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Influence of enzymatic pretreatment on yield and chemical composition of *Rosmarinus officinalis* essential oil

Małgorzata Dzięcioł

West Pomeranian University of Technology, Szczecin, Faculty of Chemical Technology and Engineering, Department of Chemical Organic Technology and Polymeric Materials, Piastów Ave. 42, 71-065 Szczecin, Poland
**Corresponding author: e-mail: malgorzata.dzieciol@zut.edu.pl*

Effect of enzymatic pretreatment before hydrodistillation process on yield and composition of *Rosmarinus officinalis* essential oil was studied. Results obtained by using two selected commercial enzymes applied in food and beverage industry were compared. Control process with non-enzymatic pretreatment in analogous conditions was also performed for proper interpretation of results. Application of gas chromatography with mass selective detector (GC-MS) enabled analysis and comparison of essential oils composition. Moreover, total phenolic content (TPC) was determined spectrophotometrically in post-processing hydrolates, which are also valuable products e.g. for cosmetic applications. Modifications of isolation process by pretreatment with selected enzymes resulted in significant increase in essential oil yields in comparison to conventional hydrodistillation and control process with non-enzymatic pretreatment in analogous conditions. No substantial changes in the composition of obtained essential oils were observed. In post-processing hydrolates higher values of total phenolic content (TPC) were found both after enzymatic and non-enzymatic pretreatment.

Keywords: *Rosmarinus officinalis* essential oil, *Rosmarinus officinalis* hydrolate, modification of hydrodistillation, enzymatic pretreatment, total phenolic content (TPC).

INTRODUCTION

Rosmarinus officinalis (rosemary) is popular aromatic plant originating from the Mediterranean region. It is widely used as a spice and traditional remedy for treatment in the case of various symptoms including anxiety, depression, memory problems, or arthritic pain. Rosemary extracts contain a wide variety of biologically active substances, mainly from the group of phenolic compounds, and are known for their antioxidant and antimicrobial potential. They are accepted in the European Union as safe food additives applied to prevent oxidation and microbial contamination of food and beverages[1–3]. Besides extracts, essential oils obtained from rosemary are applied as active ingredients in cosmetics, food products, medicine, and aromatherapy. These complex mixtures of terpenes and terpenoids, including 1,8-cineole, camphor, α- and β-pinene, camphene, borneol, and many other minor components, are valuable natural products not only because of their characteristic aromatic properties but also due to their antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, hepatoprotective, antiproliferative and anticancer activity[4–10].

The current trends related to the use of natural substances as safe substitutes for synthetic biological active substances lead to an interest in various methods of enhancing the processes of their isolation. In the case of essential oils obtained by hydrodistillation or steam distillation, the effects of various physical factors like microwaves[11–13] or ultrasounds[13–14] as well as chemical modifications including the addition of salts, acids, or ionic liquids[13, 15–18] on yield, composition, and biological activity were investigated. For *Rosmarinus officinalis* essential oil modification of hydrodistillation process by application of 5% citric acid significantly enhanced yield (by 21%), as well as its antioxidant and antifungal activity against various fungi strains: *Alternaria alternata*, *Botrytis cinerea*, *Phythophtora cactorum*, *Rhizoctonia solani*, *Phythophtora infestans*, *Sclerotinia sclerotiorum* and *Ascosphaera apis*[17]. Good results were also obtained by the addition of ionic liquids (ILs) during hydrodistillation of rosemary essential oil, what improved its yield by 25%[16]. In turn, replacing water with 5% trisodium citrate was less effective allowing only for 12% increase in yield[17], and the application of 5% sodium chloride did not affect yield[16]. An interesting and effective method of enhancing the yield of valuable compounds originated from plants can be also elicitation. In this process application of different types of abiotic and biotic elicitors induce response of the plant which can result in modulation of metabolism, what enables the changes in the essential oil yield and its chemical composition. Choosing and applying of most suitable elicitors (e.g. salts, hormones, lysates of pathogens) can improve the production of selected terpenes by plants[19].

Relatively new strategy is enzymatic pretreatment of plant materials before isolation process of essential oil, using various enzymes in proper conditions for their activity. Enzymatic degradation of plant tissues secretory structures can enhance releasing of its volatile components both by removing physical barriers and breaking chemical bonds. It is known from the literature that breaking glycosidic bonds which can keep some volatile compounds bounded in non-volatile form may improve hydrodistillation yield[20–22]. Enzymatic pretreatment of orange, grapefruit, and lemon waste peels using cellulase resulted in a several-fold increase in the yield of citrus essential oils isolated by hydrodistillation[23]. Positive effect on the yield of *Lavandula angustifolia* essential oil was also obtained by plant material pretreatment using cellulase[24]. Application of enzymatic pre-treatment with a mixture of cellulase, β-glucanase, pectinase, and xylanase resulted in an improvement of black pepper essential oil yield from 0.9 to 1.8% and cardamom essential oil yield from 1.9 to 2.5%, what was accompanied by significant changes in their composition[25]. Positive effect of enzymatic treatment using cellulose and pectinase in enhancing of pervaporative (PV) process of limonene, linalool, and linalyl acetate separation from bergamot

oil was also described[26]. Moreover, it was reported that enzymatic pretreatment of rosemary before hydrodistillation using cellulase, hemicellulase, and their combination allowed for the enhancement of rosemary essential oil yields by 5, 50, and 20%, respectively[27]. In essential oils obtained in these enzymatically modified processes, significant changes in composition were observed. They were characterized by lower content of 1,8-cineole and enhanced activity against various pathogenic microorganisms, including *Salmonella typhimurium*, *Escherichia coli,* and *Candida albicans*[27]. It is important that in some cases the beneficial effects of enzymatic pretreatment can be caused not by enzymes activity but by the acidic pretreatment of the plant material during their application, therefore appropriate control experiments should always be performed to elucidate the observed effects[28].

In this research, the effect of enzymatic pretreatment before hydrodistillation on the yield and composition of *Rosmarinus officinalis* essential oil was studied by using two commercial enzymes applied in the food and beverage industry: Hazyme DCL (amyloglucosidase) and Rapidase Fiber (pectinase enriched in arabinolytic and cellulytic activities). Control process of non-enzymatic pretreatment in analogous conditions was also performed for comparison of results and their interpretation. The influence of these pretreatments on the yield and composition of rosemary essential oils was examined. Moreover, total phenolic content (TPC) in post-processing hydrolates, which are also valuable fraction for different potential applications, were analyzed and compared.

EXPERIMENTAL

Isolation of essential oils

Dried and cut leaves of rosemary (*Rosmarinus officinalis*) (producer: KOL-POL, origin: Morocco) were used in the experiments. Rosemary essential oils were obtained by 1-hour hydrodistillation (HD) process using Deryng apparatus. Prior to selection of experiment conditions, studies on influence of hydrodistillation time (0.5, 1, 2, 3 h) on yield of essential oil were performed. It was found that essential oil yield increased with time (1.68; 2.07; 2.60; 2.72% v/w, respectively). The 1-hour time of hydrodistillation was selected because in these conditions isolation process of essential oil was not complete, what allowed to study of the possibility of increasing the yield by application of enzymatic pretreatment. Before starting the isolation process, 50.00 g of plant material and 500 mL of deionized water were placed in a 1000 mL round bottom flask and connected to Deryng apparatus. After completion of the process, the essential oil was separated and the yield in % (v/w) was calculated from its volume read from the scaled tube in the apparatus and the weight of dry plant material used for the experiment.

Possibility of increasing the yields of essential oils by enzymatic pretreatment before hydrodistillation was investigated, using two liquid commercial enzymes applied in the food and beverage industry: Hazyme DCL and Rapidase Fiber (producer: DSM Food Specialties, USA). The optimal conditions for enzymes activity (pH, temperature, time) were used in accordance with the technical information provided by the manufacturer (Table 1). Both enzymes were applied at 1% (w/w) dosage, using 0.50 g per 50.00 g of plant material, in a total amount of 500 mL of water acidified to pH 4.5.

For each pretreatment process, 500 mL of acidified water solution with a pH adjusted to 4.5 with citric acid (Stanlab, Poland) was prepared and used instead of deionized water. In experiments with enzymatic pretreatment, 50.00 g of plant material was placed in a 1000 mL round bottom flask and contacted under a reflux condenser for 2 minutes with 250 mL portion of hot acidified water preheated to 95 °C. After this short blanching of plant material, another portion (200 mL) of acidified water was added, what resulted in a decrease in the temperature to 50 °C. Finally, 50 mL of acidified water solution containing 0.50 g of enzyme was added and kept in a water bath at 50 °C under a reflux condenser for 1 hour, stirring the flask content with a magnetic stirrer. After 1-hour pretreatment, the reflux condenser was replaced with a Deryng apparatus, and process of 1-hour hydrodistillation was carried out as described above.

The analogous procedure was applied in the control pretreatment process, but without using enzymes: 2 min of plant material blanching at 95 °C and then 1-hour stirring under a reflux condenser at 50 °C with an acidified water solution, pH = 4.5. This enabled the comparison of results and checking whether the observed effects are related to enzymes activity or other conditions of pretreatment: acidification of water, blanching, or soaking of plant material at elevated temperature.

GC-MS analysis of essential oils composition

To determine *Rosmarinus officinalis* essential oils composition, gas chromatography with mass selective detector method (GC-MS) was applied. For this purpose, 20 μL of essential oil was dissolved in 1 mL of acetone in three replications and analyzed using a 6890 N gas chromatograph (Agilent Technologies) equipped with a 5973N Mass Selective Detector and a 7683 Series Injector Autosampler, in optimized conditions which were described in details elsewhere[17]. The components of essential oils were identified by comparison of their mass spectra with NIST02 MS library. Identification was confirmed using the calculated values of the linear reten-

Table 1. Characteristic of enzymes and optimal conditions for their application, provided by manufacturer (DSM Food Specialties, USA)

Enzyme	Description	Industrial applications	pH	Temperature	Time
Hazyme DCL	amyloglucosidase from *Aspergillus niger*	starch hydrolysis in fruit processing	4.0–5.0	50 °C	1–2 h
Rapidase Fiber	pectinase enriched in arabinolytic activities from *Aspergillus niger* and cellulolytic activities from *Trichoderma longibrachiatum*	fruit and vegetable processing enzymatic filter cleaning	4.0–5.0	50 °C	1–2 h

tion indices (LRI) compared with the literature values obtained on the same type of chromatographic column using programmed temperature[29] and by comparison of retention times with the available chromatographic standards. The experimental LRI values were determined using the C7-C30 n-alkanes standard mixture which contained 1000 μg/mL of each component in hexane (Supelco), analyzed in the same chromatographic conditions. Quantitative analysis was performed by internal normalization method and the relative contents of particular compounds were determined as the peak area percentages in Total Ion Chromatogram.

Total phenolic content

Total phenolic content (TPC) in hydrolates - aqueous fractions after isolation of *Rosmarinus officinalis* essential oils - was determined by the method with Folin–Ciocalteu (F–C) reagent[30]. For this purpose, 0.5 mL of hydrolate sample, diluted previously 1:20 with deionized water, 0.5 mL of F–C reagent (Chempur) and 1.5 mL of freshly prepared sodium carbonate solution (C = 200 mg/mL) were introduced to volumetric flask and filled up to 25 mL with deionized water. The content of the flask was mixed and then shaken occasionally during 30-min incubation at room temperature for blue color development. After incubation, the absorbance was measured by using UV–VIS 1600 PC spectrophotometer (VWR) in 1-cm cuvettes at 760 nm wavelength. A blank sample which contained 0.5 mL of deionized water and all other reagents was prepared analogously and analyzed. The absorbance was measured for 3 independent samples prepared for each hydrolate and blank sample and the results were averaged. The calibration curve was prepared using gallic acid (Sigma-Aldrich) as a reference standard in the concentration range of 0.05–0.50 mg/mL. The total phenolic content (TPC) was calculated using a linear regression equation of the calibration curve and expressed as mg of gallic acid equivalent per 1 mL of hydrolate (mg GAE/mL).

Statistical analysis

The experimental data are presented as the mean values from the three replications, with standard deviations (s). Statistical analysis was performed using Microsoft Excel 2016 (Microsoft) and TIBCO Statistica 13.3 (TIBCO Software Inc.), assuming a normal probability distribution. The analysis of variance (ANOVA) was performed to analyze the obtained yields of essential oils and total phenolic content (TPC) in hydrolates. The differences among mean values of experimental data were evaluated by using Tukey's HSD post-hoc test at a 5% significance level.

RESULTS AND DISCUSSION

The performed studies showed that plant material pretreatment using Hazyme DCL and Rapidase Fiber in applied conditions positively influenced the yields of obtained rosemary essential oils isolated by 1-hour hydrodistillation. The best results were obtained by using of Hazyme DCL, which caused 26% enhancement of essential oil yield, from 2.07±0.09 to 2.61±0.7% (v/w). It is worth noticing that in comparison to studies presented by Hosni et al.[27], application of Hazyme DCL, which contains amyloglucosidase from *Aspergillus niger*, was more effective than the results obtained for cellulase (5%) and a combination of cellulase/hemicellulase (20%), but less effective than for hemicellulase (50%). Pretreatment with Rapidase Fiber, possessing mixed activity of pectinase/arabinase/cellulase increased essential oil yield to 2.32±0.07% (v/w), so the enhancing effect was weaker (12%), but still better than reported in the literature for cellulase[27]. The obtained results with the application of both enzymes significantly differ compared to conventional hydrodistillation. In contrary, the control process with non-enzymatic pretreatment resulted in a similar yield (2.01±0.08) to hydrodistillation process. It confirms that the increased effectiveness of the essential oils isolation process is connected with the activity of applied enzymes. The influence of various pretreatments applied before hydrodistillation process on the yield of *Rosmarinus officinalis* essential oil is presented in Figure 1.

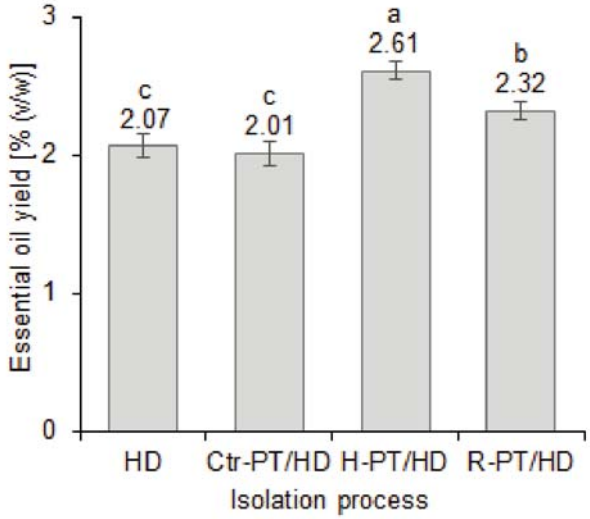

Figure 1. Influence of enzymatic pretreatments using Hazyme DCL (H-PT/HD) and Rapidase Fiber (R-PT/HD) on yields of *Rosmarinus officinalis* essential oils obtained in 1-hour hydrodistillation (HD) and in control process with non-enzymatic pretreatment (Ctr-PT/HD). Values marked with the same letter are not significantly different at the 0.05 level

Application of GC-MS method enabled the identification and quantification of 19 components of *Rosmarinus officinalis* essential oils. The two predominant constituents were 1,8-cineole (42–43%) and camphor (17–19%), significant share had also α-pinene (8–11%), borneol (~6%), and α-terpineol (~6%). All identified compounds with their retention times, linear retention indices, and relative contents are shown in Table 2.

By analyzing the contents of this table, it can be concluded that the composition of the essential oils obtained in conventional hydrodistillation and hydrodistillation preceded by enzymatic and non-enzymatic pretreatment is similar and only slightly differ in relative contents of some compounds. Looking closely at these small differences it can be noticed that the use of both enzymatic and non-enzymatic pretreatment resulted in a slight reduction in the content of some volatile compounds such

Table 2. Composition of *Rosmarinus officinalis* essential oils obtained by hydrodistillation (HD), process with control non-enzymatic pretreatment (Ctr-PT/HD) and with enzymatic pretreatment using Hazyme DCL (H-PT/HD) and Rapidase Fiber (R-PT/HD)

No.	Compound	RT [min]	LRI	Relative content ± s [%]			
			Exp / Lit	HD	Ctr-PT/HD	H-PT/HD	R-PT/HD
1	Tricyclene	4.85	920 / 923	0.18±0.01	0.14±0.01	0.16±0.01	0.16±0.01
2	α-Pinene	5.11	933 / 936	10.84±0.18	8.56±0.19	8.94±0.23	8.46±0.26
3	Camphene	5.44	946 / 950	4.57±0.09	4.05±0.08	4.33±0.10	4.22±0.01
4	β-Pinene	6.14	974 / 978	2.24±0.02	1.68±0.03	1.81±0.04	1.70±0.05
5	β-Myrcene	6.61	993 / 989	1.61±0.01	1.27±0.06	1.50±0.02	1.46±0.19
6	α-Phellandrene	7.01	1005 / 1004	0.19±0.01	0.21±0.07	0.20±0.03	0.22±0.03
7	α-Terpinene	7.43	1017 / 1017	0.54±0.05	0.45±0.03	0.51±0.03	0.47±0.10
8	1,8-Cineole	7.77	1033 / 1032	42.67±0.40	43.45±0.53	42.49±0.60	43.17±0.64
9	γ-Terpinene	8.59	1058 / 1060	0.30±0.01	0.24±0.02	0.20±0.01	0.23±0.02
10	Linalool	10.00	1101 / 1099	1.31±0.17	1.62±0.08	1.53±0.07	1.62±0.14
11	Fenchol	10.45	1112 / 1115	0.27±0.03	0.29±0.01	0.28±0.02	0.27±0.04
12	Camphor	11.30	1145 / 1143	17.81±0.14	18.69±0.17	18.29±0.46	19.22±0.30
13	Borneol	11.97	1167 / 1166	5.89±0.07	6.38±0.31	6.64±0.20	6.67±0.24
14	Terpinen-4-ol	12.41	1180 / 1177	1.81±0.10	1.98±0.05	2.06±0.06	2.05±0.18
15	α-Terpineol	12.86	1194 / 1190	6.25±0.08	6.39±0.27	6.75±0.35	6.74±0.22
16	Verbenone	13.54	1213 / 1206	0.70±0.02	0.67±0.04	0.84±0.02	0.78±0.12
17	Isobornyl acetate	15.84	1285 / 1286	0.86±0.02	1.04±0.02	1.03±0.04	0.94±0.04
18	β-Caryophyllene	20.02	1418 / 1420	0.51±0.01	0.24±0.01	0.33±0.01	0.29±0.02
19	Caryophylene oxide	24.98	1576 / 1581	0.13±0.01	0.12±0.01	0.18±0.01	0.15±0.02

RT – retention time; LRI – linear retention index: Exp – experimentally determined on HP-5MS capillary column; Lit – value from the literature[29]; Relative content – percentage of the peak area in Total Ion Chromatogram; s – standard deviation (n=3).

as α- and β-pinene and an increase in the case of some less volatile compounds like camphor or borneol. The possible reason for this may be a slight loss of volatile components during pretreatment, namely when removing the reflux condenser and replacing it with a Deryng apparatus. The biggest changes were observed in the content of α-pinene (up to 2.5% reduction) and camphor (up to 1.5% increase), for other compounds, including the main component 1,8-cineole, differences did not exceed 1%. It can be concluded that the observed variations in the composition of essential oils are not significant and are not caused by the use of enzymes during the pretreatment. This is advantageous compared to pretreatment with cellulase and hemicellulase described in the literature[27], which lead to significant changes in the content of individual components of rosemary essential oils - especially hemicellulase, which has drastically changed their composition.

The hydrolates, as by-products of the distillation process, are also of increasing interest for cosmetics and other products because of the high content of valuable biologically active compounds. Among them, an important group is phenolic compounds, known for their antioxidant properties. The phenolic compounds are compounds with monomeric, oligomeric, or polymeric rings with one or more hydroxyl groups in aromatic ring. They are widely distributed in plants and their content is directly correlated with antioxidant activity. Due to their good solubility in water, they are extracted from plant material during the hydrodistillation process and remain in the hydrolates, enriching them with antioxidant properties. The performed analyses have shown that all obtained hydrolates were rich in phenolic compounds and their content varied in a range of 2.74–3.76 mg GAE/mL, what was shown in Figure 2.

The results indicate that all applied pretreatments of plant material before hydrodistillation, both enzymatic and non-enzymatic, positively influenced the extraction of phenolic compounds to the water phase. There was a significant, about 35% increase in total phenolic content (TPC) in all processes with pretreatment. Because TPC values in the hydrolates obtained in processes with enzymatic and control non-enzymatic pretreatment were not statistically different, it can be concluded that the observed improvement of phenolics extraction is not caused by the activity of enzymes but by other conditions of pretreatment including prolonged contact of plant material with water at elevated temperature or acidification of water phase.

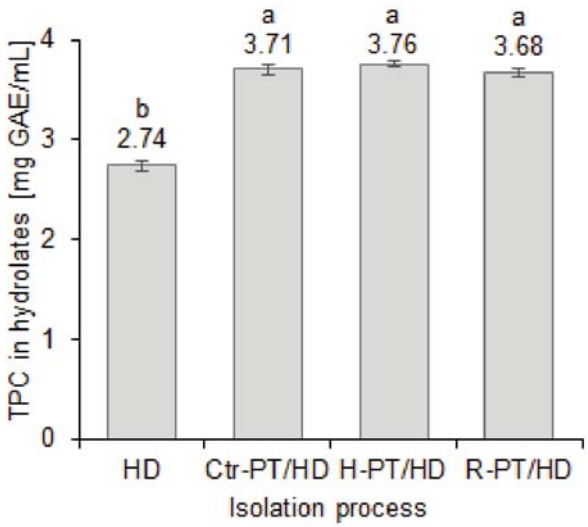

Figure 2. Influence of enzymatic pretreatments using Hazyme DCL (H-PT/HD) and Rapidase Fiber (R-PT/HD) and control non-enzymatic pretreatment (Ctr-PT/HD) on total phenolic content (TPC) in post-processing hydrolates obtained in 1-hour hydrodistillation of *Rosmarinus officinalis* (HD). Values marked with the same letter are not significantly different at the 0.05 level

CONCLUSIONS

Analyzing all results it can be concluded that applied modification of hydrodistillation process by using enzymatic pretreatment positively influenced the yields of

essential oils from *Rosmarinus officinalis*. More effective was application of Hazyme DCL (amyloglucosidase) which resulted in 26% increase in the yield of essential oil, then Rapidase Fiber (pectinase enriched in arabinolytic and cellulolytic activities) increasing the yield by 12%. Very important is that applied pretreatment did not cause significant changes in the composition of obtained essential oils, so their quality has not deteriorated. The differences observed in relative contents of particular compounds were lower than 2.5% and in most cases below 1%. They were caused rather by pretreatment conditions instead of enzymes activity and they did not exceed the typical variations in composition observed in natural products such as essential oils. An additional benefit was the improvement of the antioxidant properties of the post-processing hydrolates, where significantly higher total phenolic contents (35% increase) were found both after enzymatic and non-enzymatic pretreatment. Summarizing, the application of proposed enzymatic pretreatment before hydrodistillation can be useful biotechnological procedure for improving the efficiency of rosemary essential oils isolation and improvement of post-processing hydrolates quality. It allows to increase in the yields of essential oils with no negative effect on their composition and supports the extraction of phenolic compounds to the water phase. The obtained results encourage further in-depth research on the use of various enzymes to support the processes of isolation of precious biologically active compounds from plants.

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