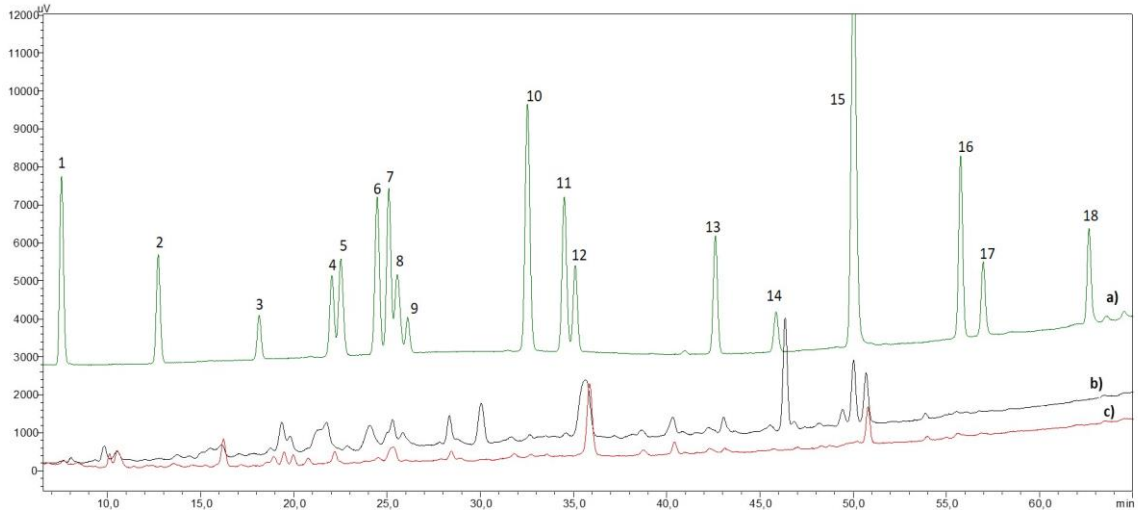


Figure 14 - HPLC chromatogram at 280 nm for a) polyphenols standard mixture of 5 mg/L, and apple extracts after QuEChERS extraction with b) methanol and c) water; (1) gallic acid, (2) protocatechuic acid, (3) (+)-catechin, (4) chlorogenic acid, (5) vanillic acid, (6) caffeic acid, (7) (-)-epicatechin, (8) syringic acid, (9) β-resorcylic acid, (10) p- coumaric acid, (11) ferulic acid, (12) sinapic acid, (13) naringin, (14) rutin, (15) cinnamic acid, (16) naringenin, (17) quercetin and (18) kaempferol

The phenolic compounds identified and quantified in apple samples after QuEChERS extraction with salt S1 are listed in Table 8.

Table 8 - Phenolic compounds identified in different extracts from apple after QuEChERS extraction (LOQ, limit of quantification; LOD, limit of detection)

Sample	Compound	Concentration (mg/L)
Methanol	Chlorogenic acid	<LOD
	Caffeic acid	<LOD
	Naringin	<LOD
Water	Vanillic acid	<LOD
	Caffeic acid	<LOD
	p-coumaric acid	<LOD
	Sinapic acid	<LOD

In the HPLC analysis from the extracts of QuEChERS extraction, none of the compounds could be quantified due to its low concentration. As previously mentioned for the CE apple extracts,

a concentration step prior HPLC analysis could be useful in the possible characterization of the phenolic composition.

Golden Red Delicious – Salt S1 and S2

This extraction technique was described by Anastassiades *et al.* (2003) applied on multiresidue analysis of pesticides in fruits and vegetables using acetonitrile prior to the salts. Some trials were performed with another type of apple to study the influence of this solvent in this matrix. Since the results in Golden Delicious were viable to analysis in CE, an additional test was performed with two salts and acetonitrile as solvent extraction.

In Table 9 is presented the FRAP and TPC values for solvent acetonitrile with 0,1% of AF. The extractions were made with the two salts mentioned in the section 2.2.2. The matrix used was a red apple from the specie Golden Red Delicious (Figure 15). This sample was bought on 30th May, 2016, the sample preparation was made in the same day.

Figure 15 - Red Delicious Apple

Table 9 - FRAP and TPC values for the Golden Delicious Apple extracts using QuEChERS as extraction and two salts

Solvent	Salt	TPC		FRAP	
		(µg GAE/g of sample)*	RSD (%)	(µg AAE/g of sample)*	RSD (%)
Acetonitrile	S1	29.0 ± 0.8	3	14.9^{a} ± 0.1	1
	S2	N.D - N.D	N.D	24.3^{b} ± 2.0	8

N.D – No data; AAE – ascorbic acid equivalent; GAE – gallic acid equivalent; EE – epicatechin equivalent; RSD – relative standard deviation; *Values are means of 3 replicates; Values followed by different letters within each column are significantly different ($p < 0.05$)

A comparison on the FRAP and TPC values when it was used two different salts (S1 and S2) is present in Figure 16.

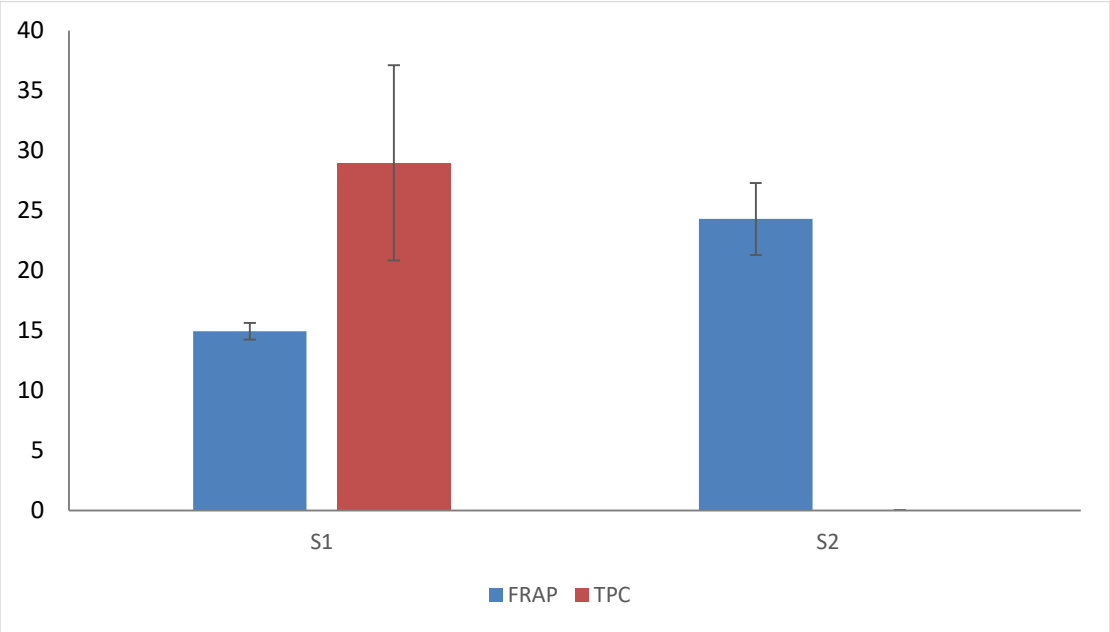


Figure 16 - Comparison between S1 and S2 onto the FRAP and TPC values

The results obtained in this trials show that acetonitrile can extract antioxidants compounds, however, the quantities found are very low. Wang *et al.* (2015), measured the TPC and AA in red apples extracts and concluded that TPC and AA levels were significantly higher for general red apple varieties than for white apple varieties (Gala and Golden Delicious). In fact, specifically in Red Delicious there are almost no data regarding the TPC as noted too by Kalinowska *et al.* 2014.

Despite this, FRAP and TPC results, it was expected that, red apples extracts presented TPC and FRAP values higher than Golden Delicious extracts, however this did not occur when it was used both salts. Maybe, it is possible to conclude that the acetonitrile solvent is not a good solvent to be used in QuEChERS for the antioxidant extraction. Perhaps, due to the lower polarity presented by acetonitrile, when compared with water, and with the effect of the salts acting together the extraction has a lower success, inducing the final results to be much lower than in Golden Delicious.

4. Conclusions and future work suggestions

This thesis intended to develop and optimize extractions of antioxidant compounds in apple matrices by QuEChERS. Apple was chosen due to its large consumption within several fruits. To be able to compare this type of extraction, a conventional extraction (CE) was also performed. To evaluate and quantify the antioxidants, three analytical methods were chosen: TPC, TFC and FRAP.

In comparison, QuEChERS extraction with salt S1 shows a greater efficiency, as can be seen by the FRAP, TPC and TFC values. This lead to one conclusion, effectively the salt S1 was able to exclude interferences enhancing the extraction species of interest. Despite this, acetonitrile was not found to be efficient as solvent in the QuEChERS extraction. Contrary, in CE extraction this solvent was able to extract considerable quantities of antioxidant compounds. The main difference between these methodologies was the salt addition in the QuEChERS extraction. Being so, the results achieved lead to believe that the compounds of the salt may annul the effect of the polarity that the solvent presented in CE, where was the second solvent with the highest efficiency within the different optic methods.

Regarding the other solvents, in both extraction methods, water was the solvent that shows the highest efficiency for this type of compounds. However once again, in QuEChERS extraction the AA and the contents of phenolics and flavonoids were higher than in CE, which proves the promoting action of the salts in the extraction of such compounds.

Considering statistical analysis, in CE it was possible to conclude that, for TPC and TFC, the three solvents under study promoted significantly different results, while in FRAP, the use of water or acetonitrile wouldn't generate these differences. In QuEChERS extraction, the three solvents in study, in all optical methods, were identified as a factor that would produce significantly differences.

Concerning HPLC analysis, in QuEChERS extraction none of the compounds detected (chlorogenic acid, caffeic acid, naringin, vanillic acid, *p*-coumaric acid and sinapic acid) was able to be quantified due to concentrations under LOD or LOQ. In apple extracts from CE, also few compounds were detected and only two of them (naringin and β-resorcyclic acid) were successfully quantified (0.64 ± 0.03 and 1.94 ± 0.09 mg/L, respectively).

It's important to refer that the samples were bought in different places and seasons, and that these parameters can modify the amounts of antioxidants in apples. In fact, some studies point out that the cultivar and harvest may induce some changes in the fruit (Drogoudi et al. 2008), as the phenolic content and the antioxidant activity of fruits seem to be regulated by

environmental and post-harvest factors, including fruit season, fruit maturity, light exposure, storage and processing (Khanizadeh et al. 2008).

As suggestion to future work, it will be useful to compare the results of antioxidants extraction of other different apple species, from different harvest seasons, with other solvents, as well as alternative salt mixtures made especially for that trials.

It would be also very interesting to study were these antioxidant compounds could be applied (e.g. cosmetic, food and pharmaceutical industries).

References

Alothman, M., Bhat, R., & Karim, A. A. (2009). Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry*, *115*(3), 785–788. http://doi.org/10.1016/j.foodchem.2008.12.005

Anastassiades, M., Lehotay, S. J., & Schenck, F. J. (2003). Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and " Dispersive Solid-Phase Extraction " for the Determination of Pesticide Residues in Produce. *Journal of AOAC International*, *86*(2).

Anastassiades, M., Tasdelen, B., Scherbaum, E., & Stajnbaher, D. (2007). *Recent developments in QuEChERS methodology for pesticide multiresidue analysis*. BOOK, Wiley-VCH: Weinheim.

Andrean, D., Prasetyo, S., Kristijarti, A. P., & Hudaya, T. (2014). The Extraction and Activity Test of Bioactive Compounds in Phaleria Macrocarpa as Antioxidants. *Procedia Chemistry*, *9*, 94–101. http://doi.org/10.1016/j.proche.2014.05.012

Asl, a H., & Khajenoori, M. (2013). *Subcritical Water Extraction*.

Barroso, M. F., Noronha, J. P., Delerue-Matos, C., & Oliveira, M. B. P. P. (2011). Flavored Waters: Influence of Ingredients on Antioxidant Capacity and Terpenoid Profile by HS-SPME/GC-MS. *Journal of Agricultural and Food Chemistry*, *59*(9), 5062–5072. http://doi.org/10.1021/jf1048244

Barroso, M. F., Ramalhosa, M. J., Alves, R. C., Dias, A., Soares, C. M. D., Oliva-Teles, M. T., & Delerue-Matos, C. (2016). Total antioxidant capacity of plant infusions: Assessment using electrochemical DNA-based biosensor and spectrophotometric methods. *Food Control*, *68*, 153–161. http://doi.org/10.1016/j.foodcont.2016.03.029

Benzie, I. F. F. F. (2003). Evolution of dietary antioxidants. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *136*(2), 113–126. http://doi.org/10.1016/S1095-6433(02)00368-9

Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of “Antioxidant Power”: The FRAP Assay. *Analytical Biochemistry*, *239*(1), 70–76. http://doi.org/10.1006/abio.1996.0292

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism*, *49*(2), 3–8. http://doi.org/10.1016/S0026-0495(00)80077-3

Biparva, P., Ehsani, M., & Hadjmohammadi, M. R. (2012). Dispersive liquid–liquid microextraction using extraction solvents lighter than water combined with high performance liquid chromatography for determination of synthetic antioxidants in fruit juice samples. *Journal of Food Composition and Analysis*, *27*(1), 87–94. http://doi.org/10.1016/j.jfca.2012.04.002

Carabias-Martínez, R., Rodríguez-Gonzalo, E., Revilla-Ruiz, P., & Hernández-Méndez, J. (2005). Pressurized liquid extraction in the analysis of food and biological samples. *Journal of Chromatography A*, *1089*(1–2), 1–17. http://doi.org/10.1016/j.chroma.2005.06.072

Cardoso-Ugarte, G. A., Sosa-Morales, M. E., Ballard, T., Liceaga, A., & San Martín-González, M. F. (2014). Microwave-assisted extraction of betalains from red beet (Beta vulgaris). *LWT - Food Science and Technology*, *59*(1), 276–282. http://doi.org/10.1016/j.lwt.2014.05.025

Carocho, M., & Ferreira, I. C. F. R. (2013). A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association*, *51*, 15–25. http://doi.org/10.1016/j.fct.2012.09.021

Chen, M., Zhao, Y., & Yu, S. (2015). Optimisation of ultrasonic-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from sugar beet molasses. *Food Chemistry*, *172*, 543–50. http://doi.org/10.1016/j.foodchem.2014.09.110

Dhillon, G. S., Kaur, S., & Brar, S. K. (2013). Perspective of apple processing wastes as low-cost substrates for bioproduction of high value products: A review. *Renewable and Sustainable Energy Reviews*, *27*, 789–805. http://doi.org/10.1016/j.rser.2013.06.046

Drogoudi, P. D., Michailidis, Z., & Pantelidis, G. (2008). Peel and flesh antioxidant content and harvest quality characteristics of seven apple cultivars. *Scientia Horticulturae*, *115*(2), 149–153. http://doi.org/10.1016/j.scienta.2007.08.010

Durance, T. (2002). *Handbook of Food Preservation*. (M. S. Rahman, Ed.)*Food Research International* (Second, Vol. 35). CRC Press. http://doi.org/10.1016/S0963-9969(00)00143-5

Eskilsson, C. S., & Björklund, E. (2000). Analytical-scale microwave-assisted extraction. *Journal of Chromatography A*, *902*(1), 227–250. http://doi.org/10.1016/S0021-9673(00)00921-3

Fornaro, A., & Coichev, N. (1998). ÁCIDO L-ASCÓRBICO: REAÇÕES DE COMPLEXAÇÃO E DE ÓXIDO-REDUÇÃO COM ALGUNS ÍONS METÁLICOS DE TRANSIÇÃO, *21*(5).

Franquin-Trinquier, S., Maury, C., Baron, A., Le Meurlay, D., & Mehinagic, E. (2014). Optimization of the extraction of apple monomeric phenolics based on response surface methodology: Comparison of pressurized liquid–solid extraction and manual-liquid extraction. *Journal of Food Composition and Analysis*, *34*(1), 56–67. http://doi.org/10.1016/j.jfca.2014.01.005

Halliwell, B., & Gutteridge, J. M. C. C. (1995). The definition and measurement of antioxidants in biological systems. *Free Radical Biology and Medicine*, *1*(18), 125–126. JOUR.

http://doi.org/10.1016/0891-5849(95)91457-3

He, L., Zhang, X., Xu, H., Xu, C., Yuan, F., Knez, Ž., … Gao, Y. (2012). Subcritical water extraction of phenolic compounds from pomegranate (Punica granatum L.) seed residues and investigation into their antioxidant activities with HPLC–ABTS+ assay. *Food and Bioproducts Processing*, *90*(2), 215–223. http://doi.org/10.1016/j.fbp.2011.03.003

Hedrick, J. L., Mulcahey, L. J., & Taylor, L. T. (1992). Supercritical fluid extraction. *Mikrochimica Acta*, *108*(3–6), 115–132. http://doi.org/10.1007/BF01242421

Herrero, M., Cifuentes, A., & Ibañez, E. (2006). Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. *Food Chemistry*, *98*(1), 136–148. http://doi.org/10.1016/j.foodchem.2005.05.058

Iqbal, S., Younas, U., Chan, K. W., Zia-Ul-Haq, M., & Ismail, M. (2012). Chemical Composition of Artemisia annua L. Leaves and Antioxidant Potential of Extracts as a Function of Extraction Solvents. *Molecules*, *17*(12), 6020–6032. http://doi.org/10.3390/molecules17056020

Kalinowska, M., Bielawska, A., Lewandowska-Siwkiewicz, H., Priebe, W., & Lewandowski, W. (2014). Apples: Content of phenolic compounds vs. variety, part of apple and cultivation model, extraction of phenolic compounds, biological properties. *Plant Physiology and Biochemistry : PPB / Societe Francaise de Physiologie Vegetale*, *84C*, 169–188. http://doi.org/10.1016/j.plaphy.2014.09.006

Khanizadeh, S., Tsao, R., Rekika, D., Yang, R., Charles, M. T., & Vasantha Rupasinghe, H. P. (2008). Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. *Journal of Food Composition and Analysis*, *21*(5), 396–401. http://doi.org/10.1016/j.jfca.2008.03.004

Kim, A.-N., Kim, H.-J., Kerr, W. L., & Choi, S.-G. (2016). The effect of grinding at various vacuum levels on the color, phenolics, and antioxidant properties of apple. *Food Chemistry*, *216*, 234–242. http://doi.org/10.1016/j.foodchem.2016.08.025

Lü, J.-M., Lin, P. H., Yao, Q., & Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *Journal Of Cellular And Molecular Medicine*, *14*(4), 840–860. JOUR. http://doi.org/10.1111/j.1582-4934.2009.00897.x

Massias, A., Boisard, S., Baccaunaud, M., Calderon, F. L., & Subra-Paternault, P. (2014). Recovery of phenolics from apple peels using CO2+ethanol extraction: Kinetics and antioxidant activity of extracts. *The Journal of Supercritical Fluids*, *98*, 172–182. http://doi.org/10.1016/j.supflu.2014.12.007

Modey, W. K., Mulholland, D. A., & Raynor, M. W. (1996). Analytical Supercritical Fluid Extraction of Natural Products. *Phytochemical Analysis*, *7*(1), 1–15.

http://doi.org/10.1002/(SICI)1099-1565(199601)7:1<1::AID-PCA275>3.0.CO;2-U

Mustafa, A., & Turner, C. (2011). Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. *Analytica Chimica Acta*, *703*(1), 8–18. http://doi.org/10.1016/j.aca.2011.07.018

Penha, M. P. da, Rocha-Leão, M. H. M. da, & Leite, S. G. F. (2012). SUGARCANE BAGASSE AS SUPPORT FOR THE PRODUCTION OF COCONUT AROMA BY SOLID STATE FERMENTATION (SSF). *BioResources*, *7*(2), 2366–2375. http://doi.org/10.15376/biores.7.2.2366-2375

Peschel, W., Sánchez-Rabaneda, F., Diekmann, W., Plescher, A., Gartzía, I., Jiménez, D., … Codina, C. (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chemistry*, *97*(1), 137–150. http://doi.org/10.1016/j.foodchem.2005.03.033

Povilaitis, D., Šulniūtė, V., Venskutonis, P. R., & Kraujalienė, V. (2015). Antioxidant properties of wheat and rye bran extracts obtained by pressurized liquid extraction with different solvents. *Journal of Cereal Science*, *62*, 117–123. http://doi.org/10.1016/j.jcs.2014.11.004

Prestes, O. D., Friggi, C. A., Adaime, M. B., & Zanella, R. (2009). QuEChERS: um método moderno de preparo de amostra para determinação multirresíduo de pesticidas em alimentos por métodos cromatográficos acoplados à espectrometria de massas. *Química Nova*, *32*(6), 1620–1634. http://doi.org/10.1590/S0100-40422009000600046

Progress, A., Prakash, A., Rigelhof, F., & MIller, E. (2011). Antioxidant Activity. *European Review for Medical and Pharmacological Sciences*, *15*(4), 376–378. http://doi.org/10.1016/j.bmcl.2010.12.025

Ramalho, V. C., & Jorge, N. (2006). Antioxidantes utilizados em óleos, gorduras e alimentos gordurosos. *Quimica Nova*, *29*(4), 755–760. http://doi.org/10.1590/S0100-40422006000400023

Rao, S., Sireesha, K., Aparna, Y., & Sadanandam, M. (2011). Free Radicals and Tissue Damage: Role of Antioxidants. *Free Radicals and Antioxidants*, *1*(4), 2–7. http://doi.org/10.5530/ax.2011.4.2

Reis, S. F., Rai, D. K., & Abu-Ghannam, N. (2012). Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds. *Food Chemistry*, *135*(3), 1991–8. http://doi.org/10.1016/j.foodchem.2012.06.068

Reverchon, E., & De Marco, I. (2006). Supercritical fluid extraction and fractionation of natural matter. *The Journal of Supercritical Fluids*, *38*(2), 146–166. http://doi.org/10.1016/j.supflu.2006.03.020

Rezaee, M., Yamini, Y., & Faraji, M. (2010). Evolution of dispersive liquid-liquid microextraction method. *Journal of Chromatography. A*, *1217*(16), 2342–57.

http://doi.org/10.1016/j.chroma.2009.11.088

Rubilar, M., Pinelo, M., Shene, C., Sineiro, J., & Nuñez, M. J. (2007). Separation and HPLC-MS Identification of Phenolic Antioxidants from Agricultural Residues: Almond Hulls and Grape Pomace. *Journal of Agricultural and Food Chemistry*, *55*(25), 10101–10109. http://doi.org/10.1021/jf0721996

Sies, H. (Ed.). (1996). *Antioxidants in Disease Mechanisms and Therapy: Antioxidants in Disease Mechanisms and Therapeutic Strategies* (1997th ed.). Academin Press.

Sigma-Aldrich. Supel™ QuE Multi-Residue Pesticide Analysis in Food and Agricultural Products using the QuEChERS Method. Retrieved March 31, 2016, from http://www.sigmaaldrich.com/catalog/search?interface=All&term=Supel+QuE&N=0&mode=match+partialmax&focus=documents&lang=pt&region=PT

Silva, C. L., Haesen, N., & Câmara, J. S. (2012). A new and improved strategy combining a dispersive-solid phase extraction-based multiclass method with ultra high pressure liquid chromatography for analysis of low molecular weight polyphenols in vegetables. *Journal of Chromatography A*, *1260*, 154–163. http://doi.org/10.1016/j.chroma.2012.08.082

United Chemical Technologies, I. (2016). QuEChERS informational booklet. Retrieved October 10, 2016, from https://sampleprep.unitedchem.com/technical-information/documents/index/?industry_type_filter=default&search=quechers&type_filter=brochures

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*(1), 44–84. http://doi.org/10.1016/j.biocel.2006.07.001

Vendruscolo, F., Koch, F., Pitol, L. de O., & Ninow, J. L. (2007). PRODUÇÃO DE PROTEÍNA UNICELULAR A PARTIR DO BAGAÇO DE MAÇÃ UTILIZANDO FERMENTAÇÃO EM ESTADO SÓLIDO. *Revista Brasileira de Tecnologia Agroindustrial*.

Vieira, F. G. K., Borges, G. D. S. C., Copetti, C., Di Pietro, P. F., Nunes, E. da C., & Fett, R. (2011). Phenolic compounds and antioxidant activity of the apple flesh and peel of eleven cultivars grown in Brazil. *Scientia Horticulturae*, *128*(3), 261–266. http://doi.org/10.1016/j.scienta.2011.01.032

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry — A review. *Innovative Food Science & Emerging Technologies*, *9*(2), 161–169. http://doi.org/10.1016/j.ifset.2007.04.014

Wang, X., Li, C., Liang, D., Zou, Y., Li, P., & Ma, F. (2015). Phenolic compounds and antioxidant activity in red-fleshed apples. *Journal of Functional Foods*, *18*, 1086–1094. http://doi.org/10.1016/j.jff.2014.06.013

Wu, D., Gao, T., Yang, H., Du, Y., Li, C., Wei, L., … Bi, H. (2015). Simultaneous

microwave/ultrasonic-assisted enzymatic extraction of antioxidant ingredients from Nitraria tangutorun Bobr. juice by-products. *Industrial Crops and Products*, *66*, 229–238. http://doi.org/10.1016/j.indcrop.2014.12.054

Wu, H., Zhu, J., Diao, W., & Wang, C. (2014). Ultrasound-assisted enzymatic extraction and antioxidant activity of polysaccharides from pumpkin (Cucurbita moschata). *Carbohydrate Polymers*, *113*, 314–24. http://doi.org/10.1016/j.carbpol.2014.07.025

APPENDIX

Appendix A. Calibration Curves of TPC method

Figure A.1 - Calibration curve for CE (Golden Delicious)

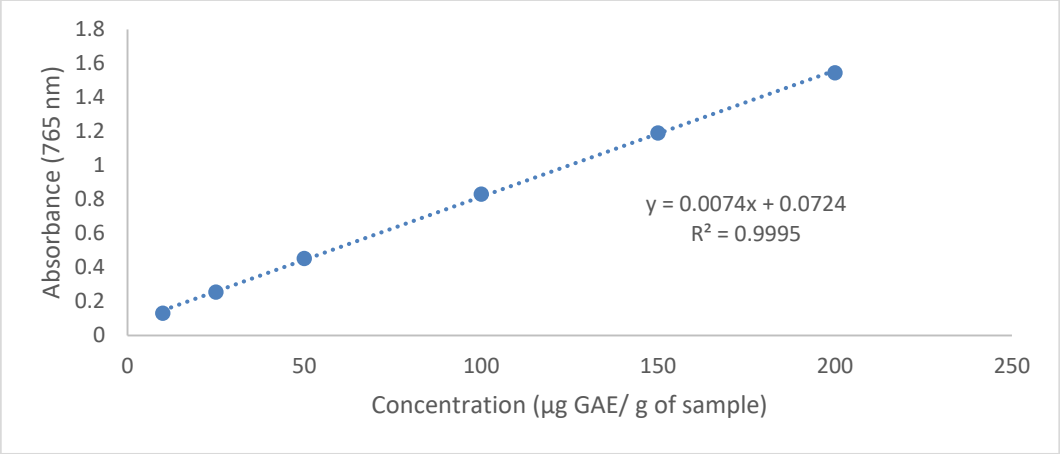


Figure A.2 - Calibration curve for QuEChERS extraction (Golden Delicious)

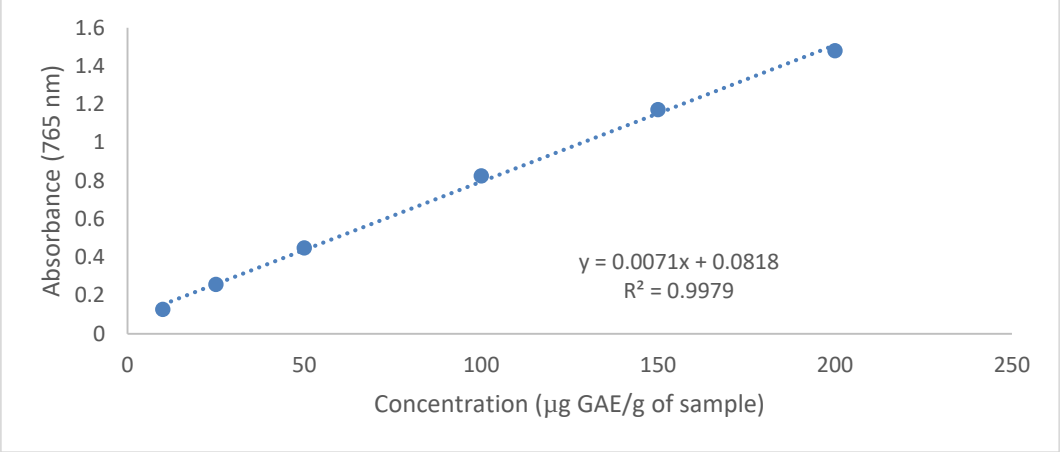
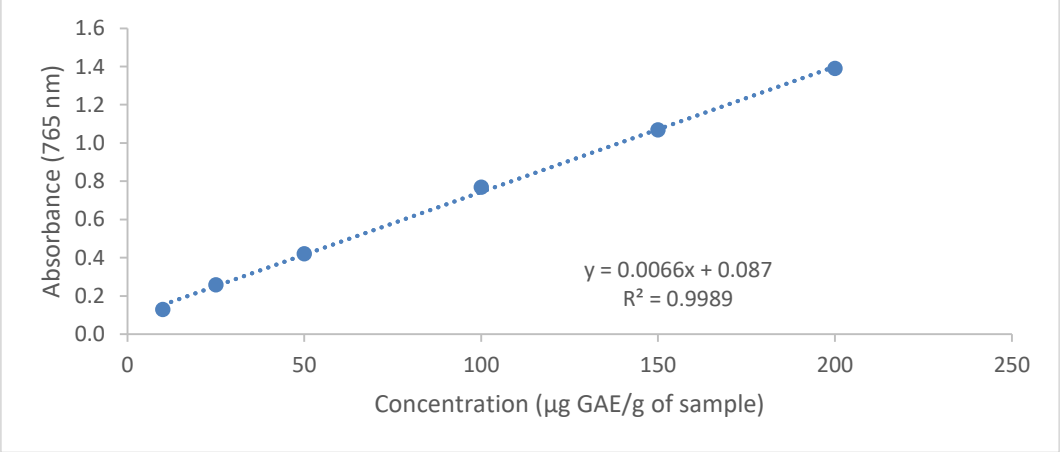


Figure A.3 - Calibration curve for QuEChERS extraction (Golden Red Delicious)



Appendix B. Calibration Curves of TFC method

Figure B.1 - Calibration curve for CE (Golden Delicious)

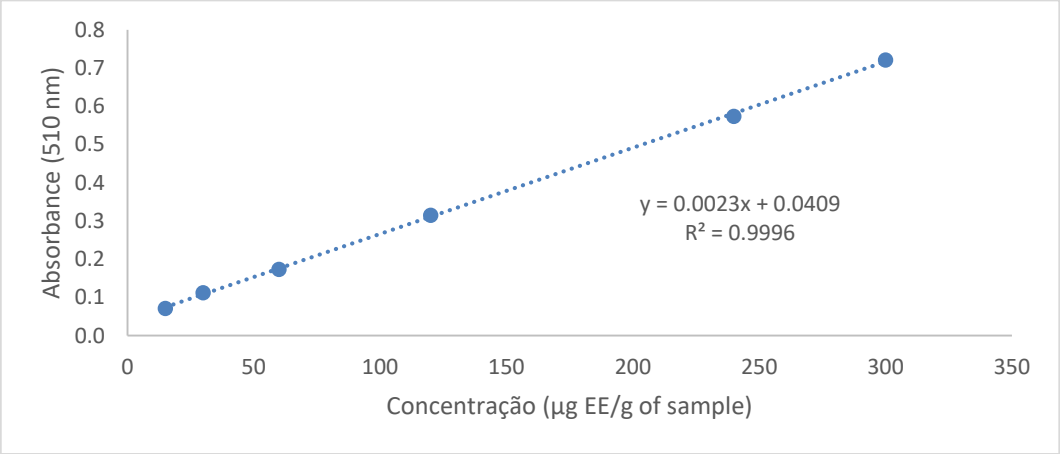
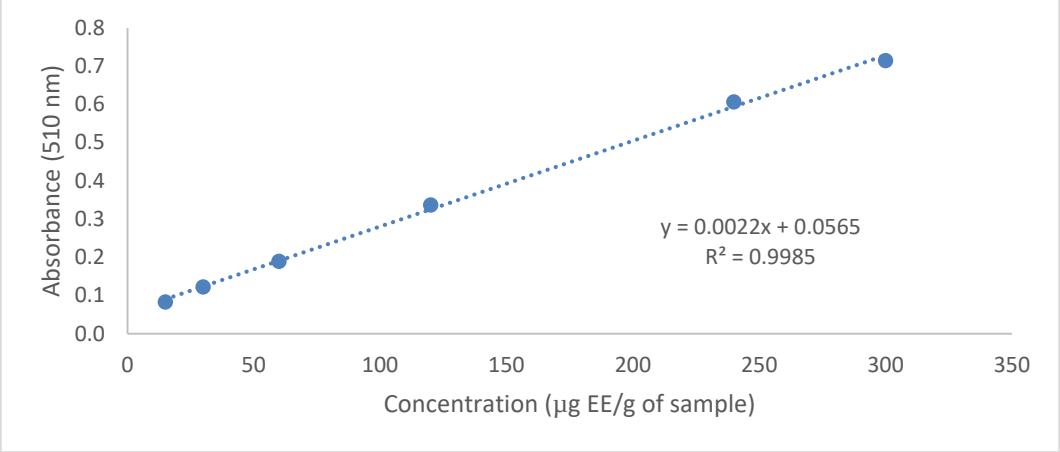


Figure B.2 - Calibration curve for QuEChERS extraction (Golden Delicious)



Appendix C. Calibration Curves of FRAP method

Figure C.1 - Calibration curve for CE (Golden Delicious)

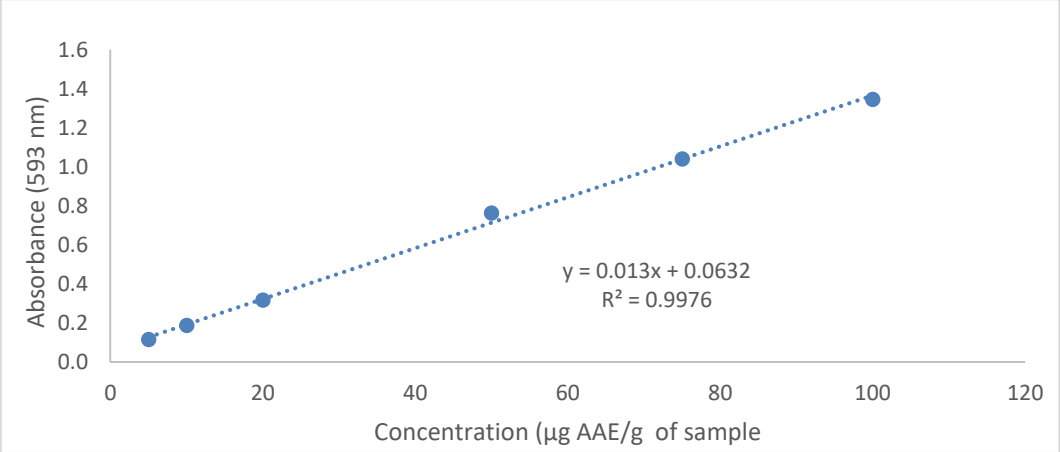


Figure C.2 - Calibration curve for QuEChERS extraction (Golden Delicious)

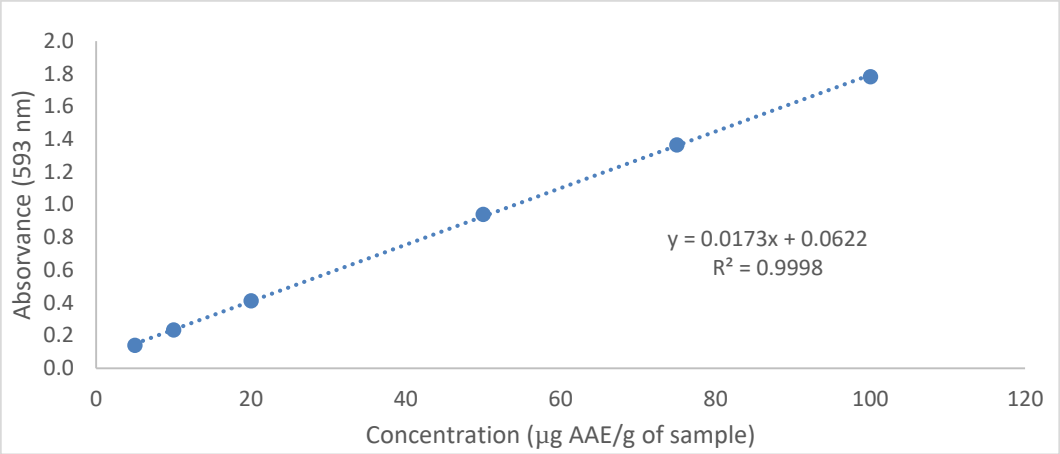


Figure C.3 - Calibration curve for QuEChERS extraction (Golden Red Delicious)