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Optimization of quercetin extraction from onion skin

Determination of antioxidant capacity and anti-diabetic activity

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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 °C. 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.

1. Introduction

Brown onion, also known as yellow onion (*Allium cepa* L.) is a biennial herbaceous originating from the territory of western and central Asia. In the European Union, 500 000 t 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].

Quercetin has beneficial effects on human health because of its antioxidant, anti-inflammatory, antimicrobial, antiviral, anti-allergic, cardioprotective, vasodilatory and anticancer 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 [2]. It has also been claimed that quercetin reduces blood pressure in hypertensive subjects [3].

Quercetin is found in many medicinal plants, fruits and vegetables [2] and it is known that dry outer skin of brown onion is one of the richest sources of free quercetin [4], being 77-times more plentiful in the inedible parts of onions than in the edible parts [3].

Quercetin has the basic skeleton of a flavonoid, as seen in Figure 1, with two benzene rings (A and B) connected with each other via a three-member carbon fragment (C) looped via oxygen [2].

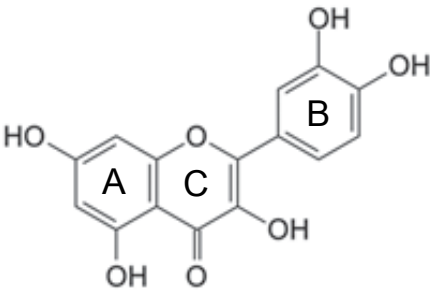

Figure 1: Chemical structure of quercetin and representation of rings A, B and C. [5].

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 [6]. 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 [7]

Quercetin often occurs in nature not only in its free form but also in the form of glycosides, the most common being rutin.

1.1. Extraction Methods

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]. A brief description of each method is presented below:

- CME: A weighed portion of the crushed solid plant sample is placed in a vessel, the 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 [3] [8].
- 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 [8].
- 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 [9].

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].

Regarding the solubility of quercetin in organic solvents, 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 [10]. Quercetin is therefore not soluble in water, partially soluble in ethanol and soluble in acetic acid and alkali, among others [2] [11]. Experimental data show that: at a constant temperature, the solubility of quercetin in methanol solutions and ethanol solutions increase with increasing methanol and ethanol contents; the solubility of quercetin in both solution mixtures increase smoothly with increasing temperature; and the highest solubility is obtained when ethanol solutions are used as solvent [12].

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 growth of the 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 [13].

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 [3].

There are several previous scientific results regarding optimization of quercetin extraction from onion skin. Jin *et al.* (2011) [9] optimized various procedures such as CME, USAE and MAE. 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 °C and 59.3% ethanol. However, the most productive method was MAE, in which the maximum extraction yield was 20.3% and 30.8% higher than USAE and CME, respectively.

Jang *et al.* (2012) [13] investigated extraction under sonication (USAE) conditions. 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 °C, 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 mass-to-liquid ratio of 1:64. The quercetin content in these extracts was 28.5 mg/g of the dry plant material.

1.2. 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). 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, polyphenols, among which quercetin, have been reported to improve diabetic status [14]. 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 [15].

Previous scientific studies regarding this subject related polyphenols to anti-diabetic effects. Bahadoran *et al.* (2013) [15] summarized the current knowledge on the impact of polyphenols, and specifically quercetin, in DM. Quercetin could interact with 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.

Snyder *et al.* (2016) [16] 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 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.

1.3. Applications and Challenges

Quercetin possesses several bioactivities, such as inhibition of proliferation of different types of 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 also has applications as a dietary supplement to improve the organoleptic quality and stability, as well as to extend the shelflife of food [6].

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. 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 [6] [17].

A possible negative effect of quercetin was described by Aguirre *et al*. (2011) [14]. Although quercetin has consistently failed to demonstrate adverse effects in animal studies, it appears to have inhibitory effects on cytochrome P450. These monooxygenase 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.

2. Materials and Methods

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 (Scale EXACTA 2200 EB Tehtnica, Slovenia) in beaker cups and frozen with liquid nitrogen before freeze-drying in a lyophiliser (ALPHA 1-2 LD Plus CHRIST, Germany) at -50 °C and 0.12 mbar for 3 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 (A11 Basic IKA, Germany) and stored in the freezer (-20 °C) until used.

All results of further analysis are expressed per gram of freeze-dried onion skin (named as g of dry matter), obtained by this procedure.

2.2. Chemical Reagents

DPPH• (2,2-diphenyl-1-picrylhydrazyl), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), formic acid (>98%) and quercetin (>95%) were obtained from Sigma Aldrich, Germany. Ethyl acetate (99.5%), acetonitrile (>99.9%) and ethanol (96%) were obtained from Honeywell, Riedel-de Haen, Germany. Absolute ethanol and absolute methanol were obtained from Emsure, Germany. Sodium hydroxide was obtained from Kemika, Zagreb. Sodium potassium tartrate tetrahydrate and maltose were obtained from Kemika, Slovenia. DNSA (2-hydroxy-3,5-dinitrobenzoic acid) and soluble starch were obtained from MERCK, Germany and finally, the enzyme α-amylase from hog pancreas (43.6 U/mg) was obtained from Fluka, Switzerland.

2.3. 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.

Note: for the following sections the mass of dry matter and reagents was weighted in an analytical scale (Mettler Toledo, AT201 Tehtnica, Switzerland).

2.3.1. Test A: solvent selection

For this test, a mass-to-liquid ratio of 1:10 was obtained by putting 100 mg of the previously powdered solids into test tubes and adding 1 mL of the following solvents: 2% acetic acid, 50% ethanol, 70% ethanol, absolute ethanol, absolute methanol, and absolute ethyl acetate.

The samples underwent conventional maceration extraction (CME) by being agitated in a thermo-shaker (TS1, Biometra, Germany) at 25 °C during four different timings: 15 min, 30 min, 1 h, and 2 h. They also underwent ultrasound-assisted extraction (USAE) on ultrasonic bath (Ultrasonic Cleaner 100W SHESTO, UK) at full mode at 25 °C 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 °C from the desired temperature. The samples were then centrifuged (Centrifuge Centric 200 Tehtnica, Slovenia) 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 °C) 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.

2.3.2. Test B: mass-to-liquid ratio selection

Three additional mass-to-liquid ratios were tested: 1:20, 1:50 and 1:100. For each mass-to-liquid ratio 1 mL of 50% ethanol solution was added to 50 mg, 20 mg and 10 mg of dry matter, respectively. Both CME and USAE were performed at 25 °C for four different timings (15 min, 30 min, 1 h, and 2 h) with only a single stage extraction.

2.3.3. Test C: temperature selection

CME was performed for temperatures of 40 °C and 60 °C, for four different timings (15 min, 30 min, 1 h, and 2 h) and single stage extraction with 50% ethanol solution and 1:50 mass-to-liquid ratio.

2.4. Determination of antioxidant capacity

The antioxidant capacity was measured using the free radical-scavenging DPPH• assay, which is based on the monitorization of the capacity of a sample to neutralize the stable free radical DPPH•, using a spectrophotometer UV/vis at 517 nm [18].

The calibration curve was obtained using sample solutions of different trolox concentrations from 5 to 50 μL using 1.10 mM trolox and supplementing with 2% acetic acid solution until 50 μL. The samples were prepared in duplicates. A control sample was also prepared containing 50 μL of 2% acetic acid solution. The samples were treated with 1 mL of 0.11 mM DPPH• solution, mixed and incubated at room temperature in the dark for 1 hour. Afterward, the absorbance was measured at 517 nm using an UV-VIS spectrophotometer (89090A, Agilent, USA) and 2% acetic acid solution was used as the blank. The absorbance of each sample was subtracted from the absorbance of the control sample and the calibration curve was plotted.

An additional calibration curve for the extracts with ethyl acetate was prepared, due to different response of this solvent, using 20 μL of ethyl acetate, 5 to 30 μL of 1.10 mM trolox and 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.

For the extract samples of Test A (excluding ethyl acetate samples), 1:10 dilutions of extracts using 2% acetic acid solution were performed. Afterward, 20 μL of the 10-times diluted extract was added to 30 μL of 2% acetic acid solution and mixed with 1 mL of 0.11 mM DPPH• solution. For the ethyl acetate samples, 20 μL of the extracts were mixed with 30 μL of 2% acetic acid solution and 1 mL of 0.11 mM DPPH• solution.

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

The samples were analyzed in two repetitions and treated as described above for the calibration curve procedure. The average values of the absorbance of the samples were subtracted from the absorbance of the control sample, 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 as 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 1. V represents the volume of solvent added to the dry matter in mL and $m_{\text{dry matter}}$ represents the mass of dry matter of onion skin weighted in mg.

$$c_{\text{TEAC in dry matter}} = \frac{c_{\text{TEAC in extracts}} \times V \times 10^3}{m_{\text{dry matter}}} \quad (1)$$

2.5. Quantification of quercetin by HPLC/DAD

The quantification of quercetin in the extracts was obtained using HPLC/DAD system Agilent 1260 Infinity, 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 × 12 mm, particle size 5 μm) [19]. The autosampler temperature was set to 10 °C, the column temperature was set to 35 °C, 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.

A mobile phase solution was prepared with 50 mL of solution B with 150 mL of solution A and used to dilute samples for analysis. A stock solution of quercetin 0.9 mg/mL was obtained by weighing 45.09 mg of quercetin and dissolving it in 50 mL of absolute ethanol.

For the calibration curve, 15 solutions with quercetin concentrations ranging from 0.15 μg/mL to 180 μg/mL were prepared by using different volumes of 0.9 mg/mL stock solution and supplementing with mobile phase solution until 1 mL. The samples were analyzed by HPLC/DAD and the chromatograms of the samples were recorded from 254 to 400 nm, whereas quercetin was determined at 370 nm; the area signals of the samples were then correlated with the concentration of quercetin in order to obtain the calibration curve.

Dilutions of onion skin 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. The samples were then centrifuged at 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.

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. V represents the volume of solvent added to the dry matter in mL and $m_{\text{dry matter}}$ represents the mass of dry matter in mg.

$$w = \frac{c_{\text{quercetin in extracts}} \times V}{m_{\text{dry matter}}} \quad (2)$$

2.6. Anti-diabetic activity

The anti-diabetic activity was assessed using the α-amylase inhibition assay, which measures the concentration of reducing sugars spectrophotometrically at 540 nm using dinitrosalicylic acid (DNSA) as the color reagent [20]. 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 [21].

The calibration curve was prepared from maltose stock solution (1.8 mg/mL), by preparing 10 dilutions ranging from 0.25 to 2.5 μmol/mL. 400 μL of each dilution was pipetted into the 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 °C in a dry bath heating system (Star Lab, Taiwan), 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 absorbance of the samples and the maltose concentration was plotted.

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 analyzed in this assay were dried in a vacuum concentrator centrifuge (UNIVAPO 100 H, UNIEQUIP) for 3-6 hours until completely dried and redissolved in the respective solvent in order to obtain the same final concentration of 10 mg of dry extract per mL of solvent. 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 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride (SPB). Test tubes were prepared in duplicate with 100 μL of each concentration and 100 μL of the enzyme α-amylase solution 0.1 mg/mL (AAS). Each of these tubes had a corresponding control tube (without enzyme) with 100 μL of each sample and 100 μL of SPB. Another control test tube corresponding to 100% activity of the enzyme, was also prepared in duplicate by adding 100 μL of SPB and 100 μL of AAS. The blank for this control had 200 μL of SPB.

All the test tubes as well as 1% starch solution (SS) were incubated for 15 minutes at 25 °C. Afterward, 200 μL of SS 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. Afterward, the procedure was conducted as described for the calibration curve. The absorbance of the control of each sample was subtracted from the respective sample absorbance. 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 3. $c_{100\%\ \text{activity}}$ 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.

$$\%\ \text{Inhibition} = \frac{c_{100\%\ \text{activity}} - c_{\text{sample}}}{c_{100\%\ \text{activity}}} \times 100 \qquad (3)$$

3. Results and Discussion

There are four variables studied in this work in order to optimize the quercetin extraction from onion skin: solvent, mass-to-liquid ratio, time and temperature. For each section, the results from DPPH• assay and 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.

3.1. Solvent selection

This experiment was conducted according to Test A described in section 2.3.1, in order to obtain the optimal solvent. The CME and USAE, as well as single and double stage extraction are also compared in this section.

3.1.1. Conventional maceration extraction

The results of TEAC, determined by the DPPH• assay and mass fraction of quercetin, determined by HPLC/DAD, obtained after first stage extraction with CME, are presented in Figure 2 and Figure 3, respectively.

As seen in Figure 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, 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 quercetin displays an amphipathic behavior [10], as described in section 1.1 of Introduction, which means it will have a higher solubility in solvents that also display this behavior, such as ethanol/water solutions [13].

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 higher for 15 min and 30 min extractions. However, the results are mostly inside the experimental error. Due to the presented results, the following experiments with USAE were performed with 15 minutes or 30 minutes extraction.

As seen in Figure 3, the results of quercetin mass fraction follow a similar pattern as the TEAC results (Figure 2). The higher values of quercetin were obtained for 50% ethanol (from 2.7 to 4.0 mg/g) and 70% ethanol (from 3.8 to 4.0 mg/g) extracts, whereas the lower values of quercetin were determined for 100% ethyl acetate (from

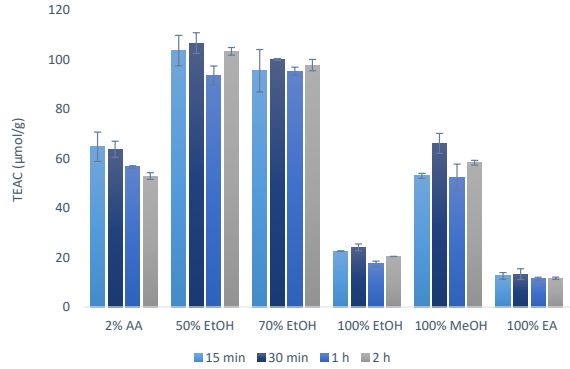

Figure 2: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after first stage conventional maceration extraction at 25 °C, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in μmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

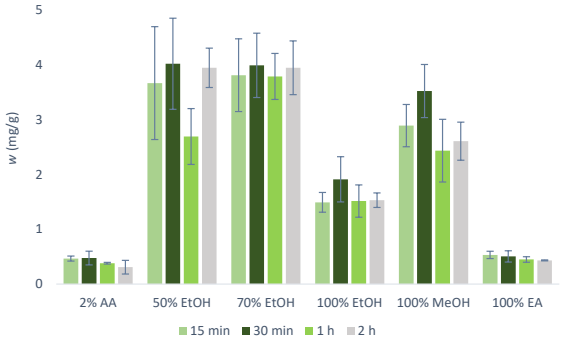

Figure 3: Mass fraction (*w*) of quercetin in extracts obtained after first stage conventional maceration extraction at 25 °C, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate

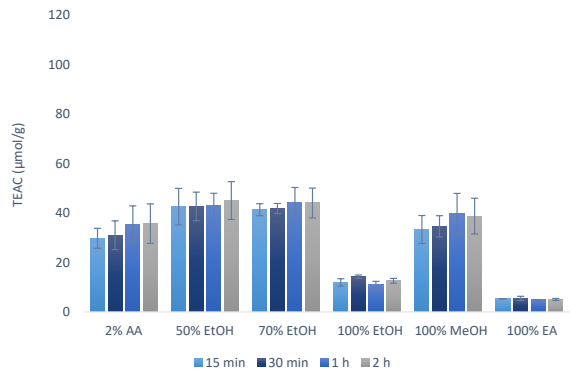

Figure 4: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after second stage conventional maceration extraction at 25 °C, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in μmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

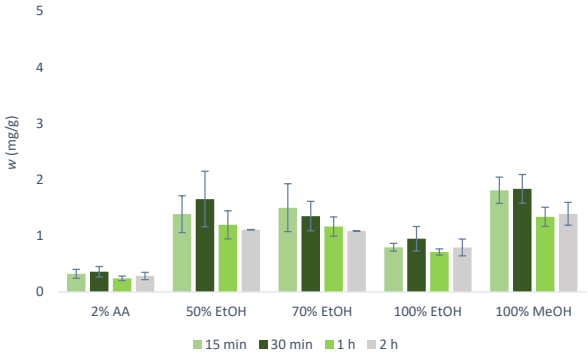

Figure 5: Mass fraction (*w*) of quercetin in extracts obtained after second stage conventional maceration extraction at 25 °C, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. The ethyl acetate results are not presented since the HPLC peak areas results were below the limit of detection AA - acetic acid; EtOH - ethanol; MeOH - methanol.

0.43 to 0.53 mg/g) and 2% acetic acid (from 0.31 to 0.47 mg/g).

The observed correlation between the antioxidant capacity (TEAC) 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 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 [2] [11], which is the main constituent of 2% acetic acid solvent.

The results of TEAC, determined by the DPPH• assay and mass fraction of quercetin, determined by HPLC/DAD, obtained after second stage extraction with CME, are presented in Figure 4 and Figure 5, respectively.

As seen in Figure 4, fewer antioxidants (lower TEAC) were present in onion skin extracts after second stage compared to first stage extracts, however, the values are not negligible. As seen in Figure 5, the results of quercetin mass fraction follow the same pattern as the TEAC results of Figure 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 percentage of TEAC and mass fraction determined in samples after second stage extraction, in comparison with the first stage extraction, was also calculated. All samples obtained after second stage extraction contain a considerable amount of antioxidants, expressed as TEAC (43% to 64%) and a considerable amount of quercetin, expressed as mass fraction (33% to 75%). This means that it can be 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: for the mass fraction results, the solvent with the highest remaining percentage of quercetin is 2% acetic acid (75±12%), which does not happen for the TEAC results (56±10%). This result can be explained by the poor solubility of quercetin in water [2] [11], 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 more important for 2% acetic acid extraction solvent than for 50% or 70% ethanol extraction solvent.

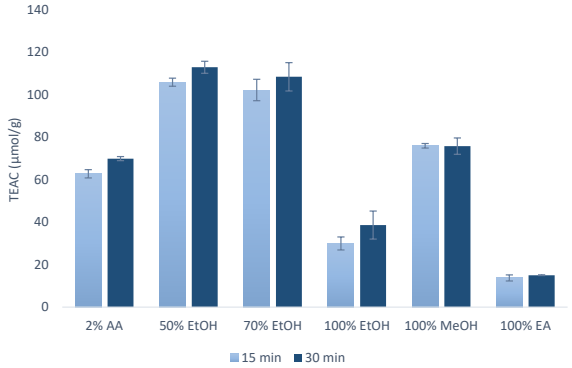

Figure 6: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after first stage ultrasound-assisted extraction (USAE) at 25 °C, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in μmol of TEAC per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

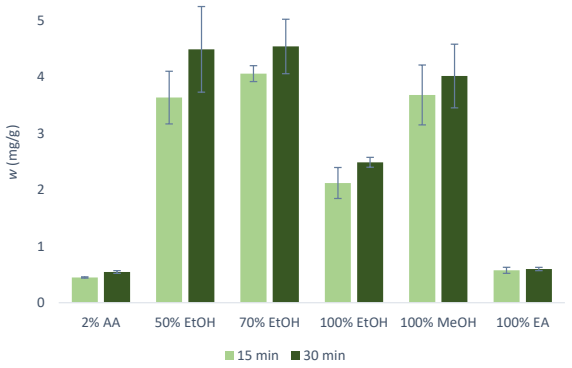

Figure 7: Mass fraction (w) of quercetin in extracts obtained after first stage ultrasound-assisted extraction (USAE) at 25 °C, for 1:10 mass-to-liquid ratio, with different solvents and different timings. Results are expressed in mg of quercetin per g of dry matter. AA - acetic acid; EtOH - ethanol; MeOH - methanol; EA - ethyl acetate.

3.1.2. Ultrasonic assisted extraction

The results of TEAC, determined by the DPPH• assay and mass fraction of quercetin, determined by HPLC/DAD, obtained after first stage extraction with USAE, are presented in Figure 6 and Figure 7, respectively.

As seen in Figures 6 and 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 [8]. 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). 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.

3.2. 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 the process acceptability by reducing health concerns regarding the final product. 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 [3], therefore, the solvent 50% ethanol was chosen as the optimal solvent for the following experiments.

This experiment was conducted according to Test B described in section 2.3.2, in order to obtain the optimal mass-to-liquid ratio. The results of TEAC, determined by the DPPH• assay and mass fraction of quercetin, determined by HPLC/DAD, obtained after single stage extraction with CME, are presented in Figure 8 and Figure 9, respectively.

As seen in Figure 8 the best mass-to-liquid ratio regarding TEAC values is 1:50. 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.

As seen in Figure 9 the mass fraction distribution of quercetin in onion skin extracts considering the mass-to-liquid ratio is slightly different compared to the TEAC distribution in Figure 8. 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. 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.

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.

3.3. Temperature selection

This experiment was conducted according to Test C described in section 2.3.3, in order to obtain the optimal extraction temperature. The results of TEAC, determined by the DPPH• assay and mass fraction of quercetin, determined by HPLC/DAD, obtained after single stage extraction with CME, are presented in Figure 10 and Figure 11, respectively.

As seen in Figure 10 and Figure 11, 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

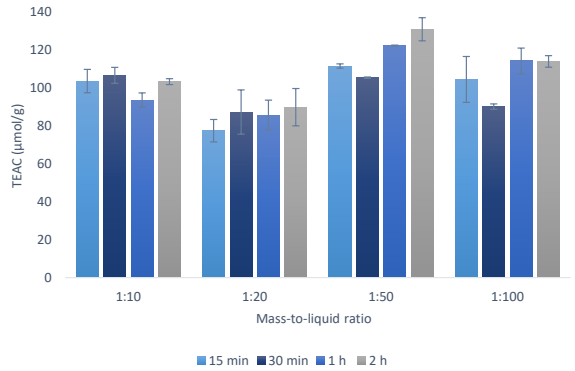

Figure 8: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after single stage conventional maceration extraction at 25 °C, with 50% ethanol, with different mass-to-liquid ratios and different timings. Results are expressed in µmol of TEAC per g of dry matter.

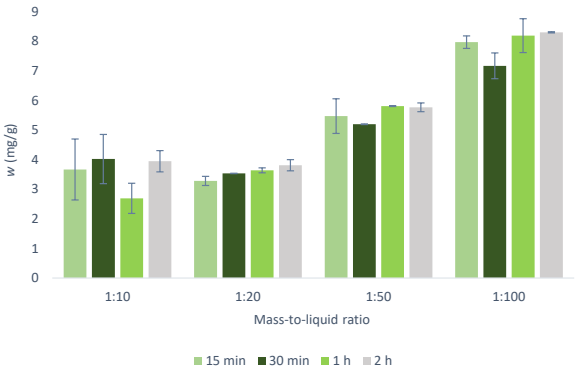

Figure 9: Mass fraction (*w*) of quercetin in extracts obtained after single stage conventional maceration extraction at 25 °C, with 50% ethanol, with different mass-to-liquid ratios and different timings. Results are expressed in mg of quercetin per g of dry matter.

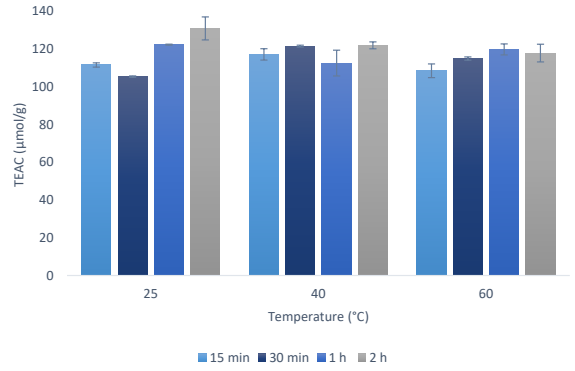

Figure 10: Trolox equivalent antioxidant capacity (TEAC) of extracts obtained after single stage conventional maceration extraction with 50% ethanol and 1:50 mass-to-liquid ratio, with different temperatures and different timings. Results are expressed in µmol of TEAC per g of dry matter.

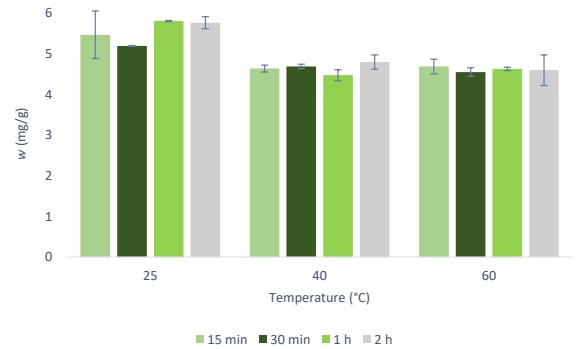

Figure 11: Mass fraction (*w*) of quercetin in extracts obtained after single stage conventional maceration extraction with 50% ethanol and 1:50 mass-to-liquid ratio, with different temperatures and different timings. Results are expressed in mg of quercetin per g of dry matter.

quercetin is slightly higher at 25 °C 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 Introduction [12]. 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 °C to 40 °C does not result in an increase in extraction yield.

3.4. 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. 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) [13], 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.

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) [9]. In this study, 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 ob-

tained in this thesis.

The study by Jang *et al*. (2012) [13] investigated the extraction of quercetin with aqueous ethanol solutions from onion solid waste under sonication (USAE) conditions. 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.

3.5. Anti-diabetic activity

As described in section 2.6, it was necessary to dry the extracts in order to re-dilute them to the desired dry extract concentration for the α-amylase assay. The anti-diabetic activity results are presented in Figure 12.

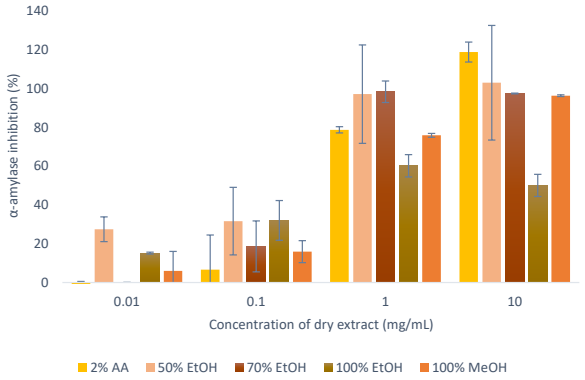

Figure 12: Anti-diabetic activity expressed in percentage of α-amylase inhibition, of extracts with different concentrations of dry extract. AA - acetic acid; EtOH - ethanol; MeOH - methanol

The inhibition graph presented in Figure 12 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 extracts had no anti-diabetic effect at the lowest concentration tested (0.01 mg/mL).

Extracts obtained with 50% ethanol and 70% ethanol appear to completely inhibit α-amylase already at 1 mg/mL and for 100% ethanol the inhibition 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 in general 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 [15] [16]. However, the results of this thesis demonstrate 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.

4. Conclusions

The optimal extraction conditions of quercetin from onion skin, according to the results of quercetin mass fraction, were obtained with CME, 50% ethanol as a solvent, 1:100 mass-to-liquid ratio, extraction time of 15 min and extraction temperature of 25 °C. Under these conditions, the antioxidant capacity expressed as 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 [13] [9].

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 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 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. The comparison of CME and USAE concluded that both the results of TEAC and mass fractions of quercetin in onion skin extracts are slightly higher after USAE compared to CME.

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.

Regarding anti-diabetic activity, 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. 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.

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