TÉCNICO LISBOA

Optimization of quercetin extraction from onion skin

Determination of antioxidant capacity and anti-diabetic activity

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Thesis to obtain the Master of Science Degree in

Integrated Master in Biological Engineering

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October 2019

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

The work presented in this thesis was performed at the Department of Food Science and Technology at Biotechnical Faculty of University Ljubljana (Ljubljana, Slovenia), during the period February-September 2019, under the supervision of Prof. Lea Pogačnik, and within the frame of the Erasmus+ program. The thesis was co-supervised at Instituto Superior Técnico by Prof. José Santos.

Acknowledgments

I would sincerely like to thank my supervisor Assoc. Prof. Dr. Lea Pogačnik for guidance through the practical work, advice, knowledge, constructive critique and quick responsiveness.

I would also like to thank my co-supervisor, Professor José Santos, for the continuous feedback, high-quality and quick review of this thesis and the help throughout the Erasmus process.

I would also like to acknowledge the Department of Food Science and Technology from the Biotechnical Faculty, University Ljubljana, for giving me the opportunity to perform this thesis at their laboratories and giving the means to perform my research. A special thanks to Assistant Professor Dr. Mihaela Skrt for the knowledge and guidance while using the HPLC/DAD system.

Thank you to my laboratory colleagues Luka, Dejan, Špela, Polona, Miha and Ivona, for helping me with laboratory devices and making me feel integrated at your faculty.

I am also thankful for the opportunity to attend the International Summer School "Food safety and healthy living (FSHL) 2019" in the Transylvania University of Brasov, Romania, where I had the opportunity to learn more about the fields of food safety and antioxidants, which was important in the construction of this thesis' background.

Finally, special thanks to my family and friends for their help and support during the time of this study. During a rather stressful and emotional period due to a medical emergency, my parents (Alice and Vítor) and Dominik provided the support and comfort I needed to overcome it and be able to continue the work of this thesis. I will be eternally grateful to you.

Abstract

In recent years, there has been an increasing concern in reducing the ecological impact of industrial waste caused by fruits and vegetables. In order to contribute to the cost reduction of onion waste disposal, while obtaining value-added products, onion skin can be used to extract quercetin, a naturally present flavonoid with antioxidant, anti-inflammatory and anti-cancer effects [1].

The goal of this study was the optimization of quercetin extraction from brown onion skin (*Allium cepa* L.) through a systematic study of the effects of different parameters on the quercetin yield. The ultrasound-assisted extraction (USAE) and conventional maceration extraction (CME) methods were compared and the operational parameters investigated were: solvent type, mass-to-liquid ratio, extraction time and temperature.

Antioxidant capacity was assessed by DPPH• radical scavenging assay and quercetin yield was determined using high-performance liquid chromatography with a diode-array detector (HPLC/DAD). The anti-diabetic activity of onion skin extracts was also investigated using the α-amylase inhibition assay.

The optimal extraction conditions of quercetin from onion skin were obtained with CME, solvent 50% ethanol, 1:100 mass-to-liquid ratio, extraction time of 15 min and extraction temperature of 25 ℃. Under these conditions, the antioxidant capacity obtained, expressed as trolox equivalent antioxidant capacity (TEAC) was 104.5 μmol/g and the mass fraction of quercetin was 7.96 mg/g. The onion skin extracts exhibited a dose-dependent relation between the concentration of dry extracts and the α–amylase inhibition, confirming that onion skin extracts can be considered anti-diabetic agents.

Keywords: *Allium cepa* L.; Quercetin; Extraction optimization; Antioxidant capacity; Anti-diabetic activity.

Resumo

Ultimamente tem havido uma preocupação crescente em reduzir o impacto ecológico do desperdício industrial de frutas e legumes. Com o objetivo de reduzir o custo da eliminação de desperdícios de cebola e obter produtos de valor acrescentado, as cascas de cebola podem ser utilizadas para extrair quercetina, um flavonoide naturalmente presente com propriedades antioxidantes, anti-inflamatórias e anticancerígenas [1].

O objetivo deste trabalho consistiu em otimizar a extração de quercetina a partir de cascas de cebola (*Allium cepa* L.) através do estudo do efeito de diferentes parâmetros, como o tipo de solvente, relação massa-solvente, tempo e temperatura de extração, no rendimento de quercetina. Os métodos de extração assistida por ultrassom (USAE) e extração de maceração convencional (CME) foram também comparados.

A capacidade antioxidante foi determinada usando o método de sequestro de radicais livres DPPH• e o rendimento em quercetina foi determinado usando cromatografia líquida de alta eficiência com detetor de arranjo de díodos (HPLC/DAD). A atividade antidiabética dos extratos de casca de cebola foi analisada usando o método de inibição de α-amilase.

As condições ótimas de extração de quercetina a partir de cascas de cebola foram obtidas usando CME, 50% etanol, relação massa-solvente 1:100, 15 min e 25 ℃. Para estas condições, a capacidade antioxidante obtida, expressa em equivalentes de trolox (TEAC), foi de 104,5 μmol/g e a fração mássica de quercetina foi de 7,96 mg/g. Os extratos exibiram uma correlação dependente da dose entre concentração e inibição de α–amilase, confirmando que os extratos podem ser considerados agentes antidiabéticos.

Palavras-chave: *Allium cepa* L.; Quercetina; Optimização de extração; Capacidade antioxidante; Atividade antidiabética.

Contents

List of Tables

List of Figures

List of Abbreviations

∆A	Absorbance
AA	Acetic acid
AMPK	AMP-activated protein kinase
AU	Area units
CE	Capillary electrophoresis
CME	Conventional maceration extraction
DAD	Diode-array detector
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalicylic acid
EA	Ethyl acetate
ET	Electron transfer
EtOH	Ethanol
GC-MS	Gas chromatography coupled with mass spectrometry
GRAS	Generally-Recognized-as-Safe
HAT	Hydrogen atom transfer
HPLC	High-performance liquid chromatography
MAE	Microwave-assisted extraction
MeOH	Methanol
MIPs	Molecularly imprinted polymers
MT	Metric ton
MW	Molecular weight
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SFE	Supercritical fluid extraction
SWE	Subcritical water extraction
TEAC	Trolox equivalent antioxidant capacity
USAE	Ultrasound-assisted extraction
UV	Ultra-violet

Chapter 1

Introduction

This chapter gives a brief overview and current state-of-the-art concerning polyphenols and more specifically quercetin proprieties. The recent extraction techniques of quercetin are presented, as well as the methods used to identify and quantify it, and the applications and challenges involved in the commercial use of this compound.

1.1 Scope of the work and hypothesis

Recently there has been a concern in finding feasible and green alternatives for the recovery and conservation of bioactive compounds from industrial wastes of fruit and vegetable processing plants. The goal being to contribute to the cost reduction of waste disposal and improve sustainable productions, while obtaining added value products.

The quality of extracts and contents of active ingredients are deeply influenced by factors such as extraction procedure, solvent used for extraction and solvent ratio. Therefore, to obtain high efficiency for medicinal plant extraction, it is necessary to optimize extraction procedures [2].

The aim of this study was therefore to optimize the extraction of quercetin, a flavonol with powerful antioxidant capacity, from onion skin through a systematic study of the effects of different parameters on the quercetin yield and comparing ultrasound-assisted extractions (USAE) with conventional maceration extraction (CME).

A range of operational parameters was investigated for both USAE and CME extraction methods: solvent type, onion skin mass-to-solvent volume ratio, and extraction time and temperature. Antioxidant capacity was assessed with DPPH• radical scavenging assay and quercetin yield was determined using high-performance liquid chromatography with a diode-array detector (HPLC/DAD). The anti-diabetic effects of onion skin extracts was investigated using the α-amylase inhibition assay.

The research hypothesis of this work is:

- The extraction yield is influenced by the extraction solvent, extraction method, extraction time, temperature and mass-to-liquid ratio.
- The extracts possess antioxidant capacity as well as anti-α-amylase activity.
- Besides quercetin there are other bioactive compounds present that contribute to antioxidant capacity and anti-diabetic properties of extracts.

1.2 Literature Review

Brown onion, also known as yellow onion (*Allium cepa* L.) is a biennial herbaceous originating from the territory of western and central Asia. In recent years, the production of onion has increased around the world at least by 25%, reflecting its medicinal and nutritive value. In the European Union, 500 000 MT of onion waste is produced annually (comprising stalk, skin, small and damaged onions), which represents an ecological problem. The onion waste is disposed because it is not suitable to be used as animal feed. However, onion skin can be used to extract naturally present bioactive compounds, such as quercetin, a strong antioxidant from the flavonoids group [1].

1.2.1 Antioxidants

Oxidative stress occurs when there is an excess of free radicals, which results in oxidative alteration of biological macromolecules such as lipids, proteins and nucleic acids. It is considered to play a pivotal role in the pathogenesis of aging and degenerative diseases. In order to cope with oxidative stress, human bodies have developed sophisticated mechanisms for maintaining redox homeostasis, such as scavenging or detoxification of reactive oxygen species (ROS), sequestration of transition metals, as well as antioxidant defenses produced in the body (endogenous) and supplied with the diet (exogenous). The last ones are usually inserted in the diet through plants, since these have an innate ability to biosynthesize a wide range of non-enzymatic antioxidants in order to protect themselves from microbial pathogens and animal herbivores and to respond to environmental stress conditions [3].

Dietary polyphenols have been widely studied for their strong antioxidant capacities and other properties by which cell functions are regulated. They represent a group of secondary metabolites which widely occur in fruits, vegetables, wine, tea, extra virgin olive oil, chocolate and other cocoa products [4].

Flavonoids are the most abundant polyphenols in human diet and possess a wide spectrum of biological activities: antioxidant, immunostimulating, anti-cancer, cardio- and hepatoprotective, antithrombotic, anti-allergic, anti-inflammatory and antiviral effect. Figure 1.1 represents the basic structure of flavonoids.

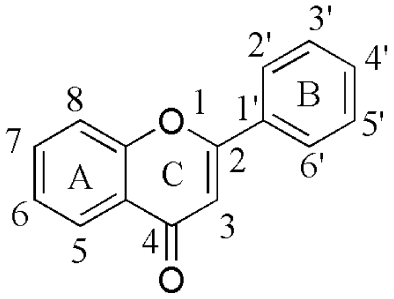

Figure 1.1: Basic skeleton of flavonoids and representation of rings A, B and C [5].

These molecules are heterocyclic oxygen-containing compounds, including two benzene rings (A and B) connected with each other via a three-member carbon fragment (C), usually looped via oxygen [6]. Flavonoids are mainly divided into anthocyanins, present in colorful flowers and fruits and anthoxanthins, which are a group of colorless compounds further divided in several categories, including flavones, flavans, flavonols, flavanols, isoflavones, and their glycosides [4].

1.2.2 Quercetin

Most of flavonoids are flavonols and among these, quercetin is the dominant one. Quercetin (molecular weight 302.25 g/mol) is found in many medicinal plants, fruits and vegetables and is mostly present in the leaves of green tea (255.55 mg/100g), black tea (204.66 mg/100g), red onions (19.93 mg/100g), brown onions (13.27 mg/100g) and cranberries (14.02 mg/100g) [6]. It is known that dry outer skin of brown onion is one of the richest sources of free quercetin, while in other plant tissues quercetin is present as glycosides only [7]. Quercetin is also 77-times more plentiful in the inedible parts of onions than in the edible parts [8].

Quercetin is recognized as an integral part of a healthy diet: its daily dose ranges from 4 to 68 mg. It is introduced into the composition of many dietary supplements and certain medications [6]. It has beneficial effects on human health because of its antioxidant, anti-inflammatory, antimicrobial, antiviral,

anti-allergic, cardioprotective, vasodilatory and anti-cancer activity [1]. It also stabilizes cell membranes, inhibits the aging process of skin, cornea, and myocardium and positively affects the function of the cardiovascular system [6]. It has also been claimed that quercetin reduces blood pressure in hypertensive subjects [8].

Quercetin's structure can be observed in Figure 1.2.

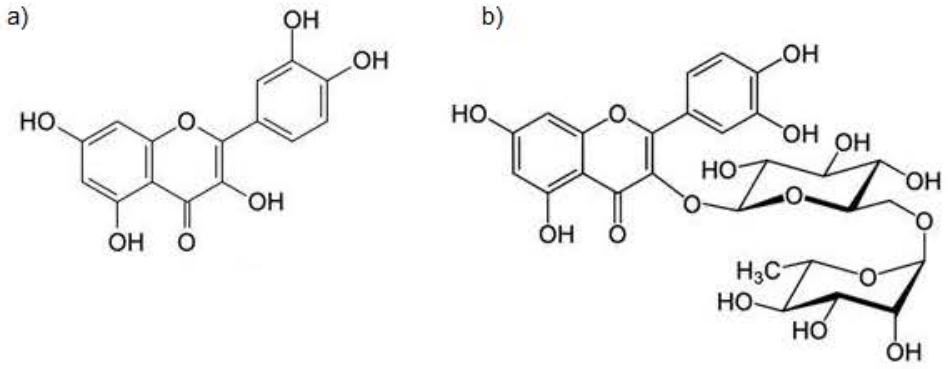

Figure 1.2: Chemical structures of (a) quercetin and (b) of its glycoside rutin [9].

The antioxidant capacity of quercetin is ascribed to: (a) a catechol group in the B-ring; (b) a 2,3-double bond in conjugation with a 4-oxo function in the C-ring; and (c) -OH group at positions 5 and 7 in A-ring [10]. One of the functions of the catechol moiety in the B-ring is the possible chelation of transition metal ions that may otherwise cause radical oxygen species formation. The unsaturated bonds localized in the C-ring act enhancing the electron-transfer and radical scavenging actions through electron-delocalization. Finally, the presence of -OH groups in the A-ring enables the formation of stable quinone structures upon flavonoid oxidation [11].

Quercetin often occurs in nature not only in its free form but also in the form of glycosides, in which one or more hydroxyl group is replaced by different types of sugar groups. Among 180 different glycosides of quercetin, the most common is rutin (quercetin-3-rutinoside), where the disaccharide rutinose is present in the position 3 of ring C, as represented in Figure 1.2.

1.2.3 Extraction methods of quercetin

Extraction procedures of quercetin and its glycosides from plant materials have been intensively developed and optimized in recent years. The most common methods of extraction in the literature are conventional maceration extraction (CME), ultrasound-assisted extraction (USAE) and microwave-assisted extraction (MAE) [1].

Given that quercetin exists in the glycoside and aglycone forms, extraction needs to be performed in the presence of a mineral acid (e.g. hydrochloric acid) to assure hydrolysis of the glycoside bonds [1].

The description of some of the most known extraction methods is presented bellow:

- CME: this method is used to extract quercetin from fruits, vegetables, and other plant materials. A weighed portion of the crushed solid sample is placed in a vessel, the selected solvent is added, and the mixture is stirred for a certain time, at room temperature or with gentle heat (digestion). The phases are separated by filtration. One advantage of this method is the non-requirement of special equipment and the main disadvantages are the high time consumption (from hours up to several days) and the use of large solvent volumes [8] [2].

- USAE: solid particles are vibrated, biological membranes are collapsed and extractable compounds are released into the solvent under ultrasonic waves. The main advantages of this method are the decrease in reaction time and its simplicity, while the disadvantage is that in some cases, active ingredients could be decomposed by ultrasound waves [2].

- MAE: this method rapidly delivers the energy both to the overall volume of solvent and to the solid matrix of the plant. Because water within the plant matrix absorbs microwave energy, the internal superheating promotes cell disruption, which facilitates desorption of chemicals from the matrix. Its main advantages are being efficient and homogeneous [12].

- Soxhlet method: the finely powdered plant is placed in a cellulose pocket in an extractor which is placed between a flask and a reflux condenser. The extracting solvent is added to the flask and heated in order to reflux and continuously extract the extractable compounds. The main disadvantages are the use of large amounts of solvent and thermal decomposition of the target compound [2].

- Supercritical fluid extraction (SFE): can be used to significantly improve the efficiency of the extraction of unstable compounds from plant materials. Supercritical carbon dioxide is usually used and since the polarity of CO_2 is low, a polar modifier, such as ethanol, is often added.

- Subcritical water extraction (SWE): Water is normally used at a temperature below the critical value of water (374 ℃) but above 100 ℃, at a pressure that is sufficiently high ($>$40 bar) to maintain the liquid state. Its main advantages are temperature-dependent selectivity, efficiency, lower cost, environmentally acceptable and safe since it eliminates the use of organic solvent [8].

- Traditional adsorption methods: Recently adsorption pre-concentration methods have been increasingly applied, often carried out under dynamic conditions by passing or pumping the test solution through a microcolumn, a pre-concentrating cartridge or a disc. These are filled with a relatively small amount of adsorbent (solid-phase extraction) and the most used adsorbents are silica gels modified with hydrophobic alkyl groups. The advantage of this methods are the need for less solvent for the subsequent desorption of the compounds and thus less need for evaporation; and the convenience of using commercially available cartridges and microcolumns.

- Novel adsorption methods: In recent years, molecularly imprinted polymers (MIPs) have been increasingly used for the selective adsorption of quercetin and other flavonoids. MIPs allow the possibility of non-covalent interactions between the functional monomer and the functional groups of quercetin, leading to the formation of a stable associate of monomer–template. The most common functional monomers are 4-vinylpyridine, acrylamide, meth-acrylic acid and 2-(dimethylamino)ethyl methacrylate [6].

The solubility of organic compounds in different solvents also plays an important role in their separation and purification applications. Studies have indicated that quercetin displays an amphipathic behavior with two phenyl rings forming the hydrophobic part of the molecule and the hydroxyl groups constituting the polar portion [13].

Quercetin is therefore not soluble in water, partially soluble in ethanol and soluble in acetic acid and alkali, among others [6] [14]. The most common way of conducting quercetin extraction is using ethanol or aqueous-based ethanol and methanol solutions. Other solvents such as ethyl acetate and dimethylformamide are also used [8].

Regarding the solubility in ethanol and methanol solutions, experimental data show that: at a constant temperature, the solubility of quercetin in water and methanol mixtures and water and ethanol

mixtures increase with increasing methanol and ethanol contents; the solubility of quercetin in both mixtures increase smoothly with increasing temperature; and the highest solubility is obtained when a water and ethanol mixture is used as solvent [15].

The high extraction yields of quercetin for ethanol and methanol aqueous solutions can be explained by a balance between lipophilic and hydrophilic properties of mixed solvents. The increase in the water percentage results in a higher solubility of the more hydrophilic glucosides, whereas higher ethanol fraction enhances the solubility of the more lipophilic aglycone. Besides, a certain amount of water provided by the aqueous part is necessary for effective swelling of plant tissues, which helps to increase the surface area for solid-solvent contact [16].

It is important to note that although ethanol is classified as a Generally-Recognized-as-Safe (GRAS) solvent, its utilization in this application is restricted by the long extraction time and the strict legal statutes that exist in many countries [8].

Several studies regarding optimization of quercetin extraction from onion skin were performed recently in order to increase its yield.

Horbowicz (2002) [7] concluded that 4 hours extraction by shaking with cold ethyl acetate is an effective, fast and simple method of isolation crude quercetin from dry onion skin with a purity of 70%. The extraction with ethanol solutions resulted in oily crude quercetin, containing up to 78% of contaminants, therefore needing further purification.

Jin *et al*. (2011) [12] optimized various procedures such as CME, USAE and microwave assisted extraction (MAE) using response surface methodology (RSM). The highest quercetin yield for CME (3.42 mg/g) was obtained for the extraction time of 16.5 min, the temperature of 59.2 ℃ and 59.3% ethanol. However, the most productive method was MAE, in which the maximum extraction yield was 20.3 mg/g and 30.8% higher than USAE and CME, respectively.

Jang *et al*. (2012) [16] investigated extraction under sonication (USAE) conditions, using rational experimental design methodologies. This study concluded that ethanol concentration and temperature are the most influential parameters compared to the remaining parameters studied, such as pH, mass-to-liquid ratio and extraction time. The quercetin mass fraction obtained in this study was 11.08 mg/g of the dry weight of onion solid waste, for the optimal conditions (59% ethanol, 49 ℃, pH 2, 1:60 mass-to-liquid ratio and 35 min).

Savic-Gajic *et al*. (2018) [1] obtained the optimal extraction conditions for 47.3 min using 80% ethanol (pH 1.0) and a liquid-to-solid ratio of 64 mL/g (which is equal to a mass-to-liquid ratio of 1:64). The quercetin content in these extracts was 28.5 mg/g of the dry plant material.

Min-Jung *et al*. (2011) [8] studied the extraction using SWE and the maximum yield of quercetin obtained was 16.29 mg/g of onion skin for 165 ℃ and 15 min. It was also concluded that the quercetin yield obtained by SWE was over eight-, six-, and four times greater than those obtained using the ethanol, methanol, and water-at-boiling-point extraction methods, respectively.

Yoon *et al*. (2004) [17] studied membrane processing as a downstream processing technique for the recovery of quercetin after extraction from onion skin with 60% ethanol. The results suggested that membrane processing with ultrafiltration and reverse osmosis could be successfully used for partial purification and concentration of quercetin from onion skin.

1.2.4 Determination methods

Antioxidants

Several *in vitro* antioxidant capacity assessment methods are often used to screen and confer antioxidant potential of plant extracts. Based on the inactivation mechanism, antioxidant capacity assessment methods are classified into hydrogen atom transfer (HAT) and electron transfer (ET) reaction-based

methods. HAT-based methods measure the ability of an antioxidant to scavenge free radicals via hydrogen donation to form stable compounds. SET-based methods measure the ability of an antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and free radicals. Some methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•) assays involve both mechanisms. Another common assay is the total phenolic content assay by Folin-Ciocalteu reagent, which is a ET reaction-based method.

Within *in vivo* assays, plant antioxidants are generally assessed for their effects on the activity of endogenous antioxidant enzymes or oxidative damage biomarkers before and after induction of oxidative stress in experimental animals. The formation of specific end products resulting from interaction of ROS with biologically important macromolecules such as DNA, protein and lipids is measured by quantifying oxidative damage biomarker methods [3].

Quercetin

The main methods for the determination of quercetin are spectroscopic, chromatographic, and electrophoretic. To determine quercetin in samples of a relatively simple composition, such as pharmaceutical preparations and dietary supplements, spectrophotometry and luminescence are often used, whereas in natural samples and biological fluids, this compound is determined using high-performance liquid chromatography (HPLC), gas chromatography coupled with mass spectrometry (GC-MS), and capillary electrophoresis (CE). In addition, various electrochemical methods have been increasingly used in recent years to determine quercetin.

HPLC in combination with different detection methods is one of the most used and effective methods for separation, identification, and determination of flavonoids. Usually reversed-phase HPLC is used and various hydrophobic adsorbents with immobilized alkyl radicals are applied as stationary phases. As a rule, binary solvent systems like acetonitrile-water or methanol-water, containing different amounts of the organic component, are used as the stationary phases. To suppress the dissociation of flavonoids with low values of pKa, formic, acetic, or phosphoric acid is introduced in the mobile-phase. The use of gradient elution allows a rapid separation of complex mixtures of flavonoids. HPLC can have different types of detectors: DAD (diode-array detector), UV-VIS (photometric detector), MS (mass-spectrometric detector), C (coulometric detector), and Amp (amperometric detector).

Capillary electrophoresis can be used for the determination of quercetin and other flavonoids, usually with an amperometric detector. The advantages of the method are the high efficiency of separation, rapidness, simplicity, small sample volume and a lower consumption of reagents. This method is usually applied after the pre-concentration by solid-phase extraction [6].

1.2.5 Diabetes and α-amylase

Diabetes mellitus (DM) is a chronic metabolic disorder which results in disturbances of carbohydrate, protein and lipid metabolism, due to either a lack of insulin secretion (type I) or increased cellular resistance to insulin (type II). Under normal physiological conditions, blood glucose levels are tightly regulated by the secretion of insulin by specialized β-cells in pancreas Langerhans's islets.

Early treatment and prevention play a pivotal role in reducing burden of diabetes on the population. Lifestyle changes, such as exercising and dietary pattern modifications, are recommended, but these behavioral measures are difficult to maintain in the long term. The benefits of pharmaceutical factors to treat the disease aggressively in its early stages have been recommended, but medications may have unwanted side effects. In this context, flavonoids, among which quercetin, have been reported to improve diabetic status [18].

Based on several *in vitro*, animal models and some human studies, dietary plant polyphenols and polyphenol-rich products modulate carbohydrate and lipid metabolism, attenuate hyperglycemia, dyslipidemia and insulin resistance, improve adipose tissue metabolism, and alleviate oxidative stress and stress-sensitive signaling pathways and inflammatory processes.

Among the many hypoglycemic effects of polyphenols, one of them is the inhibition of α-glucosidase and α-amylase, key enzymes responsible for digestion of dietary carbohydrates into glucose. Polyphenols, by inhibiting these enzymes, delay carbohydrate digestion which results in a decrease in glucose absorption thereby reducing the postprandial plasma glucose rise [19]. In fact, one of the therapeutic targets currently introduced in the management of type II DM is inhibition of these two enzymes to decrease the re-absorption of glucose in the intestine.

α-amylase (α-1,4-glucan-4-glucanohydrolases) is a prominent secretory product of the pancreas and salivary gland responsible for the initial step in the hydrolysis of complex carbohydrate to a mixture of oligosaccharides and disaccharides in the intestinal mucosa. These sugars are further digested to monosaccharide by the action of α-glucosidase [20].

Phenolic compounds such as flavonoids bind covalently to the 4-oxopyrane structure of α-amylase via the hydroxyl groups of ring B, and change its activity due to the ability to form quinones or lactones that react with nucleophilic groups on the enzyme molecule [20]. These bonds can happen with the polar groups in the allosteric site which is close to the catalytic site. The results of this interaction would change the enzyme's molecular configuration and its hydrophilic and hydrophobic properties, causing a decrease in enzyme activity [21].

The current α-amylase and glucosidase inhibitors in clinical use are associated with side effects such as hypoglycemia, diarrhea, flatulence, and bowel bloating that limit their use in the treatment of diabetes and its complications. There is, therefore, an urgent need to search for complementary and alternative therapies with minimal side effects that can serve as an alternative to the management of diabetes [20].

Previous scientific studies regarding this subject related polyphenols to anti-diabetic effects. Bahadoran *et al*. (2013) [19] summarized the current knowledge on the impact of polyphenols, and specifically quercetin, in DM. It was concluded that quercetin can decrease the intestinal absorption of glucose via inhibition of Na^{+}-dependent glucose transporters, SGLT1 and SGLT2. This decrease of intestinal absorption of the dietary carbohydrate helps to improve glucose homeostasis and insulin resistance. *In vitro* studies also show that quercetin improved insulin-dependent glucose uptake in muscle cells and adipocytes by translocation of glucose transporter, GLUT4, to plasma membrane mainly through induction of the AMP-activated protein kinase (AMPK) pathway. AMPK has a key role in metabolic control; activation of this pathway is considered as a new treatment for obesity, type II DM and metabolic syndrome.

Rasouli *et al*. (2017) [22] evaluated the α-amylase and α-glucosidase inhibitory activity of 26 polyphenols using molecular docking and virtual screening studies. This analysis revealed that among the docked compounds, quercetin showed high binding affinities for interaction with the α-glucosidase active site, which indicates that it can decrease or fully inhibit this enzyme activity. The enzyme α-glucosidase is present on the small intestine and has the same role as α-amylase, which is excreted by pancreas.

Snyder *et al*. (2016) [23] further analyzed this effect with *in vivo* tests, where mice were fed polyphenol-rich fruit extracts and quercetin in order to observe the effects on adiposity and blood glucose regulation. The conclusion was that in a cell culture model, quercetin was shown to reduce intracellular lipid accumulation in a dose-dependent manner and mice had significantly lower blood glucose concentrations after food deprivation when given quercetin-rich extracts.

The previous studies indicate a possible relation between quercetin and anti-diabetic effects, however, no direct relation between this compound and α-amylase was discovered and no experiments were conducted with onion skin extracts.

1.2.6 Applications and Challenges

Quercetin possesses several bioactivities, such as inhibition of proliferation of different types of cancer cells (e.g. colorectal cancer cells, prostate cancer cells, liver cancer cells, pancreatic cancer cells and lung cancer cells) by modulating their cellular processes and restraining them from growing. Due to its potential health benefits for humans, quercetin has come into the focus of utilization as a nutraceutical ingredient in food and pharmaceutical industries. It has already applications as dietary supplement to improve the organoleptic quality and stability, as well as to extend the shelflife of food.

However, quercetin has low water solubility, resulting in difficulties to directly incorporate high levels of quercetin into water-based food matrixes. It also has low bioavailability, chemical instability and short biological half-life, which may reduce its efficacy when used in the food and pharmaceutical fields.

When digested in the human body (e.g., mouth, small intestine, liver, kidneys), quercetin undergoes glucuronidation, sulfation or methylation. During the food processing and storage, many factors such as heat, pH or metal ions, could affect the chemical stability (including oxidation and degradation) of quercetin. A possible solution to these challenges is the utilization of delivery systems including lipid-based carriers, nanoparticles, inclusion complexes, micelles and conjugates-based encapsulation, which have the potential to improve both the stability and bioavailability and thus health benefits of quercetin [10].

A study by Elsebaie *et al*. (2017) [24] focused on this issue by analyzing the extraction of biological compounds from red onion peels and the microencapsulation using maltodextrin, soybean protein isolate, and a complex of both compounds by freeze drying manner. Furthermore, there was also a quality evaluation of cake integrated with microencapsulated polyphenols, which had improved qualitative properties and increased polyphenol content when compared with cake integrated with extract due to the encapsulation protective effect pending baking.

A possible negative effect of quercetin was described by Aguirre *et al*. (2011) [18]. Although quercetin has consistently failed to demonstrate adverse effects in animal studies, it appears to have inhibitory effects on cytochrome P450. These monooxygenases enzymes are important in hepatic drug metabolism, which is crucial for the elimination of many therapeutic drugs. Therefore, quercetin may influence the patient's response to drug therapy.

Chapter 2

Materials and Methods

The scope of the work included plant material preparation by freeze-drying and extraction optimization. The antioxidant capacity of the extracts was quantified with 2,2-diphenly-1-picrylhydrazyl (DPPH•) scavenging capacity assay and the quercetin present in the extracts was identified and quantified by high performance liquid chromatography coupled with diode-array detector (HPLC/DAD) system. The anti-diabetic properties of extracts was also determined by α-amylase assay

2.1 Onion Skin Preparation

The skin from brown onions (*Allium cepa* L.) was collected in February 2019 from Celje, Slovenia. The skin was weighed in beaker cups and each freeze-drying process included 6 cups. The samples were then frozen with liquid nitrogen and freeze-dried in a lyophiliser at -50 ℃ and 0.12 mbar for 1-4 days. After that, the beaker cups were weighed again to obtain the average percentage of evaporated water. The dried samples were powdered using an analytic mill and stored in the freezer (-20 ℃) until used.

2.2 Chemical Reagents

- Liquid nitrogen (N_2) (Messer Greisheim, Germany)
- DPPH• (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich, Germany)
- Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma Aldrich, Germany)
- Ethyl acetate 99.5% purity (Honeywell, Riedel-de Haen, Germany)
- Absolute ethanol (Emsure, Germany)
- Absolute methanol (Emsure, Germany)
- Formic Acid $>$ 98% (Sigma Aldrich, Germany)
- Acetonitrile $>$ 99.9% (Honeywell, Riedel-de Haen, Germany)
- Quercetin $>$ 95% (Sigma Aldrich, Germany)
- Ethanol 96% (v/v) (Honeywell, Riedel-de Haen, Germany)
- Sodium hydroxide (Kemika, Zagreb)
- DNSA (2-hydroxy-3,5-dinitrobenzoic acid) (MERCK, Germany)
- Sodium potassium tartrate tetrahydrate (Kemika, Slovenia)
- Soluble starch (Merck, Germany)
- Maltose (Kemika, Slovenia)
- Enzyme α-amylase from hog pancreas 43.6 U/mg (Fluka, Switzerland)

2.3 Laboratory equipment

- Lyophilizer ALPHA 1-2 LD Plus (CHRIST, Germany)
- Water bath (Kambic, Laboratorijska oprema)
- Thermo-shaker TS1 (Biometra, Germany)
- Ultrasonic Cleaner 100W (SHESTO, UK)
- Analytical scale Mettler Toledo, AT201 (Tehtnica, Switzerland)
- Scale EXACTA 2200 EB (Tehtnica, Slovenia)
- Centrifuge Centric 200 (Tehtnica, Slovenia)
- UV-VIS Spectrophotometer 89090A (Agilent, USA)
- Magnetic mixer RotaMix S-10 (Libra, Slovenia)
- Vortex mixer MS3 basic (IKA, Germany)
- Analytical mill A11 Basic (IKA, Germany)
- HPLC 1260 Infinity system (Agilant Technologies, Germany)
- Vacuum Concentrator Centrifuge UNIVAPO 100 H (UNIEQUIP)
- Dry Bath Heating System (Star Lab, Taiwan)

2.4 Preparation of extracts

This section contains a description of the extraction conditions used for tests A, B and C.

Note: For tests A, B and C two repetitions of each experiment were performed in order to access the coherence of results.

2.4.1 Test A: solvent selection

For this test, a mass-to-liquid ratio of 1:10 was used by measuring 100 mg of the previous powdered solids into test tubes and adding 1 mL of the following solvents:

1. Aqueous solution of 2% acetic acid
2. Aqueous solution of 50% ethanol
3. Aqueous solution of 70% ethanol
4. Absolute ethanol
5. Absolute methanol
6. Absolute ethyl acetate

The samples underwent conventional maceration extraction (CME) by being agitated in a thermo shaker at 25 ℃ during four different timings: 15 min, 30 min, 1 h, and 2 h. They also underwent ultrasound-assisted extraction (USAE) on ultrasonic bath at full mode at 25 ℃ for 15 min and 30 min. The temperature in the ultrasonic bath was unable to be precisely controlled, therefore there was an increase of 5-10 ℃ from the desired temperature.

The samples were then centrifuged at a Relative Centrifugal Force (RCF) of 25230 x g for 5 min, and the solvent was extracted with a pipette and stored in the freezer (-20 ℃) until used.

A second-stage extraction was performed by adding 1 mL of the same solvent to the test tubes containing the remaining solids after the first extraction. The process was repeated as described above.

2.4.2 Test B: mass-to-liquid ratio selection

Three additional mass-to-liquid ratios were tested and prepared as followed, using 50% ethanol solution:

- 1:20 ratio: 1 mL of solvent was added to 50 mg of powdered onion skin.
- 1:50 ratio: 1 mL of solvent was added to 20 mg of powdered onion skin.
- 1:100 ratio: 1 mL of solvent was added to 10 mg of powdered onion skin.

Both CME and USAE were performed at 25 ℃ for the four timings with only a single stage extraction.

2.4.3 Test C: temperature selection

CME was performed for temperatures of 40 ℃ and 60 ℃, for the four timings and single stage extraction with 50% ethanol solution and 1:50 mass-to-liquid ratio.

2.5 Determination of antioxidant capacity - Free radical-scavenging ability using DPPH• radical

The DPPH• assay is a very common, simple and rapid method to evaluate the antioxidant capacity of different biological samples. The method is based on the monitorization of the capacity of a sample to neutralize the radical, using a spectrophotometer.

Free radicals contain an unpaired electron and are usually unstable and very reactive. However, DPPH• is a stable free radical due to resonance stabilization caused by its three aromatic rings. During the assay the extract is mixed with the DPPH• solution and the antioxidants in the extracts donate a hydrogen to DPPH•, generating the reduced form of the radical (DPPH-H). This creates a change of the solutions' color from violet to pale yellow, which can be followed in a spectrophotometer UV/vis at 517 nm. Essentially, a lower absorbance indicates a higher concentration of antioxidants in the sample. This method should be conducted in the dark since light influences this reaction by accelerating the decrease of absorbance [25].

2.5.1 Preparation of reagents

- 2% acetic acid: 5 mL of acetic acid was supplemented with distilled water to the 250 mL mark and mixed.
- 1.10 mM trolox: 2.76 mg of trolox was dissolved in 500 μL of absolute ethanol and supplemented with the 2% acetic acid to the 10 mL mark. The solution was kept in the fridge (5 ℃) until used.

- 0.11 mM DPPH•: 3.01 mg of radical was dissolved in 72 mL of methanol and mixed on a magnetic stirrer for 20 min. The solution was protected against light with aluminium foil and used if its absorbance at 517 nm was equal or greater than 1.

2.5.2 Calibration curve

The calibration curve was prepared by measuring sample solutions of different trolox concentrations. The samples contained 5 to 50 μL of 1.10 mM trolox and were supplemented with 2% acetic acid solution until 50 μL. A control sample was also prepared containing 50 μL of 2% acetic acid solution. 1 mL of DPPH• solution was added to the samples following mixing in the vortex and incubation at room temperature in the dark for 1 hour. Afterward, the solutions were transferred into 1.5 mL polystyrene cuvettes and the absorbance was measured at 517 nm. 2% acetic acid solution was used as the blank. The samples were analyzed in replicates. The absorbance of each sample was subtracted from the absorbance of the control sample and the calibration curve was plotted (Appendix A, Figure A.1).

An additional calibration curve for the extracts with ethyl acetate was prepared, due to different response of this solvent. The samples contained 20 μL of ethyl acetate, 5 to 30 μL of 1.10 mM trolox and were supplemented with 2% acetic acid until 30 μL. A control sample was also prepared containing 20 μL of ethyl acetate and 30 μL of 2% acetic acid. The remaining procedure was conducted as described above and the calibration curve was plotted (Appendix A, Figure A.2).

2.5.3 Sample analysis

Each extract was analyzed in 2 repetitions. For Test A, 1:10 dilutions of extracts (excluding ethyl acetate samples) were performed by diluting 50 μL of extracts with 450 μL of 2% acetic acid solution. 20 μL of the 10-times diluted extract was added to 30 μL of 2% acetic acid solution and mixed with 1 mL of DPPH• solution in the vortex. The samples were treated same way as the standard solutions of trolox, used to obtain calibration curve.

For the ethyl acetate samples, there was no need for dilution: 20 μL of the extracts were mixed with 30 μL of 2% acetic acid solution and 1 mL of DPPH• solution. The samples were treated same way as the standard solutions of trolox, used to obtain calibration curve. For Test B, 20 μL of 10-times diluted extracts were used for the 1:20 mass-to-liquid ratio assay, 50 μL of 10-times diluted extracts were used for the 1:50 mass-to-liquid ratio assay, whereas 20 μL of undiluted extract were used for the 1:100 mass-to-liquid ratio assay. The samples were treated the same way as the standard solutions of trolox, used to obtain the calibration curve.

For Test C, the procedure was the same as test B for 1:50 mass-to-liquid ratio for both temperatures.

The average values of the absorbance of the samples were subtracted from the control absorbance, and by using the slope of the calibration curve as well as the dilution factors it was possible to obtain the concentration of antioxidant compounds in the extracts ($c_{\text{TEAC in extracts}}$) expressed in mmol of trolox equivalent antioxidant capacity (TEAC) per liter of extract. The concentration of TEAC in dry matter ($c_{\text{TEAC in dry matter}}$) expressed as μmol TEAC per gram of dry matter was obtained using equation 2.1. V_{solvent} represents the volume of solvent added to the dry matter in mL and $m_{\text{dry matter}}$ represents the mass of dry powder of onion skin weighted in mg.

$$c_{\text{TEAC in dry matter}} = \frac{c_{\text{TEAC in extracts}} \times V_{\text{solvent}} \times 10^3}{m_{\text{dry matter}}} \tag{2.1}$$

The DPPH• assay was also conducted for standard solution of quercetin. A stock solution of quercetin with concentration of 0.367 mg/mL was prepared in 50% ethanol in order to mimic the concentration of quercetin in extracts of 1:10 mass-to-liquid ratio, 50% ethanol solution and 15 min. The

solution was diluted 10 times and the method described above was performed in order to obtain the TEAC results for isolated quercetin.

2.6 Quantification of quercetin by HPLC/DAD

High-performance liquid chromatography (HPLC) is the most widely used separation technique for phenolic compounds in food matrices. The HPLC conditions mainly include the use of C18 reverse-phase columns, a binary solvent gradient, and diode array detector or mass spectrometry. The mobile phase usually consists of an aqueous solution of acid and an organic solvent (usually acetonitrile or methanol). The phenolic compounds are eluted according to their polarity and molecular size, and a gradient elution system with the mobile phase is often used [26]. The quantification of quercetin was obtained using HPLC/DAD method based on the comparison with quercetin standard.

For the quantification of quercetin from onion skin the HPLC/DAD system Agilent 1260 Infinity was used, which consists of binary pump G1312B (Agilent 1260), vacuum degasser G1322A (Agilent 1260), thermostated autosampler G1367E (Agilent 1260 HiPALS), thermostat for the column G1316A (Agilent 1260 TCC) and diode array detector G4212B (Agilent 1260 DAD).

For analysis the Agilent ZORBAX Eclipse Plus Column C18 with an inner diameter of 4.6 mm, a length of 150 mm and particle size of 3.5 μm was used. The column was connected to a precolumn ZORBAX Eclipse XBD-C18 (4.6 mm $\times$ 12 mm, particle size 5 μm). [26].

The autosampler temperature was set to 10 ℃, the column the temperature was set to 35 ℃, the injection sample volume was 20 μL and the flow rate was set to 0.8 mL/min. The solvent system was composed of 0.1% formic acid in water (A) and acetonitrile with 0.1% formic acid (B) with the following gradient elution: 75% A and 25% B initially, raised to 30% B in 10 min, 65% B in 20 min, 100% B in 21 min, maintained for 1 min, returned to 25% B in 23 min and maintained at 25% B for 4 min. Data was acquired with HPLC 2D Chemstation Agilent software, revision B.04.03.

2.6.1 Preparation of reagents:

- Solution A - 0.1% (v/v) formic acid (HCOOH): in a 2 L flask, 2 mL of formic acid was added together with mQwater to the 2 L mark and mixed for 5 minutes.
- Solution B - acetonitrile + 0.1% formic acid: in a 1 L flak, 1 mL of formic acid was added together with acetonitrile to the 1 L mark and mixed for 5 minutes.
- Mobile phase solution: 200 mL of mobile phase was prepared by mixing 50 mL of solution B with 150 mL of solution A. This solution was used to dilute samples for analysis.
- Stock solution of quercetin (0.9 mg/mL): 45.09 mg of quercetin was weighed and dissolved in 50 mL of absolute ethanol.

2.6.2 Calibration curve

The calibration curve was prepared from 0.9 mg/mL stock solution of quercetin. 15 solutions with quercetin concentrations ranging from 0.15 μg/mL to 180 μg/mL were prepared by mixing different volumes of the stock solution and supplemented with mobile phase solution until 1 mL. The samples were analyzed in HPLC/DAD and the chromatograms of the samples were obtained from a range of 254 to 400 nm and quercetin was determined at 370 nm. For each concentration the samples were injected three times and the average area of the peaks of the three repetitions were correlated with the concentration of quercetin in order to obtain the calibration curve (Appendix B, Figure B.1).

2.6.3 Sample analysis

Dilutions of extracts ranging from 1:4 to 1:40 were prepared using the mobile phase solution in order to obtain the signal response within the calibration curve.

Samples were then mixed in the vortex, centrifuged at a RCF of 25230 x g for 5 minutes in order to remove non-soluble particles. The samples were then transferred to vials and analyzed on HPLC/DAD system. Each sample was injected once and each sample had two corresponding repetitions analyzed.

The chromatograms were recorded in the wavelength range of 254 to 400 nm, whereas quercetin was determined at 370 nm, close to its absorption maximum. An example of a chromatogram obtained in this thesis can be observed in Figure 2.1.

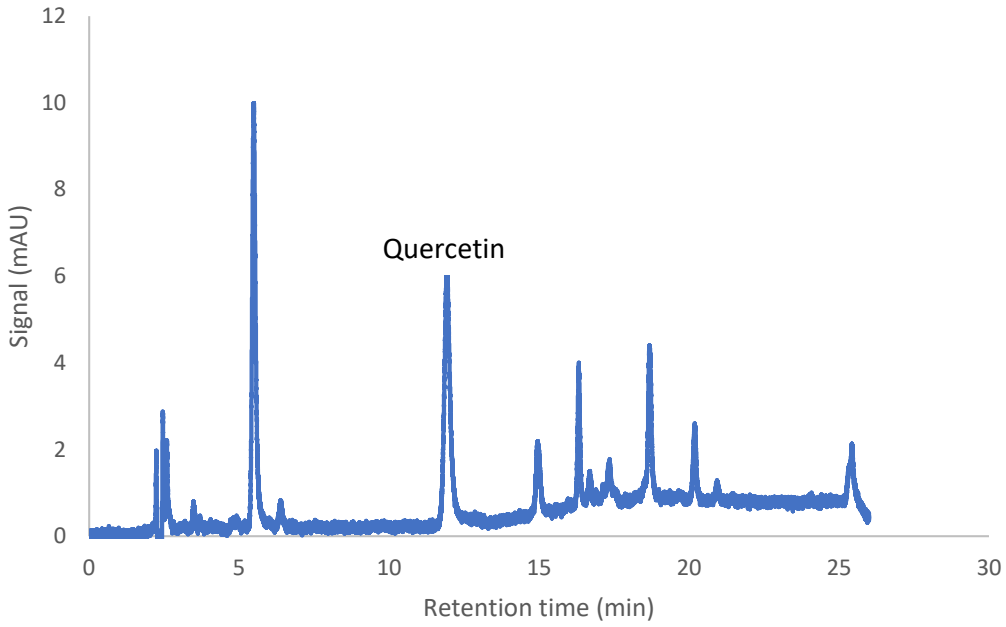

Figure 2.1: Chromatogram of onion skin extract analyzed by HPLC/DAD. The graph represents the signals measured at 370 nm and expressed in Area Units (mAU) in function of retention time in minutes. The sample analyzed in this chromatogram was extracted under the following conditions: 1:100 mass-to-liquid ratio, 15 minutes, 50% ethanol and 25℃

The peak corresponding to quercetin (in Figure 2.1 at 11.9 minutes) was identified by cross referencing previous chromatograms of standard solutions of quercetin. Using the area signals given by the chromatograms, the slope of the calibration curve and the dilution factors, it was possible to obtain the concentration of quercetin in the extracts ($c_{\text{quercetin in extracts}}$) expressed as μg of quercetin per mL of extract. The mass fraction of quercetin (w) expressed in mg of quercetin per g of dry matter, was calculated by using equation 2.2. V_{solvent} represents the volume of solvent added to the dry matter in mL and $m_{\text{dry matter}}$ represents the mass of dry powder of onion skin weighted in mg.

$$w = \frac{c_{\text{quercetin in extracts}} \times V_{\text{solvent}}}{m_{\text{dry matter}}} \tag{2.2}$$

2.7 Anti-diabetic assay by inhibition of α-amylase activity

The enzyme α-amylase catalyzes the hydrolysis of α-1,4-glycosidic linkages of starch components in a retaining fashion to produce oligosaccharides, such as maltose [27].

One of the main assays used to determine the activity of α-amylase is based on measuring the amount of reducing sugars by the dinitrosalicylic acid (DNSA) assay. DNSA is an aromatic compound that reacts with the aldehyde group of reducing sugars to form 3-amino-5-nitrosalicylic acid. The concentration of the reducing sugars can therefore be determined spectrophotometrically at 540 nm [28].

2.7.1 Preparation of reagents

- 0.02 M Sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride
- 2 M Sodium hydroxide (NaOH)
- Dinitrosalicylic acid (DNSA) color reagent: prepared by dissolving 1 g of 2-hydroxy-3,5-dinitrobenzoic acid in 50 mL of distilled water. After that, 30 g of sodium potassium tartrate tetrahydrate was added slowly to the mixture, together with 20 mL of 2 M NaOH. Complete dissolving was ensured by using ultrasound bath. The mixture was then diluted to a final volume of 100 mL with distilled water. The final solution was protected with aluminum foil and stored for no longer than 2 weeks.
- 1% Starch: prepared by dissolving 1 g of soluble starch in 90 mL of sodium phosphate buffer. The mixture was brought to a gentle boil to dissolve, cooled and adjusted to 100 mL with distilled water.
- Maltose stock solution 1.8 mg/mL: prepared by dissolving 180 mg of maltose (MW 360.3) in 100 mL of distilled water in a volumetric flask.
- Enzyme solution 0.1 mg/mL: 1 mg of enzyme α-amylase was dissolved in 10 mL of distilled water. The absorbance was accessed at 280 nm with UV cuvette.

2.7.2 Calibration Curve

The calibration curve was prepared from the maltose stock solution, by preparing 10 maltose dilutions ranging from 0.25 to 2.5 μmol/mL. 400 μL of each dilution was pipetted into test tubes, together with a control tube with 400 μL of distilled water. The tubes were prepared in duplicate and after pre-incubation at 25 ℃, 200 μL of DNSA color reagent was added. The test tubes were then incubated in a boiling water bath for 15 minutes and then cooled on ice. The reaction mixture was then diluted by adding 1.8 mL of distilled water following which the absorbance was measured at 540 nm. The calibration curve relating the difference between the absorbance of the samples and the absorbance of the control was plotted in relation to the maltose concentration (Appendix C, Figure C.1).

2.7.3 Sample Analysis

This assay was conducted for the samples of Test A (mass-to-liquid ratio of 1:10 and for all solvents) for 15 min extraction time. (Note: the sample of 100% ethanol did not have enough solids for the desired final concentration, therefore the sample of 2 h extraction time of the same solvent was used. The sample of ethyl acetate was not used since for all the times tested it did not contain enough dry extract to obtain the most concentrated extract (10 mg/mL)). All the results were the combination of the two repetitions.

The extracts were dried in a vacuum concentrator centrifuge for 3-6 hours until completely dried and re-dissolved in the respective solvent in order to obtain the same final concentration of 10 mg of dry

extract per mL of solvent, as represented in table 2.1. $V_{extract}$ represents the initial volume of extracts collected and transferred to test tubes to be dried; $m_{dry\ extract}$ represents the mass of dry extract after the drying process and $V_{solvent}$ represents the volume of each solvent necessary to be added to the dry extract to obtain a final concentration of 10 mg/mL.

Table 2.1: Volumes of extracts used of each sample for the α-amylase assay, the respective mass of dry extract and the volume of solvent used to re-dilute the samples to obtain a concentration of dry extract of 10 mg/mL. The samples are from Test A with a mass-to-liquid ratio of 1:10, for all the solvents and specific timings.

Sample	$V_{extract}$ (µL)	$m_{dry\ extract}$ (mg)	$V_{solvent}$ (µL)
2% AA, 15 min	400	4.50	450
50% EtOH, 15 min	800	12.10	1210
70% EtOH, 15 min	900	9.90	990
100% EtOH, 2 h	1000	4.25	425
100% MeOH, 15 min	900	5.52	552

Four concentrations of dry extract (10 mg/mL, 1 mg/mL, 0.1 mg/mL and 0.01 mg/mL) were prepared for each sample. The last three concentrations were prepared by diluting each concentrated solution (10 mg/mL) in the sodium phosphate buffer. Test tubes were prepared in duplicate with 100 µL of each concentration and 100 µL of the enzyme solution (c = 0.1 mg/mL). Each of these tubes had a corresponding control tube (without enzyme) with 100 µL of each sample and 100 µL of the sodium phosphate buffer.

Another control test tube corresponding to 100% activity of the enzyme, was also prepared in duplicate by adding 100 µL of sodium phosphate buffer and 100 µL of the enzyme solution. The blank for this control had 200 µL of the sodium phosphate buffer.

All the test tubes as well as 1% starch solution were incubated for 15 minutes at 25 ℃.

After pre-incubation, 200 µL of 1% starch solution was added to each tube at 5 s intervals. The mixture was incubated for exactly 3 minutes and afterward 200 µL of DNSA color reagent was added also in 5 s intervals to stop the reaction. The test tubes were then incubated in a boiling water bath for 15 minutes, cooled with ice and 1.8 mL of distilled water was added. The absorbance of each sample was measured at 540 nm and the absorbance of the respective control was subtracted from the result,

The concentration of maltose in each tube was obtained by using the calibration curve of maltose and the percentage of inhibition of α-amylase activity was obtained using equation 2.3. $c_{100\%\ activity}$ represents the concentration of maltose in the test tube of 100% activity of α-amylase and c_{sample} represents the concentration of maltose in the samples.

$$\%\ \text{Inhibition} = \frac{c_{100\%\ activity} - c_{sample}}{c_{100\%\ activity}} \times 100 \tag{2.3}$$

The test was also conducted for standard solution of quercetin. A stock solution of quercetin with concentration of 0.243 mg/mL was prepared in 50% ethanol in order to mimic the concentration of quercetin in concentrated solution (10 mg/mL). Dilutions with concentrations of 2.43×10^{-2} mg/mL, 2.43×10^{-3} mg/mL and 2.43×10^{-4} mg/mL, were prepared using the sodium phosphate buffer and the method described above was repeated.

Chapter 3

Results and Discussion

This chapter comprises the results and discussion of optimization process of quercetin extraction from onion skin. The optimal time of freeze-drying procedure was chosen and the optimal solvent, mass-to-liquid ratio and temperature were obtained through an analysis of antioxidant capacity and mass fraction of quercetin in the extracts. The mass fraction of quercetin in dry extracts was also determined. Furthermore, the anti-diabetic activity of onion skin extracts was determined as well as the contribution of pure quercetin to total antioxidant capacity and anti-diabetic activity of onion skin extracts.

3.1 Freeze-drying of onion skin

As described in section 2.1 of Materials and Methods chapter, the first step of onion skin extraction was the freeze-drying process. It was important to determine the minimum duration of freeze-drying necessary to obtain completely dried samples, in order to be able to compere results from different extraction procedures. Therefore, the percentage of evaporated water from the onion skin was followed during the freeze-drying process, as seen in Figure 3.1.

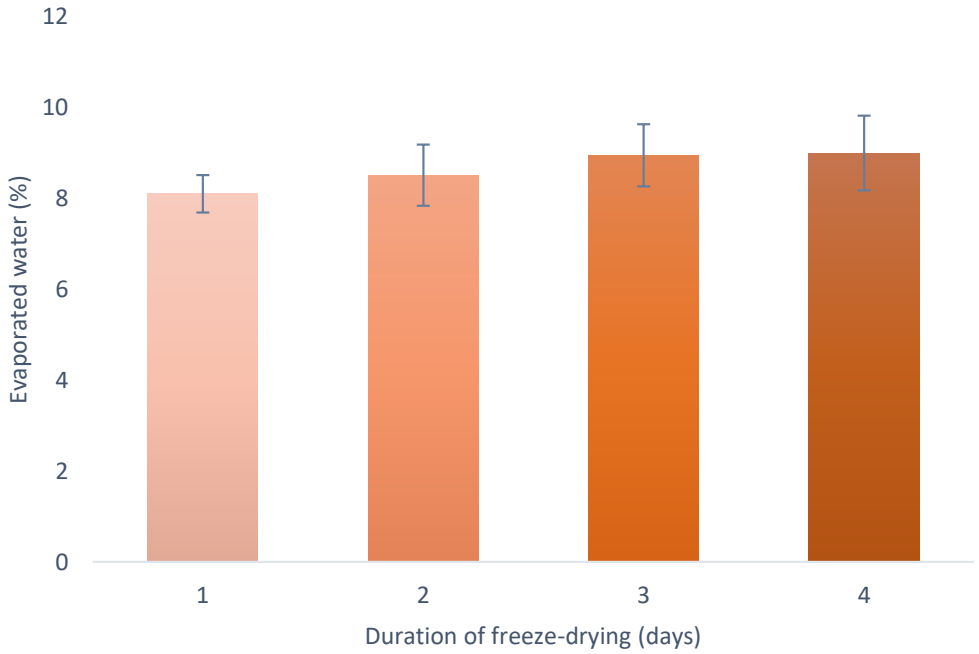

Figure 3.1: The effect of duration of freeze-drying process on evaporated water from the onion skin.

As seen in the graph of Figure 3.1, the difference between the percentage of evaporated water from day 1 and day 3 is considerable, however, between day 3 and day 4 the percentage of evaporated water seems to stabilize. Therefore, it was concluded that the minimum duration of freeze-drying procedure is 3 days, since having an extra day would not yield a considerable difference and the energy costs would be higher. Therefore, all the samples used in the following analysis were dried for 3 days and had an average of percentage of evaporated water of 9.42%. All results of further analysis are expressed per

gram of freeze-dried onion skin (named as g of dry matter), obtained by this procedure.

3.2 Solvent selection

There are four variables studied in this work in order to optimize the quercetin extraction from onion skin:

- solvent,
- mass-to-liquid ratio,
- time,
- temperature.

For the initial experiment (Test A) a fixed temperature (25 ℃) and mass-to-liquid ratio (1:10) were selected, whereas different solvents and extraction times were checked, in order to obtain the best extraction solvent and the optimal duration of extraction.

As described in section 2.4.1 of Materials and Methods chapter, both conventional maceration extraction (CME) and ultrasonic assisted extraction (USAE) methods were tested, as well as single and double stage extraction.

For each section, the results from DPPH• assay and High-Performance Liquid Chromatography with Diode-Array Detector (HPLC/DAD) are presented. The results are expressed per gram of freeze-dried onion skin (dry matter). The values presented in all Figures were obtained as an average of repetitions of each experiment, with the respective standard deviation.

3.2.1 Conventional maceration extraction

The conventional maceration extraction (CME) was performed in two stages, the results are presented separately (fist stage and second stage extraction).

First stage extraction

The results of trolox equivalent antioxidant capacity (TEAC), determined by the DPPH• assay, for extracts obtained after first stage of CME are presented in Figure 3.2.

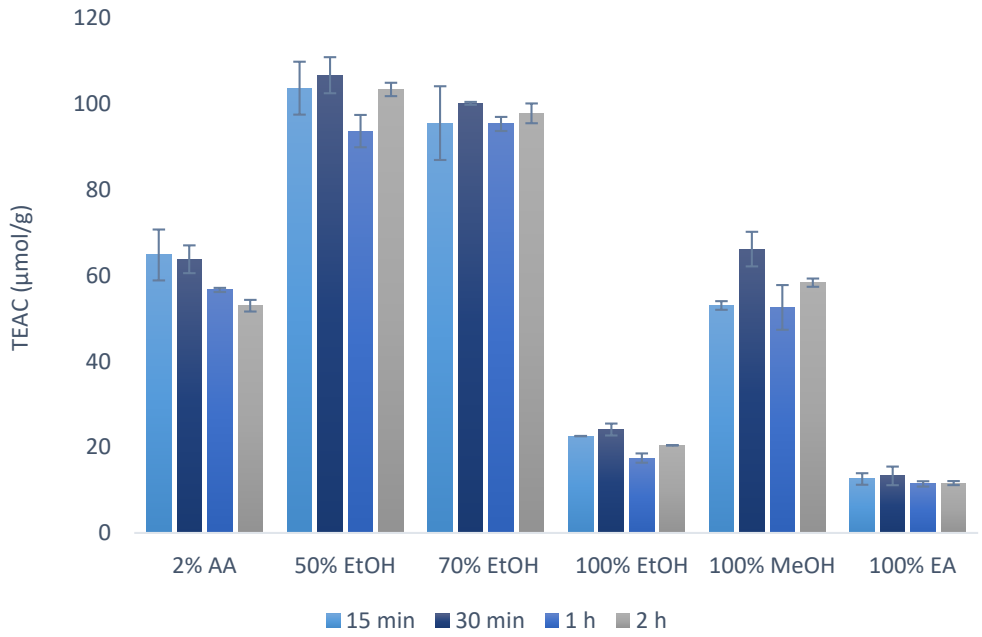

Figure 3.2: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after first stage conventional maceration extraction at 25℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in µmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

As seen in Figure 3.2, the higher TEAC values were determined for 50% ethanol (from 93.6 to 106.6 µmol/g) and 70% ethanol (from 95.3 to 100.1 µmol/g) extracts, followed by 100% methanol (from 52.5 to 66.1 µmol/g) and 2% acetic acid (from 52.9 to 64.8 µmol/g), whereas the lower TEAC values were determined for 100% ethanol (from 17.4 to 24.1 µmol/g) and 100% ethyl acetate (from 11.4 to 13.3 µmol/g).

These results are expected since the best solvents described in the literature are aqueous-based ethanol solutions [15]. As described in section 1.2.3 of Introduction, quercetin displays an amphipathic behavior [13], which means it will be most soluble in solvents that also display this behavior. Ethanol is mostly a polar solvent, but also contains non-polar moiety, and water displays only polar proprieties. These two solvents together generate a solvent with amphipathic behavior, ideal for extraction of quercetin and other phenolic compounds with the same characteristics [16]. On the contrary, 2% water solution of acetic acid is too polar solvent and ethyl acetate too hydrophobic solvent, which appears to justify the negative influence in the extraction yield of polyphenols, including quercetin.

The results also show that the TEAC values for most of the selected solvents are not strongly influenced by the duration of extraction, generally being slightly high for 15 min and 30 min extractions. However, the results are mostly inside the experimental error. The more pronounced decrease of TEAC in 2% acetic acid for the 1 h and 2 h compared to shorter extraction times might be explained by the decrease in stability of antioxidants in this solvent. Due to the presented results, the following experiments with USAE were performed with 15 minutes or 30 minutes extraction.

The results of the HPLC/DAD determination of quercetin obtained after first stage CME are presented in Figure 3.3.

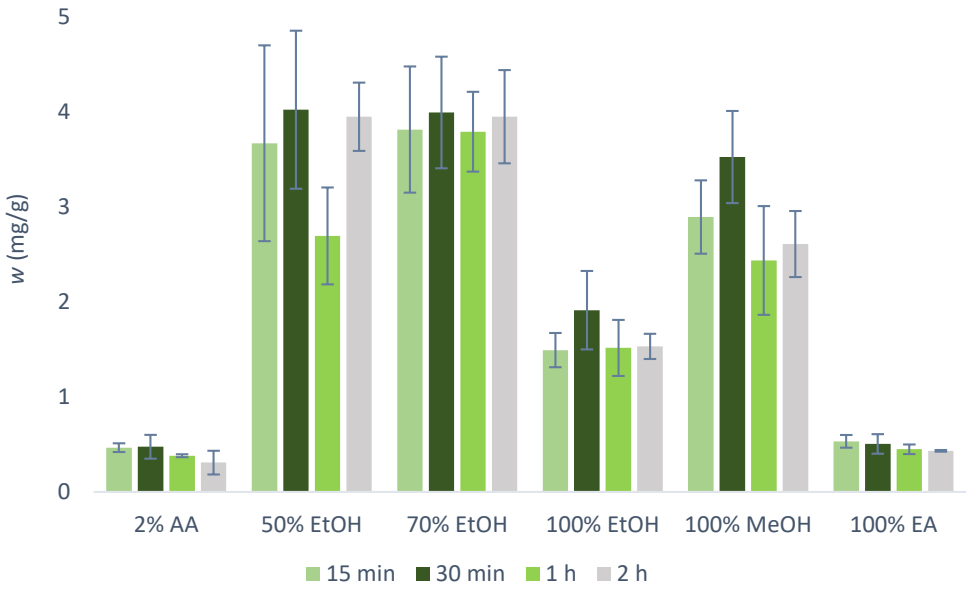

Figure 3.3: Mass fraction (*w*) of quercetin in extracts obtained after first stage conventional maceration extraction at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

As seen in Figure 3.3, the results of quercetin mass fraction follow a similar pattern as the TEAC results (Figure 3.2). The higher values of quercetin are obtained for 50% ethanol (from 2.7 to 4.0 mg/g) and 70% ethanol (from 3.8 to 4.0 mg/g) extracts, followed by 100% methanol (from 2.4 to 3.5 mg/g) and 100% ethanol (from 1.5 to 1.9 mg/g), whereas the lower values of quercetin were determined for 100% ethyl acetate (from 0.43 to 0.53 mg/g) and 2% acetic acid (from 0.31 to 0.47 mg/g).

The observed correlation between the TEAC results and the amount of quercetin in extracts given by HPLC/DAD analysis indicates that DPPH• assay can be used as a screening test to evaluate and predict the extraction efficiency of quercetin.

However, it can also be observed that the correlation factor between mass fraction of quercetin and TEAC is lower in 2% acetic acid compared to other solvents, which leads to the logic interpretation that the extraction with 2% acetic acid is quite satisfactory for polar antioxidants, but it is much worse for extraction of less polar quercetin. This result was expected since quercetin is poorly soluble in water [6] [14] which is the main constituent of 2% acetic acid solvent.

Second stage extraction

The results of TEAC, determined by the DPPH• assay, for extracts obtained after second stage CME are presented in Figure 3.4.

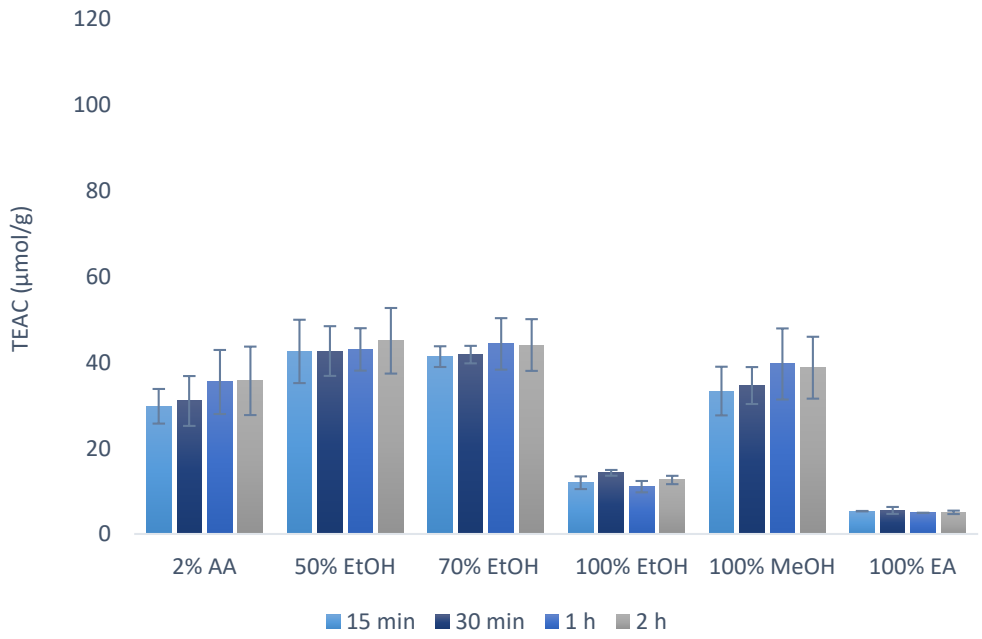

Figure 3.4: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after second stage conventional maceration extraction at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in µmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

As seen in Figure 3.4, fewer antioxidants (lower TEAC) were present in onion skin extracts after second stage CME compared to first stage extracts, however, the values are not negligible.

The higher TEAC values were determined for 50% ethanol (from 42.5 to 45.0 µmol/g) and 70% ethanol (from 41.3 to 44.3 µmol/g) extracts, followed by 100% methanol (from 33.3 to 39.6 µmol/g) and 2% acetic acid (from 29.8 to 35.7 µmol/g), whereas the lower TEAC values were determined for 100% ethanol (from 11.0 to 14.2 µmol/g) and 100% ethyl acetate (from 4.9 to 5.5 µmol/g).

In order to conclude if there are any differences between solvents regarding efficiency of first and second stage extraction, the percentage of TEAC determined in samples after second stage extraction, in comparison with the first stage extraction, for each solvent was calculated (Table 3.1).

Table 3.1: The percentages of trolox equivalent antioxidant capacity (TEAC) after second stage extraction in comparison to first stage extraction, for each solvent. The values represent the averages for the four different timings. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

Solvent	TEAC after second stage extraction (%)
2% AA	56 ±10
50% EtOH	43 ±3
70% EtOH	44 ±2
100% EtOH	59 ±5
100% MeOH	64 ±10
100% EA	43 ±1

It can be observed from the Table 3.1 that all samples obtained after second stage extraction contain a considerable amount of antioxidants, expressed as TEAC (43% to 64%). This means that it is worthwhile not only to perform a single stage extraction but to proceed also with the second stage in order to extract more antioxidants present in onion skin.

However, there are some notable differences among solvents, 100% methanol (64±10%), 100% ethanol (59±5%) and 2% acetic acid (56±10%) being the solvents with the highest percentage of antioxidants still present in the samples after second stage extraction compared to first stage extraction.

This result can indicate that more water-soluble antioxidants stay in the samples after first stage extraction, being retrieved in higher percentage in the second stage extraction by the solvent 2% acetic acid.

The results of the HPLC/DAD determination of quercetin obtained after second stage CME are presented in Figure 3.5.

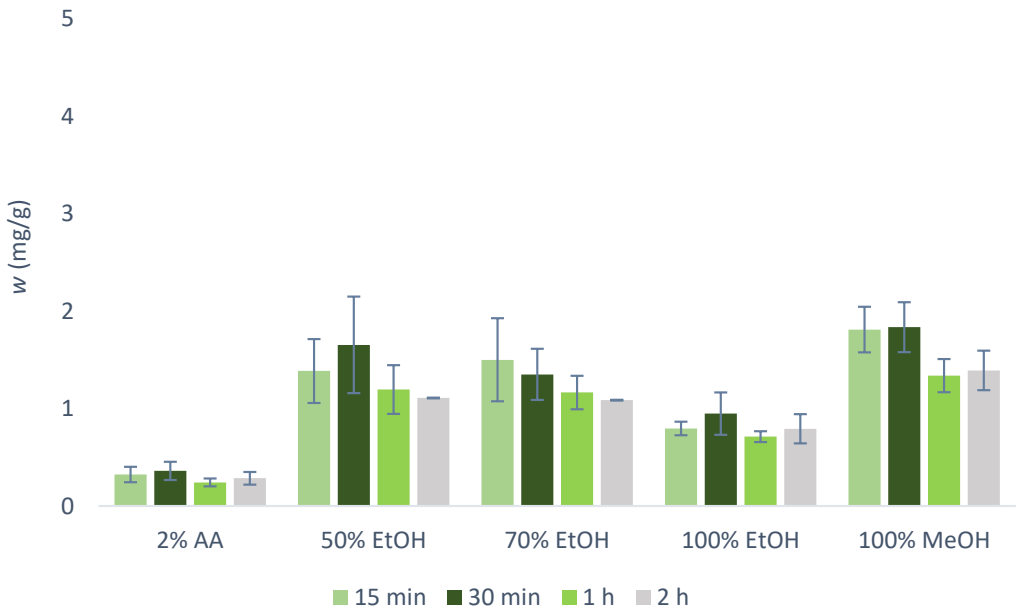

Figure 3.5: Mass fraction (w) of quercetin in extracts obtained after second stage conventional maceration extraction at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol.

As seen in Figure 3.5, the results of quercetin mass fraction follow the same pattern as the TEAC results of Figure 3.4, except for 100% methanol which has a higher mass fraction than the ethanol solutions, meaning that more quercetin and less other antioxidants were extracted by 100% methanol. The values of mass fraction for the ethyl acetate samples are not present in the graphic since the peak areas of HPLC results were below the limit of detection, even without any dilution, making the use of the calibration curve impossible.

In order to conclude if there are any differences between solvents regarding efficiency of first and second stage extraction, the percentage of w determined in samples after second stage extraction, in comparison with the first stage extraction, for each solvent was calculated (Table 3.2).

Table 3.2: The percentages of mass fraction (*w*) after second stage extraction in comparison to first stage extraction, for each solvent. The values represent the averages for the four different timings. AA - acetic acid; EtOH - ethanol; MeOH - methanol.

Solvent	*w* after second stage extraction (%)
2% AA	75 ±12
50% EtOH	38 ±7
70% EtOH	33 ±5
100% EtOH	50 ±3
100% MeOH	56 ±5

As seen in Table 3.2, the percentages of mass fraction after second stage extraction are similar to the percentages of TEAC values, presented in Table 3.1. The samples obtained after second stage extraction also contain a considerable amount of quercetin, expressed as mass fraction (33% to 75%), but the differences among different solvents are more pronounced.

The solvents with the highest percentage of quercetin mass fraction still present in the samples after second stage extraction compared to first stage extraction, are similar to those determined by TEAC assay (Table 3.1), however, in this case the solvent with the highest remaining percentage of quercetin is 2% acetic acid (75±12%). This result can be explained by the poor solubility of quercetin in water [6] [14], resulting in a high amount of this compound left after the first stage extraction. The results show that making double stage extraction in order to extract the highest quantity of quercetin is much more important for 2% acetic acid extraction solvent than for 50% or 70% ethanol extraction solvent.

3.2.2 Ultrasound-assisted extraction

The ultrasound-assisted extraction (USAE) was performed in two stages, the results are presented separately (fist stage and second stage extraction). Due to results of extraction time in section 3.2.1 of this Chapter, only 15 minutes and 30 minutes extractions were tested for this purpose.

First stage extraction

The results of TEAC, determined by the DPPH• assay, for extracts obtained after first stage USAE are presented in Figure 3.6.

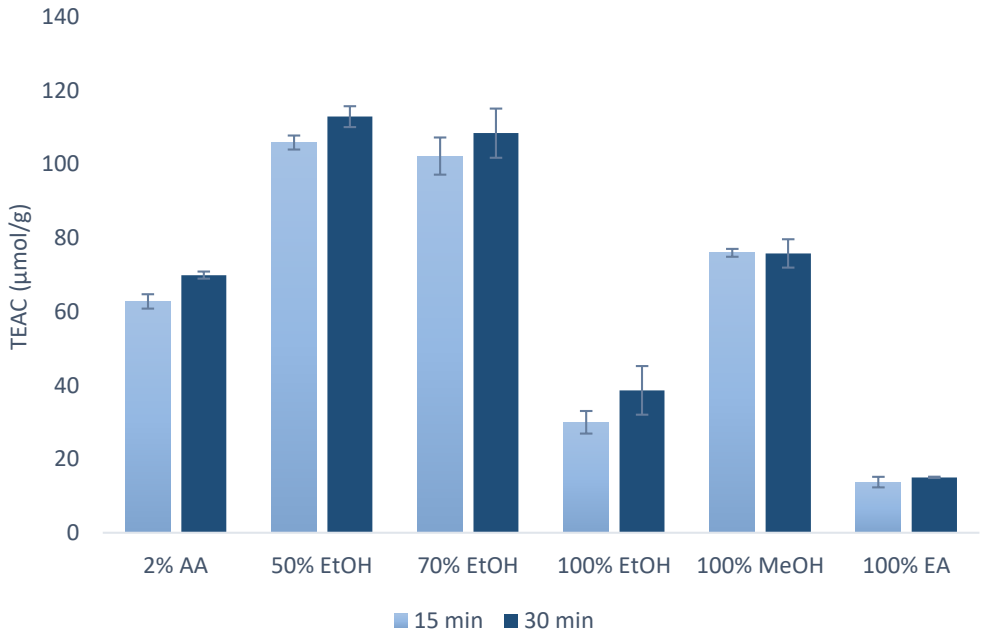

Figure 3.6: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after first stage ultrasound-assisted extraction (USAE) at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in µmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

The results of the HPLC/DAD determination of quercetin obtained after first stage USAE are presented in Figure 3.7.

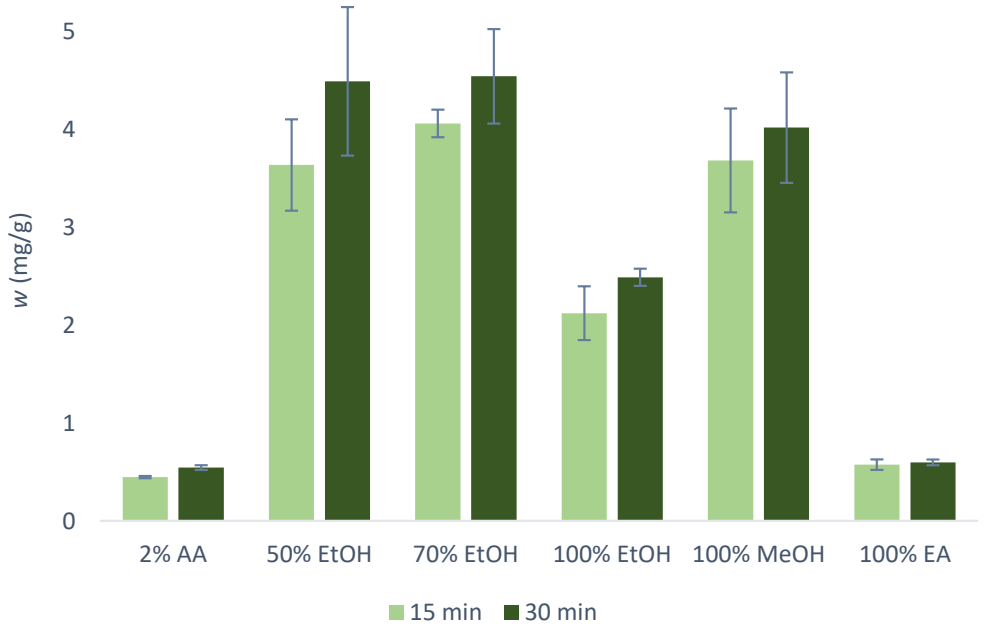

Figure 3.7: Mass fraction (w) of quercetin in extracts obtained after first stage ultrasound-assisted extraction (USAE) at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

As seen in Figures 3.6 and 3.7, the TEAC and mass fractions of quercetin in onion skin extracts are slightly higher after USAE compared to CME. These differences are more pronounced in ethanol solutions and pure methanol. This result was expected since USAE promotes larger agitation (cavitation) and generally improves the extraction efficiency compared to CME [2]. Since the process generates an increase in temperature that is hard to control, it was plausible that this slight increase in extraction yield could be due to the higher temperature instead of the USAE method itself (hypothesis denied in the following sections).

Second stage extraction

The results of TEAC, determined by the DPPH• assay, for extracts obtained after second stage USAE are presented in Figure 3.8.

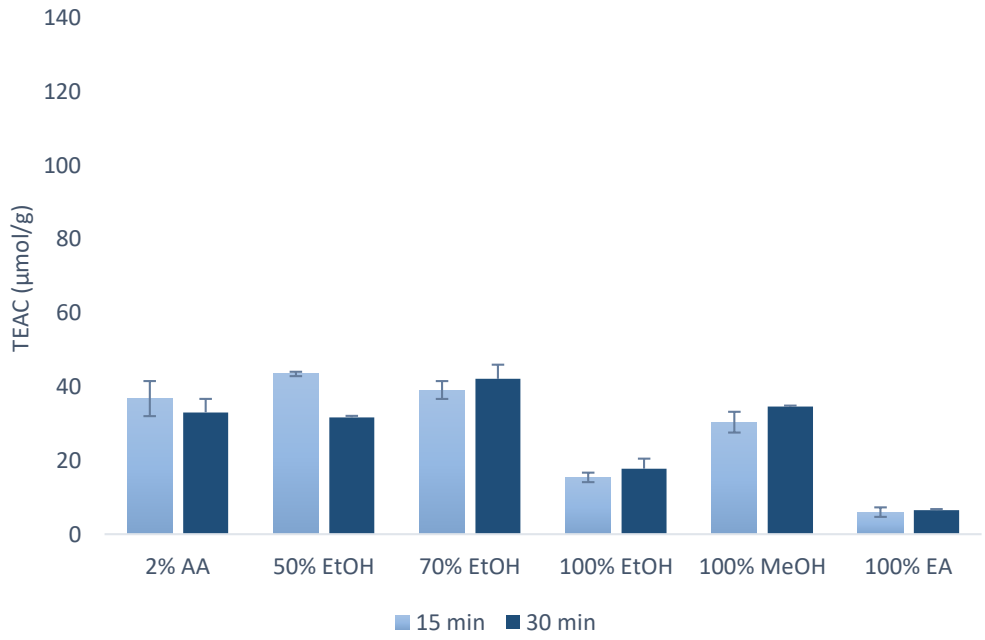

Figure 3.8: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after second stage ultrasound-assisted extraction (USAE) at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in µmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

The results of the HPLC/DAD determination of quercetin obtained after second stage USAE are in Figure 3.9.

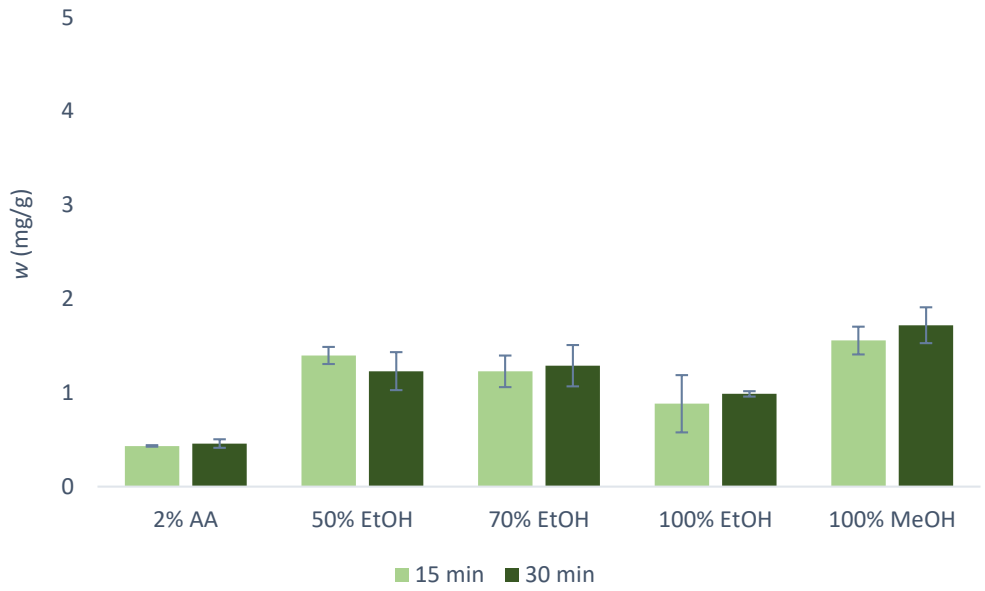

Figure 3.9: Mass fraction (w) of quercetin in extracts obtained after second stage ultrasound-assisted extraction (USAE) at 25 ℃, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

As seen in Figures 3.8 and 3.9, the results of TEAC and mass fraction of quercetin in onion skin extracts after the second stage extraction with USAE are very similar to the results obtained after second stage extraction using CME, which indicates that for the second stage extraction CME is sufficient to obtain the same results.

3.3 Mass-to-liquid ratio selection

In the previous section, it was concluded that both 50% and 70% ethanol solutions were the best extraction solvents. Considering the future applications of this extraction, it was considered that the lower the content of ethanol in the solvent the better to reduce costs and increase health acceptability. As described in the Introduction chapter, although ethanol is classified as a Generally-Recognized-as-Safe solvent, its utilization in this application is restricted by strict legal statutes that exist in many countries [8], therefore the solvent 50% ethanol was chosen as the optimal solvent for the following experiments.

For this experiment (Test B) a fixed temperature (25 ℃) and solvent (50% ethanol) were selected, whereas different mass-to-liquid ratios and extraction times were checked, in order to obtain the best mass-to-liquid ratio and the optimal duration of extraction.

As described in section 2.4.2 of Materials and Methods chapter, the mass-to-liquid ratios tested were 1:10 (the results were used from test A), 1:20, 1:50 and 1:100, and both CME and USAE were tested for single stage extraction.

3.3.1 Conventional maceration extraction

The results of TEAC, determined by the DPPH• assay, for extracts obtained after single stage of CME are presented in Figure 3.10.

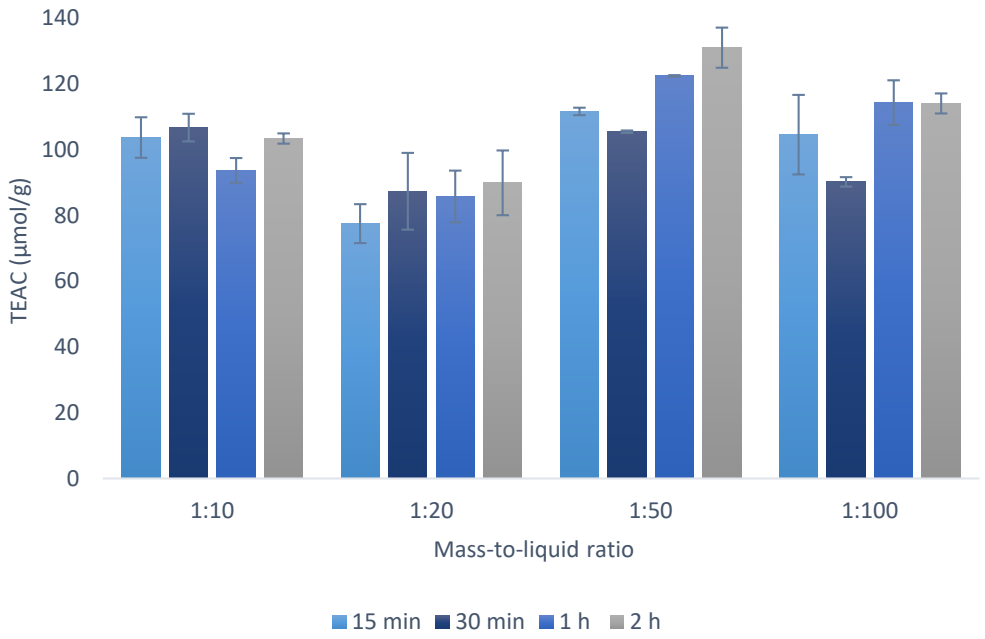

Figure 3.10: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after single stage conventional maceration extraction at 25 ℃, with 50% ethanol, with different mass-to-liquid ratios and different timings. Results are expressed in µmol of TEAC per g of dry matter.

As seen in Figure 3.10 the best mass-to-liquid ratio regarding TEAC values is 1:50. The optimal extraction times vary according to the ratio, however, for the 1:50 mass-to-liquid ratio the optimal time is the longest tested, 2 h.

This result is reasonable since the higher the volume of solvent in relation to the mass of solids, the higher the concentration of antioxidants that can be extracted. However, there must be a balance, since lower amount of solids may also mean lower total amount of antioxidants present to be extracted.

The results of the HPLC/DAD determination of quercetin obtained after single stage CME are presented in Figure 3.11.

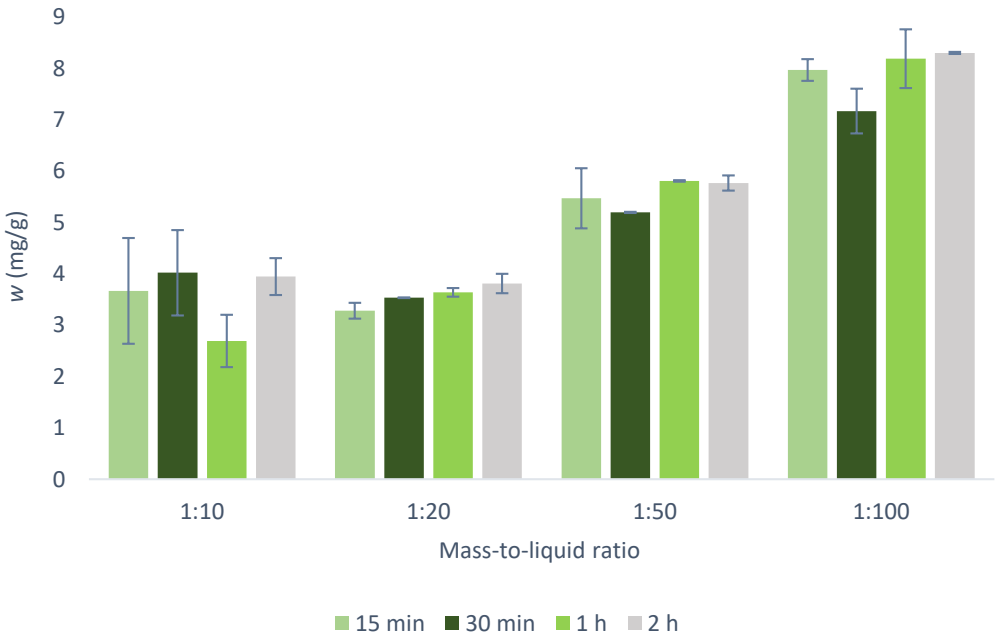

Figure 3.11: Mass fraction (w) of quercetin in extracts obtained after single stage conventional maceration extraction at 25 °C, with 50% ethanol, with different mass-to-liquid ratios and different timings. Results are expressed in mg of quercetin per g of dry matter.

As seen in Figure 3.11 the mass fraction distribution of quercetin in onion skin extracts considering the mass-to-liquid ratio is slightly different compared to the TEAC distribution (Figure 3.10). The mass fraction of quercetin increases with the increase of solvent volume to solids mass, resulting in an optimal extraction for the 1:100 mass-to-liquid ratio among tested ratios. There are some differences among results of different extraction timings, but they are generally within experimental standard deviation. The results suggest that quercetin requires a higher volume of solvent to solids ratio in order to be efficiently extracted from onion skin compared to other antioxidants that were determined in higher quantity with 1:50 mass-to-liquid ratio.

3.3.2 Ultrasound-assisted extraction

The results of TEAC, determined by the DPPH• assay, for extracts obtained after single stage of USAE are presented in Figure 3.12.

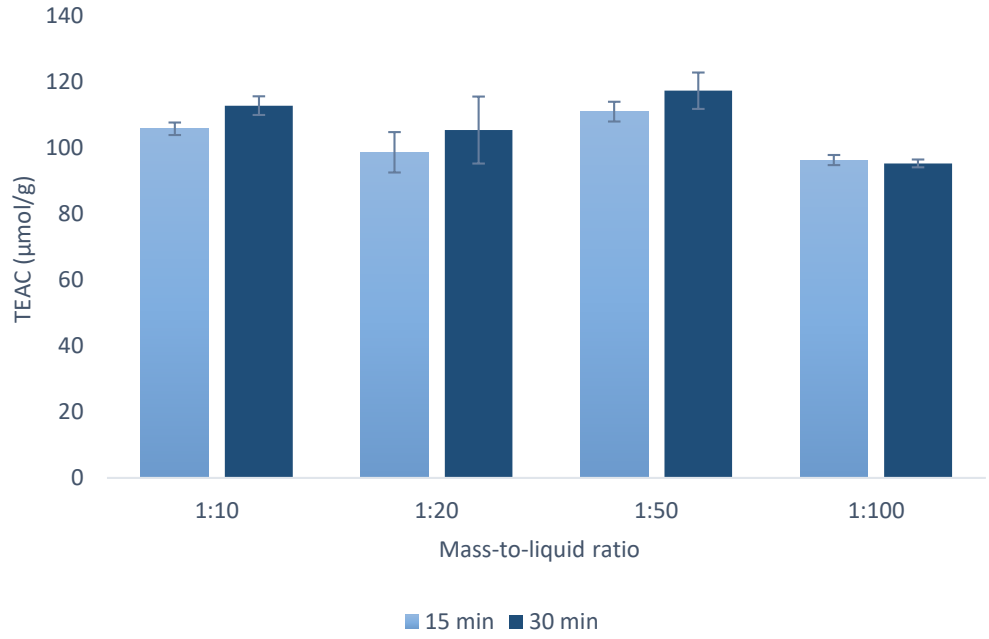

Figure 3.12: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after single stage ultrasound-assisted extraction (USAE) at 25 ℃, with 50% ethanol, with different mass-to-liquid ratios and different timings. Results are expressed in µmol of TEAC per g of dry matter.

The results of the HPLC/DAD determination of quercetin obtained after single stage USAE are presented in Figure 3.13.

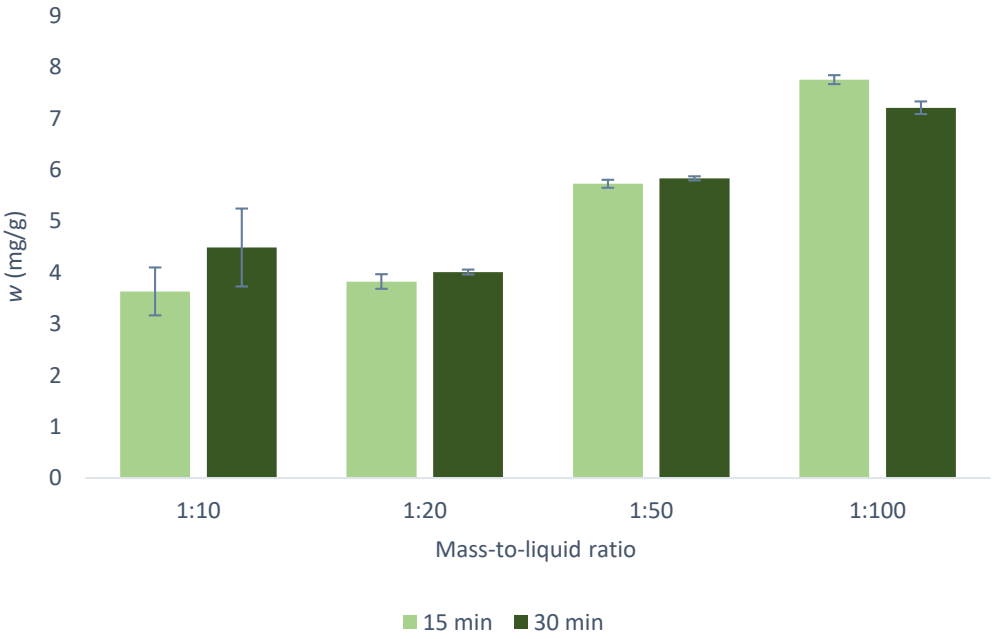

Figure 3.13: Mass fraction (w) of quercetin in extracts obtained after single stage ultrasound-assisted extraction (USAE) at 25 ℃, with 50% ethanol, with different mass-to-liquid ratios and different timings. Results are expressed in mg of quercetin per g of dry matter.

Both in Figures 3.12 and 3.13 the TEAC and mass fraction results for extracts obtained with USAE are slightly higher than those of CME, which was expected as mentioned previously, except for the 1:100 mass-to-liquid ratio which has the same or slightly inferior results. For USAE the optimal mass-to-liquid

ratio regarding TEAC equals to 1:50, whereas the highest mass fraction of quercetin was obtained with 1:100 mass-to-liquid ratio, which is in accordance with results obtained by CME.

During the laboratory work, the DPPH• assay was conducted before the HPLC determination and used as a prediction for HPLC results. Therefore, for the further experiment of temperature selection, the 1:50 mass-to-liquid ratio was used as the optimal extraction ratio, being the optimal extraction for all antioxidants, determined by DPPH• assay. Since the increase in extraction yield with USAE method is only slightly higher than CME, it was decided to use CME for further experiments as it reduces the costs associated with USAE equipment and the temperature is easier to control.

3.4 Temperature selection

For this experiment (Test C) a fixed solvent (50% ethanol) and mass-to-liquid ratio (1:50) were selected, whereas different temperatures and extraction times were checked, in order to obtain the best extraction temperature and the optimal duration of extraction.

As described in section 2.4.3 of Materials and Methods chapter, the temperatures tested were 25 ℃ (from previous test B), 40 ℃ and 60 ℃, and CME was tested for single stage extraction.

The results of TEAC, determined by the DPPH• assay, for extracts obtained after single stage of CME are presented in Figure 3.14.

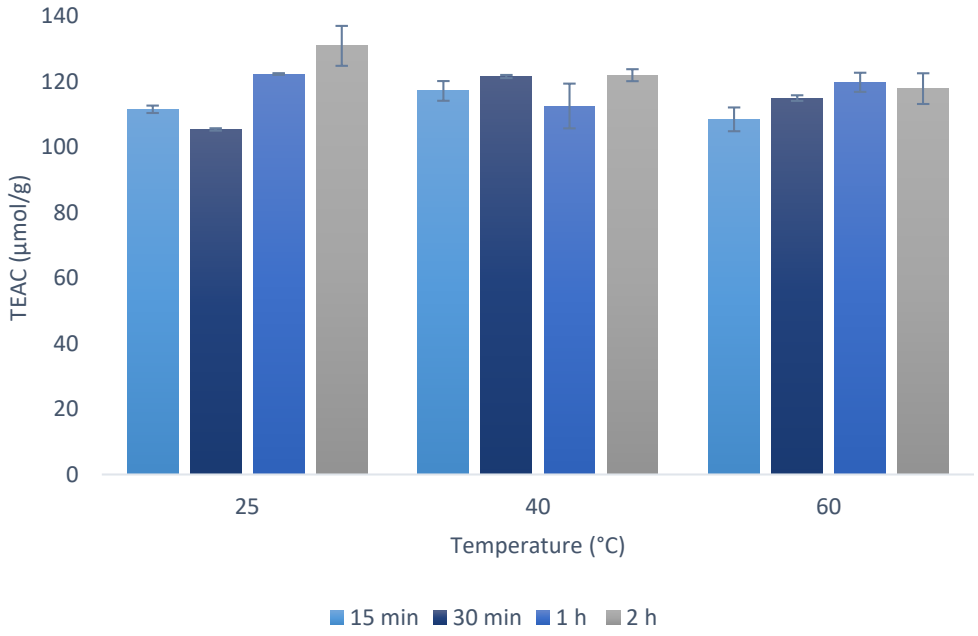

Figure 3.14: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after single stage conventional maceration extraction with 50% ethanol and 1:50 mass-to-liquid ratio, with different temperatures and different timings. Results are expressed in µmol of TEAC per g of dry matter.

The results of the HPLC/DAD determination of quercetin obtained after single stage CME are presented in Figure 3.15.

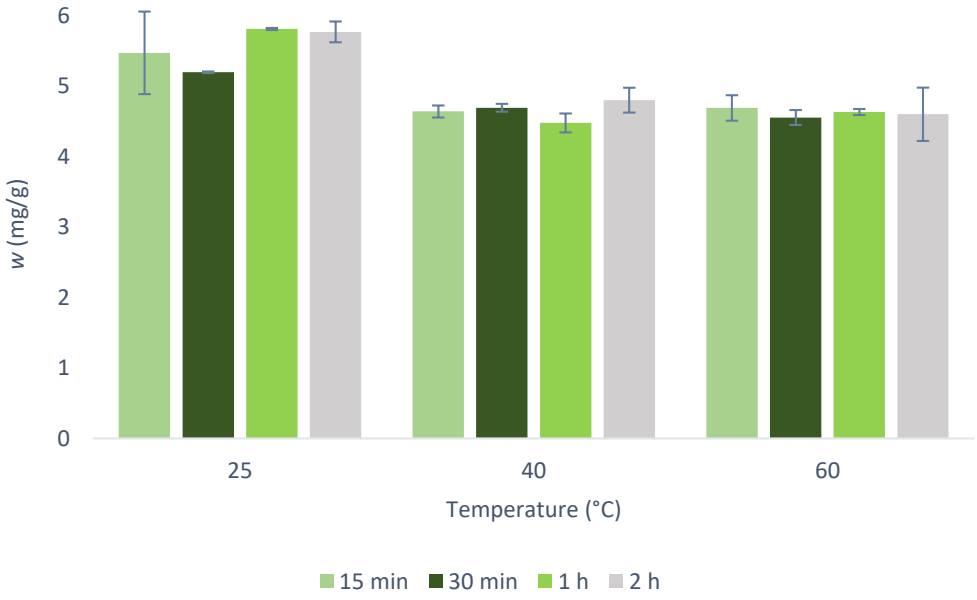

Figure 3.15: Mass fraction (w) of quercetin in extracts obtained after single stage conventional maceration extraction with 50% ethanol and 1:50 mass-to-liquid ratio, with different temperatures and different timings. Results are expressed in mg of quercetin per g of dry matter.

As seen in Figure 3.14 and Figure 3.15, the TEAC and mass fraction of quercetin do not vary considerably with the extraction temperature, however, there are some differences between both determinations. The results of TEAC for the tree temperatures tested are similar and the variations are contained inside the experimental standard deviation, whereas the mass fraction of quercetin is slightly higher at 25 ℃ compared to higher temperatures tested in this research.

It is well known that solubility of quercetin in mixtures of ethanol increase smoothly with increasing temperature, as described in section 1.2.3 of Introduction [15]. However, with the increase in temperature the connections and structure of quercetin may destabilize, which may affect the mass fraction of quercetin, resulting in its slight decrease with higher temperatures.

These results confirm that the increase in extraction yields in USAE compared to CME methods is in fact due to the method and not due to the increase in temperature, since the increase in temperature from 25 ℃ to 40 ℃ does not result in an increase in extraction yield.

3.5 Optimized quercetin extraction

Regarding the parameters used as variables in this thesis, it was noticeable that the variation of solvent type had a higher impact and significant differences in yield of antioxidants and quercetin when compared to the remaining variables in study, namely mass-to-liquid ratio, temperature, time and extraction method. Therefore, the selection of solvent is the most important parameter to be defined when considering the extraction of quercetin from onion skin and should be analyzed at the beginning of the approach, as done in this thesis.

This is in coherence with the results of Jang *et al.* (2012) [16], that concluded that ethanol concentration and temperature are the most influential parameters compared to the remaining parameters studied, such as pH, mass-to-liquid ratio and extraction time. However, the impact of temperature in the results of this thesis was not so influential. This is also explained by Jang *et al.* (2012), since it was concluded that for USAE the extraction yield increases with temperature, while for CME (the method

used to assess the temperature influence in this thesis) the temperature seems to exert a monotonous effect on the extraction yield.

In conclusion, focusing on the results of quercetin mass fraction, the most efficient extraction was performed with CME by using 50% ethanol as a solvent and 1:100 mass-to-liquid ratio. The optimal time was 15 min since there was no notable difference in results for 15 min, 30 min, 1 h, and 2 h extraction, hence the shortest time is the most economically feasible. The extraction temperature optimization experiment showed that the optimal temperature was 25 °C, considering that the results for 1:50 mass-to-liquid ratio are similar to the results obtained with 1:100 mass-to-liquid ratio. For these optimal extraction parameters, the TEAC was 104.5 μmol/g and the mass fraction of quercetin was 7.96 mg/g.

The optimal extraction parameters obtained in this thesis are coherent with the ones obtained by Jin *et al*. (2011) [12]. This study optimized various procedures such as CME, USAE and microwave assisted extraction (MAE) for quercetin extraction from onion skin using response surface methodology (RSM). The highest quercetin yield for CME was obtained for the extraction time of 16.5 min, the temperature of 59.2 °C and 59.3% ethanol. All the optimal parameters are very similar to the ones obtained in this thesis, however, the optimal temperature obtained varies slightly. In this study, USAE results were also very similar to CME, which corresponds to the results in this thesis. The quercetin yield for conventional solvent extraction was 3.42 mg/g, which is inferior to the one obtained in this thesis.

The study by Jang *et al*. (2012) [16] investigated the extraction of quercetin with aqueous ethanol solutions from onion solid waste under sonication (USAE) conditions, using rational experimental design methodologies. The quercetin mass fraction obtained in this study was 11.08 mg/g of the dry weight of onion solid waste, for the optimal conditions (59% ethanol and 49 °C), which represents a very similar mass fraction obtained in this thesis.

Another relevant study by Savic-Gajic *et al*. (2018) [1] obtained the optimal extraction conditions of quercetin from onion skin for 47.3 min, 80% ethanol and mass-to-liquid ratio of 1:64. In the extract obtained under these conditions, the quercetin content was 28.5 mg/g, which represent a much higher content than the one obtained in this thesis. However, there were some experimental differences that can influence this results such as the moisture content of the onion skin and the fact that the extraction of quercetin was performed under reflux at the boiling point of the solvent. It is important to note that this study optimized the quercetin extraction using a numerical optimization method - central composite design (CCD), which allowed for a correlation of more variables to obtain the exact optimal extraction. While in this thesis work, a traditional optimization method was applied (one factor-at-a-time, OVAT), where the analysis of interactions between independent variables is not possible. And finally the results are also difficult to compare due to the fact that each of the research was performed with different plant materials, which is most likely the main reason for the different results.

Another important parameter for the optimal solvent choice is the quercetin purity in dry extracts, since in large-scale application of this process the extracts could be dried to remove the organic solvent and the amount of bioactive compound in the powder is relevant for its application.

In order to determine this parameter, the mass fraction of quercetin in dry extracts was calculated using data from dry extracts of Table 2.1 in section 2.7 of Materials and Methods chapter and the results of HPLC determination of quercetin.

As described in section 2.7 of Materials and Methods, the extracts were dried and the mass of dry extract ($m_{\text{dry extract}}$) was obtained. The concentration of solids in the original extracts ($c_{\text{solids in extract}}$) could also be obtained by dividing the mass of dry extract ($m_{\text{dry extract}}$) by the initial volume of extract before the drying process (V_{extract}). This data is presented in Table 3.3.

Table 3.3: Volumes of extracts used of each sample for the drying process, the respective mass of dry extract and the concentration of solids in extracts. The samples are from Test A with a mass-to-liquid ratio of 1:10, for all the solvents and specific timings. AA - acetic acid; EtOH - ethanol; MeOH - methanol.

Sample	V_{extract} (µL)	$m_{\text{dry extract}}$ (mg)	$c_{\text{solids in extract}}$ (mg/mL)
2% AA, 15 min	400	4.5	11.3
50% EtOH, 15 min	800	12.1	15.1
70% EtOH, 15 min	900	9.9	11.0
100% EtOH, 2 h	1000	4.2	4.3
100% MeOH, 15 min	900	5.5	6.1

As seen in Table 3.3 the mass of dry extract is much lower than the original mass of onion skin (dry matter) used for the extraction (100 mg), which means that most of the solids did not dissolve in any of the solvents, but were discarded. The sample with the highest amount of dry extract is the 50% ethanol extract, which corresponds to the best extraction for the test A.

Finally, the mass fraction of quercetin in the dry extracts ($w_{\text{quercetin in dry extract}}$) was calculated using data from Table 3.3 and Table 3.4, as seen in equation 3.1. The values of concentration of quercetin in the original liquid extracts ($c_{\text{quercetin in extract}}$) are also presented in Table 3.4, and were obtained after HPLC analysis using the peak areas, the calibration curve and the dilution factors, representing the average of the two repetitions tested.

$$w_{\text{quercetin in dry extract}}\ (\text{mg/g}) = \frac{c_{\text{quercetin in extract}}\ (\text{mg/mL})}{c_{\text{solids in extract}}\ (\text{mg/mL}) \times 10^{-3}} = \frac{m_{\text{quercetin}}\ (\text{mg})}{m_{\text{dry extract}}\ (\text{g})} \tag{3.1}$$

Another parameter that can be analyzed is the mass of quercetin that could be retrieved from the dry extracts per the initial mass of dry matter used ($w_{\text{quercetin retrieved}}$), using the $w_{\text{quercetin in dry extract}}$ obtained previously, the mass of dry extract ($m_{\text{dry extract}}$) represented in Table 3.3 and the mass of dry matter initially used for the extracts that where subsequently dried ($m_{\text{dry matter}}$). This parameter can therefore be obtained using equation 3.2.

$$w_{\text{quercetin retrieved}}\ (\text{mg/g}) = \frac{w_{\text{quercetin in dry extract}}\ (\text{mg/g}) \times m_{\text{dry extract}}\ (\text{mg}) \times 10^{-3}}{m_{\text{dry matter}}\ (\text{g})} \tag{3.2}$$

Table 3.4: Concentration of quercetin in extracts ($c_{\text{quercetin in extract}}$), obtained by HPLC analysis; mass fraction of quercetin in dry extract ($w_{\text{quercetin in dry extract}}$) and mass of quercetin retrieved from the dry extract per mass of dry matter ($w_{\text{quercetin retrieved}}$). The samples are from Test A with mass-to-liquid ratio of 1:10, for all the solvents and specific timings. AA - acetic acid; EtOH - ethanol; MeOH - methanol.

Sample	$c_{\text{quercetin in extract}}$ (mg/mL)	$w_{\text{quercetin in dry extract}}$ (mg/g)	$w_{\text{quercetin retrieved}}$ (mg/g)
2% AA, 15 min	0.0467	4.15	0.0931
50% EtOH, 15 min	0.367	24.3	1.47
70% EtOH, 15 min	0.383	34.8	1.71
100% EtOH, 2 h	0.153	35.9	0.612
100% MeOH, 15 min	0.291	47.4	1.30

As seen in Table 3.4, the values of mass fraction of quercetin in dry extract are higher than the values of mass fraction of quercetin in dry matter obtained through HPLC/DAD analysis, expressed in Figure 3.3 of section 3.2.1 of this chapter. This means that quercetin was purified with the drying of the extracts, since it is more concentrated in the dry extracts than it was in the liquid extracts, when expressed as dry matter.

The highest mass fraction of quercetin in dry extract belongs to 100% methanol extract (47.4 mg/g). However, considering the scalability of the process, not only this parameter is relevant but also the total mass of quercetin that can be retrieved from the dry extracts. The higher values of $w_{\text{quercetin retrieved}}$ are attributed to the extracts 50% ethanol (1.47 mg/g) and 70% ethanol (1.71 mg/g). This result indicates that for the same initial amount of dry matter, these two solvents result in an optimal amount of quercetin in the dry extracts, that could be separated in future steps.

As justified above in section 3.3 of this chapter, it was considered that the lower the content of ethanol in the extracts the better to reduce costs and increase the process acceptability by reducing health concerns regarding the final product. Therefore, the solvent 50% ethanol could be considered one of the optimal solvents regarding quercetin purity in the dry extract, confirming the previous conclusion of the optimal solvent.

Horbowicz (2002) [7] optimized the extraction of quercetin from powdered onion skin and also obtained the mass fraction of quercetin in dry extracts. The results of percentage of mass fraction of quercetin in dry extracts for extraction with 1:50 mass-to-liquid ratio, cold 60% ethanol for 4 hours is 45%. The results of this thesis for 50% ethanol of $w_{\text{quercetin in dry extract}}$ in percentage is 2.43%, which represents a significantly lower value than the one obtained by Horbowicz (2002). This difference might be explained by the different extraction procedures used and it is also important to note that no other scientific papers with results of purity of quercetin in dry extracts were found, therefore a real comparison with the literature was not possible.

The values of $c_{\text{quercetin in extract}}$ and $w_{\text{quercetin in dry extract}}$ of 50% ethanol extract will be relevant in further sections in order to compare the antioxidant and anti-diabetic results of this extract with the pure quercetin that it contains.

3.6 Anti-diabetic activity

As described in section 2.7 of Materials and Methods, it was necessary to dry the extracts in order to redilute them to the desired dry extract concentration for the α-amylase assay. These results are already presented in Table 2.1.

The anti-diabetic activity of the selected concentrated extracts was obtained my measuring α-amylase activity through the amount of reducing sugars by the dinitrosalicylic acid (DNSA) assay after addition to different concentrations of dry extracts (0.01 mg/mL, 0.1 mg/mL, 1 mg/mL and 10 mg/mL).

As described in section 2.7 of Materials and Methods, samples of 1:10 mass-to-liquid ratio, 15 min and all solvents except ethyl acetate were used for this experiment.

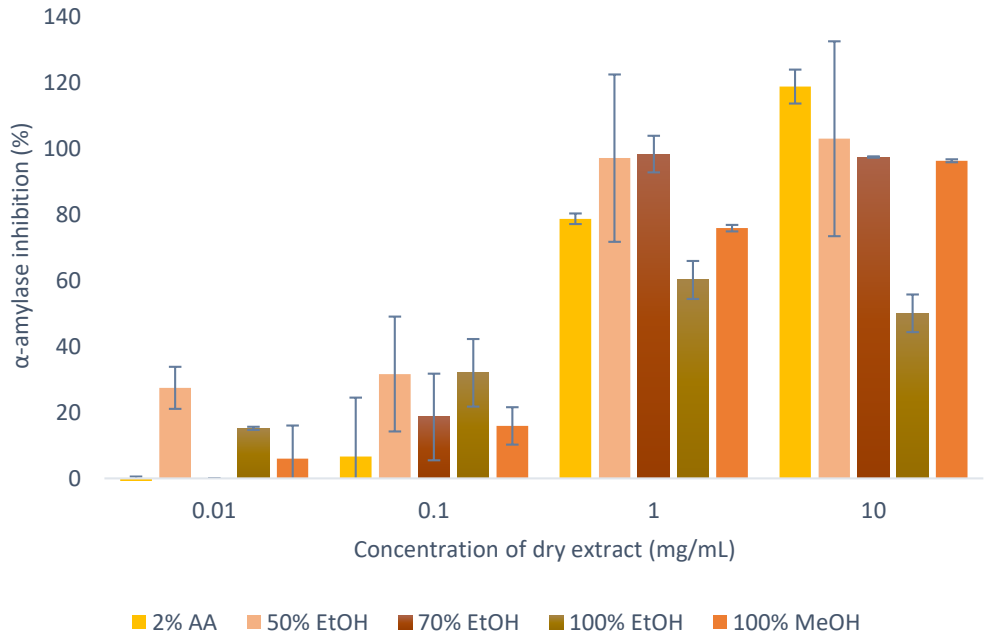

Figure 3.16: Anti-diabetic activity expressed in percentage of α-amylase inhibition, of extracts with different concentrations of dry extract. AA - acetic acid; EtOH - ethanol; MeOH - methanol

The inhibition graph presented in Figure 3.16 showed for all extracts a dose-dependent relation: the higher the applied concentration, the higher the inhibition of α–amylase. However, considerable differences among extracts with different solvents were observed. Extracts obtained with 50% ethanol, 100% ethanol and 100% methanol decreased α–amylase activity in all the concentration range tested, while 2% acetic acid and 70% ethanol had no effect at the lowest concentration tested (0.01 mg/mL).

For all solvents there is a progressive dose-dependent relation, except for extracts obtained with 50% ethanol and 70% ethanol that appear to completely inhibit α–amylase already at 1 mg/mL. For solvent 100% ethanol the inhibition also does not increase at the highest tested concentration (10 mg/mL), since the maximum inhibition is reached already at 1 mg/mL (60%). Therefore, at the highest extract concentration (10 mg/mL), the enzyme is completely inhibited by all solvents except for 100% ethanol, which seems to be the worst extraction solvent for substances with anti-diabetic activity.

It can thus be concluded that all onion skin extracts are potent inhibitors of α–amylase and can, therefore, be considered as anti-diabetic agents.

The optimal solvent regarding α–amylase inhibition is 50% ethanol when considering the tested concentration range. This result is coherent with the TEAC results since, for the samples of mass-to-liquid ratio 1:10 used in this test, 50% ethanol was considered the optimal solvent with the highest value of TEAC. This high value of antioxidants can be assumed to be due to the high polyphenol content in the extract, which react with α-amylase resulting in the highest inhibition.

The conclusion that onion skin extracts can be considered anti-diabetic agents can be partly based in previous studies that concluded that polyphenols have this effect. For instance, Bahadoran *et al.* (2013) [19] concluded that polyphenols affect carbohydrate metabolism by inhibiting of α-glucosidase and α-amylase, the key enzymes responsible for digestion of dietary carbohydrates to glucose.

Snyder *et al.* (2016) [23] also concluded that mice had significantly lower blood glucose concentrations after food deprivation when given quercetin-rich extracts.

However, the results of this thesis demonstrated that onion skin extracts containing quercetin have a direct impact on the enzyme α-amylase, which was not shown by previous studies. These results reinforce the possibility of onion skin extracts to be used in the diet and food supplements as an alternative

to the management of diabetes. However, studies regarding the safety and dosage in humans are still required for its safe application.

3.7 The impact of pure quercetin in onion skin extracts

As concluded in section 3.5 of this chapter, the optimal balance of mass of dry extracts and mass fraction of quercetin in dry extracts belongs to the extracts with 50% ethanol. The values of $c_{\text{quercetin in extract}}$ and $w_{\text{quercetin in dry extract}}$ for this solvent are 0.367 mg/mL and 24.3 mg/g respectively.

The goal of this section was to study the real impact that quercetin has in these extracts comparatively to other existing compounds, regarding antioxidant and anti-diabetic activity.

3.7.1 Antioxidant capacity

As described section 2.5 of Materials and Methods, the DPPH• assay was also conducted for pure quercetin by preparing a quercetin standard solution with a concentration of 0.367 mg/mL in 50% ethanol in order to mimic the amount of quercetin present in the 50% ethanol extract.

The results obtained from this analysis are presented in Table 3.5.

Table 3.5: Trolox equivalent antioxidant capacity (TEAC) of standard quercetin solution and the 50% ethanol extract (1:10 mass-to-liquid ratio, 15 min). The results represent the average of two repetitions. EtOH - ethanol.

	TEAC (mmol/mL)
Standard quercetin solution (0.367 mg/mL)	1.75
Extract (50% EtOH, 15 min)	10.4

As seen in Table 3.5 the antioxidant capacity of pure quercetin (1.75 mmol/mL) represents 17% of the total antioxidant capacity of the extract (10.4 mmol/mL). This result indicates that the majority of antioxidant capacity of the onion skin extracts are possibly due to other polyphenols or antioxidant compounds and not only due to quercetin.

This result is supported by Elsebaie *et al*, (2017) [24], since it was observed through HPLC analysis that the major phenolic compounds in the methanol extract were vanillic (84.78%), ellagic (6.84%), and protocatchuic acid (2.41%). Another study by Cheng *et al*. (2013) [29] determined that the main phenolic compounds also in methanol extracts of brown onions are gallic acid, followed by quercetin, which was identified as the second major compound. Therefore, it is possible that the major contribution to overall antioxidant capacity of the extracts can be attributed to these polyphenols that were not identified and quantified in the framework of this thesis.

3.7.2 Anti-diabetic activity

As described in section 2.7 Materials and Methods the α-amylase assay was also conducted for pure quercetin. The concentrated extracts for sample analysis had a dry extract concentration equal to 10 mg/mL. In order to obtain a concentration of stock standard solution of quercetin that mimics the concentration of quercetin in this extract, equation 3.3 was used together with the results of mass fraction of quercetin in dry extract for the sample of 50% ethanol presented in Table 3.4 ($w_{\text{quercetin in dry extract}}$ = 24.3 mg/g).

$$0.0243\left(\frac{\text{mg quercetin}}{\text{mg dry extract}}\right) \times 10\left(\frac{\text{mg dry extract}}{\text{mL solvent}}\right) = 0.243\left(\frac{\text{mg quercetin}}{\text{mL solvent}}\right) \tag{3.3}$$

Therefore, the concentration of stock standard solution of quercetin is 0.243 mg/mL, which corresponds to the concentration of quercetin in concentrated 50% ethanol extract with 10 mg/mL of dry extract concentration.

The stock standard solution of quercetin was diluted 10-, 100- and 1000-times in order to mimic the concentrated extracts with dry extract concentrations of 1 mg/mL, 0.1 mg/mL and 0.01 mg/mL, respectively.

The results of the α-amylase inhibition are presented in Figure 3.17.

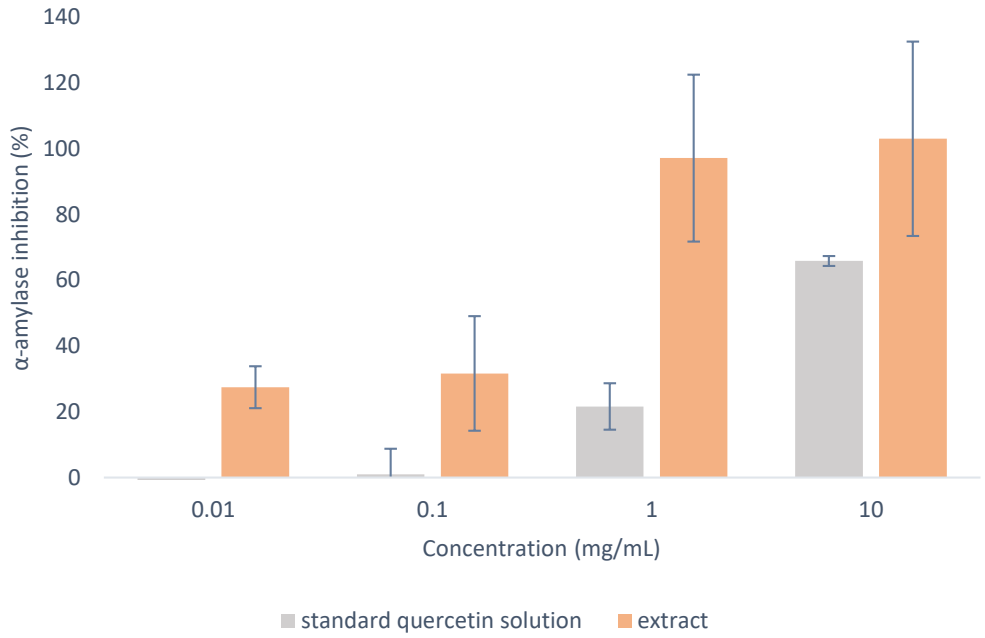

Figure 3.17: Anti-diabetic activity expressed as percentage of α-amylase inhibition, for different concentrations of concentrated 50% ethanol extracts (1:10 mass-to-liquid ratio, 15 min) and corresponding standard quercetin solutions.

As seen from Figure 3.17 the α-amylase inhibitions are lower for the standard quercetin solution compared to corresponding concentrated onion skin extracts, which was expected and is in accordance with the previous results regarding TEAC of pure quercetin (section 3.7.1 of this chapter). The results show that the extracts contain other substances that can also interact with this enzyme.

At the highest concentration, the standard solution of quercetin inhibits 65% of α-amylase activity; therefore, the remaining 35% of inhibition can be attributed to other substances present in the extract. These results also indicate that although quercetin represents only 17% of the total antioxidant capacity, it is more relevant for the α-amylase inhibition (65 %) than the remaining bioactive substances.

A study by Oboh *et al.* (2014) [21] showed that glycosylation increased the inhibitory ability of quercetin on key enzymes linked to type II diabetes (α-amylase and α-glucosidase), and a combination of quercetin and rutin (glycosylated form of quercetin) had higher synergistic inhibitory abilities on the enzymes than the individual flavonoids. This fact can also be the explanation for the higher inhibition obtained by concentrated onion skin extracts compared to pure quercetin solutions in this thesis, since the concentrated extracts probably contain a mixture of both types of quercetin (also the glycosylated form) as well as other bioactive substances, resulting in higher inhibition.

Previous reports have also shown that the excessive inhibition of α-amylase could lead to abnormal bacterial fermentation of undigested starch in the colon and therefore low α-amylase inhibitory activity is useful [21]. Since it was demonstrated in this thesis that quercetin inhibited α-amylase activity in a

dose-dependent manner, it would be possible to regulate the exact concentration of quercetin (or onion skin extract), necessary for diabetes control but low enough to diminish the effects of this abnormalities.

Chapter 4

Conclusion

The main goal of this study was accomplished by optimizing the extraction of quercetin from onion skin through a systematic study of the effects of different parameters.

Regarding the freeze-drying of onion skin, it was concluded that the optimal duration of the freeze-drying procedure is 3 days.

The optimal extraction conditions of quercetin from onion skin, according to the results of quercetin mass fraction, were obtained with conventional maceration extraction (CME), 50% ethanol as a solvent, 1:100 mass-to-liquid ratio, extraction time of 15 min and extraction temperature of 25 ℃. Under these conditions, the antioxidant capacity expressed as trolox equivalent antioxidant capacity (TEAC) was 104.5 μmol/g and the mass fraction of quercetin was 7.96 mg/g. The mass fraction result is in agreement with some of the previous scientific studies in this field [16] [12].

The optimal solvent results are in agreement with previous literature results since it is generally accepted that aqueous-based ethanol solutions are the optimal solvents. It was also concluded that the solvent type had a higher impact in yield of antioxidants and quercetin, when compared to the remaining variables in study, and therefore is an important parameter to be defined at the beginning of extraction experiments. It is important to note that both 50% and 70% ethanol were deemed the optimal solvents, however, considering the future applications of this extraction and in order to reduce possible costs and increase the health acceptability, the solvent 50% ethanol was chosen as optimal. However, this evaluation must be done for each process considering real economic and regulatory parameters.

Regarding the detection methods, DPPH• radical scavenging assay was used to analyze the antioxidant capacity of extracts through TEAC and high-performance liquid chromatography with diode-array detector (HPLC/DAD) was used to asses quercetin mass fraction in the onion skin dry powder (dry matter).

It was concluded that DPPH• assay can be used as a screening test to evaluate and predict the extraction efficiency of quercetin, since there is a correlation between TEAC results and the amount of quercetin in extracts given by HPLC/DAD analysis.

Regarding the comparison of single and double stage extraction, it was concluded that it can be worthwhile not only to perform a single stage extraction but to also proceed with the second stage in order to extract more antioxidants and quercetin present in the onion skin. For antioxidant capacity and quercetin mass fraction it is more worthwhile to perform second stage extraction for the extracts with 100% methanol and 2% acetic acid, respectively.

The comparison of CME and ultrasound-assisted extraction (USAE) showed that both the results of TEAC and mass fractions of quercetin in onion skin extracts are slightly higher after USAE compared to CME. The differences are more pronounced in the ethanol solutions and pure methanol. Therefore, the extraction method decision must be evaluated on a case-by-case basis to conclude if this slight increase in product yield generates a worthwhile profit/expenses ratio. For the purpose of this study, it was considered that CME was good enough regarding extraction efficiency.

Regarding the results after second stage extraction with USAE, it was concluded that both TEAC and mass fraction of quercetin in onion skin extracts results were very similar to the second stage extraction results with CME. Therefore, for the second stage extraction procedure, the CME method is enough to obtain the same results.

The mass-to-liquid ratio optimization concluded that 1:50 is the optimal ratio regarding TEAC results

and 1:100 is the optimal ratio regarding mass fraction of quercetin. These results suggest that quercetin requires higher volumes of solvent in order to be efficiently extracted from onion skin compared to other antioxidants. Since the goal of the work was to optimize quercetin extraction, the mass-to-liquid ratio of 1:100 was considered optimal.

The temperature optimization concluded that the TEAC and mass fraction of quercetin results do not vary considerably with the extraction temperature. However, the mass fractions of quercetin obtained with extraction at 25 ºC are slightly higher compared to the other temperatures studied.

Regarding the mass fraction of quercetin in the dry extracts, it was concluded that quercetin was purified during all extraction procedures tested, and that the mass of quercetin that can be retrieved from the dry extracts per mass of dry matter is the highest for the extracts obtained with 50% and 70% ethanol. Both these solvents were deemed optimal regarding quercetin purification in the context of dry extracts.

The results of the optimization of quercetin extraction are generally in agreement with most of the literature. There has been an extensive effort to optimize extraction conditions of quercetin from onion skin and other plant materials and this thesis contributes to this research as it confirms the optimal extraction solvent, combines an integrative study of a high number of experimental variables and includes the study of solvents not usually studied, such as 2% acetic acid and ethyl acetate.

Regarding future work, more studies should be conducted on the bioavailability, dosage, and impact of excessive quercetin on the human body, before its application in the food supplement and food industry market. Further studies regarding quercetin recovery and purification from ethanol onion skin extracts should also be conducted.

The onion skin extracts were also subjected to the α–amylase inhibition assay. It was concluded that all the analyzed extracts exhibit a dose-dependent relation between the concentration of dry extracts and the α–amylase inhibition, confirming the hypothesis that onion skin extracts containing quercetin could be used as anti-diabetic agents. The extracts with 50% ethanol showed to optimally inhibit α–amylase. This result represents a contribution to previous studies, which demonstrated other effects of quercetin on diabetes, confirming its potential to be used as an alternative therapy. However, studies regarding *in vivo* effects on α–amylase, safety, and dosage in humans are still required for its safe application.

Finally, the real impact of quercetin in onion skin extracts was studied in comparison to other existing compounds, regarding antioxidant capacity and anti-diabetic activity. The antioxidant capacity of pure quercetin represents 17% of the total antioxidant capacity of the extract. This result indicates that most of the antioxidant capacity of the onion skin extracts are possibly due to other polyphenols or antioxidant compounds and not only due to quercetin.

At the highest concentration, the standard solution of quercetin inhibits 65% of α-amylase activity while the extract inhibits 100% of α-amylase activity, which indicates that quercetin is the main compound responsible for anti-diabetic effects in the onion skin extracts.

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Appendix A

Calibration Curve - DPPH• assay

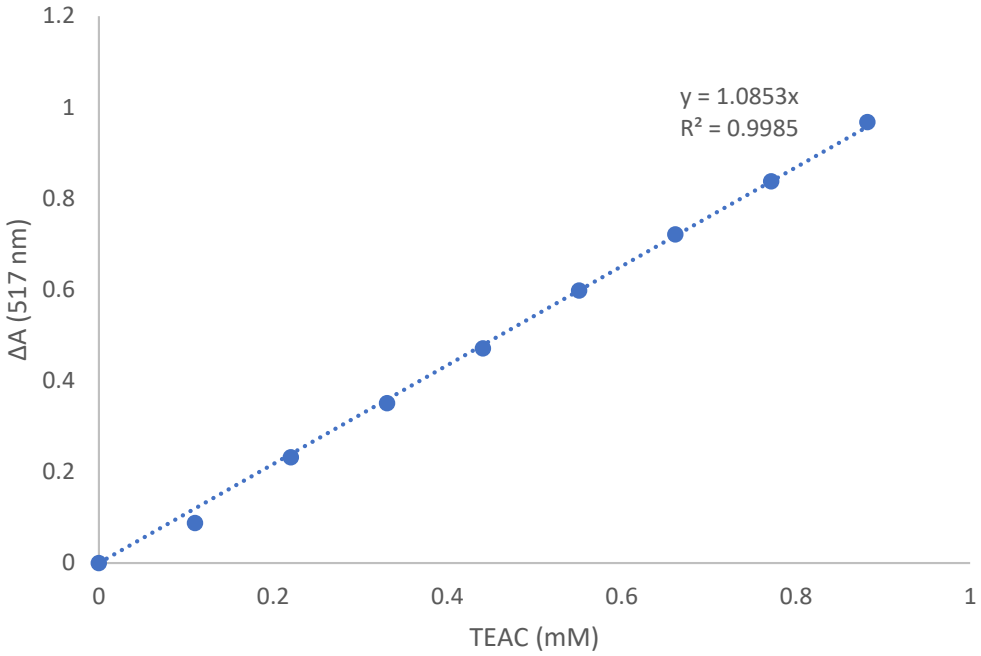

Figure A.1: DPPH• assay calibration curve for all solvents except ethyl acetate. ΔA represents the difference between the absorbance of the control and the absorbance of the sample, measured at 517 nm, and TEAC represents the trolox equivalent antioxidant capacity expressed as mM of trolox. The slope of the calibration curve is 1.08 Absorbance/mM.

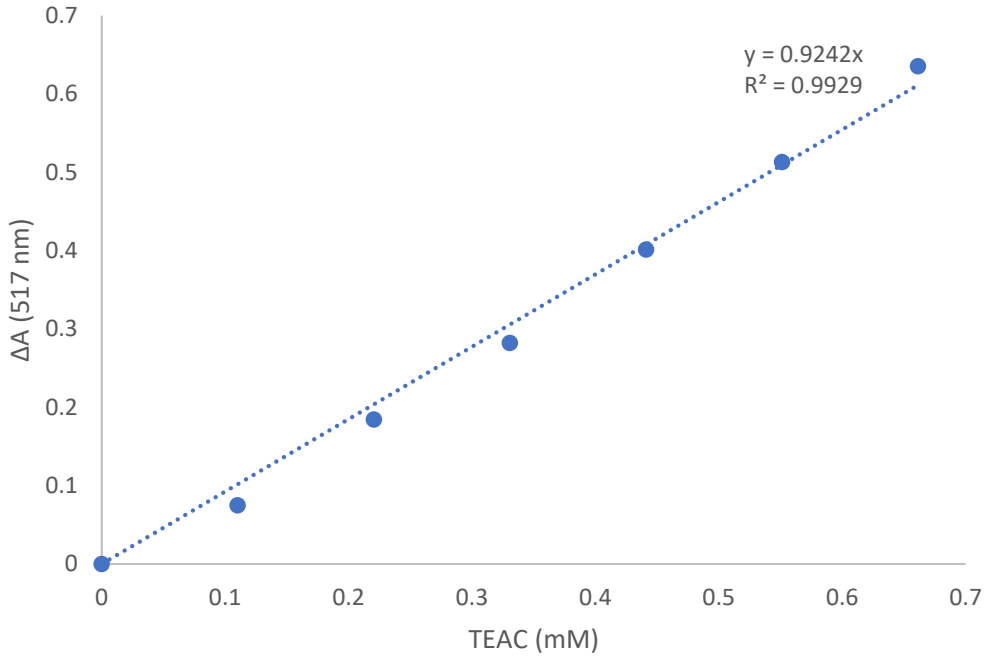

Figure A.2: DPPH• assay calibration curve for solvent ethyl acetate. ΔA represents the difference between the absorbance of the control and the absorbance of the sample, measured at 517 nm, and TEAC represents the trolox equivalent antioxidant capacity expressed as mM of trolox. The slope of the calibration curve is 0.924 Absorbance/mM.

Appendix B

Calibration Curve - HPLC/DAD determination

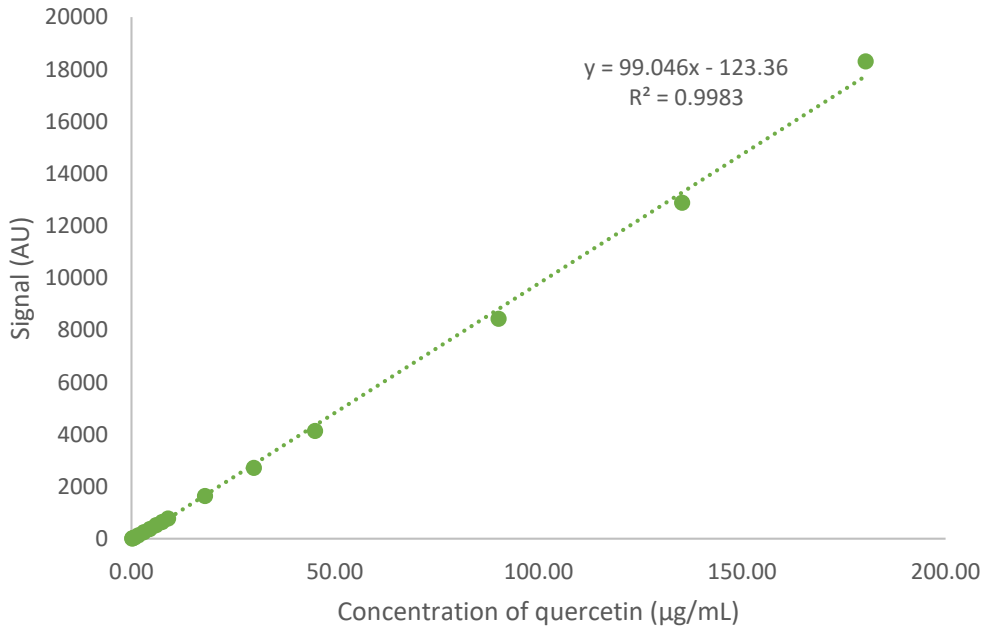

Figure B.1: HPLC/DAD calibration curve that correlates the signal of quercetin expressed in Area Units (AU) measured at 370 nm and the quercetin concentration of the standard solutions, expressed in µg/mL.

Appendix C

Calibration Curve - α-amylase inhibition assay

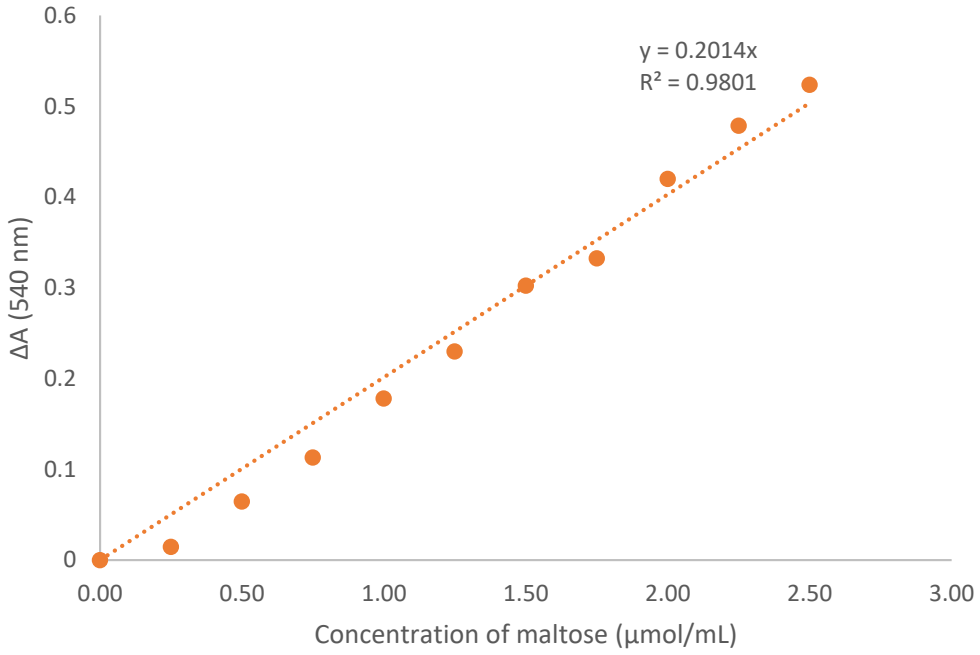

Figure C.1: α-amylase inhibition assay calibration curve. ΔA represents the difference between the absorbance of the sample and the absorbance of the control, measured at 540 nm, and the concentration of maltose is expressed in μmol of maltose per mL. The slope of the calibration curve is 0.201 Absorbance.mL/μmol.