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The effect of commercial enzyme preparation-assisted maceration on the yield, quality, and bioactivity of essential oil from waste carrot seeds (*Daucus carota* L.)

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SUMMARY: Eight enzyme preparations were screened with a view to maximizing the yield of carrot seed essential oil. Three of the eight enzyme preparations investigated, lipase from *Mucor circinelloides*, XPect® pectinase, and Esperase® protease, significantly influenced the amount of essential oil obtained, with Esperase® being the most effective. The Taguchi method was applied to optimize the processing conditions for the Esperase® protease. Under the optimum conditions, the essential oil yield increased by approximately 48%. The main constituent compounds in the oil are: carotol (OeA: 40.80%–OeB: 46.17%), daucol (OeA: 7.35%–OeB: 6.22%), sabinene (OeA: 5.12%–OeB: 6.13%), alpha-pinene (OeA: 4.24%–OeB: 5.11%) and geranyl acetate (OeA: 4.50%–OeB: 3.68%). As compared to the control sample, the essential oil obtained from enzyme-pretreated carrot seeds has the same biological activity against *Bacillus subtilis* and *Candida* sp., lower activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and higher activity against *Aspergillus niger* and *Penicillium expansum*.

KEYWORDS: *Carrot; Enzyme preparation; Essential oil; Taguchi method*

RESUMEN: ***Efecto de la preparación mediante maceración con enzima asistida comercial sobre el rendimiento, la calidad, y la bioactividad de aceite esencial de residuos de semillas de zanahoria*** **(*Daucus carota L.*)**. Ocho preparados enzimáticos fueron seleccionados con el fin de maximizar el rendimiento de aceites esenciales de semillas de zanahoria. Tres de los ocho preparados de las enzimas investigadas, lipasa de Mucor circinelloides, Xpect® pectinasa y Esperase® proteasa, influyeron de manera significativa sobre la cantidad de aceite esencial obtenido, siendo Esperase® el más eficaz. El método de Taguchi se aplicó para optimizar las condiciones del procesamiento para esta última. Bajo las condiciones óptimas, el rendimiento de los aceite esenciales aumentó aproximadamente un 48%. Los principales compuestos constituyentes del aceite son: carotol (OEA: 40.80%–OeB: 46,17%), ducol (OEA: 7,35%–OeB: 6,22%), sabineno (OEA: 5,12%–OeB: 6,13%), alfa-pineno (OEA: 4,24%–OeB: 5,11%) y acetato de geranilo (OEA: 4,50%–OeB: 3,68%). En comparación con la muestra control, el aceite esencial obtenido a partir de las semillas de zanahoria mediante enzima-pretratada tiene la misma actividad biológica frente a Bacillus subtilis y Candida sp., menor actividad frente a Staphylococcus aureus, Escherichia coli, y Pseudomonas aeruginosa, y una mayor actividad contra Aspergillus niger y Penicillium expansum.

PALABRAS CLAVE: *Aceite esencial; Método Taguchi; Preparación enzimática; Zanahoria*

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1. INTRODUCTION

Essential oils are mixtures of volatile organic compounds obtained by steam distillation, hydrodistillation, or cold pressing. Along with their olfactory qualities, they also have some cytotoxic properties: antibacterial, antiviral, antifungal, insecticidal, and antiparasitic (Burt 2004), and sometimes even anticarcinogenic. They do not themselves carry a risk of genotoxicity, so they are safe for consumers (Bakkali *et al*., 2008). Because of these properties, essential oils are often used in perfumery, cosmetics, and food, and even in medicine and aromatherapy (Roldán-Gutiérrez *et al*., 2008).

An example of an essential oil with such a range of applications is carrot seed essential oil. It is used mainly in the food industry as a flavoring for soups, concentrates, grape wine, and nonalcoholic beverages, and also in the cosmetic and fragrance industry as a fixative (Saad *et al*., 1995; Surburg and Panten, 2006). It has fungicidal and antibacterial properties (Batt *et al*., 1983; Dwivedi *et al*., 1991; Giraud-Robert, 2005; Kilibarda *et al*., 1996; Staniszewska *et al*., 2005), and it has also been proven to be a hypotensive agent and a cardiac and central nervous system depressant (Saad *et al*., 1995).

Carrot seed essential oil is obtained by means of hydro-distillation of ground waste seeds. The extraction of this oil, similar to the extraction of essential oils from other plants with endogenous oil bodies, is often difficult as the structure of plant tissue inhibits the migration of the extractant (water) and the release of intracellular metabolites (Pinelo and Meyer, 2008). Due to these difficulties, new technologies are sought to improve the extraction process. Enzymes which catalyze the hydrolysis of glycosidic bonds in plants (mainly cellulases, hemicellulases, and pectinases) can be used for this purpose (Puri *et al*., 2012). It has been found that such enzymes, as well as proteinases, facilitate the release of bioactive compounds (Pinelo and Meyer, 2008), speed up extraction, and reduce the consumption of energy and extraction solvent (Puri *et al*., 2012), which makes the process environmentally friendly. For the same reasons, it is advisable to pretreat plant material with enzymatic hydrolysis prior to essential oil extraction.

This study presents a method of plant material pretreatment prior to hydro-distillation, leading to a higher yield of essential oil and reduced production costs. The experiment involved the pretreatment of waste carrot seeds (*Daucus carota* L.) var. Koral with commercial enzyme preparations.

2. MATERIALS AND METHODS

2.1. Materials

Waste carrot seeds which lost their ability to germinate were obtained from a local producer (W. Legutko Breeding and Seed Company, Wielkopolska, Poland).

Three lipases (Lipex® and two lipases produced by *Rhizomucor miehei* and *Aspergillus niger*), pectate lyase (XPect®), amylase (Stainzyme®), cellulase (Celluclean®), and serine protease (Esperase®) (Novozymes, Bagsvaerd, Denmark) were donated by UNIVAR. The fourth, a noncommercial lipase, (specially prepared mycelium of *Mucor circinelloides*) was obtained from the Institute of Technical Biochemistry, Lodz University of Technology.

2.2. Essential oil extraction

2.2.1. Seed preparation

Carrot seeds (100.0 g) were ground in a buhr mill and homogenized in 450.0 mL of water at 20.0 °C for 5.0 min using an MPW–324 homogenizer (Mechanika Precyzyjna, Warsaw, Poland).

2.2.1.1. Enzymatic pretreatment

a) Screening: Ground and homogenized seeds were subjected to the action of eight enzyme preparations at a concentration of 1.0 g·100.0 g^{-1} of seeds. The application of enzyme preparations was preceded by pH regulation in the appropriate range, as suggested by the manufacturer (Beckman Φ71, Beckman Instruments, Inc., 1981) using citric acid (70.0 g citric acid·100.0 mL^{-1} water). The slurry was then thoroughly shaken in a bioreactor (Reactor-Ready, Radleys equipped with a Heidolph RZR 2102 stirrer) for 18 hours, at the temperature suggested by the manufacturer.
b) Optimization: Ground and homogenized seeds were subjected to the action of *Esperase*® at a concentration of 0.5, 1.0, and 2.0 mL·100.0 g^{-1} of seeds. The application of the enzyme preparation was preceded by pH regulation in the range of 7.0–12.0 (Beckman Φ71, Beckman Instruments, Inc., 1981) using citric acid (70.0 g

citric acid·100.0 mL^{-1} water). The slurry was then thoroughly shaken in a bioreactor (Reactor-Ready, Radleys equipped with a Heidolph RZR 2102 stirrer) at 25–45 °C for 0.5–18 hours (according to the Taguchi method).

c) Optimization: the actions undertaken were analogous to those from a), applying mutatis mutandis: enzyme preparation – Esperase®; Esperase® concentration 0.5–2.0 mL·100.0 g^{-1} of seeds; pH range 7.0–12.0, shaking conditions 25–45 °C, 0.5–18 hours.

2.2.2. Hydro-distillation

Plant material prepared according to step 2.2.1. (control sample – OeA) or 2.2.1.1. (OeB – sample pretreated with Esperase®) was placed in a 2 L flask, to which 550.0 mL of water was added. Hydro-distillation was performed in triplicate for 5.0 h in a hydro-distillation apparatus providing very good separation of phases through an odorless operation (Śmigielski *et al.*, 2009).

2.3. Physicochemical properties of carrot seed essential oil

The refractive index of the carrot seed essential oil obtained by hydro-distillation from the control and enzyme-pretreated carrot seeds was measured using an Abbemat refractometer (Dr. Kernchen); optical rotation was measured using an Autopol IV polarimeter (Rudolph Research).

2.4. GC-MS analysis

The equipment used in the study consisted of a Trace GC Ultra gas chromatograph coupled with a DSQ II mass spectrometer from Thermo Electron Corporation, an Rtx-1ms capillary column from Restek (60 m long, internal diameter of 0.25 mm, film thickness of 0.25 μm). Signals from two detectors (FID, MS) were simultaneously collected using an MS-column flow splitter from SGE. The following parameters and conditions used were: programmed temperature 50–(3 min)–300 °C (30 min), temperature gradient 4 °C·min^{-1}, injector temperature (SSL) 280 °C, detector temperature (FID) 300 °C, carrier gas – helium, carrier gas flow rate at a constant pressure of 200 kPa, split of 1:20. The parameters of the mass spectrometer were as follows: ionization energy 70 eV, ion source temperature 200 °C, full scan mode in the mass range of 33–420. The flavor compounds in carrot essential oil were identified by GC–MS according to the mass fragmentation pattern and spectral comparison with standards from the NIST, Wiley 8th edition and Adams Libraries as well as by comparison of retention indices with data from the NIST, Wiley 8th edition, Adams Libraries, and the Pherobase (http://www.pherobase.com/).

2.5. Near-infrared spectroscopy

The spectrophotometer used was an FT-IR Nicolet 6700; number of sample scans 32, collection length 15.76 sec, resolution 8.000, levels of zero filling 1, number of scan points 8480, number of FFT points 16,384, laser frequency 15,798.3 cm^{-1}, interferogram peak position 4096, apodization–Happ-Genzel, phase correction–Mertz, number of background scans 32, background gain 1.0, wave range (wavenumber) 11,000–4000 cm^{-1}, detector–InGaAs, beam splitter: CaF_2, source–white light. The supplied software OMNIC and TQ Analyst were used for both the control of the spectrophotometer's work and the analysis of the obtained results.

2.6. Assessment of the antimicrobial activity of the essential oil

2.6.1. Microorganism cultivation and inoculum preparation

The following strains were used in the study: the Gram-positive bacteria *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 1803; the Gram-negative bacteria *Escherichia coli* ATCC 1627 and *Pseudomonas aeruginosa* ATCC 1555; the yeast *Candida* sp. LOCK 0008; and the molds *A. niger* LOCK 0436 and *Penicillium expansum* LOCK 0535. The microorganisms were obtained from the American Type Culture Collection ATCC and the Center of Industrial Microorganisms Collection of the Institute of Fermentation Technology and Microbiology, Lodz University of Technology, Poland, WDCM 105. The stock cultures of bacteria were maintained on Trypticase Soy Agar (TSA, Oxoid) slants, while those of the yeast and molds on Sabouraud Dextrose Agar (SDA, bioMerieux) slants at 4 °C. Before each experiment, the strains were twice subcultured in Trypticase Soy Broth (TSB, Oxoid) and Sabouraud Dextrose Liquid Broth (SDLB, bioMerieux). Inoculated broths of *B. subtilis* were incubated at 30 °C for 24 h, and those of *E. coli*, *S. aureus*, and *P. aeruginosa* at 37 °C for 24 h. Broths inoculated with yeast and molds were incubated at 25 °C for 24–72 h. Freshly prepared slant cultures were used for the experiments. An inoculum of each strain was prepared in a standard saline solution (0.85% NaCl) and adjusted to a final concentration of approximately 10^7 CFU·mL^{-1}.

2.6.2. Antimicrobial test conditions

The antimicrobial activity of the essential oils was estimated by the impedimetric method using a Bactometer M64 (bioMerieux). The procedure was as follows: 0.1 mL of a standardized inoculum of the tested strain was placed in a Bactometer well containing a chemical agent at the tested concentration

and a growth medium, adjusted to a final volume of 1 mL. The essential oils were added in the concentration range of 50 to 400 μL·mL^{-1} at 50 μL increments for *P. aeruginosa*, from 1 to 15 μL·mL^{-1} at 1 μL increments for *B. subtilis*, *S. aureus, E. coli, A. niger* and *P. expansum* and from 0.1 to 1 μL·mL^{-1} at 0.1 μL increments for *Candida* sp. *B. subtilis*, *S. aureus*, and *P. aeruginosa* strains were cultivated in GPM (General Purpose Medium) and the *E. coli* strain in EM (Entero Medium); yeast and molds were grown in YMM (Yeast Moulds Medium). GPM, EM, and YMM media are designated for the impedimetric evaluation of specified microorganisms by the manufacturer of the Bactometer, bioMerieux. Cell suspensions of 0.1 mL in 0.9 mL of the appropriate medium served as positive controls. Negative controls were bacteria and yeast/mold cultures with 0.5 μg·mL^{-1} of Novobiocin and 0.2 μg·mL^{-1} of cycloheximide, respectively. The microorganisms were incubated for 72 h at their optimal growth temperatures as indicated above. After incubation in the Bactometer, each culture was checked for microorganism viability by streaking on Plate Count Agar (PCA, bioMerieux). Plates were incubated for 72 h for bacteria and 120 h for yeast and molds at the optimal growth temperatures of particular microorganisms.

Minimal inhibitory concentration (MIC) was calculated as the minimal concentration of a chemical agent inhibiting microbial growth in Bactometer wells (all microorganisms were grown on PCA plates simultaneously).

All experiments were conducted in triplicate.

2.7. Sensory assessment

Sensory assessment was based on a forced-choice test (Baryłko-Pikielna and Matuszewska, 2009). The respondents were asked to decide which of the coded essential oils (i.e., the essential oil from seeds pretreated with Esperase® or the essential oil from the control sample) was lighter and smelled more similar to the essential oil from carrot seeds obtained by another research team via the classical method. The analysis took place in a special sensory evaluation laboratory.

2.8. Statistical evaluation

2.8.1. The Taguchi method

The optimization of the enzymatic pretreatment of waste carrot seeds prior to hydro-distillation was performed according to the Taguchi experimental design approach, which allows for reducing costs and time consumption by evaluating several process factors at the same time with the smallest number of experimental runs based on a table known as the orthogonal array (Cukor *et al*., 2011; Chen *et al*., 2011; Tadayon *et al*., 2012; Benito-Román *et al*., 2011).

For the problem under consideration, a L9 orthogonal array (Statistica 7.0) was adopted, consisting of 9 systems. The following factors which may influence enzyme activity were identified as critical: enzyme preparation loading (0.5; 1.0; 2.0 mL·100.0 g^{-1} seed), time (0.5; 2.0; 18.0 h), solution pH (7.0; 10.5; 12.0), and temperature (25.0; 35.0; 45.0 °C). The parameters of carrot seed enzymatic modification prior to hydro-distillation were optimized in terms of the levels of the above factors. The aim of optimization was to determine such levels of these factors that would ensure the highest efficiency of hydro-distillation, with oil yield chosen as the output factor. The study was randomized to avoid systematic errors and all the experiments were carried out in triplicate. The signal-to-noise ratio (S/N ratio, Eta) was calculated from the experimental data using a loss function, which gave a function transforming repetitive data to other values and was used as a measure of the variation present in the experiment. The characteristics of the maximum desired value were adopted and optimization was done by calculating the S/N ratio (Eta) (controllable factors/confounders) according to the formula:

$$\text{Eta} = -10 \cdot \log_{10} [(1/n) \cdot \Sigma(1/y_i^2)] \quad (1)$$

where n is the number of iterations and y_i is the value of the output variable (the yield of the isolated essential oil).

The theoretical amount of essential oil was calculated for the determined optimum process conditions on the basis of the expected S/N ratio.

3. RESULTS AND DISCUSSION

3.1. Selection of enzyme type

The aim of this study was to develop an effective method of essential oil extraction which provides higher efficiency due to enzymatic hydrolysis. For this purpose, screening was performed on eight different enzyme preparations: four lipases (from *M. circinelloides*, *R. miehei*, and *A. niger*, as well as Lipex®), which are thought to cleave ester bonds both in cuticle polyesters and cell membrane phospholipids, as well as glycosidic bonds in cell wall polysaccharides; two preparations which degrade the cell wall and its main components, cellulose and pectin: Celluclean classic® cellulase hydrolyzing β-1,4-glycosidic bonds in cellulose and XPect® pectinase hydrolyzing α-1-4 linked polygalactosyluronic acid; Esperase® serine protease cleaving peptide bonds present in cell membrane proteins, and Stainzyme® amylase hydrolyzing endo-1,4 bonds in starch, both in amylose and amylopectin.

Carrot seeds were subjected to the action of these eight enzyme preparations at a concentration of 1.0 g·100.0 g^{-1} of seeds for 18 hours under

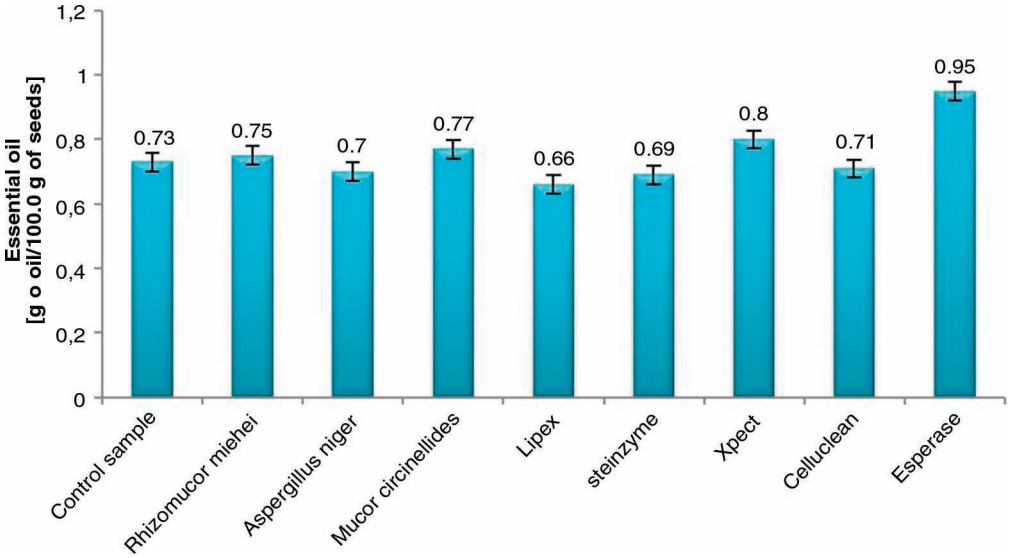

FIGURE 1. Efficiency of carrot seed essential oil extraction according to the enzyme preparation used.

conditions suggested by the manufacturer. The results of the screening are shown in Figure 1.

Only three of the eight enzyme preparations investigated, *M. circinelloides* lipase, XPect® pectinase, and Esperase® protease, significantly influenced the efficiency of the essential oil extraction. The highest yield, higher by nearly 30% than that of the control sample, was afforded by Esperase®, so this preparation was chosen for further studies.

3.2. Optimization of the process for enzyme-assisted hydro-distillation

The selection of appropriate conditions to ensure the optimum activity of the enzyme preparations used for the treatment of plant material, is a key step because the effectiveness of enzymatic digestion influences the efficiency of essential oil extraction. The application of the Taguchi method for the optimization of enzymatic processing conditions considerably accelerated this step.

Following the results of preliminary screening, Esperase® protease was investigated with a view to optimize its processing conditions. The results obtained from 9 experiments conducted in triplicate according to the L_9 orthogonal array are presented in Table 1.

The ANOVA statistical analysis at a significance level of $p = 0.05$ showed that all input factors influenced the yield of carrot seed essential oil, with pH being the most significant one (contribution of 44.83%) (Table 2).

The results of the statistical analysis of the relationship between input factor levels and mean Eta values (3 replicates) are visually presented in Fig. 2. The optimum levels of input parameters adopted for enzyme-pretreated carrot seeds were as follows: time 2.0 h, pH 10.5, temperature 35 °C and an enzyme preparation loading of 2.0 mL·100.0 g^{-1} of seeds.

The theoretical amount of OeB was calculated for the determined optimum process conditions on the basis of the expected S/N ratio. The S/N value under optimal conditions is 0.800612, hence $y_i = 1.096$ from equation (*1*).

To verify the optimum levels of input parameters, 3 experiments were performed, showing the yield of OeB (1.08 ± 0.004 g·100 g^{-1}) to be approximately 48% higher than that of OeA (0.73 ± 0.001 g·100 g^{-1}).

3.3. Physicochemical properties of carrot seed essential oils

The specific rotation and refractive index of the obtained essential oils did differ significantly, according to the Mann-Whitney test. Essential oil from the control sample revealed a specific rotation of -8.038 ± 0.0453 and a refractive index of n^{D}_{20}: 1.488635, while the corresponding figures for the essential oil from enzyme-pretreated seeds were -6.606 ± 0.031 and n^{D}_{20}: 1.488982, respectively. Commercially available essential oils from carrot seeds are not standardized in terms of refractive index and optical rotation, and there is little information about these parameters in the literature (Özcan and Chalchat, 2007; Pigulevskii and Kovaleva, 1955; Pigulevskii *et al*., 1965).

3.4. Organoleptic observations

Essential oil from the control sample was a clear, oily liquid, amber-yellow in color, characterized by a heavy herbal-earthy scent.

TABLE 1. Plan of optimization of carrot seed (*Daucus carota* L.) enzymatic modification prior to hydrodistillation by the Taguchi method; L9 orthogonal array – input factors and mean output factors

No of tests	Time [h]	Enz. prep. [mL·100.0 g^{-1} of seeds]	Temperature [°C]	pH	Ess. oil obtained [g·100.0 g^{-1} of seeds]	S/N ratio [Eta]
1	0.5	1.0	35.0	10.5	0.94	−0.57410
2	18.0	2.0	45.0	10.5	0.83	−1.58545
3	2.0	2.0	25.0	10.5	1.01	0.08387
4	18.0	0.5	25.0	12.0	0.73	−2.77458
5	0.5	1.0	45.0	12.0	0.99	−0.05878
6	0.5	2.0	25.0	7.0	0.70	−3.09863
7	18.0	0.5	35.0	7.0	0.79	−2.08480
8	2.0	0.5	35.0	12.0	0.89	−0.98022
9	2.0	1.0	45.0	7.0	0.77	−2.23379

TABLE 2. ANOVA analysis of statistical significance of input factors for enzymatic pretreatment of carrot seeds (*Daucus carota* L.) according to the Taguchi method

Input factor	Sum of squares SS	Fisher F criterion	p-Value	Contribution* [%]
pH	14.84627	170.7958	0.000000	44.83
Enz. prep.	8.46981	97.4392	0.000000	25.58
Time	6.23699	71.7522	0.000000	18.84
Temperature	2.77789	31.9576	0.000001	8.39
Residual	0.78232			2.36

*Contribution is defined as 100×(pooled sum of squares/total sum of squares).

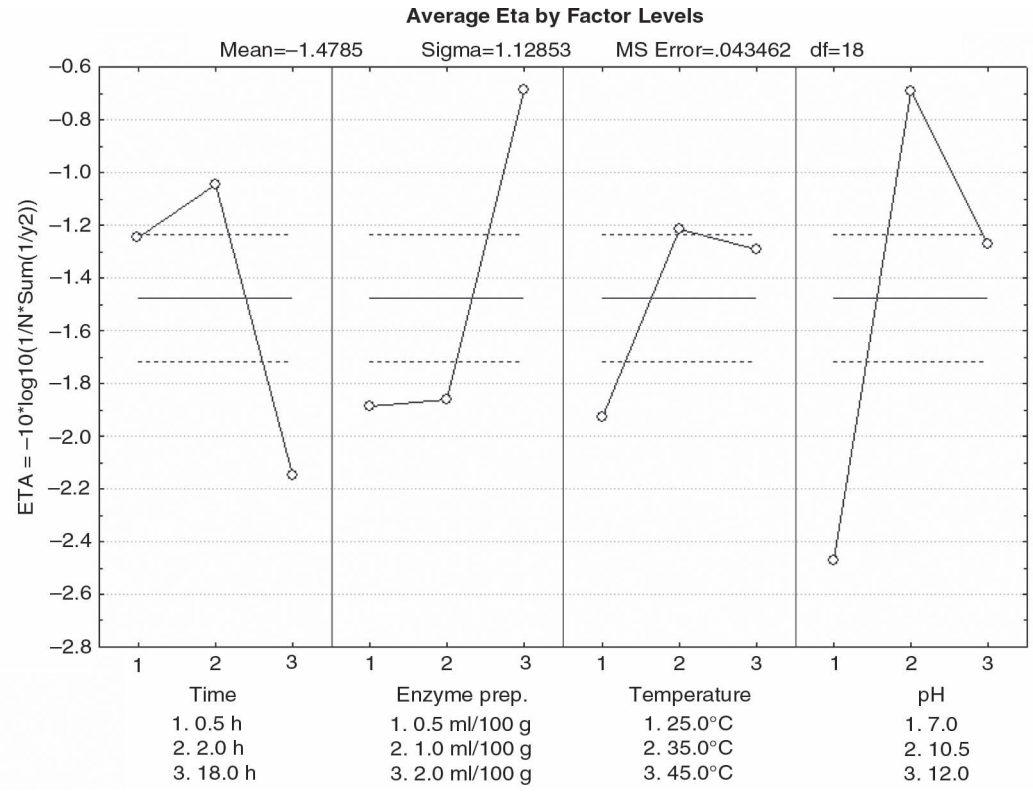

FIGURE 2. The effect of input factors in the process of enzymatic pretreatment of carrot seeds (*Daucus carota* L.) on the S/N ratio.

Essential oil from enzymatically pretreated carrot seeds was slightly lighter. Its scent was characteristic of essential oils from this kind of plant material; however, a slightly sour note was present.

3.5. Characterization of flavor compounds in carrot seed essential oils

75 (OeB) to 86 (OeA) chemical compounds were identified in the essential oils, which corresponded to 97.59% (OeA) and 98.95% (OeB) of their total composition. The composition of the essential oils was compared using a statistical tool (Mann-Whitney test) (StatSoft electronic manual, 2012, www.statsoft.com).

The content of the main compound, carotol, differed among the essential oils and was significantly increased (by 13%) by enzymatic hydrolysis (OeA: 40.80–OeB: 46.17%). The action of serine protease not only cleaves peptide bonds but also ester linkages (Topf *et al*., 2001), releasing carotol from its possible combinations with proteins. It is also possible that the degradation of protein quaternary structure increases the availability of carotol and facilitates its extraction.

Given that there was no other significant change in the concentration of oxygenated sesquiterpenes, this statistically significant increase in carotol content suggests that differences in the content of other compounds are likely to result from the enhanced extraction of this terpenoid, which changes the proportions in the chemical composition of the oil. Hence, a subsequent decrease in the percentage fractions of all analyzed groups of compounds (except for oxygenated sesquiterpenes) can be noted. Some statistically significant changes were observed for oxygenated monoterpenes: geraniol (OeA: 0.71–OeB: 0.42) and terpinen-4-ol (OeA: 0.95–OeB: 0.29); and sesquiterpenes: alpha-amorphene (OeA: 0.47–OeB: 0.22) and (Z)-beta-farnesene (OeA: 0.56–OeB: 0.47).

3.6. Similarity of the oils as determined by NIRS analysis

Near-infrared spectroscopy (NIRS) was applied to compare the essential oils OeA and OeB. Despite some differences observed in the composition of the essential oil from enzyme-pretreated carrot seeds, as revealed by the statistical analysis (Table 3), the high correlation coefficient obtained (86.29%) indicates very high similarity of the quantitative contents of the main chemical compounds in the essential oils studied.

3.7. Antimicrobial activity

The antimicrobial activity of the essential oils derived from carrot seeds is shown in Table 4. Both essential oils showed significantly higher activity against the tested Gram-positive bacteria (*B. subtilis*, *S. aureus*) than against Gram-negative bacteria (*E. coli*, *P. aeruginosa*), which is characteristic of all essential oils, possibly due to the existence of an outer membrane surrounding the cell walls of Gram-negative bacteria, limiting the diffusion of hydrophobic substances, such as oils, through the lipopolysaccharide layer of the wall (Burt, 2004).

The growth of Gram-positive bacteria was inhibited at concentrations of 2–5 $\mu L \cdot mL^{-1}$, with the activity of OeB against *S. aureus* (5 $\mu L \cdot mL^{-1}$) being lower than that of OeA (2 $\mu L \cdot mL^{-1}$). The lower activity of OeB against bacteria in the genus *Staphylococcus* is associated with a significantly reduced content of camphene and linalool, which are compounds with high biostatic activity (Alma *et al*., 2004; Soković *et al*., 2010). Their reduced content may also lower the activity of the essential oil from carrot seeds pretreated with Esperase® against Gram-negative bacteria.

On the other hand, OeB showed greater antifungal activity than OeA, which may result from the increased amount of carotol, which is a potent fungicide (Abad *et al*., 2007) with an efficiency comparable to that of commercial agents such as Funaben T (Jasicka-Misiak *et al*., 2004), and limonene (Omran *et al*., 2011). The activity of OeB against both molds (2 $\mu L \cdot mL^{-1}$) is 2.5 times greater than that of the control sample (5 $\mu L \cdot mL^{-1}$), while the sensitivity of *Candida* sp. to the tested essential oils remains the same, and is the highest among all the tested microorganisms (MIC 0.6 $\mu L \cdot mL^{-1}$).

These results show carrot seed essential oil to have good fungistatic activity against yeasts in the genus *Candida* and Gram-positive bacteria. Tavares and others (2008) and Maxia and others (2009) emphasized the antifungal properties of the essential oil obtained from the umbels of *D. carota* L. subsp. *halophilus* and *D. carota* L. subsp. *carota*, respectively.

4. CONCLUSIONS

It was proven that the use of *M. circinelloides* lipase, Xpect pectinase and Esperase® serine protease prior to the hydro-distillation of carrot seeds results in an increased efficiency of essential oil extraction, with the highest yield afforded by Esperase®.

The optimal conditions for the enzymatic pretreatment of carrot seeds were selected by means of the Taguchi method. It was shown that the use of the enzyme preparation Esperase® to treat waste carrot seeds prior to hydro-distillation under the optimal conditions (2.0 h; pH 10.5; 35 °C; enzyme preparation loading of 2.0 mL·100.0 g^{-1} seeds) improves the diffusion of the extractant and increases the efficiency of essential oil extraction by 48%. The presented method is free from defects typical of conventionally applied methods associated with insufficient degradation of plant tissue, which is especially

TABLE 3. Chemical composition of essential oils obtained from carrot seeds (*Daucus carota* L.)
OeA – essential oil from the control sample
OeB – essential oil from seeds pretreated with Protease®

No.	Compound	Content [%] OeA	Content [%] OeB	RI Rtx-1	RI lit. Rtx-1
1.	α-Thujene*	0.31	0.39	923	923
2.	α-Pinene*	4.24	5.11	931	932
3.	Camphene*	0.39	0.41	943	944
4.	Verbenene*	0.10	0.08	946	944
5.	Sabinene*	5.12	6.13	966	966
6.	β-Pinene	1.51	1.45	970	971
7.	3-Methylnonane*	0.08	0.00	971	970
8.	β-Myrcene*	0.42	0.35	983	981
9.	Car-2-ene*	0.14	0.05	1006	1008
10.	p-Cymene*	1.89	2.29	1012	1012
11.	Limonene*	0.64	0.45	1021	1022
12.	β-Ocimene*	0.05	0.00	1030	1034
13.	γ-Terpinene*	0.29	0.08	1049	1049
14.	trans-Sabinene hydrate*	0.18	0.37	1053	1053
15.	trans-Linalool oxide*	0.02	0.06	1058	1054
16.	cis-Linalool oxide *	0.01	0.09	1075	1078
17.	α-Terpinolene*	0.04	0.00	1079	1080
18.	cis-Sabinene hydrate *	0.14	0.34	1083	1083
19.	Linalool*	0.38	0.16	1085	1083
20.	Thujol*	0.16	0.32	1089	1095
21.	Thujone*	0.03	0.05	1097	1099
22.	α-Campholene aldehyde *	0.09	0.11	1104	1103
23.	Nopinone*	0.10	0.14	1106	1110
24.	trans-Pinocarveol*	0.02	0.38	1122	1121
25.	cis-Verbenol*	0.41	0.00	1124	1127
26.	Sabinene ketone*	0.33	0.22	1127	1132
27.	trans-Verbenol*	1.67	1.83	1129	1128
28.	Isopinocamphone*	0.05	0.00	1136	1141
29.	Pinocarvone*	0.29	0.31	1139	1139
31.	cis-Sabinol*	0.13	0.06	1150	1147
32.	p-Mentha-1,5-dien-8-ol*	0.09	0.00	1147	1149
33.	Terpinene-4-ol*	0.95	0.29	1162	1162
34.	p-Cymene-8-ol*	0.02	0.00	1164	1162
35.	Myrtenal	0.31	0.28	1171	1170
36.	α-Terpineol*	0.12	0.06	1173	1175
37.	Verbenone*	0.69	0.75	1179	1176
38.	Carveol*	0.09	0.07	1201	1197
39.	Carvone*	0.00	0.05	1231	1230
40.	Cumin aldehyde*	0.09	0.03	1240	1241
41.	Geraniol*	0.71	0.42	1235	1239
42.	Bornyl acetate	0.16	0.18	1267	1270
43.	Perillyl alcohol*	0.00	0.09	1277	1278
44.	Carvacrol*	0.06	0.00	1279	1278
45.	p-Cymenol*	0.00	0.06	1286	1289
46.	p-Mentha-1,4-dien-7-ol*	0.05	0.02	1312	1302

TABLE 3 (*Continued*)

No.	Compound	Content [%] OeA	Content [%] OeB	RI Rtx-1	RI lit. Rtx-1
47.	γ-Terpinyl acetate	0.06	0.06	1331	1333
48.	Neryl acetate	0.02	0.01	1340	1342
49.	Geranyl acetate*	4.50	3.68	1359	1361
50.	Farnesyl acetate*	0.17	0.00	1369	1370
51.	Daucene	1.61	1.79	1376	1377
52.	β-Cubebene*	0.18	0.00	1377	1372
53.	β-Elemene*	0.07	0.08	1384	1389
54.	Sativene*	0.02	0.04	1394	1396
55.	cis-α-Bergamotene*	0.20	0.17	1408	1407
56.	β-Caryophyllene*	2.70	2.63	1414	1420
57.	β-Cedrene*	0.05	0.02	1423	1422
58.	γ-Elemene*	0.01	0.08	1430	1432
59.	(Z)-β-Farnesene*	0.57	0.47	1429	1434
60.	β-Funebrene*	0.08	0.02	1434	1431
61.	(E)-β-Farnesene	1.83	1.72	1444	1446
62.	α-Humulene*	0.13	0.00	1448	1448
63.	α-Amorphene*	0.12	0.00	1455	1457
64.	γ-Muurolene*	0.35	0.15	1469	1471
65.	α-Curcumene	0.06	0.05	1468	1468
66.	Germacrene D*	0.03	0.39	1470	1468
67.	Dauca-5,8-dien*	0.00	0.04	1471	1468
68.	β-Selinene*	2.17	2.15	1479	1485
69.	α-Selinene*	0.33	0.00	1488	1490
70.	α-Himachalene*	0.70	0.99	1490	1494
71.	β-Bisabolene*	1.49	1.22	1498	1502
72.	γ-Cadinene*	0.05	0.00	1505	1507
73.	β-Sesquiphellandrene*	0.24	0.19	1512	1516
74.	Calamene*	0.01	0.00	1515	1517
75.	α-Chamigrene*	0.09	0.11	1520	1526
76.	α-Bisabolene*	0.25	0.05	1540	1534
77.	Spathulenol*	1.12	0.75	1546	1553
78.	Caryophyllene oxide*	4.18	3.95	1567	1551
79.	Carotol*	40.80	46.17	1589	1593
80.	Humulene epoxide II*	0.61	0.36	1592	1602
81.	Cubenol*	0.14	0.17	1601	1605
82.	Muurola-4,10(14)-dien-1-beta-ol*	0.68	0.23	1617	1620
83.	Daucol*	7.35	6.22	1620	1630
84.	α-Cadinol*	0.10	0.17	1632	1638
85.	Ledene oxide*	0.17	0.20	1646	1646
86.	α-Eudesmol*	0.04	0.00	1649	1657
87.	β-Eudesmol*	0.56	0.40	1651	1655
88.	Longifolenaldehyde*	0.63	0.55	1657	1651
89.	α-Bisabolol*	0.00	0.11	1668	1670
90.	Juniper camphor*	0.39	0.35	1677	1682
91.	α-Cyperone	0.16	0.18	1725	1727

TABLE 3 (*Continued*)

No.	Compound	Content [%] OeA	Content [%] OeB	RI Rtx-1	RI lit. Rtx-1
	Total amount	97.59	98.95		
	Monoterpenes	15.14	16.23		
	Ox. Monoterpenes*	12.09	10.54		
	Sesquiterpenes*	13.35	12.38		
	Ox. Sesquiterpenes*	56.93	59.80		

*Statistically significant differences (Mann-Whitney test; p = 0.05).

TABLE 4. Antimicrobial activity of essential oils obtained from carrot seeds (*Daucus carota* L.) expressed as the minimal concentration of the oil inhibiting microbial growth (MIC) in $\mu L \cdot mL^{-1}$ *
OeA – essential oil from the control sample
OeB – essential oil from seeds pretreated with Esperase®

	Gram-positive bacteria		Gram-negative bacteria		Yeast	Molds	
Essential oil	Bacillus subtilis ATCC 6633	Staphylococcus aureus ATCC 1803	Escherichia coli ATCC 1627	Pseudomonas aeruginosa ATCC 1555	Candida sp. LOCK 0008	Aspergillus niger LOCK 0436	Penicillium expansum LOCK 0535
OeA	5	2	5	100	0.6	5	5
OeB	5	5	10	250	0.6	2	2

*μL of oil$\cdot mL^{-1}$ of growth medium.

important in the case of raw materials that contain small amounts of oil or are not readily available. What needs to be emphasized is the fact that the content of carotol in the enzyme-pretreated essential oil is higher by 13% than in the control sample.

The essential oil obtained from enzyme-pretreated carrot seeds has good antifungal properties (with its activity against molds being 2.5 times higher than that of the control sample) and lower bacteriostatic activity against Gram-negative bacteria. Its qualitative composition is similar to that of the control sample, as confirmed by NIRS analysis (a correlation coefficient of 86.29%); hence, it can be used in the food industry as a flavoring , and in pharmaceutical and cosmetic preparations.

REFERENCES

Abad MJ, Ansuategui M, Bermejo P. 2007. Active antifungal substances from natural sources. *ARKIVOC* **7**, 116–145.

Alma MH, Nitz S, Kollmannsberger H, Digrak M, Efe FT, Yilmaz N. 2004. Chemical composition and antimicrobial activity of the essential oils from the gum of Turkish pistachio (*Pistacia vera* L.). *J. Agric. Food Chem*. **52**, 3911–3914. http://dx.doi.org/10.1021/jf040014e.

Bakkali F, Averbeck S, Averbeck D, Idaomar M. 2008. Biological effects of essential oils – A review. *Food Chem. Toxicol*. **46**, 446–475. http://dx.doi.org/10.1016/j.fct.2007.09.106.

Baryłko-Pikielna N, Matuszewska I. 2009. *Sensoryczne badania żywności*. Cracow*:* Wydawnictwo Naukowe PTTŻ.

Batt C, Solberg M, Ceponis M. 1983. Effect of volatile components of carrot seed oil on growth and aflatoxin production by *Aspergillus parasiticus*. *J. Food Sci*. **48**, 762–768. http://dx.doi.org/10.1111/j.1365–2621.1983.tb14893.x.

Benito-Román O, Alonso E, Lucas S. 2011. Optimization of the β-glucan extraction conditions from differentwaxy barley cultivars. *J. Cereal Sci*. **53**, 271–276. http://dx.doi.org/10.1016/j.jcs.2011.01.003.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. *Intern. J. Food Microbiol*. **94**, 223–253. http://dx.doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Chen HH, Chung CC, Wang HY, Huang TC. 2011. Application of Taguchi method to optimize extracted ginger oil in different drying conditions. pp. 310–316. In: IPCBEE May 7–9; Bangkok. Singapoore: IACSIT Press (2011).

Cukor G, Jurković Z, Sekulić M. 2011. Rotatable central composite design of experiments versus Taguchi method in the optimization of turning. *Metalurgija* **50**, 17–20.

Dwivedi SK, Pandey VN, Dubey NK. 1991. Effect of essential oils of some higher plants on *Aspergillus flavus* link infesting stored seeds of guar (*Cyamopsis tetragonaloba* L.). *Flavour Frag. J*. **6**, 295–297.

Giraud-Robert AM. 2005. The role of aromatherapy in the treatment of virial hepatitis. *Int. J. Aromather*. **15**, 183–192. http://dx.doi.org/10.1016/j.ijat.2005.10.005.

Jasicka-Misiak I, Lipok J, Nowakowska EM, Wieczorek PP, Młynarz P, Kafarski P. 2004. Antifungal activity of the carrot seed oil and its major sesquiterpene compounds. *Z. Naturfors*. **59c**, 791–796.

Kilibarda N, Nanusević N, Dogivić N, Ivanić R, Savin K. 1996. Content of the essential oil of the carrot and its antibacterial activity. *Pharmacie* **51**, 777–778.

Maxia A, Marongiu B, Piras A, Porcedda S, Tuveri E, Gonçalves MJ, Cavaleiro C, Salgueiro L. 2009. Chemical characterization and biological activity of essential oils from *Daucus carota* L. subsp. *carota* growing wild on the Mediterranean coast and on the Atlantic coast. *Fitoterapia* **80**, 57–61. http://dx.doi.org/10.1016/j.fitote.2008.09.008.

Omran SM, Moodi MA, Amiri SMBNA, Mosavi SJ, Saeed SAMGM, Shiade SMJS, Kheradi E, Salehi M. 2011. The Effects of Limonene and Orange Peel Extracts on Some Spoilage Fungi. *Int. J. Molec. Clin. Microbiol*. **1**, 82–86.

Özcan MM, Chalchat JC. 2007. Chemical composition of carrot seeds (*Daucus carota* L.) cultivated in Turkey:

characterization of the seed oil and essential oil. *Grasas Aceites* **5** (4), 359–365.

The Pherobase: Database of pheromones and semiochemicals [Internet]. Available from http://www.pherobase.com/ Accessed Mar. 11, 2014.

Pigulevskii GV, Kovaleva VI. 1955. Essentials oil of Carrot (*Daucus carota* L.). *Trudy Botan*, **5**, 7–20.

Pigulevskii GV, Kovaleva VI, Motskus DV. 1965 Essential oils obtained from the fruit of the wild carrot *Daucus carota* L., collected in different regions. *Rast Resursy* **1**, 227–230.

Pinelo M, Meyer AS. 2008. Enzyme-assisted extraction of antioxidants: release of phenols from vegetal Matrixes. *J Env. Agricult. Food Chem*. **7**, 3217–3220.

Puri M, Sharma D, Barrow CJ. 2012. Enzyme-assisted extraction of bioactives from plants. *Trends Biotechnol*. **30**, 37–44. http://dx.doi.org/10.1016/j.tibtech.2011.06.014.

Roldán-Gutiérrez JM, Ruiz-Jiménez J, Luque de Castro MD. 2008. Ultrasound-assisted dynamic extraction of valuable compounds from aromatic plants and flowers as compared with steam distillation and superheated liquid extraction. *Talanta*, **75**, 1369–1375. http://dx.doi.org/10.1016/j.talanta.2008.01.057.

Saad H-EA, El-Sharkawy SH, Halim AF. 1995. Essential oils of *Daucus carota* L. spp. *maximus*. *Pharm. Acta. Helv*. **70**, 79–84. http://dx.doi.org/10.1016/0031-6865(94)00054-Y.

Soković M, Glamočlija J, Marin PD, Brkić D, van Griensven LJLD. 2010. Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. *Molecules*, **15**, 7532–7546. http://dx.doi.org/10.3390/molecules15117532.

Staniszewska M, Kula J, Wieczorkiewicz M, Kusewicz D. 2005. Essential oil of cultivated carrots-the chemical composition and antimicrobial activity. *J. Essent. Oil Res*. **17**, 579–583. http://dx.doi.org/10.1080/10412905.2005.9699002.

StatSoft: Electronic Manual online [Internet]. Available from https://www.statsoft.com/textbook/ Accessed Mar. 11, 2014.

Surburg H, Panten J. 2006. *Common fragrance and flavor materials. Preparation, properties and uses*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Tadayon F, Motahar S, Hosseini M. 2012. Application of Taguchi method for optimizing the adsorption of lead ions on nanocomposite silica aerogel activated carbon. *Acad. Res. Int*. **2**, 42–48.

Tavares AC, Gonçalves MJ, Cavaleiro C, Cruz MT, Lopes MC, Canhoto J, Ribeiro Salgueiro L. 2008. Essential oil of *Daucus carota* L. subsp. *halophilus*: Composition, antifungal activity and anti-inflammatory properties. *J. Etnopharm*. **130**, 593–598. http://dx.doi.org/10.1016/j.jep.2010.05.054.

Topf M, Varnai P, Graham RW. 2001. QM/MM study of the deacylation step in elastase: Exploring active site dynamics and the effect of quantum region size. *Abstr. Papers Am. Chem. Soc*. **222** (1–2): Comp 061.

Śmigielski K, Raj A, Krosowiak K, Gruska R. 2009. Chemical composition of the essential oil of *Lavandula angustifolia* cultivated in Poland. *J. Essenti. Oil Bear. Pl*. **12** (3), 338–347. http://dx.doi.org/10.1080/0972060X.2009.10643729.