A STRATEGY FOR ENHANCING THE EXTRACTION YIELD OF POLYPHENOLS FROM SEA BUCKTHORN LEAVES

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In this paper, the influence of enzymatic pretreatment on the microwave assisted extraction (MAE) of polyphenols from sea buckthorn leaves is described. Different parameters, such as enzyme concentration, extraction time, and type of enzyme were studied. Comparative extractions without enzymatic pretreatment were carried out. The best results were achieved for an enzyme concentration of 1% and an extraction time of 300 s. The Glucanex enzyme led to a higher total phenolic content (TPC) compared with Ultrazyme and Carezyme.

Keywords: enzymatic pretreatment, microwave, polyphenols, sea buckthorn

1. Introduction

Sea buckthorn (*Hippophae Rhamnoides* L.), which belongs to *Elaeagnaceae* family, is a nitrogen-fixing bush widely found in temperate and subtropical areas. All its parts are rich in a wide range of bioactive compounds, such as tocopherols, carotenoids, polyphenols, sterols, vitamins, lipids, and minerals [1, 2]. These components are useful in medicinal and pharmaceutical applications due to the antioxidant, anti-inflammatory, antibacterial, antiviral, and antitumor properties [3].

Polyphenols are valuable metabolites synthesized by plants which can act as antioxidants, protective agents against UV light, phytoalexins and attractants for pollinators [4-6]. Conventional extraction methods of non-volatile bioactive compounds are Soxhlet extraction and maceration. These methods present low efficiency due to high temperatures or long extraction times that could lead to degradation of valuable constituents and require high consumption of organic

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solvents [7]. In recent years, more efficient extraction processes, such as ultrasound and microwave assisted extractions [8, 9], simultaneous ultrasonic-microwave assisted extraction [10-12], pressurized and supercritical fluid extractions [13, 14], pulsed electric field [15, 16], microwave and enzyme co-assisted extraction [17] have been developed.

MAE is an alternative process used for polyphenols extraction from vegetal material, being an environmentally friendly process. The advantages of MAE are a shorter extraction time, better extraction yield, high extraction efficiency and selectivity, good control of heating process, and low energy consumption [18-21].

The distribution of phenolic compounds in plants is not uniform. Insoluble compounds are found in the cell walls, while soluble components are within the plant cell vacuoles [22, 23]. Thus, the structure of the plant matrix is an important factor influencing the extraction efficiency and any strategy to modify it to enhance the extraction yield is attractive/desirable. Such a strategy is represented by the enzymatic pretreatment [24, 25]. It can soften or break the cell walls, allowing the solvent to easier access the valuable constituents [26]. Enzymes such as glucuronidases, cellulases, hemicellulases, pectinases, glucanases, amylases, and tannases have already been used to break the carbohydrate linkages and to disrupt the cell wall structure [27]. The aim of this work was to study the influence of enzymatic pretreatment on the MAE of polyphenols from sea buckthorn leaves.

2. Material and methods

2.1. Materials

The sea buckthorn leaves (*Hippophae Rhamnoides* L.) were harvested in the summer of 2017 at Hofigal S.A., in Furculesti. The fresh leaves were dried in an airflow heating oven at 60 °C to a constant weight. The dried leaves were ground using an electric grinder and sieved to a particle size under 1 mm. The ground sea buckthorn leaves were dosed in samples of 25 g (in sealed plastic vessels) and stored at 4-5 °C until they were used for the extraction of polyphenols. Folin-Ciocalteu reagent (Merck), ethanol and sodium carbonate were of analytical grade. The enzymes (Glucanex – mixture of β-glucanase, cellulase, protease, and chitinase; Carezyme 5000T – cellulase; Ultrazyme 100T – pectinase) were kindly donated by Novozymes A/S (Denmark).

2.2. Enzymatic pretreatment procedure

To maintain the pH value at 5 during the enzymatic pretreatment, sea buckthorn leaves were mixed with a buffer solution containing 0.1 M citric acid and 0.2 M $Na_2HPO_4 \cdot 2H_2O$. The experiments were performed in a 1:1.1 (v:v) ratio of citric acid to sodium phosphate dihydrate. Enzymes were added in the

pretreatment mixture and stirred at 300 rpm for 30 min at 40 °C. The weight percentage concentration of enzymes related to substrate was 1, 5, and 10% for Glucanex and 1% for the other ones. After pretreatment, the mixture was submitted to MAE of polyphenols. Control samples, without enzymatic pretreatment, were also performed.

2.3. MAE procedure

MAE of polyphenols was performed in a microwave applicator (Biotage®Initiator). The extractions were carried out using a mixture of 50% ethanol in buffer solution, a 20:1 (v:w) ratio of solvent to plant and a temperature of 60 °C. Individual experiments were performed considering the following extraction times: 200, 300, and 450 s. After extraction, the mixture was centrifuged at 3000 rpm for 10 min at room temperature and the fresh supernatant was further analyzed. The polyphenolic extracts were analyzed in order to determine the TPC.

2.4. Determination of total phenolic content

TPC of extracts was colorimetrically evaluated using the Folin-Ciocalteu method according to International Standard ISO 14502-1 with minor modifications (see our previous work [28]). The results were quantified as milligrams of gallic acid equivalents per 1 gram of dry matter (mg GAE/g DM) using the standard curve corresponding to 1-5 mg/mL gallic acid solution.

3. Results and discussion

Enzyme assisted extraction technique offers multiple advantages, but there are some major limitations, such as lower efficiency and high extraction time. These shortcomings can be overcome by the combined microwave-assisted enzymatic extraction. Considering the extraction of polyphenols from plants, this procedure is a powerful technique, due to a number of advantages, such as reduced extraction time, high extraction efficiency, environmental compatibility, and lower solvent consumption [29].

MAE is a commonly used green technique for bioactive compounds extraction. The microwave extractions combined with enzymatic treatment depend on various parameters, such as absorption of the microwave energy by polar solvents, stirring rate, and temperature. Since enzymes can be inactivated due to severe conditions, all the MAEs of polyphenols were performed at 300 rpm and 60 °C, using a mixture of 50% ethanol in water as extraction solvent [30].

3.1. Enzyme screening for pretreatment of polyphenols extraction

Plant cell walls comprise of a series of components, such as cellulose, hemicellulose and lignin, as well as pectin and proteins. Bioactive compounds are linked to the cell wall components by hydrophobic interactions and hydrogen

bonds. These interactions ensure cell stability and resistance to the extraction of the biocomponents. Therefore, enzymes with different specific hydrolytic properties (glucanases, cellulases, pectinases, and proteases and their combinations) are used to hydrolyze the bonds between the cell polysaccharides, and to improve the extraction of polyphenols. Glucanex (mixture of β-glucanase, cellulase, protease, and chitinase), Carezyme 5000T (cellulase) and Ultrazyme 100T (pectinase) were chosen for the screening procedure.

Glucanex can release phenolic acids from plant material by hydrolyzing the celluloses and β-glucans, by modifying the protein functionality, and by solubilizing proteins and their aggregates. Carezyme 5000T and Ultrazyme 100T are used to improve the extraction yield by breaking down the cell walls through cellulose and pectin hydrolysis.

The results regarding the influence of enzyme type on the TPC of sea buckthorn leaves are shown in Fig. 1.

It can be noticed that, compared with Ultrazyme and Carezyme, the extraction is more efficient when a pretreatment with Glucanex is performed, the TPC being approximatively 30% higher. These results can be explained by the enzyme complexity (Glucanex), whose components exhibit different lyase roles. The lower efficiencies of both Carezyme and Ultrazyme can be caused by the proteins which may be found in the extracts after enzymatic degradation of the cell walls. These proteins can form complexes with phenolic compounds, leading to a TPC decrease [31]. Regardless of the type of enzyme used, the enzymatic pretreatment leads to better results compared with the extraction without pretreatment (see Fig. 1). Since Glucanex led to a higher TPC value compared with the other enzymes, all further experiments were performed using Glucanex.

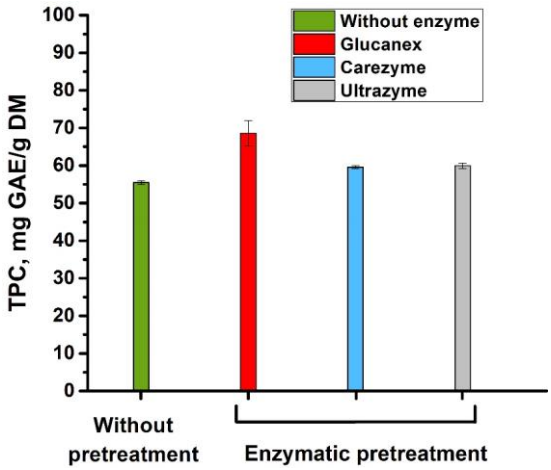

Fig. 1. Influence of different enzymes on the TPC of sea buckthorn leaves for an enzyme concentration of 1% and an extraction time of 200 s.

3.2. Effect of enzyme loading

Enzyme-assisted extraction of polyphenols from plants has some limitations, such as the cost of enzymes and the difficulty to scale up. They are relatively expensive for processing large volumes of plant material (when a high amount of enzyme is required) and exhibit sensibility at various environmental conditions: temperature, pH, and the percentage of dissolved oxygen. Thus, the next step of this study was to establish the enzyme amount needed for the pretreatment.

As shown in Fig. 2, the best results were achieved for an enzyme concentration of 1 and 5%. Also, it can be noticed that for all enzyme concentrations the TPC was 18-25% higher than for the extraction without enzymatic pretreatment. When the amount of enzyme is increased up to 10%, the polyphenolic content decreases, due to substrate saturation based on an excessively high enzyme concentration, resulting in a waste of enzyme. Considering the economic aspects mentioned above, although the polyphenols content for an enzyme concentration of 1 and 5% are similar, all further experiments were performed using the 1% concentration.

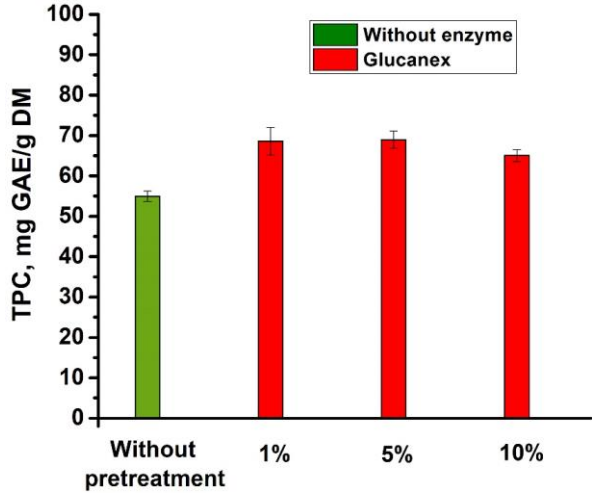

Fig. 2. Influence of enzyme concentration on the TPC of sea buckthorn leaves for an extraction time of 200 s using Glucanex.

3.3. Effect of extraction time

The extraction of phenolic compounds can be influenced by the extraction time. It is dependent on the enzymatic concentration. Recent studies showed that the enzyme amount can be reduced up to half its initial value if the extraction time is increased twice [31]. However, a prolonged microwave treatment can lead to the degradation of polyphenols (due to long exposure at elevated temperatures), while increasing the energy consumption proportionally. The extraction time

influence on the TPC of sea buckthorn leaves are shown in Fig. 3. It can be noticed in Fig. 3 that the polyphenols content increases with the extraction time. The best results are achieved for 300 and 450 s. For all the extraction times, the enzymatic pretreatment leads to a higher polyphenolic content compared with the extractions without pretreatment (25-40% higher). Due to the insignificant difference between TPC values for 300 and 450 s and considering the energy consumption, the optimal extraction time is 300 s. Also, using a short extraction time, the degradation of polyphenols can be avoided.

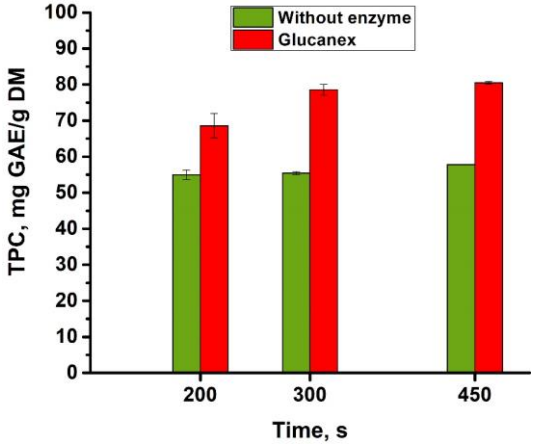

Fig. 3. Influence of extraction time on the TPC of sea buckthorn leaves for an enzyme concentration of 1% using Glucanex.

4. Conclusions

Enzymatic pretreatment is an efficient strategy to enhance the polyphenolic compounds extraction yield. The enzyme disrupts the plant cell walls; therefore, the polyphenols are released more easily from the vegetal material. The influence of different parameters – enzyme concentration, extraction time, type of enzyme – on the extraction efficiency were studied. The enzymatic pretreatment led to a 40% higher TPC value compared with the extraction without pretreatment. The best results were achieved using the Glucanex enzyme, with a concentration related to the substrate of 1%, and an extraction time of 300 s.

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