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Essential Oils of *Taxodium distichum* Winter Leaves Obtained by Supercritical Carbon Dioxide Extraction Method and Hydrodistillation

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Abstract: The extraction of *Taxodium distichum* needles was done using supercritical carbon dioxide extraction and hydrodistillation. SC CO_2 extraction of *T. distichum* winter leaves was conducted at different pressures and temperatures using a central composite rotatable design. The optimal extraction conditions concerning the yield and α-tocopherol relative amount were determined by response surface methodology. The optimal conditions for the highest extraction yield of 3.97% were at 17.79 MPa and 62.70 °C and the highest α-tocopherol relative amount of 85.99 mg per 100 g of the plant material was at 22.07 MPa and 35.86 °C. The essential oil obtained by hydrodistillation contained 62 compounds and (-)-caryophyllene oxide was dominant (55.55%). The *T. distichum* essential oil obtained by SC CO_2 extraction and hydrodistillation had a different composition. The desirable compounds influence the choice of the extraction method.

Keywords: *Taxodium distichum*; leaves; SC CO_2 extraction; hydrodistillation; α-tocopherol

Citation: Djapic, N. Essential Oils of *Taxodium distichum* Winter Leaves Obtained by Supercritical Carbon Dioxide Extraction Method and Hydrodistillation. *Separations* **2022**, *9*, 436. https://doi.org/10.3390/separations9120436

Academic Editors: Chunjian Zhao, Wei Wang and Meng Luo

Received: 3 November 2022
Accepted: 7 December 2022
Published: 13 December 2022

1. Introduction

Taxodium distichum L. Rich., Cupressaceae, or bald cypress, is an allochthonous coniferous species [1–4]. Taxodium is a winter deciduous conifer [4,5]. It is a conifer well-adapted to hydric habitats [6,7]. Bald cypress grows on lowlands, especially on flooded sites, where it grows in groups or individually [6,7]. Green, needle-like leaves turn orange-yellow in autumn to coppery red in winter [8,9]. Bald cypress distribution, prevalence and commonness are well described by forest scientists [1,2,10]. In urban landscapes it grows successfully [11,12].

The essential oil of needle-like bald cypress leaves during all seasons was investigated, and it compromised monoterpene hydrocarbons, mainly α-pinene, myrcene, β-pinene, limonene, camphene and α-terpineol [13]. In its essential oils, from different seasons, α-pinene was present in the amount of 81.9% up to 94.3% [13]. The essential oil, from a different geographical region, contained bornyl acetate, β-caryophyllene, α-humulene and germacene D [14]. Major constituents of the needles, of three varieties being recognized under one polymorphic species *Taxodium distichum* (L.) Rich., were found to contain α-pinene, limonene and β-phellandrene [15]. When other conifer needles are concerned, various methods were used for the extraction of monoterpenes from conifer needles *Abies grandis* and *Picea pungens* [16]. After steam distillation, solvent extraction and liquid CO_2 extraction extracts consisting of a great number of monoterpenes were obtained [16]. It was found that the most effective technique for collecting monoterpenes is steam distillation compared to solvent and liquid CO_2 extraction [16].

Carbon dioxide (CO_2), at pressures and temperatures beyond its critical point, is a solvent enabling efficient extraction of lipophilic compounds [17]. Supercritical carbon dioxide (SC CO_2) extraction is a green technology and a method that can replace extractions using solvents. SC CO_2 extracts can be obtained at low temperatures compared to extractions at elevated temperatures. SC CO_2 extraction was a method of choice for the

removal of extractives from the green needles, branches, cones and bark of Scots pine trees [18]. SC CO_2 extraction of Scots pine needles and stumps yielded resin acids, terpenes and steroids [18]. SC CO_2 extraction can separate the fatty acids from wood material [19]. SC CO_2 has been applied for the extraction of volatiles from conifer needles, *Pinus sylvestris* and *Picea abies* [20]. The most abundant compounds in needles were bornyl acetate and camphene [20]. SC CO_2 has been utilized for the extraction of polyprenol from *Picea sitchensis*, *Cedrus atlantica* "Glauca", *Pinus sylvestris* and *Taxus baccata* conifer species [21]. Data on the lipophilic phytochemicals present in *T. distichum* needles SC CO_2 and hydrodistillation extracts is not known.

The aims of the research were: to evaluate the effect of pressure and temperature on SC CO_2 extraction of *T. distichum* needles, to optimize the total extraction yield and the α-tocopherol relative amount by response surface methodology (RSM) and to identify the phytochemicals present in essential oil obtained by hydrodistillation.

2. Materials and Methods

2.1. Chemicals

The CO_2 used for the extraction was 99.97% pure (w/w) (Messer, Tehnogas AD, Serbia). The α-tocopherol (98.2%) was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). All solvents were of analytical reagent grade (J. T. Baker, Phillipsburg, NJ, USA). The anhydrous sodium sulfate was purchased from Centrohem, Stara Pazova, Serbia.

2.2. Plant Material

T. distichum coppery red needles were collected in February 2022 in Futoski park, Novi Sad, Serbia (45°14′60″ N, 19°49′42″ E). The needles were air dried at room temperature for three days. Prior to extractions, the needles were grounded using a laboratory mill. The percent of moisture in the needles was determined in accordance with the AOAC Official Method 925.40 and was 2.28 ± 0.11% [22]. The pulverized plant material was sieved through 0.5 mm, 0.4 mm, 0.315 mm and 0.2 mm mesh sieves. The pulver remaining on each sieve was used to compute the fragments' distribution and were normalized to the total mass. The sieve analysis results were determined using Rosin–Rammler distribution [23]. The average particle size was computed to be 0.371 mm ± 0.12.

2.3. Extractions

The SC CO_2 experiments were performed on the laboratory-made system described previously [24,25]. The grounded plant material (50 g) was placed in the extraction cell. The SC CO_2 continuous flow passed through the sample. After each extraction obtained extracts were weighted. The obtained extracts' yield was weighted on the balance with the precision of ±0.0001 g. The extraction yield was calculated in percentages (grams of extract per 100 g of the dry pulverized plant material). The extractions were performed at different extraction conditions determined by the RSM and obtained by the central composite rotational design (CCRD) [26]. The 90 min extraction time and the CO_2 flow rate of 1.94 $kg \cdot h^{-1}$ were kept constant during all experiments. In CCRD, the extraction pressure (X_1) and temperature (X_2) were independent variables in coded values investigated to evaluate their effect on the extraction yield and α-tocopherol relative amount (Y). The statistical analysis of experimental data and three-dimensional response surface plots were generated using Minitab LLC®, State College, PA, USA, 2021. The goodness of fit was based on the R^2 determination coefficient. There are no previous data describing the influence of moisture on the SC CO_2 extraction of *T. distichum* needles. Moisturizing of pulverized plant material with water induces the extraction of alkaloids from leaves that contain them. For leaves containing alkaloids, moisture of up to 20% is acceptable. Higher moisture induces the increase of extractor pressure to insecure levels inducing the obstruction of flow in the metering valve and blockage of the in-line filter placed after the extractor. Due to the obstacles mentioned, the pulverized plant material moisture was kept at 2.28 ± 0.11%.

Hydrodistillation was performed according to the standard procedure described in Ph. Iug. IV [27]. Pulverized plant material (50 g) was used for hydrodistillation. Bright brown essential oil was dried over anhydrous sodium sulphate. After drying, the obtained oil was dissolved in *n*-hexane and subjected to GC-MS analysis.

2.4. GC-MS Analysis

The GC-MS analyses were performed using an Agilent 7890A GC equipped with Agilent 5975 MSD (Agilent Technologies, Palo Alto, CA, USA). The GC-MS was equipped with a HP-5MS (Agilent J&W 19091S-433) column (30 m × 0.25 mm ID, film thickness 0.25 µm). The carrier gas was He, adjusted to a flow of 1.0 mL·min^{-1}. The injection port temperature was 250 °C. The HP-5MS column temperature was linearly programmed from 40 °C at the rate of 3 °C·min^{-1}. The split ratio was 1:50. The ionization energy was 70 eV with a scan time of 1 s and mass scan range of 30–450 mass units. The component percentage was calculated from the GC peak areas. Components were identified by matching mass spectral data and retention times with those in a mass spectra library (Wiley 275.1) and using literature [28,29]. The quantitative analyses have been provided based on calibration curves. The standard solutions of α-tocopherol were prepared in *n*-hexane. Six different standard solutions were prepared. The R^2 for the calibration curve was 0.999. All analyses were performed in duplicate.

3. Results

The CCRD was used to optimize two coded operating variables, the pressure and temperature of the SC CO_2 extraction, to achieve the highest extraction yield and α-tocopherol relative amount. The pressure range was chosen due to the plant material with low oil content. For plant material with low oil content, it was not appropriate to apply pressure values higher than 22.07 MPa. The temperature range was from 36 °C up to 64 °C due to the phytochemicals present in the plant material. The higher temperatures would reduce the relative amount of some phytochemicals present in the plant material. The operating conditions were defined in five levels and the design was done with thirteen experiments with five replicates for the central point (Table 1). The extraction yield was from 1.93% to 4.08% depending on the applied process parameters (Table 1). The lowest yield was at 7.93 MPa and 50 °C and the highest in the experiments performed at 15 MPa and 64 °C.

Table 1. The CCRD experimental design and results for the *T. distichum* needles total extraction yield [%].

Run	Pressure [MPa], X_1	Temperature [°C], X_2	Extraction Yield [%]
1.	22.07	50	3.86
2.	15	64	4.08
3.	7.93	50	1.93
4.	10	40	2.84
5.	15	50	3.97
6.	15	50	3.91
7.	20	60	3.92
8.	10	60	2.98
9.	15	36	3.64
10.	15	50	4.02
11.	20	40	3.80
12.	15	50	3.86
13.	15	50	3.98

The response surface regression coefficients of the polynomial function for the *T. distichum* needles extraction yield obtained by SC CO_2 are depicted in Table 2.

Table 2. The response surface regression coefficients of the polynomial function for the *T. distichum* needles total extraction yield [%].

Term	Coefficient	Standard Error Coefficient	*T*-Value	*p*-Value
Intercept	3.948	0.058	67.88	0.000
X_1	0.578	0.046	12.59	0.000
X_2	0.110	0.046	2.40	0.048
$X_1 \cdot X_1$	−0.525	0.049	−10.64	0.000
$X_2 \cdot X_2$	−0.042	0.049	−0.85	0.421
$X_1 \cdot X_2$	−0.005	0.065	−0.08	0.941
$R^2 = 0.9754$				

The surface response plot of the proposed model visualizing the effect of independent variables on dependent ones is depicted in Figure 1.

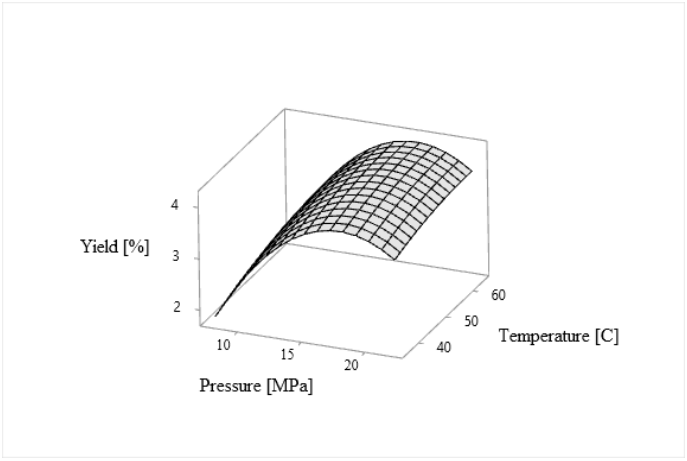

Figure 1. Surface response plot for the extraction yield in a function of extraction pressure and temperature.

The composition of SC CO_2 extracts obtained was determined by gas chromatography and mass spectrometry. The compounds determined and their relative amount in mg α-tocopherol equivalents per 100 g of the plant material are depicted in Table 3.

Table 3. The compounds determined in the SC CO_2 extracts of *T. distichum* needles and their relative amount in mg α-tocopherol equivalents per 100 g of leaves.

No.	Compound	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Run 11	Run 12	Run 13
1.	Caryophyllene oxide	-	0.92	-	-	-	-	1.00	-	-	0.94	-	1.01	1.05
2.	Hexahydrofarnesyl acetone	0.91	0.86	0.31	-	1.32	0.42	0.88	0.86	-	0.82	-	0.90	0.92
3.	Neophytadiene	39.64	75.93	84.71	17.64	28.72	20.58	75.82	23.50	35.80	76.38	67.34	77.03	76.27
4.	Eicosane	7.81	13.08	4.36	14.27	1.68	-	13.71	-	-	12.91	-	13.56	14.02
5.	Stearyl aldehyde	18.93	18.39	21.06	5.78	15.41	9.62	18.62	22.36	8.64	17.83	11.53	18.54	18.67
6.	1-Octadecanol	3.37	2.56	4.18	1.83	-	-	2.71	1.62	0.84	2.77	-	2.92	2.68
7.	Phytol	45.06	26.78	27.30	11.19	41.12	48.72	24.98	36.34	11.17	25.06	38.16	25.93	26.11
8.	Sandaracopimaradiene	3.26	2.93	6.13	5.32	4.87	3.96	2.76	4.82	2.68	3.14	2.93	2.85	3.05
9.	Geranylgeraniol	2.36	4.96	3.54	26.92	2.77	-	4.85	3.92	3.39	5.18	10.76	5.02	5.34
10.	Ferruginol	4.91	1.04	4.39	-	4.60	-	0.91	-	-	1.09	5.86	0.99	1.19
11.	*m*-Pentadecylphenol	10.76	7.46	21.86	6.91	8.07	5.80	7.02	6.61	16.39	7.23	7.36	7.68	7.63
12.	α-Tocopherol	32.85	39.83	20.96	68.03	22.98	50.08	40.54	77.36	19.07	39.94	45.87	40.68	40.03
13.	β-Sitosterol	-	2.21	3.60	-	-	-	1.83	4.12	-	1.96	6.58	2.07	2.71

The response surface regression coefficients of the polynomial function for the α-tocopherol relative amount of *T. distichum* needles obtained by SC CO_2 is depicted in Table 4.

The response surface plot showing the influence of independent variables in coded values on α-tocopherol relative amount is depicted in Figure 2.

Table 4. The response surface regression coefficients of the polynomial function for the α-tocopherol relative amount of *T. distichum* needle leaves (mg α-tocopherol equivalents per 100 g of plant material).

Term	Coefficient	Standard Error Coefficient	*T*-Value	*p*-Value
Constant	40.20	1.78	22.53	0.000
X_1	9.38	1.41	6.65	0.000
X_2	−17.27	1.41	−12.24	0.000
$X_1 \cdot X_1$	−2.03	1.51	−1.34	0.221
$X_2 \cdot X_2$	3.98	1.51	2.63	0.034
$X_1 \cdot X_2$	−4.35	1.99	−2.18	0.065
$R^2 = 0.9676$				

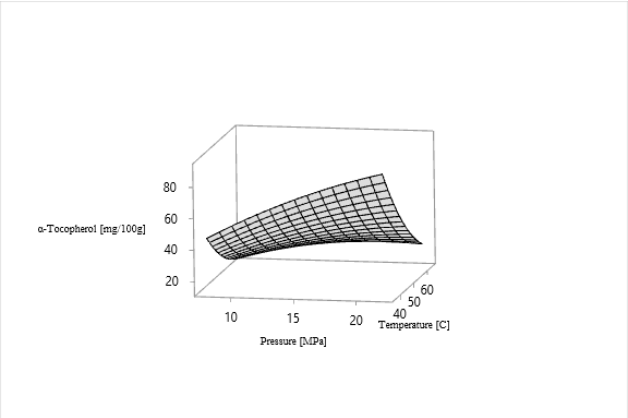

Figure 2. Surface response plot for the α-tocopherol relative amount in a function of independent variables.

The chemical composition of the *T. distichum* needles obtained by hydrodistillation is depicted in Table 5.

Table 5. The chemical composition of *T. distichum* needles obtained by hydrodistillation.

No.	Compound	RI	%
1.	*n*-nonanal	1100	0.36
2.	α-Campholenal	1122	0.19
3.	*trans*-Pinocarveol	1135	0.22
4.	Borneol	1165	1.57
5.	*p*-Mentha-1,5-dien-8-ol	1166	0.09
6.	Terpinen-4-ol	1174	0.05
7.	α-Terpineol	1186	0.31
8.	Myrtenol	1194	0.73
9.	Verbenone	1204	0.88
10.	*trans*-Carveol	1215	0.14
11.	Bornyl acetate	1287	11.36
12.	*trans*-Pinocarvyl acetate	1298	0.44
13.	Myrtenyl acetate	1324	0.31
14.	*trans*-Carvyl acetate	1339	0.28
15.	α-Terpinyl acetate	1346	0.34
16.	Silphiperfol-4,7(14)-diene	1358	0.03
17.	Ethyl decanoate	1395	0.11
18.	*trans*-β-Caryophyllene	1417	0.20
19.	*trans*-α-Ionone	1428	0.19
20.	α-Humulene	1452	0.11
21.	Geranyl acetone	1453	0.41
22.	2-Isopropenyl-4,8-dimethyl octahydronaphthalene	1473	0.10
23.	ar-Curcumene	1479	0.29
24.	*trans*-β-Ionone	1487	0.09

Table 5. *Cont.*

No.	Compound	RI	%
25.	α-Selinene	1498	0.08
26.	α-Muurolene	1500	0.09
27.	β -Bisabolene	1505	0.16
28.	γ-Cadinene	1513	0.25
29.	*trans*-Calamenene	1521	0.23
30.	α -Cadinene	1537	0.11
31.	α -Calacorene	1544	0.06
32.	Italicene epoxide	1549	1.46
33.	Salviadienol	1549	0.55
34.	*trans*-Nerolidol	1561	0.23
35.	Caryophyllene oxide	1582	55.56
36.	4(14)-Salvialene-1-one	1592	0.57
37.	Humulene epoxide I	1593	0.84
38.	Humulene epoxide II	1608	5.71
39.	Isoaromadendrene epoxide	1612	0.33
40.	Humulene epoxide III	1626	0.22
41.	allo-Aromadendrene epoxide	1639	0.60
42.	Caryophylla-4(12),8(13)-dien-5-α-ol	1639	2.28
43.	α-Muurolol (=Torreyol)	1644	0.32
44.	β-Eudesmol	1649	0.12
45.	α-Cadinol	1652	0.38
46.	14-Hydroxy-(*Z*)-caryophyllene	1666	2.41
47.	4-Hydroxy-9-epi-(*E*)-caryophyllene	1668	3.65
48.	Germacra-4(15),5,10(14)-trien-1-α-ol	1680	0.48
49.	α-Costol	1765	0.27
50.	14-Hydroxy-α-muurolene	1779	0.06
51.	8-Cedren-13-ol acetate	1788	0.49
52.	2-α-Acetoxy-amorpha-4,7(11)-diene	1805	0.09
53.	Khusinol acetate	1823	0.11
54.	Hexahydrofarnesyl acetone	1838	0.14
55.	(5*E*,9*E*)-Farnesyl acetone	1913	0.08
56.	Pimaradiene	1948	0.07
57.	Ethyl hexadecanoate	1992	0.10
58.	Abietatriene	2055	0.02
59.	Phytol	2111	0.07
60.	Ethyl linoleate	2151	0.04
61.	Ethyl oleate	2171	0.11
62.	Ethyl octadecanoate	2196	0.03

4. Discussion

The RSM-CCRD approach with two variables, pressure and temperature, was used to optimize SC CO_2 extraction to achieve the highest extraction yield and α-tocopherol relative amount. The CCRD provided information on experimental variable effects and experimental error in the least possible number of necessary runs. The extraction yield varied from 1.93% to 4.08% depending on the applied process parameters (Table 1). The *p*-value showed that the linear term of pressure and temperature had a statistically significant influence on the extraction yield (Table 2). The regression analysis was significant for the extraction yield ($p < 0.05$) with the coefficient of determination, $R^2 = 0.9754$. The extraction yield in terms of pressure and temperature is depicted in Figure 1. The linear behavior of temperature on the extraction yield can be noticed. The extraction yield increases to the pressure of 17.79 MPa, and with further increase of pressure it decreased. The maximum extraction yield was at a pressure of 17.79 MPa and temperature of 62.70 °C. Under these operating variables the extraction yield was 3.97%. The relative amount of α-tocopherol varied from 19.07 to 77.36 mg per 100 g of plant material (Table 3). The *p*-value showed that the linear term of pressure and temperature and quadratic term of temperature had a significant influence on the α-tocopherol relative amount (Table 4). The optimal conditions

for obtaining the highest α-tocopherol relative amount were at the pressure of 22.07 MPa and temperature of 35.86 °C. Under these conditions the α-tocopherol relative amount was 85.99 mg per 100 g of the plant material.

There are no previous SC CO_2 extractions of *T. distichum* winter needles. The α-tocopherol relative amount in olive leaves was 10.10 mg per 100 g of the plant material at process parameters of 25 MPa, 40 °C, CO_2 flow rate of 1 SL·min^{-1}, particle diameter of 1.5 mm and extraction time of 120 min [30]. At the same process parameters, with an extraction time of 60 min, the α-tocopherol relative amount was 6.94 mg per 100 g of leaves [30]. Comparing the temperatures, the optimal extraction temperature for olive leaves was at 40 °C and for *T. distichum* needles at 35.86 °C, and it can be noticed that a higher relative amount of α-tocopherol is obtained at lower temperatures. The α-tocopherol relative amount in *Eugenia involucrata* leaves was the highest, 68.27 mg per 100 g of leaves, at the pressure of 20 MPa, at 60 °C and under a CO_2 flow rate of 4 mL·min^{-1} [31]. Up to now, general process parameters and extraction conditions for the highest α-tocopherol relative amount cannot be drawn. The present study indicates that the α-tocopherol relative amount is higher at lower temperatures.

The phytochemical content present in essential oils varies according to geographic location, season, year and harvesting method and has a diversity that is not infinite. The content can be attributed to temperature, humidity, light intensity, soil moisture and nutrient availability, and the most vital for leaf production is photosynthesis, influencing the total biomass production. Many abiotic and biotic factors reduce the leaf area, influence leaf productivity and can reduce the contribution of the leaf area to biomass production and accumulation. The recovery of SC CO_2 extracts revealed the presence of a limited number of phytochemicals. The SC CO_2 extracts consisted of: caryophyllene oxide, hexahydrofarnesyl acetone, neophytadiene, eicosane, stearyl aldehyde, 1-octadecanol, phytol, sandaracopimaradiene, geranylgeraniol, ferruginol, *m*-pentadecylphenol, α-tocopherol and β-sitosterol. Different extraction parameters influenced their presence in the extracts (Table 3). Chlorophyll biodegradation induces the formation of phytol and is essential in α-tocopherol biosynthesis [32]. The α-tocopherol biosynthesis uses phytol formed after the phytyl ester hydrolysis of the chlorophyll propionate side chain, and the other pathway involves *de novo* biosynthesis from geranylgeranyl diphosphate [33]. It can only be observed, from the results obtained, that the α-tocopherol biosynthesis proceeds in coppery red *T. disctichum* needles.

Differences were found among the SC CO_2 extracts and essential oil (Table 5). The predominant compounds in the essential oil analyzed were: caryophyllene oxide (55.55%), bornyl acetate (11.36%), humulene epoxide II (5.71%) and 4-hydroxy-9-epi-(*E*)-caryophyllene (3.65%). Caryophylla-4(12),8(13)-dien-5-α-ol (2.28%) and 14-hydroxy-(*Z*)-caryophyllene (2.41%) were present in more than 2%. The most abundant oxygenated monoterpene was borneol (1.57%). Three sesquiterpenes were present in a higher amount than 0.20%: ar-curcumene (0.29%), γ-cadinene (0.25%) and *trans*-calamenene (0.23%). Oxygenated sesquiterpenes were the most abundant essential oil constituents: caryophyllene oxide (55.56%), humulene epoxide II (5.71%), 4-hydroxy-9-epi-(*E*)-caryophyllene (3.65%), 14-hydroxy-(*Z*)-caryophyllene (2.41%), caryophylla-4(12), 8(13)-dien-5-α-ol (2.28%), italicene epoxide (1.46%); other oxygenated sesquiterpenes were detected in less than 1%. Diterpenes detected in the essential oil were: pimaradiene (0.07%), ethyl hexadecanoate (0.10%), abietatriene (0.02%), phytol (0.07%), ethyl linoleate (0.04%), ethyl oleate (0.11%) and ethyl octadecanoate (0.03%). Phytol was detected in the quantity of 0.07%, and α-tocopherol was not detected due to its degradation at elevated temperatures.

In SC CO_2 extracts the α-tocopherol relative amount depends on the extraction temperature, with a higher relative amount at lower temperatures. α-Tocopherol is not present in essential oil obtained by hydrodistillation, indicating that its isolation is influenced by the extraction temperature. The methods where the experiments are done at lower temperatures can be used for the recovery of α-tocopherol. Hydrodistillation is a technique of choice for obtaining oxygenated sesquiterpenes. The results suggested that SC CO_2 can be used for

obtaining extracts containing α-tocopherol when operating at low extraction temperatures and hydrodistillation is a method of choice for obtaining oxygenated sesquiterpenes.

5. Conclusions

In SC CO_2 extraction, the influence of pressure and temperature on the total extraction yield and α-tocopherol relative amount was analyzed. Experimentally the total extraction yield and α-tocopherol were significantly influenced by pressure and temperature. The optimized process variables for the total extraction yield were at 17.79 MPa and 62.70 °C. The optimal extraction conditions, considering the highest α-tocopherol relative amount, were at 22.07 MPa and 35.86 °C. In the essential oil obtained after hydrodistillation, 62 compounds were determined with the caryophyllene oxide, which represented 55.55% of the total essential oil. The winter *T. distichum* needles essential oil obtained by hydrodistillation can be used for the isolation of caryophyllene oxide.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The author declares no conflict of interest.

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