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Hydrodistillation and Steam Distillation of Fennel Seeds Essential Oil: Parameter Optimization and Application of Cryomilling Pretreatment

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Abstract: The aim of this study was to examine the effectiveness of cryomilling (CM) pretreatment on the isolation of fennel seeds essential oil (EO). Therefore, the study included (i) the process optimization and comparison of the efficiencies of hydrodistillation (HD) and steam distillation (SD); (ii) the evaluation of the effect of the CM application prior to the distillation method (selected based on the results obtained in the first part) on the EO yield; (iii) the evaluation of possible quality differences of the EOs obtained with the performed isolation methods. The obtained results showed that HD (at a solid to liquid ratio 1:10 and a distillation time of 120 min) was more efficient in terms of the EO yield compared to SD (at a pressure of 0.83 bar and distillation time of 117 min as optimal conditions). Moreover, an increased EO yield or even reduced distillation time was observed when HD was combined with a 3 min or 5 min CM pretreatment. GC-MS analysis showed no qualitative differences in chemical composition upon any of the applied isolation procedures, although higher amounts of volatiles were found in the cryomilled samples. The results of this study could be of interest to academia and the EO industry, as CM showed a positive aspect in EO isolation that could provide economic benefits in terms of higher yields or energy savings.

Keywords: *Foeniculum vulgare* Mill.; essential oil isolation; cryogenic grinding; optimal conditions; yield; volatiles

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1. Introduction

Fennel (*Foeniculum vulgare* Mill.) is a wild or cultivated, aromatic, perennial herbaceous plant belonging to the Apiaceae family characterized by a small light golden flower and slightly curved seeds of yellowish to greenish color. The application of fennel seeds in the food and cosmetic industry, phytotherapy, and other fields is continuously growing due to the presence of various phytochemicals, i.e., secondary plant metabolites, consisting of various bioactive compounds with beneficial properties [1].

Many extensive studies on phytochemicals from the plants of the Apiaceae family have shown the presence of various important components such as volatiles, flavonoids, phenolic compounds, fatty acids, hydrocarbons, and amino acids [2]. Mallik et al. (2020) [3] reported that saponins, alkaloids, coumarins, tannins, flavonoids, and steroids are present in fennel seeds. In addition to non-volatiles, fennel seeds are abundant with essential oil (EO), which is rich in volatile compounds belonging to various chemical groups [4,5].

More than 87 constituents have been identified in fennel EO [2]. Among them, terpenes predominate and are responsible for the characteristic EO odor and taste. Furthermore, the same authors reported that the major volatile constituents of fennel EO are *trans*-anethole and fenchone, while the third most abundant constituent varies regardless of the growing

climate [2]. In accordance with these findings, Ghasemian et al. (2019) [5] confirmed that *trans*-anethole (78.47–79.64%) and fenchone (8.4–10.5%) were the major components among the 21 components identified in EO from fully mature seeds of *F. vulgare* Mill. from Iranian regions, followed by monoterpene hydrocarbons (5.6–6.7%) and sesquiterpene hydrocarbons (0.35%). Other studies documented *trans*-anethole and estragole (methylchavicol), followed by fenchone and D-limonene as the most abundant constituents of fennel seeds EO [6,7]. Javed et al. (2020) [8] also reported the presence of α-pinene and camphene. Belabdelli et al. (2020) [9] found that sweet fennel EO contains higher levels of *trans*-anethole and estragole, while Kalleli et al. (2019) [7] described *trans*-anethole as the most abundant in var. *dulce* and estragole in var. *vulgare*. Furthermore, bitter fennel EO contains more estragole, fenchone, α-pinene, and limonene [9].

The concentration of these compounds depends on several factors, seed species [6], growing conditions [8], geographic origin [1], environmental conditions [6], yield [9], and accumulation of volatiles during vegetation [1,9], highlighting that the content of EO decreases with seed maturity [1] and extraction methods [1,9].

EOs are usually isolated by conventional methods such as hydrodistillation (HD) and steam distillation (SD). HD is an extraction method that involves three main physicochemical processes to obtain EO from plant material: hydrodiffusion, hydrolysis, and decomposition by heat. In general, the distillation time can vary from 3 to 6 h depending on the type of plant material and, together with the plant-to-water ratio and the heating time, can have a great influence on the yield and the exact composition of EO [10]. Mimica-Dukić et al. (2003) [11] reported that the ratio of fennel seeds to water and distillation time during HD significantly affected the EO yield, i.e., a lower seeds/water ratio and longer distillation time resulted in a higher EO yield. However, the qualitative and quantitative composition of the oils obtained under different HD conditions were only slightly affected. The major drawback of this method is that the EOs are exposed to prolonged boiling water and the high temperature cannot be controlled, which promotes polymerization of aldehydes, hydrolysis of unsaturated or ester compounds, or thermal decomposition of other heat-sensitive components, leading to differences in the composition of the volatile oils being extracted [10]. On the other hand, steam is used in SD, which takes advantage of the volatility of a compound that vaporizes when heated with steam and the hydrophobicity of a compound that dissolves into an oil phase upon condensation [10]. Several factors affect the final quality of a steam-distilled EO, but the most important are time, pressure, and temperature. EOs isolated using SD may differ in composition from those naturally occurring in plants due to chemical reactions that lead to the formation of certain artificial chemicals called artefacts [12]. However, Gavahian et al. (2015) [13] reported that *Mentha piperita* L. EOs obtained using HD and SD in a similar extraction time were similar in their physical properties and chemical composition. Božović et al. (2017) [12] also reported that there is no rule for appropriate extraction time, as different plants require different time periods for EO extraction to achieve the desired quantity or quality of extract; however, higher yields due to longer distillation may lead to the accumulation of more artefacts. Although these methods have drawbacks such as a long extraction time, high energy consumption, and losses of volatiles [12], they are still the most commonly used methods for EO extraction.

In order to overcome the drawbacks of conventional isolation techniques, novel extraction methods have been developed in recent decades aimed at reducing the extraction time, achieving higher yields, and obtaining a suitable chemical composition of high quality EOs [14]. These novel extraction methods such as ultrasound-assisted extraction [15], enzyme-assisted extraction [16], microwave-assisted extraction, sub- and supercritical fluid extraction, pressurized liquid extraction, pulsed electric fields and high voltage electrical discharges [17], dielectric barrier discharge cold plasma [18] and cryomilling are used as pretreatment for distillation [19].

Cryomilling (CM) or cryogrinding is a milling technique that was previously used mainly for milling spices in food processing [20]. It is a mechanical milling process of plant material at a temperature lower than −150 °C using cryogens [20,21] where liquid nitrogen

is mostly used in food milling [21]. The process consists of two phases: the precooling phase and the milling phase. First, the plant material is cooled to extremely low temperature in a conveyor by liquid nitrogen, and in the second phase, the cooled material is milled with a hammer or a pin mill. The main advantages of CM include the retention of highly volatile compounds by applying extremely low temperatures while crushing the particles easily and quickly, shortening the time of milling and reducing powder agglomeration, contamination, and oxidation. Moreover, it is an environmentally friendly process since liquid nitrogen is harmless and no hazardous chemical substances are released. The only drawback is the risk of direct contact between a coolant such as liquid nitrogen and human skin, as it can cause cold burns. Therefore, additional safety precautions are required when manipulating during CM [20].

CM is well suited for use with spices, preserving the aroma and maintaining the health and hygiene of the spices [21]. Therefore, CM is widely used in the pharmaceutical industry [20]. The important parameters for CM are the rotor speed, feed rate, and sieve opening size [21]. Mékaoui et al. (2016) [22] showed a difference in CM compared to conventional milling on the yield of cumin seeds EO using HD or SD assisted by microwaves, where CM as a pretreatment remarkably increased the yield (6.22–14.5%). They also confirmed that CM did not modify the chemical composition of EO. Cvitković et al. (2022) [19] emphasized CM as a rapid exhausting of the plant material, which accelerates the distillation time. They reported a positive EO yield trend when using CM as a pretreatment for HD of myrtle leaves. A 3 min CM yielded 16.6% more of the total volatile compounds than the control, especially low boiling point terpenes, whose amount increased correspondingly with shorter extraction time (30 min) and less energy. Akloul et al. (2014) [23] also confirmed a shorter distillation time when using CM as a pretreatment. They reported that the major quantities of volatile oils were mostly obtained in the first 5 and 10 min of extraction of *Curcuma longa* rhizomes and *Carum carvi* L. fruits, respectively.

Although numerous studies have addressed the issue of the isolation of fennel seeds EO, the literature data on the application of CM as a pretreatment for enhancing EO yields are scarce. Moreover, to the best of the authors' knowledge, there are no data on the application of CM in the isolation of fennel seeds EO. Therefore, the main goal of this study was to examine the effectiveness of CM pretreatment in isolating fennel seeds EO. Accordingly, the study was divided into three parts: (i) process optimization and comparison of the efficiency of HD and SD as conventional extraction methods in the isolation of fennel seeds EO; (ii) evaluation of the effect of CM application prior to the distillation method (selected based on the results obtained in the first part) on the EO yield; (iii) evaluation of possible differences in the quality of EOs obtained under the conducted isolation procedures.

2. Materials and Methods

2.1. Chemicals

Purified water was of Milli-Q quality (Millipore, Bedford, MA, USA) and *n*-hexane 95% was purchased from Fisher Scientific (Loughborough, UK). Anhydrous sodium sulfate was obtained from Lach-Ner Ltd. (Neratovice, Czech Republic) and liquid nitrogen from Messer Croatia Plin Ltd. (Zaprešić, Croatia). Commercial standards of (+)-α-pinene, camphene, (−)-β-pinene, (R)-(−)-α-phellandrene, 3-carene, *p*-cymene, γ-terpinene, (+)-carvone, eucalyptol, (+)-fenchone, (±)-camphor, *p*-anisaldehyde, *trans*-anethole, and alkane standard solution C_7–C_{30} were procured from Sigma Aldrich (St. Louis, MO, USA), myrcene from Merck (Darmstadt, Germany), R-(+)-limonene and nerol from Fluka® Analytical (Munich, Germany), and α-terpinene and estragole from Dr. Ehrenstorfer GmbH (Augsburg, Germany). All chemicals and solvents were of HPLC grade.

2.2. Plant Material

Cultivated bitter fennel seeds (*F. vulgare* Mill.) were purchased from Agristar Ltd. (Višnjevac, Croatia). The seeds were grown in the Zelčin area (Baranja County, Croatia) in

March 2020. The agrotechnical measures implemented during the cultivation of the seeds were: plowing, pre-sowing preparation (disking and roto-harrowing), weed control by mowing, and harvesting with a combine harvester in September 2020. Drying of the fennel seeds was carried out on floor dryers in 30 cm thick layer of seeds at 42 °C for 20 h using natural gas as an energy source. For the optimization of the HD and SD parameters, dry fennel seeds were milled using an electric grinder (Waring WSG30, Sprzęt Laboratoryjny i Medyczny Labpartner KBS, Warsaw, Poland) for 10 s to obtain a coarse powder, while for testing the influence of CM pretreatment, fennel seeds were cryomilled according to the procedure described in Section 2.5. The moisture contents of the whole and milled seeds were determined by drying at 105 °C to a constant mass and were less than 5%.

2.3. HD

In order to optimize the HD conditions, the solid to liquid ratio (g mL^{-1}) and distillation time (min) were varied (Table 1). A proper amount of dry milled fennel seeds was mixed with 200 mL of deionized water in a round bottom flask and subjected to HD according to the experimental design (Table 1) using a Clevenger-type apparatus (Deotto Lab, Zagreb, Croatia). The distillation time was measured from the appearance of the first EO drop on the top of the condenser. The distilled EO was collected, dried over anhydrous sodium sulfate, and stored in tightly closed dark vials at −18 °C until analysis. The EO yield was calculated as the percentage of the ratio of obtained oil (mL) and initial mass (g) of the plant material (v/w).

Table 1. Fennel seeds EO yield (%) according to the CCD matrix for HD and SD.

Sample	HD			SD		
	X_1: Solid to Liquid Ratio (g: mL)	X_2: Time (min)	EO Yield (%)	X_1: Pressure (bar)	X_2: Time (min)	EO Yield (%)
1	1:20	80	5.00	0.15	40	1.64
2	1:20	80	5.12	0.15	120	1.95
3	1:10	120	5.50	0.85	40	2.63
4	1:30	40	5.10	0.85	120	2.91
5	1:34.1	80	5.46	0.01	80	1.74
6	1:10	40	4.60	0.99	80	3.02
7	1:5.9	80	5.02	0.50	23.4	1.08
8	1:20	80	5.10	0.50	136.6	2.71
9	1:20	80	5.15	0.50	80	2.08
10	1:20	80	5.05	0.50	80	1.93
11	1:20	136.6	5.10	0.50	80	2.60
12	1:20	23.4	4.40	0.50	80	2.24
13	1:30	120	5.40	0.50	80	2.70
	Mean		5.08			2.25

EO = essential oil, CCD = central composite design, HD = hydrodistillation, SD = steam distillation.

2.4. SD

SD was carried out using a laboratory-scale SD equipment (Darkol Ltd., Varaždin, Croatia). The distillation was performed with 300 g of milled fennel seeds varying the pressure and time of distillation (Table 1). After distillation, EO was collected, dried over anhydrous sodium sulfate and stored in tightly closed dark vials at −18 °C until analysis. The EO yield was calculated as stated in Section 2.3.

2.5. CM

CM of seeds was performed using a laboratory-scale Spex 6875D Freezer/Mill (Metuchen, NJ, USA). A milling chamber (50 mL) with a 10 cm stainless steel pin was filled with 40 g of fennel seeds and closed with a stainless cap. Prior to milling, each sample was pre-cooled for 2 min with a flow of liquid nitrogen. The fennel seeds were then milled

for 1, 3, 5, and 7 min with a speed rate of 14 cycles per second (cps). The powder was collected into a plastic container, tightly sealed, and stored at −18 °C until distillation.

2.6. Particle Size Distribution

The particle size distributions of the conventionally milled and cryomilled seeds were determined by laser diffraction using a Malvern Mastersizer particle size analyzer equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK). The dry dispersion unit was filled with approximately 3 to 5 g of powder sample and the measurements were carried out at a pressure of 1.5 bar and a 100% feed rate. Each measurement was performed in duplicate and the diameter of the 50th percentile (d (50), μm) was calculated using the Mastersizer 2000, v. 5.60 software with the following parameters: refractive index of 1.5, an absorption of 0.1, and the obscuration 1.4%. The median particle size (d (50), μm) was considered to determine the interdependence of CM and particle size reduction.

2.7. Scanning Electron Microscopy

To assess the effect of milling on the morphology of the fennel seeds, the high-resolution field emission scanning electron microscope (SEM) JSM-7000F (Jeol, Tokyo, Japan) at the Ruđer Bošković Institute (Zagreb, Croatia) was used. To fix the samples and ensure electrical contact with the rest of the instrument, fennel seeds ground conventionally or by CM were spread in a thin layer on a carbon tape on the SEM sample holder. Images were obtained with an accelerating voltage of 5.0 kV at a standard distance of the objective from the sample (WD = 10 mm). Photomicrographs were taken of each sample at 20 and 500× magnification and a secondary electron detector was used to produce the micrograph/image. The morphological characteristics were studied on the conventionally milled fennel seeds and those cryomilled for 5 min.

2.8. Gas Chromatography–Mass Spectrometry Analysis

The compositions of the fennel seeds EOs were analyzed by gas chromatography–mass spectrometry (GC-MS) using an Agilent Technologies 6890N network gas chromatograph system coupled to an Agilent 5973 *inert* mass selective detector (Agilent Technologies, Santa Clara, CA, USA). For the separation of compounds, a capillary column (Agilent HP-5MS ((5%-phenyl)-methylpolysiloxane; 30 m × 0.25 mm × 0.25 μm)) was used. Prior to injection, the EO samples were diluted (1:99) in a solution of *n*-hexane and an internal standard (nerol, 1.0518 mg mL^{-1}). The injection volume was 1.0 μL (Agilent 7683B autosampler injector) with a split ratio of 100:1 under 250 °C using helium as the carrier gas at a constant flow rate of 1 mL min^{-1}. The initial oven temperature was 60 °C, then 60–145 °C (3 °C min^{-1}) and 145–250 °C (30 °C min^{-1}) with a 3 min hold at 250 °C. The total run time was 34.83 min. The transfer line, MS source, and quadrupole temperatures were 280, 230, and 150 °C, respectively. The detector ionization energy was 70 eV. The qualitative analysis of the compounds was performed in scan mode (30–550 at 1 scan s^{-1}), while single ion monitoring (SIM) mode was used to quantify the compounds. The alkane solution was analyzed at the same conditions and retention indices (RI) were calculated according to Bianchi et al. (2007) [24].

The EO volatiles were confirmed by matching their retention times, RI, and mass spectra (m/z) with authentic standards, and by comparing them with m/z in the NIST database (ChemStation Data Analysis). Quantitative determination was carried out using calibration curves of the standards: α-pinene, camphene, β-pinene, myrcene, α-phellandrene, α-terpinene, *p*-cymene, D-limonene, eucalyptol, γ-terpinene, L-fenchone, camphor, estragole, carvone, *p*-anisaldehyde, and *trans*-anethole. For sabinene and *cis*-sabinene hydrate identification was performed according to their m/z, RI, and comparison with literature data, while their quantitative values were calculated according to the 3-carene calibration curve. Analysis was performed in triplicate for each sample and the results are expressed in mg mL^{-1} of EO as mean ± standard deviation.

2.9. Experimental Design and Statistical Analysis

The experimental design and statistical analysis were performed using the Statistica v. 12 (Statsoft Inc., Tulsa, OK, USA) and Design Expert 10.0 (Stat-Ease Inc., Minneapolis, MN, USA) software. In order to establish the optimal conditions of HD and SD for obtaining the maximum EO yield, a two-factor central composite design (CCD) followed by response analysis was applied, giving a total of 13 experimental trials with five replications of the central point. Independent variables that were considered for HD optimization were the solid to liquid ratio (X_1) and distillation time (X_2) at five levels (−1.41, −1, 0, 1, +1.41), namely, 1:5.9, 1:10, 1:20, 1:30, and 1:34.1 g:mL for X_1 and 23.4, 40, 80, 120, and 136.6 min for X_2. For the optimization of SD, pressure (X_1) and time of distillation (X_2) were selected as the operating variables at five levels (−1.41, −1, 0, 1, +1.41): X_1 −0.01, 0.15, 0.50, 0.85, and 0.99 bar, X_2 −23.4, 40, 80, 120, and 136.6 min. Design matrices for both extraction methods are given in Table 1. The experiments were performed in a random order arranged by the software. The regression model for each response was calculated as follows (1) [25]:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, β_0 is the model intercept; β_i, β_{ii}, and β_{ij} are the regression coefficients of the linear, square, and interaction terms, respectively, while X_i and X_j represent independent variables.

Analysis of variance (ANOVA) was carried out to determine significant differences ($p \leq 0.05$) among the applied extraction conditions. The model was fitted by multiple linear regressions (MLR) and the validity of the quadratic and linear empirical models was tested by ANOVA and lack of fit test with a confidence level of 95%. For the optimization of HD and SD, a prediction and profiling tool was applied, and optimal conditions were selected based on the desirability function (D = 0.879).

In order to evaluate the effect of CM pretreatment on the EO yield, a mixed four- and three-level full factorial design (FFD) comprising 12 experiment runs was employed, where the duration of CM (1, 3, 5, and 7 min) and distillation time (40, 80, and 120 min) were set as independent variables. Multifactorial analysis of variance followed by post hoc Tukey's HSD test were used to examine the differences in the EO yield under the applied conditions. Significant differences in the chemical compositions of fennel seeds EO obtained at optimized HD and SD conditions, as well as selected CM conditions, were tested using one-way ANOVA and post hoc Tukey's HSD test. All differences were considered significant at a level of $p \leq 0.05$.

3. Results and Discussion

3.1. Optimization of HD and SD Parameters for the Isolation of Fennel Seeds EO

The first part of this study involved optimization of the HD and SD parameters for enhanced isolation of fennel seeds EO. For this purpose, the parameters solid to liquid ratio and distillation time as well as pressure and distillation time were varied during HD and SD, respectively. The results for the fennel seeds EO yield (%) obtained using HD are presented in Table 1. The yields obtained with HD ranged between 4.40 and 5.50%, with a mean of 5.08%. The highest yield was obtained at a solid to liquid ratio of 1:10 and a distillation time of 120 min, while the lowest yield characterized HD at a solid to liquid ratio of 1:20 and a distillation time of 23.4 min. The obtained results are similar to those of Khammassi et al. (2018) [26], who reported 1.2 to 5.06% of the 16 wild edible Tunisian *F. vulgare* EOs after 4 h distillation. On the contrary, Belabdelli et al. (2020) [9] obtained only 1.42% of sweet fennel seeds EO from Algeria after 5 h distillation time, while Ahmed et al. (2019) [27] reported 1.6 and 1.1% of fennel seeds EO from Egypt and China, respectively, obtained with a solid to liquid ratio of 1:10 and distillation time of 3 h. A literature search revealed that the EO yield of fennel seeds from Pakistan was 2.81% after 3 h distillation [28], while Mimica-Dukić et al. (2003) [11] reported an EO yield in the range of 2.82–3.38% for *F. vulgare* seeds from the Balkans area.

Since the yield is of great interest for production, the influence of the examined HD parameters on the EO yield was tested. The ANOVA results are shown in Table 2. The solid to liquid ratio (X_1) and distillation time (X_2) had a statistically significant influence ($p \leq 0.05$) on the yield of fennel seeds EO, i.e., the EO yield was significantly affected by both their individual influence and their interaction (Table 2). When observing the 3D surface plot, the highest EO yield was obtained at a solid to liquid ratio of 1:10 and a longer distillation time (over 100 min) (Figure 1a). This can be attributed to the stronger presence of the plant matrix, thus requiring a longer distillation time to achieve efficient extraction. A similar trend of increased EO yield obtained at a lower solid to liquid ratio and longer distillation time was reported by Mimica-Dukić et al. (2003) [11], who obtained a higher EO yield when combining a lower solid to liquid ratio (1:2) with a longer distillation time (6 h). In addition, a higher yield was also obtained when the solid to liquid ratio exceeded 1:25 and the distillation time was about 80 min (Figure 1a), which could be due to the presence of more solvent (water) that generated more steam and, thus, enhanced the extraction of volatiles. However, in response to the lower plant load, a shorter distillation time was sufficient to extract all of the EO. Lainez-Cerón et al. (2021) [29] also reported an increased EO yield when a higher solid to liquid ratio was used during HD of eucalyptus EO. A literature search also revealed that most of the EO yield from dill seeds (74.81%) was obtained during the first 75 min of HD, while further distillation did not contribute as effectively [30]. At a solid to liquid ratio of 1:20, neither of the effects prevailed, resulting in a lower yield (Figure 1a).

As the examined HD parameters are interrelated and affect the fennel seeds EO yield, their optimization is of great importance. Accordingly, they were combined in linear, quadratic, and interaction coefficients to obtain a regression model equation describing the dependence of the fennel seeds EO yield upon the HD parameters, i.e., solid to liquid ratio and distillation time (Table 2). The coefficient of determination (R^2) obtained was 0.933, while the "lack of fit" value was non-significant ($p > 0.05$), both indicating good accuracy of the model for optimization of the HD conditions which will deliver the highest yield of fennel seeds EO. The defined HD optimal conditions were a solid to liquid ratio 1:10 and 120 min of distillation time, respectively, which predicted a 5.37% yield of EO (Table 3). To validate the model and confirm the predicted value of the EO yield, HD of fennel seeds was performed under the optimal conditions, obtaining a 5.50% yield of EO (Table 3) and confirming the validity of the obtained model.

Table 2. Influence of HD and SD parameters on the fennel seeds EO yield (%).

Source of Variation	HD		SD	
	EO Yield (%)			
	F-Value	*p*-Value	F-Value	*p*-Value
X_1	19.635	0.001 *	15.844	0.003 *
X_1^2	8.557	0.022 *	-	-
X_2	12.170	0.010 *	9.393	0.012 *
X_2^2	10.538	0.014 *	-	-
X_1X_2	8.362	0.023 *	-	-
Lack of fit	5.781	0.062	1.030	0.512
R^2	0.933		0.716	
Model	$Y = 3.6301 - 0.0032X_1 + 0.0012X_1^2 + 0.0271X_2 - 0.0001X_2^2 - 0.0004X_1X_2$		$Y = 0.8498 + 1.3499X_1 + 0.0090X_2$	

HD = hydrodistillation, SD = steam distillation, EO = essential oil. HD: X_1—solid to liquid ratio, X_2—distillation time; SD: X_1—pressure, X_2—distillation time. * Statistically significant variable at $p \leq 0.05$.

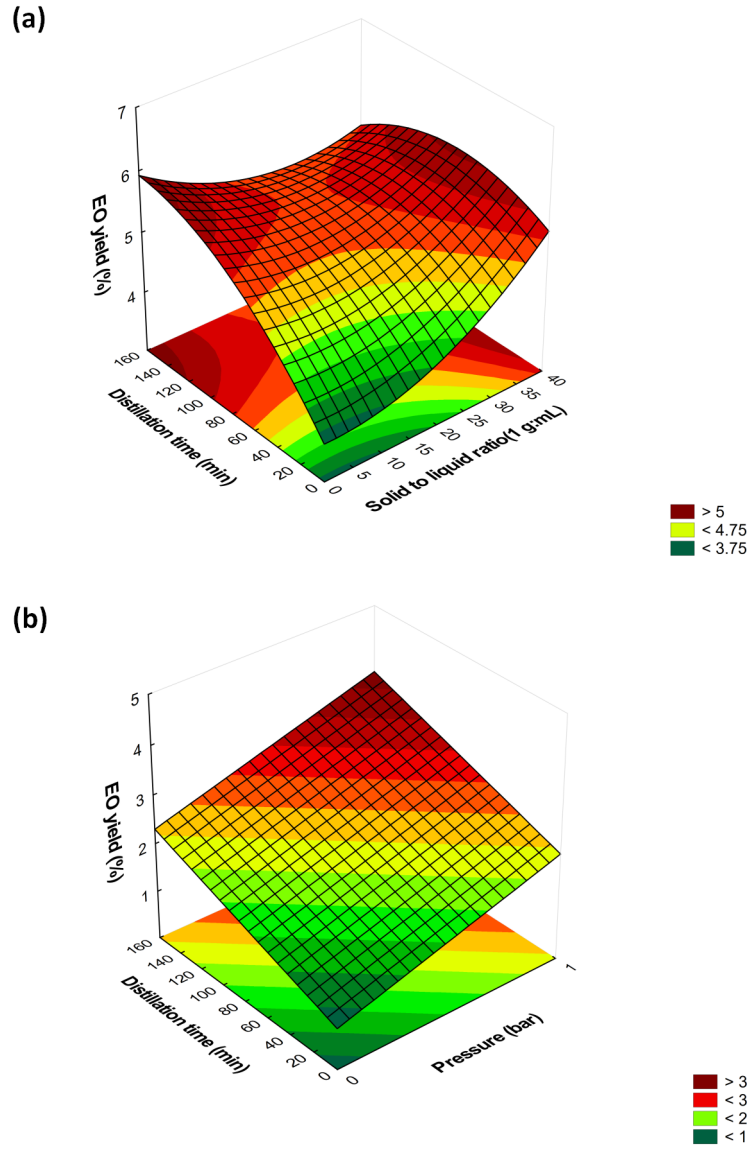

Figure 1. 3D graphs of fennel seeds EO extraction evolution during (**a**) HD and (**b**) SD.

Table 3. Predicted and experimental values for fennel seeds EO yield (%) obtained at optimal conditions of HD and SD.

	Optimized Parameters		EO Yield (%)	
			Predicted	**Experimental**
HD	**Solid to Liquid Ratio (g:mL)**	1:10	5.37	5.50
	Distillation time (min)	120		
SD	Pressure (bar)	0.83	3.03	2.95
	Distillation time (min)	117		

EO = essential oil, HD = hydrodistillation, SD = steam distillation.

In addition to optimizing the HD conditions, optimization of the SD conditions was also carried out. Table 1 shows the EO yield (%) obtained by varying the SD parameters, namely, pressure and distillation time, and it ranged between 1.08 and 3.02% with a mean of 2.25%. Compared to the EO yields obtained by using HD, one can notice remarkably lower yields obtained by SD. A pressure of 0.99 bar and distillation time of 80 min gained the highest yield (3.02%), while the lowest yield (1.08%) was obtained when a pressure of

0.50 bar and distillation time of 23.4 min were applied. Moser et al. (2014) [31] reported similar or lower EO yield values of steam-distilled fennel seeds (0.042–1.375%), where they varied the distillation duration (15–1080 min), while Damayanti and Setyawan (2012) [32] reported a fennel seeds EO yield of 2.04% obtained at 1 atm during 7.5 h of SD. Furthermore, Leal et al. (2011) [33] conducted 5 h SD of anise seeds and gained a 0.72% yield of EO. They explained the low yield as being a result of using whole seeds for the SD, since milling in SD is limited considering that too compact extraction beds lead to major head loss as the steam cannot flow through the bed, and it condenses before leaving the extractor. On the contrary, using large particles hardens the access of steam to the volatile oil located inside seeds; therefore, particle size is a very important parameter.

Considering the influence of the examined SD parameters on the fennel seeds EO yield, the ANOVA results showed a significant influence ($p \leq 0.05$) of pressure (X_1) and distillation time (X_2) on the EO yield (Table 2). Figure 1b shows the fennel seeds EO yield as affected by pressure and distillation time, where it can be seen that increases in both pressure and distillation time result in an increased EO yield, with the highest process yield obtained at their maximum applied values. As mentioned earlier, Moser et al. (2014) [31] investigated the effect of distillation time on the yield of fennel seeds EO during SD. They found significant differences in EO yield depending on the distillation time: the EO yield after 15 min was 0.042%, followed by 0.072% after 30 min, 0.157% after 60 min, 0.276% after 120 min, 0.573% after 240 min, 0.782% after 360 min, 0.923% after 480 min, 1.030% after 600 min, 1.053% after 720 min, 1.201% after 840 min, 1.353% after 960 min, and 1.375% after 1080 min. Božović et al. (2017) [12] reported the influence of distillation time (1, 2, 3, 6, 12, and 24 h) during SD on the yield of EO from fennel aerial parts collected at different phenological stages. The maximum yield (1.250%) was obtained after 24 h SD of fennel's aerial parts harvested in October, while the minimum yield (0.070%) was obtained after 1 h of distillation with fennel's aerial parts harvested in August. Furthermore, Zheljazkov et al. (2013) [34] studied the effect of SD duration (1.25, 2.5, 5, 10, 20, 40, 80, and 160 min) on the EO yield obtained from all fennel's aerial parts (above ground herbage, including stems, leaves, and umbels with immature seeds). The EO yield significantly increased with the increase in distillation time, from 0.06% after 1.25 min to 0.68% after 160 min, showing that a longer period of distillation contributes to the yield increase.

Optimization of the tested SD parameters was also performed. The statistical data revealed a linear regression model equation as the best fitted model showing the reliance of the fennel seeds EO yield on the pressure and distillation time (Table 2). The obtained non-significant ($p > 0.05$) "lack of fit" value and R^2 of 0.716 described the model as adequate for the prediction of the fennel seeds EO yield (Table 2). The calculated optimal conditions for SD were a pressure of 0.83 bar and 117 min of distillation time, which predict obtaining a 3.03% yield of fennel seeds EO (Table 3). The predicted EO yield was also confirmed experimentally with SD conducted at the optimal conditions, when 2.95% of fennel seeds EO was obtained (Table 3).

Finally, the comparison of the fennel seeds EO yield obtained under optimal conditions with HD (5.50%) or SD (2.95%) clearly shows the greater efficiency of HD as an extraction method for the isolation of EO from fennel seeds. These results can be ascribed to the material attributes, as seeds are described with a hard and compact husk demanding more exhaustive conditions such as boiling for release of volatiles from glandules given that hot water softens and penetrates the material [35]. Řebíčková et al. (2020) [36] also compared HD and SD as methods for isolating *Laurus nobilis* L. leaves EO. Their findings were similar to those in this study, i.e., HD resulted in a higher EO yield (0.95%) compared to the yield of SD (0.79%), as did Gavahian et al. (2015) [13], who also reported a higher EO yield of *M. piperita* L. using HD (2.29%) compared to SD (2.00%). They noted that mint leaves' glands ruptured during HD causing an outflow of captured EO, while in SD they were only wrinkled. In addition, all leaves were not equally accessible for steam during SD, where leaves placed in the top of a pile were in contact with the steam of lower temperature, leading to a decreased yield, opposite to leaves placed in the bottom [13].

In conclusion, HD at a solid to liquid ratio of 1:10 and 120 min of distillation time was selected as the method of fennel seeds EO isolation for the further parts of these experiments.

3.2. Effect of CM on the Isolation of Fennel Seeds EO

In addition to optimizing the distillation parameters, this study additionally aimed to improve and enhance the EO yield of fennel seeds by using CM as a pretreatment for distillation. The goal was to examine whether the application of CM further increases the EO yield or whether it ensures satisfactory EO yield in a shorter distillation time, which will consequently provide certain energy savings. From an economic point of view, both outputs are desirable and could be of interest to the EO industry. Therefore, fennel seeds were cryomilled at different time intervals and then subjected to the previously established HD conditions (solid to liquid ratio of 1:10 and 120 min of distillation time). To test a possible reduction in distillation time, HD was also conducted for 40 and 80 min.

As shown in Table 4, the EO yield after the application of CM ranged from 4.65 to 6.49% for all samples, with a mean of 5.40%. These values show an absolute yield enhancement compared to the yield obtained when HD was used without CM as a pretreatment (Table 1). Moreover, both CM and distillation time had a significant effect ($p < 0.001$) on the EO yield, as well as their interaction. When considering the influence of CM, it can be seen that the EO yield increased with the extension of CM until the 5th min of CM, when the highest yield was obtained (5.83%), while further CM (7 min) no longer contributed to the yield increase.

Table 4. Influence of CM and distillation time on the fennel seeds EO yield (%).

Source of Variation	EO Yield (%)
Cryomilling (min)	$p < 0.001$ *
1	5.05 ± 0.02 [a]
3	5.38 ± 0.02 [b]
5	5.83 ± 0.02 [c]
7	5.33 ± 0.02 [b]
Distillation time (min)	$p < 0.001$ *
40	4.96 ± 0.02 [a]
80	5.49 ± 0.02 [b]
120	5.75 ± 0.02 [c]
Cryomilling (min) × Distillation time (min)	$p < 0.001$ *
1 × 40	4.70 ± 0.04 [a]
1 × 80	4.95 ± 0.04 [b]
1 × 120	5.50 ± 0.04 [c]
3 × 40	4.65 ± 0.04 [a]
3 × 80	6.00 ± 0.04 [d]
3 × 120	5.50 ± 0.04 [c]
5 × 40	5.50 ± 0.04 [c]
5 × 80	5.50 ± 0.04 [c]
5 × 120	6.49 ± 0.04 [e]
7 × 40	5.00 ± 0.04 [b]
7 × 80	5.49 ± 0.04 [c]
7 × 120	5.50 ± 0.04 [c]
Mean	5.40

CM = cryomilling, EO = essential oil. Results are expressed as mean ± standard error. * $p \leq 0.05$. Values with different letters within the column are statistically different at $p \leq 0.05$.

To the best of the authors' knowledge, this is the first study that examines the influence of CM on the yield of fennel seeds EO. Although a literature comparison was difficult due to a lack of data, several studies support the results of this study. For example, similar behavior to that mentioned above was reported by Tischer et al. (2016) [37] on *Baccharis articulata*. They reported that prolonged CM led to trichome glands' rupture and a

decrease in the EO yield, accordingly. They also stated that the volatile compounds may be lost by volatilization at room temperature or absorption on the walls of the grinding vessel. The same behavior can be extrapolated for the 7 min CM of fennel seeds, which probably caused the rupture of all four oil glands, known as ducts and vittae, on the dorsal surface and two vittae on the commissural or ventral surface. It can be concluded that 7 min CM is not required because it unnecessarily prolongs seed rupture, resulting in lower yield and higher energy costs. Shorter 3 min or 5 min CM could be most favorable for a higher yield of fennel seeds EO.

As for the distillation time after CM, all the time intervals studied gave satisfactory EO yields, but 80 and 120 min gave the most promising results. HD with a duration of 80 min gave a 5.49% yield of EO, which is equal to the yield obtained with HD for 120 min without CM as pretreatment, meaning a reduction in distillation time of 20 min. On the other hand, 120 min of HD combined with CM provided a 5.75% yield of EO, which is an increase of 0.25% compared to the yield when HD was applied for the same duration without CM (Table 3). Furthermore, when observing the interactions of CM with distillation time, it is also important to highlight the following samples: 3 min CM × 80 min HD which yielded a 6.00% yield of EO, and 5 min CM × 120 min HD, which yielded an even higher yield of EO, 6.49%. These yields represent increases of almost 10 and 18%, respectively, compared to the EO yield obtained with 120 min HD without using CM. Cvitković et al. (2022) [19] reported the same effect of CM pretreatment on myrtle leaves combined with 30 min HD, where a 3 min CM yielded 16.6% of total volatiles compared to the control (conventionally milled leaves), but prolonged CM (6 and 9 min) resulted in a decrease in total volatiles.

Akloul et al. (2014) [23] studied and compared the effect of CM and conventional milling of *C. longa* rhizomes and *C. carvi* L. fruits as a pretreatment for microwave-assisted HD (HDAM) and microwave-assisted SD (SDAM). Concerning HDAM, they reported a 50% increase in the EO yield for *C. longa* and 25% for *C. carvi* in a shorter distillation time (for 10 and 5 min, respectively) after incorporating CM as a pretreatment. They reasoned that the extremely low temperature in the cryomill hardens the oil in the sample and makes the sample brittle so that it can be easily crumbled and milled to a finer and more uniform size. Therefore, the loss of volatiles can be significantly reduced [21,23]. The particle size measurement results obtained in this study confirm these statements. The d (50) value for conventionally milled fennel seeds was 658 µm, while the particle size was reduced more than 2-fold at 7 min CM (301 µm). Other d (50) values for cryomilled samples were 585 µm for 1 min CM, 508 µm for 3 min CM, and 358 µm for 5 min CM, from which it can be seen that each additional 2 min of CM reduced the particle size by about 15–30%. In addition, the SEM images of the fennel seeds' rupture after conventional milling and 5 min CM clearly shows a difference in particle size as well as a finer size of cryomilled seeds compared to conventionally milled ones (Figure 2). Cvitković et al. (2022) [19] reported about a 2-fold size reduction per 3 min of CM, where a 3 min CM (d (50) = 107 µm) was sufficient compared to lower yields and higher energy consumption at 6 min and 9 min CM (47 µm and 29 µm). Additionally, Mousavi et al. (2020) [38] reported increased concentrations of volatiles (D-limonene, fenchone, estragole, and anethole) in fennel seeds EO obtained from superfine powder in the fraction range 315–500 µm, while their amounts decreased when a smaller fraction (180–315 µm) or the fraction >500 µm were used. Thus, they concluded that the ideal particle size for the most efficient extraction of fennel seeds EO was between 315 and 500 µm, which is consistent with the results of this study.

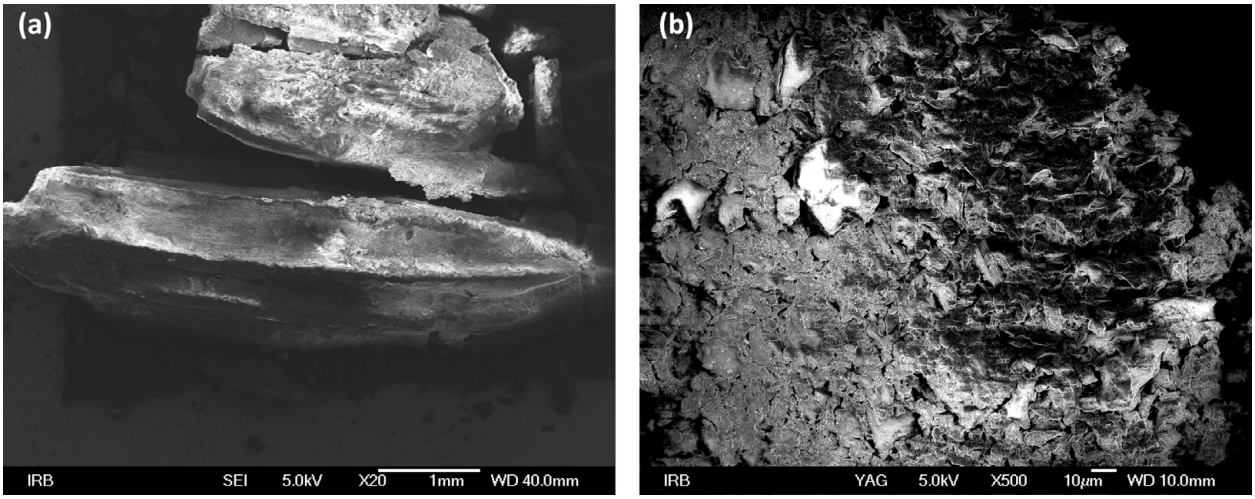

Figure 2. SEM images of the fennel seeds milled (**a**) conventionally and (**b**) by 5 min CM.

3.3. Chemical Characterization of Fennel Seeds EO

To evaluate possible differences in chemical composition with regard to the isolation procedure, the EO samples obtained under the optimal conditions of HD (1:10/120 min) and SD (0.83 bar/117 min) and under selected CM conditions (3 min/80 min; 5 min/120 min) were further analyzed using GC-MS. Their chemical profiles are shown in Table 5. A total of 18 compounds were detected in all of the EO samples analyzed, as follows: α-pinene, camphene, sabinene, β-pinene, myrcene, α-phellandrene, α-terpinene, *p*-cymene, D-limonene, eucalyptol, γ-terpinene, *cis*-sabinene hydrate, L-fenchone, camphor, estragole, carvone, *p*-anisaldehyde, and *trans*-anethole (Table 5 and Figure 3). As can be seen, the compounds belonging to monoterpene hydrocarbons were the most numerous (11), followed by oxygenated monoterpenes (4) and phenylpropanoids (2), while only one compound belonged to aromatic aldehydes (others), regardless of the sample type (Table 5). Concerning their abundance, phenylpropanoids were the most represented (67.65–72.36%) in all of the EOs, followed by oxygenated monoterpenes (16.81–19.82%), monoterpene hydrocarbons (9.58–12.10%), and others (0.43–0.49%). *Trans*-anethole was the most abundant compound in all of the samples (605.88–647.04 mg mL^{-1}). It is the most common ingredient in fennel EO belonging to the group of phenylpropanoids and is the chief aroma accountable for the characteristic sweet, distinct, anise-like flavor [39]. Furthermore, L-fenchone (145.90–191.17 mg mL^{-1}), α-pinene (29.52–47.42 mg mL^{-1}), estragole (21.79–27.79 mg mL^{-1}), myrcene (19.91–26.28 mg mL^{-1}), and D-limonene (14.01–18.89 mg mL^{-1}) also appeared in noticeable amounts, while other compounds were present in concentrations <10 mg mL^{-1}. According to the literature, bitter fennel can be classified into two chemotypes based on the relative presence of the major compounds in EO: anethol or estragole chemotypes [40,41]. Consistently, the samples of EO examined in this study can be assigned to the anetholic chemotype. However, this classification is not unique. Other studies suggested the classification of even more chemotypes, for example, Mota et al. (2015) [42] reported four chemotypes of *F. vulgare*, namely, anethole, estragole, anethole/estragole, and anethole/fenchone, while Božović et al. (2021) [43] proposed the α-terpineolic chemotype as a new chemotype of fennel.

Table 5. Composition of fennel seeds EO (mg mL^{-1}) obtained at optimal conditions of HD, SD, and CM.

9	Compound		RI	RT	*p*-Value	HD	SD	CM	
						1:10/120 min	0.83 bar/117 min	3 min/80 min	5 min/120 min
						mg mL^{-1}			
	Monoterpene hydrocarbons								
1	α-Pinene		941	5.314	<0.001 *	29.52 ± 0.33 ^{a}	33.18 ± 0.19 ^{b}	44.43 ± 0.60 ^{c}	47.42 ± 0.96 ^{d}
2	Camphene		956	5.689	<0.001 *	5.35 ± 0.26 ^{a}	6.09 ± 0.39 ^{a}	8.04 ± 0.15 ^{b}	8.40 ± 0.47 ^{b}
3	Sabinene		979	6.322	<0.001 *	1.41 ± 0.01 ^{a}	1.71 ± 0.02 ^{b}	1.70 ± 0.00 ^{b}	1.86 ± 0.06 ^{c}
4	β-Pinene		983	6.425	<0.001 *	1.44 ± 0.03 ^{a}	1.61 ± 0.02 ^{b}	1.86 ± 0.02 ^{c}	1.98 ± 0.03 ^{d}
5	Myrcene		995	6.779	<0.001 *	19.91 ± 0.07 ^{a}	21.52 ± 0.03 ^{b}	23.71 ± 0.02 ^{c}	26.28 ± 0.32 ^{d}
6	α-Phellandrene		1009	7.204	<0.001 *	4.08 ± 0.01 ^{a}	4.26 ± 0.02 ^{b}	4.63 ± 0.07 ^{c}	4.97 ± 0.05 ^{d}
7	α-Terpinene		1022	7.579	0.042 *	0.33 ± 0.06 ^{a}	0.54 ± 0.04 ^{ab}	0.51 ± 0.02 ^{ab}	0.56 ± 0.16 ^{b}
8	*p*-Cymene		1030	7.833	<0.001 *	1.22 ± 0.01 ^{a}	1.43 ± 0.02 ^{b}	1.47 ± 0.00 ^{c}	1.53 ± 0.01 ^{d}
9	D-Limonene		1034	7.969	<0.001 *	14.01 ± 0.15 ^{a}	15.10 ± 0.06 ^{b}	17.05 ± 0.38 ^{c}	18.89 ± 0.41 ^{d}
11	γ-Terpinene		1064	8.984	<0.001 *	6.25 ± 0.04 ^{a}	6.85 ± 0.04 ^{b}	7.24 ± 0.11 ^{c}	7.89 ± 0.16 ^{d}
12	*cis*-Sabinene hydrate		1072	9.271	<0.001 *	0.77 ± 0.01 ^{b}	0.69 ± 0.01 ^{a}	0.84 ± 0.01 ^{c}	0.88 ± 0.02 ^{c}
	Oxygenated monoterpenes								
10	Eucalyptol		1037	8.066	<0.001 *	1.00 ± 0.01 ^{a}	0.96 ± 0.03 ^{a}	1.09 ± 0.00 ^{b}	1.18 ± 0.03 ^{c}
13	L-Fenchone		1092	10.044	<0.001 *	157.81 ± 1.08 ^{b}	145.90 ± 0.39 ^{a}	184.05 ± 3.78 ^{c}	191.17 ± 2.02 ^{d}
14	Camphor		1149	12.180	<0.001 *	3.52 ± 0.03 ^{b}	3.27 ± 0.04 ^{a}	3.96 ± 0.10 ^{c}	4.24 ± 0.07 ^{d}
16	Carvone		1245	16.138	0.775	1.12 ± 0.03 ^{a}	1.13 ± 0.04 ^{a}	1.15 ± 0.03 ^{a}	1.14 ± 0.02 ^{a}
	Phenylpropanoids								
15	Estragole		1200	14.368	<0.001 *	21.79 ± 0.11 ^{a}	25.32 ± 0.14 ^{b}	25.72 ± 0.46 ^{b}	27.79 ± 0.58 ^{c}
18	*trans*-Anethole		1289	18.055	<0.001 *	605.88 ± 4.34 ^{a}	625.70 ± 3.47 ^{b}	637.31 ± 4.55 ^{c}	647.04 ± 2.84 ^{c}
	Others								
17	*p*-Anisaldehyde		1257	16.614	0.663	4.31 ± 0.03 ^{a}	4.38 ± 0.11 ^{a}	4.43 ± 0.01 ^{a}	4.33 ± 0.21 ^{a}
	Total (%)	*Monoterpene hydrocarbons*			<0.001 *	9.58 ± 0.03 ^{a}	10.34 ± 0.04 ^{b}	11.50 ± 0.03 ^{c}	12.10 ± 0.23 ^{d}
		Oxygenated monoterpenes			<0.001 *	18.58 ± 0.02 ^{b}	16.81 ± 0.10 ^{a}	19.63 ± 0.22 ^{c}	19.82 ± 0.20 ^{c}
		Phenylpropanoids			<0.001 *	71.35 ± 0.04 ^{c}	72.36 ± 0.13 ^{d}	68.41 ± 0.23 ^{b}	67.65 ± 0.25 ^{a}
		Others			0.002 *	0.49 ± 0.01 ^{c}	0.49 ± 0.01 ^{c}	0.46 ± 0.00 ^{ab}	0.43 ± 0.02 ^{a}

EO = essential oil, HD = hydrodistillation, SD = steam distillation, CM = cryomilling. Results are expressed as mean ± standard deviation. * $p \leq 0.05$. Values with different letters within row are statistically different at $p \leq 0.05$.

Regarding the chemical composition, the literature search revealed that Mimica-Dukić et al. (2003) [11] reported a total of 13 chemical constituents in an amount >0.1% in fennel seeds EO obtained under different HD conditions, where the main constituents were anethole (72.27–74.18%), fenchone (11.32–16.35%), and estragole (3.78–5.29%), which is consistent with the results of this study. Another study documented the presence of 18 compounds in EO from seeds of three *F. vulgare* cultivars (var. *azoricum*, *dulce* and *vulgare*) isolated with HD for 2 h [44]. Their chemical profile was quite similar to that obtained in this study, except for α-terpinene, *p*-cymene, *cis*-sabinene hydrate, eucalyptol, and carvone, which were not present in their samples. Instead, they detected *o*-cymene, β-phellandrene, linalool, fenchyl-acetate, and cuminaldehyde. They also confirmed *trans*-anethole, limonene, estragole, and fenchone as the major compounds in fennel seeds EO, with different ratios depending on the variety. On the other hand, Belabdelli et al. (2020) [9] determined 10 compounds in fennel seeds EO obtained during 5 h of HD, and the chemical profile listed in their work was similar to that in this study, although with different quantities: estragole (84.8%), limonene (7.8%), and fenchone (3.1%). Compared to the results of this work, the above mentioned differences in the amounts of individual compounds are probably due to cultivar divergency as well as genetic background, agricultural practices, and environmental conditions.

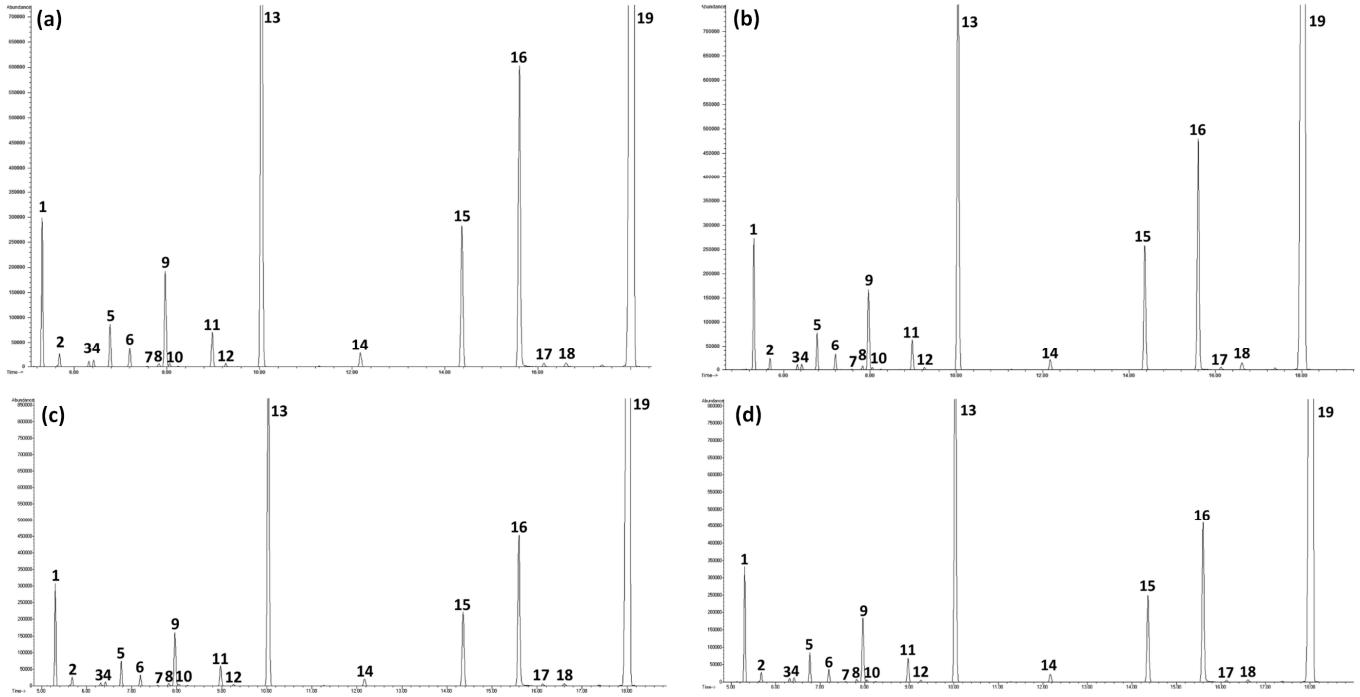

Figure 3. GC-MS chromatogram of fennel seeds EO obtained under (**a**) optimized HD conditions (1:10/120 min), (**b**) optimized SD conditions (0.83 bar/117 min), (**c**,**d**) selected CM conditions (3 min/80 min; 5 min/120 min). (1 = α-Pinene, 2 = Camphene, 3 = Sabinene, 4 = β-Pinene, 5 = Myrcene, 6 = α-Phellandrene, 7 = α-Terpinene, 8 = *p*-Cymene, 9 = D-Limonene, 10 = Eucalyptol, 11 = γ-Terpinene, 12 = *cis*-Sabinene hydrate, 13 = L-Fenchone, 14 = Camphor, 15 = Estragole, 16 = Nerol (IS), 17 = Carvone, 18 = *p*-Anisaldehyde, 19 = *trans*-Anethole).

In terms of the differentiation in EO chemical constitution according to the isolation procedure, significant differences in the presence of individual compounds were found between the examined EOs, thereby reflecting the amounts of their chemical subclasses (Table 5). As can be observed, CM expectedly caused a significant increase in the extraction of almost all detected volatiles, especially low-boiling-point ones, highlighting the 5 min CM as the process with remarkably increased amounts of individual volatiles. Compared to HD, for example, the amounts of most volatiles in the EOs from the cryomilled samples increased by about 20–30%, with increases of more than 50% recorded for some compounds (i.e., α-pinene 60%). Therefore, the total amounts of monoterpene hydrocarbons and oxygenated monoterpenes were highest in the EOs from cryomilled samples. One can also notice that in the same EO samples, the relative amount of total phenylpropanoids decreased as the total amount of monoterpene hydrocarbons and oxygenated monoterpenes increased, although the concentrations of estragole and *trans*-anethole were also highest in these samples. When comparing the influence of HD and SD, a negligible difference in the amounts of certain compounds was found, with most of them being slightly more present in the steam-distilled samples. Řebíčková et al. (2020) [36] compared the chemical composition of EOs isolated from leaves of *L. nobilis* L. and reported that EOs obtained with SD contained more compounds (73) than those obtained with HD (54). They explained this by the fact that certain compounds of the plant's EO could be thermally modified or degraded upon contact with boiling water. Furthermore, only carvone and *p*-anisaldehyde were not affected by the isolation procedure, and their levels remained quite similar regardless of the method used (Table 5).

The results confirmed that the CM conditions associated with HD, especially the longer CM pretreatment before a longer distillation time, favored the isolation of volatiles, as they are likely to evaporate during conventional milling, particularly light ones. Similar results were documented by Cvitković et al. (2022) [19] in their study on the application of

CM on myrtle leaves prior to HD. They also reported an increase in the amount of volatiles in EOs obtained from cryomilled samples compared to that isolated from conventionally milled leaves (control). More precisely, a 3 min CM yielded 16.6% more of the total volatiles compared to the control, and the concentrations of the individual compounds, especially the low-boiling-point volatiles, increased by 14.6–28.2%. On the other hand, they observed a decrease in the content of total volatiles as well as individual ones compared to the control when a longer CM was applied (6 and 9 min). Another study by Bellik et al. (2019) [45] also confirmed significant differences in the quantitative composition of *Cymbopogon schoenanthus* L. Spreng EO when using CM or conventional milling. Their results also evidenced that CM provided rapid extraction with considerable yields of monoterpene hydrocarbons, opposite to the loss of these compounds with conventional milling due to their lower boiling point.

In summary, the chemical profile of the examined EOs confirmed that the applied isolation methods did not cause qualitative changes; however, CM was definitely highlighted as an efficient pretreatment to achieve higher yields of volatiles in the extracted EO.

4. Conclusions

The results of this study defined a solid to liquid ratio of 1:10 and 120 min of distillation time as optimal conditions for the isolation of fennel seeds EO using HD, while a pressure of 0.83 bar and distillation time of 117 min were set as optimal for the isolation of fennel seeds EO by SD, with HD proving to be a more efficient extraction method (5.50% yield of EO) than SD (2.95% yield of EO). Furthermore, CM followed by HD showed a positive influence on the EO yield, where 3 min CM/80 min and 5 min CM/120 min gave increased oil yields (6.00 and 6.49%, respectively) or even reduced the distillation time, while 7 min CM proved to be too long and not effective. Regarding the chemical composition of the obtained EOs, a total of 18 compounds were detected, with phenylpropanoids being the most abundant (67.65–72.36%), followed by oxygenated monoterpenes (16.81–19.82%), monoterpene hydrocarbons (9.58–12.10%), and aromatic aldehydes (0.43–0.49%). *Trans*-anethole was the predominant compound (605.88–647.04 mg mL^{-1}) and L-fenchone (145.90–191.17 mg mL^{-1}), α-pinene (29.52–47.42 mg mL^{-1}), estragole (21.79–27.79 mg mL^{-1}), myrcene (19.91–26.28 mg mL^{-1}), and D-limonene (14.01–18.89 mg mL^{-1}) were also present in notable amounts. Moreover, GC-MS analysis revealed that none of the isolation procedures used had any effect on the qualitative composition of fennel seeds EO. Finally, CM proved to be beneficial for the isolation of fennel seeds EO, allowing certain economic benefits that could be favorable and of interest to both EO producers and the scientific community.

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