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Supercriticial fluid extraction of flavors and fragrances from *Hyssopus officinalis* L. cultivated in Iran

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Abstract

Hyssopus officinalis L. (hyssop) as a food ingredient has its own importance in flavor industry and also in sauce formulations. Supercritical fluid extraction (SFE) of hyssop, cultivated in Iran, was performed at various pressures, temperatures, extraction (dynamic and static) times and modifier (methanol) concentrations using an orthogonal array design with an $OA_{25}(5^5)$ matrix conditions. Pressure, temperature and modifier in the SFE system influenced the extraction yield. Also, the composition of the extracted oils was greatly impacted by the operating conditions. Main components of the extracts under different SFE conditions were sabinene (4.2–17.1%, w/w), *iso*-pinocamphene (0.9–16.5%) and pinocamphene (0.7–13.6%). The extraction of sabinene, for example, was favored at 100 atm, 55 °C, 1.5% (v/v) methanol, 30 min dynamic time and 35 min static time. Use of SFE under different conditions can allow targeting the extraction of different constituents.
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1. Introduction

Conventionally, the essential oils of the aromatic herbs have been isolated using hydrodistillation and/or solvent extraction (Mahmood, Kaul, & Acharya, 2004; Nickavar, Mojab, & Asgarpanah, 2005; Priestap, Van Baren, Lira, Coussio, & Bandoni, 2003). However, low yield, loss of volatile compounds, long extraction times, toxic solvent residue, degradation of unsaturated compounds and production of undesirable off-flavors (due to heat) are involved in using such techniques (Kerrola, Galambosi, & Kallio, 1994; Khajeh, Yamini, Sefidkon, & Bahramifar, 2004; Poiana, Sicari, & Mincione, 1998).

As an alternative approach, supercritical fluid extraction (SFE) has received increasing attention in the extraction of essential oils (Diaz-Maroto, Perez-Coello, & Cabezudo, 2002; Ebrahimzadeh, Yamini, Sefidkon, Chaloosi, & Pourmortazavi, 2003; Illes, Daood, Perneczki, Szokonya, & Then, 2000; Povh, Marques, & Meireles, 2001; Roy, Goto, & Hirose, 1996; Sonsuzer, Sahin, & Yilmaz, 2004). This is due to the fact that supercritical fluids can provide improved mass-transfer rates and the operation can easily be manipulated by changing the pressure and/or temperature of the system. Also, by optimizing these parameters, certain compounds can be selectively isolated from the matrix being extracted. Carbon dioxide in its supercritical state has been used in many SFE applications (Cassel et al., 2000; Floch, Rios, Tena, & Valcarcel, 1998; Morales, Berry, Mcintyre, & Aparicio, 1998).

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SFE operations always involve many variables that may affect the efficiency of the extraction. Selection of these variables and their levels is critical. Several statistical techniques such as simplex optimization and factorial design have been employed for the optimization of analytical methods (Roy et al., 1996). Factorial design has some advantages over simplex optimization in that global optimum can be provided, large amounts of quantitative information can be extracted and both discrete and continuous factors can be estimated. One obvious disadvantage of the factorial design is the large number of experiments required when several variables are examined. However, the number of the experiments can be considerably reduced by the use of orthogonal array design (Lan, Wong, Chen, & Sin, 1994; Lan, Wong, Lee, & Sin, 1995).

Supercritical carbon dioxide ($SCCO_2$) has found applications in the extraction of flavors and fragrances from their natural sources and as a result many studies have been published using this environmentally friendly extraction method. The extraction of coriander seed (Illes et al., 2000), chamomile (Povh et al., 2001), *Hyssopus officinalis* (Kerrola et al., 1994), *Zataria multiflura* (Ebrahimzadeh et al., 2003), *Thymbra spicata* (Sonsuzer et al., 2004) and *Carom copticum* (Khajeh et al., 2004) are just a few of numerous studies reported in the literature using $SCCO_2$ for the extraction of spices and medicinal herbs. Hyssop (*Hyssopus officinalis*), which is one of the most important pharmaceutical herbs, is extensively cultivated in central and south European countries such as Russia, Spain, France and Italy (Omidbaigi, 2000). Despite having a bitter taste, hyssop is used as a food flavor and also in sauce formulations (Omidbaigi, 2000). Hyssop oil possesses anti-fungal and anti-bacterial properties (Omidbaigi, 2000), which are essential in such industries as canning, beverages and cosmetics. Food industry can always benefit from the new approaches leading to more hygienic ingredients such as food flavors. The essential oil of hyssop from different origins has been reported using the traditional methods of extraction (Garg, Nagvi, Singh, Ramg, & Kumar, 1999; Gorunovic, Bogavac, Chalchat, & Chabard, 1995; Jankovsky & Landa, 2002; Schulz & Stahl-Biskup, 1991; Tsankova & Kontaktchiev, 1993). Furthermore, solvent extraction as well as other methods including SFE with single operational conditions have been employed for determination of chemical composition of the essential oils of hyssop (Kerrola et al., 1994; Mitic & Dordevic, 2000). The objectives of the present work were to investigate the effect of several SFE parameters (pressure, temperature, modifier, and dynamic and static extraction times) in the $SCCO_2$ extraction of Iranian hyssop.

2. Materials and methods

2.1. Plant materials

The plant materials already dried on a perforated tray at ambient conditions were obtained from Industrial Research Centre for Studies of Plant Pesticides and Fertilizers (Karaj, Iran) with a moisture content of 5.7% (w/w), measured according to AACC (1983) method 44-19. The roots were separated from the plants and the remaining parts were extracted. Immediately prior to SFE, the sample was ground in a blender to produce a fine powder. Particles with an average size of 0.4 mm were separated using a suitable sieve and were used in the SFE experiments.

2.2. Statistical design

The conditions in the SFE of hyssop were planned according to the Taguchi's experimental design (Roy, 1990) (Table 1). All selected factors were examined using a five-level orthogonal array design with an $OA_{25}(5^5)$ matrix. In this study, interactions among variables were not incorporated in the matrix and the focus was placed on the main effects of the five most important factors.

2.3. Hydrodistillation

Distillation on a Clevenger-type apparatus was conducted for 4 h under atmospheric pressure using 80.0 g of dried sample to obtain the essential oils. The amount of oil recovered (0.5%, w/w, dry-basis) was measured gravimetrically.

Table 1
The five-factor-five-level (OA_{25}) orthogonal array design for the supercritical fluid extraction of *Hyssopus officinalis* along with the corresponding extraction yields

Run no.	Temperature (°C)	Pressure (atm)	Dynamic time (min)	Static time (min)	Modifier volume (μL)	Extraction yield (%, w/w)
1	35	100	10	15	0.0	0.5
2	45	350	10	20	3.0	2.0
3	55	200	40	15	3.0	2.5
4	45	100	20	25	4.5	1.0
5	75	100	40	20	6.0	0.1
6	35	250	30	25	3.0	2.4
7	55	350	20	30	0.0	2.1
8	75	350	30	15	4.5	1.3
9	75	300	20	35	3.0	2.4
10	45	250	40	35	0.0	2.4
11	45	300	50	15	1.5	2.2
12	45	200	30	30	6.0	2.8
13	75	250	10	30	1.5	1.6
14	35	300	40	30	4.5	2.8
15	35	350	50	35	6.0	2.9
16	65	250	20	15	6.0	2.4
17	65	200	10	35	4.5	1.1
18	55	100	30	35	1.5	0.2
19	55	300	10	25	6.0	2.2
20	65	100	50	30	3.0	0.1
21	75	200	50	25	0.0	1.3
22	35	200	20	20	1.5	1.8
23	65	300	30	20	0.0	2.3
24	55	250	50	20	4.5	2.8
25	65	350	40	25	1.5	1.8

2.4. Supercritical fluid extraction (SFE)

A Suprex MPS/225 system (Pittsburgh, PA) in the SFE mode was used for all SFE experiments. In this study, extractions were accomplished with an 8.0-mL-volume extraction-vessel at five different temperatures (35, 45, 55, 65 and 75 °C), five different pressure levels (100, 200, 250, 300 and 350 atm), five different static times (15, 20, 25, 30 and 35 min), five different dynamic times (10, 20, 30, 40 and 50 min) and five different modifier amounts (0.0, 1.5, 3.0, 4.5 and 6.0%, v/v). A Duraflow™ manually variable restrictor was used with the SFE system to collect the extracted analytes. The $SCCO_2$ flow rate through the Duraflow restrictor was maintained at approximately 0.3–0.4 mL/min (in the compressed state). The powdered plant-material (2.5 g) was mixed with 8.0 g glass beads (2.0 mm in diameter), and charged into the 8.0-mL extraction vessel. The extraction was then carried out under various experimental conditions (Table 1) in accordance with the Taguchi's approach (Roy, 1990). The extracted materials collected in a test-tube containing 3.0 mL dichloromethane (held in an ice-bath) were then diluted to a final volume of 5.0 mL with the addition of dichloromethane. In order to measure extraction yields, 3.0 mL of this solution was transferred into a pre-weighed vial and evaporated using a gentle flow of nitrogen gas until constant weights of the samples were reached. Apparently, the extracts are non-volatile components of hyssop since when using such SFE conditions, the collected flavors were mainly the non-volatile components. To study the modifier effects, methanol was spiked directly into the extraction vessel, which was already charged with the sample prior to the extraction. After extraction, the extracts were transferred into brown glass bottles and stored in a freezer until used.

2.5. Reagents

HPLC-grade dichloromethane was purchased from Merck-Schuchardt (Darmstadt, Germany) and methanol (also HPLC-grade) was purchased from Aldrich Chemical Co. (Gillingham-Dorset, England). Carbon dioxide (99.99% purity) for SFE as well as nitrogen and helium (as carrier gases in GC and GC/MS experiments, respectively) were obtained from Daga Co. (Tehran, Iran).

2.6. GC and GC/MS analysis

All essential oils from SFE and hydrodistillation were analyzed using an HP 6890 GC-Plus gas chromatograph (Hewlett-Packard Co., Wilmington, DE) equipped with an HP-1701 column (30 m × 0.32 mm × 0.25 μm) and an FID detector. Oven temperature programming included a hold at 50 °C for 2 min, then a ramp at a rate of 10 °C/min to 120 °C and another ramp at 5 °C/min to the final temperature of 240 °C, where a hold for 5 min was allowed. The carrier gas, N_2, was adjusted to a linear velocity of 23 cm/s. Injector and detector temperatures were both set at 250 °C. The SFE samples (1.0–3.0 μL) were injected using the split mode with a split ratio of 1/60.

The GC/MS analysis for the identification of the GC peaks was carried out on an Agilent 6890 N GC/MS system equipped with an HP-5MS19091S-433 column (30 m × 0.25 mm × 0.25 μm). Oven temperature was held at 35 °C for 2 min and then increased to 95 °C at a rate of 3 °C/min, after which another ramp at 5 °C/min up to 220 °C and a hold for 5 min was applied. The temperature was then increased to 280 °C at a rate of 10 °C/min and held for 5 min. The carrier gas, He, was adjusted to a linear velocity of 40 cm/s. The transfer-line temperature was set at 280 °C. The ionization energy was set at 70 eV with a scan time of 1 s and the mass range of 30–550 amu. The components of the extracted essential oils were identified by comparing their mass spectra with those of a computer database (Wiley 7 library). Furthermore, the measured retention indices for most of the identified compounds were verified with those published in the literature. Chromatographic patterns of several samples from both analytical methods were identical.

3. Results and discussion

3.1. Optimization of the experimental conditions in the SFE

Since various parameters potentially affect the extraction process, optimization of the experimental conditions represents a critical step in the development of an SFE method. In fact, pressure and temperature of the fluid, percentage of the modifier and the dynamic and static extraction times are generally considered as the most important factors. The optimization of the method can be carried out step-by-step or by using an experimental design. The results of the SFE experiments, based on the extraction yields, are given in Table 1. The mean values of the extraction yields for the corresponding factors at each level were calculated according to the assignment of the experiment. Although it seems strange, according to this assignment, the extraction yields of the five experiments at 300 atm, for example, are used to calculate mean value of the corresponding five runs. The mean values of the five levels of each factor (e.g., pressure) reveal how the extraction yield will change when the level of that factor is changed.

3.2. Effect of pressure

Fig. 1a presents the effect of pressure on the extraction yield of hyssop in $SCCO_2$ using five pressure levels of 100, 200, 250, 300 and 350 atm. Results showed that increasing pressure from 100 to 300 atm enhanced the extraction yield, which was due to the increased $SCCO_2$ density at higher pressures. Above 300 atm, however, an increase in the pressure level led to a slight reduction in the extraction efficiency, which can probably be related to the reduced diffusion rates of the extracted materials from

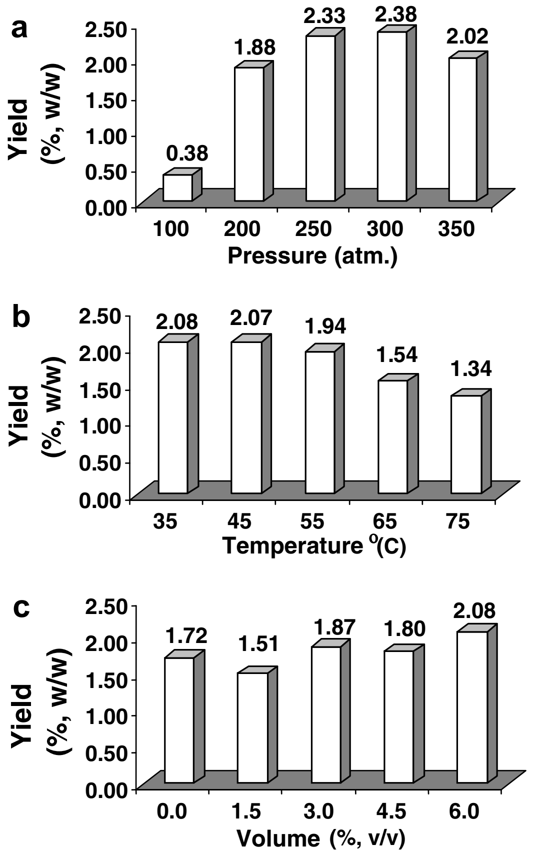

Fig. 1. The effect of pressure (a), temperature (b) and the amount of modifier (c) on the extraction yield of *Hyssopus officinalis* for 25 different samples (Table 1). Each point is the average of five different conditions according to Taguchi's statistical approach (Roy, 1990).

the sample matrix to the supercritical fluid environment. According to Rezaei and Temelli (2000), the diffusion rates of the solutes are reduced at higher pressures and in this case, it appears that at the conditions of this study (at pressures above 300 atm), diffusion has played a major role in the mass transfer rates of the extractable materials from the matrix into the supercritical fluid medium, which gives less extraction efficiency than that expected by the solubility enhancements at higher pressures. This can be justified by the limited amounts of the extractable materials present in the herbs that cannot be easily accessible to the supercritical fluid environment. Rezaei and Temelli (2001) showed that when the amounts of free solutes to be extracted were minimized, the role of solubility of the solutes in the supercritical fluid extraction was no longer a major one and extraction kinetics (i.e., the mass transfer rates) was mainly controlled by the diffusion properties of the solutes to be extracted.

3.3. Effect of temperature

Fig. 1b presents the effect of temperature on the extraction yield of hyssop in $SCCO_2$ at five temperature levels of 35, 45, 55, 65 and 75 °C. The highest extraction yield (~2.1%, w/w) was obtained at a temperature range of 35–45 °C, after which an increase in the temperature resulted in a drop in the extraction yield in a way that at 75 °C it reached to a level as low as ~1.3%. Such drop in the extraction yield can be related to the reduced $SCCO_2$ density at higher temperatures (Rezaei & Temelli, 2000), which in turn results in the reduced solubilities of the solutes in $SCCO_2$ and as a result greater influence on the extraction kinetics (or mass transfer rates) than on the higher diffusion rates of the solutes at higher temperatures.

3.4. Effect of modifier

Fig. 1c presents the effect of modifier (methanol) on the extraction yield of hyssop in $SCCO_2$ using five different levels of modifier (0.0, 1.5, 3.0, 4.5 and 6.0 μL). A drop in the extraction yield was observed once a small amount (1.5%, v/v) of the modifier was added into the system. Thereafter, the extraction yield was slowly improved with the addition of the modifier up to the 6.0% (v/v) level of this study. In general, modifier can exhibit very complicated effects on the extraction behaviors in different systems. Sometimes, the addition of a small amount of modifier may increase the extraction yield by improving the solvent power of the supercritical fluid. However, the modifier can also have an adverse effect on the textural behavior of the matrix to be extracted leading to a drop in the extraction efficiency. Moreover, in cases where the amounts of the extractable materials are limited and the extraction kinetics are controlled by the diffusion properties of the supercritical fluid (Rezaei & Temelli, 2001), the effect of modifier on the diffusion rate has to be considered as well, but this was not part of the objectives of this study.

3.5. Effect of static time

Fig. 2a presents the effect of static time (15, 20, 25, 30 and 35 min) of the extraction on the extraction yield of hyssop in $SCCO_2$. Since among the runs applied in this study (Table 1), each static time corresponds to a given dynamic time, sum of both static and dynamic times need to be considered in order to have a more realistic judgment on these results. According to the results of this study, increasing the static times of the extraction did not reveal any apparent change in the extraction yield, which was probably due to an equilibrium established between the compounds in the sample matrix and those in the supercritical fluid much earlier than 25 min (the shortest time allowed in this study) and as a result extra static time did not improve the extraction yield. With such results and considering the dynamic times applied in this study, it can be suggested that no more than 15 min of static time to be allowed when performing an extraction using such herbs as hyssop as the matrix. Obviously, when a shorter static time is applied, the applicable dynamic times for such extractions can be extended.

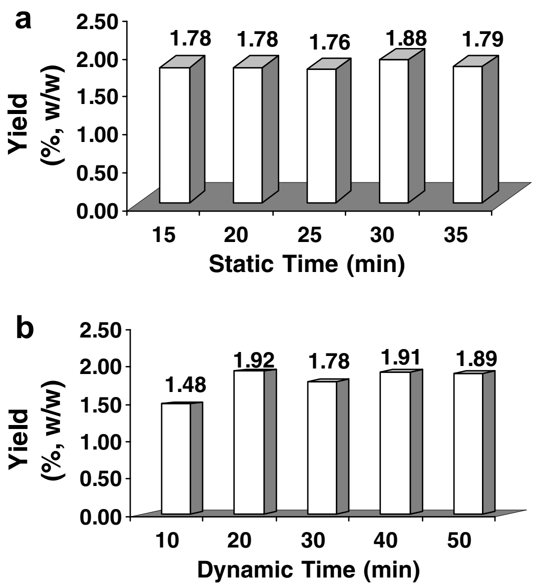

Fig. 2. The effect of static (a) and dynamic (b) times of the extraction on the extraction yield of *Hyssopus officinalis* for 25 different samples (Table 1). Each point is the average of five different conditions according to Taguchi's statistical approach (Roy, 1990).

3.6. Effect of dynamic time

Fig. 2b presents the effect of dynamic time of the extraction on the extraction yield of hyssop in $SCCO_2$ when applying five different levels of dynamic times (10, 20, 30, 40 and 50 min). The extraction yield at the 10-min dynamic time was lower than those at the other times and above 20 min of dynamic time no changes were observed on the extraction yield. When the results from Fig. 2b are compared with those shown in Fig. 2a, the optimum level of dynamic time can be estimated to be somewhere within the 10–15 min time range. It can be concluded that the optimum level of the static time to be allowed for the extraction is also 10–15 min since the level of the materials to be extracted are far below the level predicted by the solubility level of those components at the conditions of this study. Therefore, as it was the case with the static time (Fig. 2a) with an additional time (<15 min, according to Fig. 2a) all the extractable materials were extracted and any time longer than that level did not improve the extraction yield.

3.7. The identification and quantification of the extracted components

The samples extracted using SFE as well as that extracted by hydrodistillation showed a relatively simple GC–MS chromatographic pattern. Detailed identification and quantification of the compounds extracted by SFE under different conditions and also those extracted by hydrodistillation were performed using GC/MS and GC instruments, respectively (Table 2), where chromatographic

Table 2
Relative peak areas (%)[a] of the identified components from the supercritical fluid extraction of *Hyssopus officinalis* at different conditions (runs 1–25) compared to those with hydrodistillation (HD)

Compound	α-Pinene	β-Pinene	Cumene	*p*-Cymene	Sabinene	Pinocamphene	Pinocarvone	*iso*-Pinocamphene	Germacrene D
RI[b]	928	970	1017	1020	1024	1152	1154	1165	1477
HD	0.63	0.51	0.07	1.57	11.04	8.69	0.40	10.90	1.53
Run 1	0.24	0.15	0.76	0.86	5.76	1.21	0.10	1.41	0.14
Run 2	0.20	0.72	1.75	0.62	4.23	3.56	0.20	4.23	0.19
Run 3	0.84	1.43	1.89	2.32	15.90	9.31	0.59	11.30	1.82
Run 4	0.42	0.75	1.88	1.92	13.00	13.60	0.93	16.50	1.61
Run 5	0.44	0.76	1.84	2.12	14.40	2.82	0.22	3.12	0.12
Run 6	0.76	0.76	2.07	2.29	14.90	9.27	0.55	10.50	2.62
Run 7	0.42	0.78	1.89	1.00	6.70	2.78	0.06	3.18	0.81
Run 8	0.41	0.77	1.87	1.25	8.52	6.86	0.62	8.39	1.36
Run 9	0.53	0.78	1.86	1.45	9.82	8.02	0.53	9.88	1.71
Run 10	0.57	0.59	1.49	1.78	12.30	5.25	0.33	6.03	1.67
Run 11	0.51	0.77	1.79	1.96	13.00	5.55	0.45	6.68	0.60
Run 12	0.58	0.21	1.98	1.90	12.70	6.51	0.26	7.80	0.55
Run 13	0.52	0.94	1.81	1.52	9.99	7.43	0.45	9.19	1.42
Run 14	0.63	0.81	1.95	1.89	12.90	8.53	0.58	10.20	1.82
Run 15	0.33	0.99	1.75	1.62	10.90	4.91	0.47	6.04	1.05
Run 16	0.56	0.81	1.91	1.30	8.85	8.62	0.48	10.20	2.44
Run 17	0.48	0.79	1.92	1.41	9.41	3.15	0.35	3.94	0.81
Run 18	0.32	0.78	1.86	1.38	9.20	0.72	0.08	0.93	0.13
Run 19	0.31	0.75	1.79	0.80	5.33	5.57	0.35	6.60	1.37
Run 20	0.26	0.78	1.91	1.28	8.63	3.39	0.28	3.78	0.09
Run 21	0.91	1.04	1.98	2.50	17.00	4.59	0.27	5.68	2.32
Run 22	0.40	0.78	1.87	1.20	8.14	4.15	0.33	5.09	1.10
Run 23	0.96	0.98	1.90	2.37	16.30	5.76	0.25	7.09	1.41
Run 24	0.74	0.91	1.95	2.32	16.00	6.30	0.53	7.90	1.74
Run 25	0.68	0.80	1.99	1.77	12.20	4.95	0.48	6.01	2.32

[a] The relative peak areas are reported based on the peak-area normalization of the GC chromatograms.

[b] RI (retention indices) were measured relative to those of *n*-alkanes (C_9–C_{16}) on the non-polar HP-5MS column.

Table 3
The results for the analysis of variance (ANOVA, 95% confidence level) of the calculated models in supercritical fluid extraction of *Hyssopus officinalis*

Source of variance	Sum of squares	Mean square	Degree of freedom	F_{value}
Pressure	16.00	4.00	4	14.05
Temperature	2.18	0.55	4	1.92
Static time	0.41	0.10	4	1.45
Dynamic time	1.40	0.35	4	1.23
Modifier level	1.16	0.29	4	1.02
Pooled error	1.14	0.28	4	
Total	22.30		24	
$F_{\text{critical}} = 6.39$				

patterns of the samples from both analytical methods were identical. The major compounds identified were sabinene, pinocamphene and *iso*-pinocamphene, which were extracted at different levels when applying different conditions. The maximum level of sabinene, for example, was obtained in run 21, whereas the maximum levels of pinocamphene and *iso*-pinocamphene were obtained in run 4 indicating that different operational conditions need to be applied in order to target the extraction of certain components of a matrix. The minor compounds identified were pseudo-cumene, nopinone, myrtenol, *iso*-phytol, β-bourbonene, methyl eugenol, β-caryophllene, β-cubene, allo-aromadendrene, bicyclogermacrene, α-amorphene, *cis*-calamenene, elemol and spathulenol.

Table 3 shows the results for the analysis of variance (ANOVA) of the calculated models. Pressure had greater influence on the SFE of hyssop for all of the analytes and as a result the extraction yield was improved as the pressure increased. The pressure increase causes an increase in the density of the supercritical fluid, which in turn increases its solvent power.

With respect to the quantitative optimization, run #5 with 2.9% yield was the best, that is when 6 μL of modifier, 35 °C, 350 atm along with a static time of 35 min and dynamic time of 50 min were applied. However, qualitatively, run #4 was best since the highest amounts of pinocomphene (16.5%), *iso*-pinocomphene (13.6%), the major components of hyssop, were obtained.

4. Conclusions

With SFE, changing extraction variables is less tedious and also shorter extraction times can be used for the full recovery of the extracted materials. According to the results of this study, SFE offered more choices (parameters and their levels) for the extraction of different components. The five-level orthogonal array design with an $OA_{25}(5^5)$ matrix helped reduce the number of experiments to study the SFE of essential oils from hyssop using five different parameters at five different levels. The greatest effect on the extraction yield and the selectivity of the extraction was observed from pressure. For food and drug applications, there are no concerns about the toxic effects of the solvent when using $SCCO_2$ for such extractions.

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