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Original Article

The Solid-Phase of the Dynamic Microextraction of Thymol and Carvacrol Using a Porous, Low-Cost and Unbreakable Disk Coated by Polythiophene/Multiwalled Carbon Nanotubes

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Abstract

Background and Aim: In the present study, a stainless steel plate was penetrated and platinized, and it was used as the disk for the solid phase dynamic extraction (SPDE) method. The SPE disk was coated by polythiophene/multiwalled carbon nanotubes (PTh/MWCNTs) nanocomposite using in-situ electropolymerization method.

Materials and Methods: A high-surface area, porous and unbreakable platinized SPE disk was used for the dynamic extraction and determination of thymol and carvacrol by high-performance liquid chromatography (HPLC) with UV detection. The effect of various variables, including the type of elution solvent, pH, ionic strength and extraction time was optimized by the use of the central composite design (CCD) method.

Results: Under the response surface methodology, the present method has admissible calibration curves over wide linear ranges of 0.01-100 µg mL^{-1} with high coefficient of determination (0.9998) that firmly confirms the high pertinence of the present method to the quantification of analytes. Meanwhile, their limits of detection (LODs) were 0.007 and 0.005 µg mL^{-1}, and the limits of quantification (LOQs) were 0.09 and 0.01 µg mL^{-1} for thymol and carvacrol, respectively. Moreover, the enrichment factor (EF) was 87 for thymol and 54 for carvacrol, and the preconcentration factor was 142.8 for thymol and carvacrol.

Conclusion: The SPDE-PTh/MWCNTs-disk was used for the extraction of thymol and carvacrol in thyme samples, and satisfactory results were obtained.

Keywords: Polythiophene/multiwalled carbon nanotubes (PTh/MWCNTs), Solid-phase dynamic extraction (SPDE), SPE disk, Thymol, Carvacrol

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Introduction

Thyme (Thymus daenensis endemic species belongs to the Lamiaceae family) (1), contains thymol (2-isopropyl-5-methylphenol) and carvacrol (5-isopropyl-2-methylphenol) as the two principal components (Thymus vulgaris L.), and summer savory (Satureja hortensis L.) (2). These are significant anti-cancer

agents that are abundantly found in many foods, liquors, dairy desserts, antiseptic mouthwashes, perfumes and medicines because of their antitussive, antibacterial, anti-rheumatic, antifungal, antioxidant and anti-cancer properties (3-6). A separation method reduces the matrix effect and increases the extraction efficiency (7, 8). Hence, the extraction of phenolic acids and flavonoids is carried out following the consumption of large volumes of toxic solvents such as methanol, ethanol, chloroform, etc., (9, 10) which are toxic and expensive. They have low vapor pressure, and are known as environmental pollutants (11). Likewise, the extraction techniques based on the green chemistry approach are able to overcome such limitations and techniques such as direct-immersion solid-phase microextraction (DI-SPME) (12), hydrodistillation-headspace solvent microextraction (HD-HSME) (4), ultrasound assisted microextraction-nanomaterial solid phase dispersion (UAME-NMSPD) (13), and vortex-assisted surfactant-enhanced dispersive liquid-liquid microextraction (VASEDLLME) (14) that are applied for the quantification of such species from different media. Solid-phase extraction (SPE), as the most powerful and efficient, the simplest, fastest, easily auto-mated, and relatively inexpensive solventless sample preparation method is an efficient choice for the separation and enrichment of various inorganic and organic analytes (15). This method has various advantages over other techniques such as the stability and reusability of the solid phase, the reaching of high preconcentration factors, facileness of separation, the enrichment under dynamic conditions, needing no organic solvents, and minimal costs following the diminishing in the consumption of reagents (16). The key element of SPE is the stable phase which must have a porous and high contact surface, reversible absorption, high purity and acceptable chemical stability (17). Transitional silica particles (18) and organic polymers (19), as conventional sorbents of SPE, have reasonable extraction efficiency for small groups of compounds. Nowadays, SPE experiments are conducted based on three different configuration, including cartridge (20), membrane disk (21), and disk-cartridge (17) that are applicable for the analysis of organic and inorganic compounds in different matrices. So far, various compounds such as porous polymers, alumina, activated carbon, cellulose and various silicates have been used as sorbents in SPE (22-24). However, commercial SPE disks usually do not have adequate selectivity and efficiency. Small size, suitable hardness, high strength and high sorption capacity of distinct nanoparticles with respect to bulk materials, and their application in the purification, removal and separation of different combinations of various complex matrices, to overcome drawbacks of commercial SPE disks were widely considered and used. Accordingly, the development of efficient, cost-effective, porous, and green usable phases for the determination of thymol and carvacrol in thyme is a challenge to design and develop high efficiency materials for the SPE of analytes. In the present study, an unbreakable, porous and economical disk for the solid-phase dynamic measurement of thymol and carvacrol in thyme using stainless steel plate and polythiophene/multiwalled carbon nanotubes (PTh/MWCNTs) sorbent was developed, and a new disk was introduced. The proposed SPE disk that was prepared via coating the PTh/MWCNTs nanocomposite film on platinized stainless steel plate was utilized in order to extract thymol and carvacrol in aqueous solutions through the use of SPDE strategy followed by high-performance liquid chromatography-ultraviolet detection (HPLC-UV) quantification.

Materials and Methods

Reagents and Supplies

Extra pure thiophene (99.5%w/w), lithium perchlorate ($LiClO_4$) and potassium hexachloroplatinate (IV) (K_2PtCl_6) were purchased from Merck (Darmstadt, Germany). All the inorganic acids, bases and organic solvents such as acetonitrile (analytical grade) were of analytical reagent grade and provided by Merck, Darmstadt, Germany. Multi-walled carbon nanotube (MWCNT) with purity that exceeded 90%w/w (110 - 170 nm diameters and 5-9 µm length) was purchased from Sigma-Aldrich (Germany). Thymol (≥98%) and carvacrol (≥99%) standard materials were purchased from ROTH (Karlsruhe, Germany). Stock solutions of thymol and carvacrol (100 µg mL^{-1}) were prepared via dissolving adequate amounts of these materials in methanol. They were stored at 4 ºC in a refrigerator. The standard working solutions were prepared daily

from these stock solutions using double distilled water.

Instruments

HPLC was conducted using a KNAUER HPLC system that was composed of a returning pumps, a Smartline UV Detector 2600, a high pressure manual injection value (with 20 µL injection loop), and a Smartline Manager 5050 degasser. All the injections were carried out by overflowing the fixed volume loop. The separation was conducted on an RP-C18 analytical column (Eurospher 100-5 C18, with 25 cm length and 4.6 mm I.D.) packed with 5 µm particles. The mobile phase was comprised of 60:40 v/v% of acetonitrile and water that was delivered at flow rate 1 mL min^{-1}. Data collection and processing was performed with KNAUER ClarityChrom software. The UV detector wavelength was fixed at 277 nm, and the column temperature was set at 40 ºC. The retention time and the instrumental linear dynamic range of thymol and carvacrol and their accurate quantification were investigated via recording the signal assign to several solutions that were analyzed by their direct injection into the HPLC-UV system. A proper standard calibration curve (R^2>0.999) was obtained in the range of 0.5-100 µg mL^{-1}. Fourier transform infrared spectra were recorded by an FT-IR 8400 spectrometer (Shimadzu, Kyoto, Japan) in the transmittance mode, and were employed to characterize the functional groups of the nanomaterials. A VEGA\\TESCAN CM120 (Brno, Czech Republic) field-emission scanning electron microscope (FE-SEM) was utilized to examine the morphology of the PTh/MWCNTs nanocomposite coating. A Flexiflow Enteral peristaltic pump (Ross Products, Columbus, Ohio, USA) was used for circulating the sample headspace through the SPE disk system. A hand-made electrode rotator was prepared and utilized for spinning the electrode during EPD process in order to ensure the uniform deposition of the nanocomposite film on the surface of fiber. Sample stirring was carried out using a Heidolph MR 3001-K magnetic heater-stirrer (Kelheim, Germany). A peristaltic pump (Flexiflow Enteral Pump, Ross Products, Columbus, Ohio, USA) was used for aspirating the headspace into the needle device.

Fabrication of the SPDE-PTh/MWCNTs-Disk Setup

To obtain a porous and adhesive substrate, the surface of the stainless steel plate was platinized using an emended electrophoretic procedure (12). It has been demonstrated that, the application of this approach leads to the tight attachment of the coating to the substrate and results in durable, mechanically strong and chemically resistant disk coating. Subsequently, PTh/MWCNTs electrosynthesis and its simultaneous deposition onto the surface of substrate were carried out. For this purpose, 1 mL of redistilled thiophene was added to 30 mL of acetonitrile in a Pyrex flask, and was then sonicated in an ultrasonic bath for 3 min to be homogeneous. Then, 0.3 g of MWCNTs was added, and the mixture was sonicated for complete dispersion (30 min at 45°C). In the next step, the mixture was transferred into a 40-mL electrochemical cell, and the fiber and normal cases were connected to the anode and cathode of a DC power supply, respectively. A 3-V potential was applied between the electrodes (distance 1 cm, I ≈ 30 µA) and at the same time the stirring of the mixture was carried out for 30 min. Consequently, a thin layer of the PTh/MWCNTs nanocomposite was deposited on the surface of the fiber (Fig.1). The MWCNTs are embedded in PTh as thiophene monomers were migrating, polymerizing and attaching on the surface of disk. Subsequently, the platinized disk was removed, and then the electrodeposited coating of its outer surface was scraped. The washing of the disk was carried out by the use of water and methanol three times in order to remove any possible pollution and remaining monomers. In the final step, the disk was placed in an oven for 1 h at 300 °C for conditioning the electropolymerized PTh/MWCNTs film. Subsequently, a short PTFE cylinder with an inner diameter of 100 mm and a length of 5 cm that was equipped with two porous frits (as one pre filter and the other as disk support) was used as the SPE column.

SPDE-PTh/MWCNTs-Disk Procedure

The SPDE-PTh/MWCNTs-disk procedure includes two steps: dynamic SPE step and elution step (Fig. S-1). In dynamic SPE step, a 40 mL extraction vial was used as the sample container, while 10 mL of the sample solution containing 5 µg mL^{-1} of thymol and carvacrol in double distilled water at pH 3 and ionic

strength (1% w/v $NaNO_3$) was poured into the extraction vial. The tip of the SPE column and normal tube were immersed into the sample solution. The furthest part of SPE column and tube were connected to the tubes of peristaltic pump. Subsequently, the sample solution was passed through the SPE column for 20 min at a flow rate of 12 mL min^{-1}. In the elution step, and after the accomplishment of the extraction, the pump was switched off and the SPE column was removed from the extraction vial, and then it was placed inside a conical micro-vial containing 70 µL of acetonitrile for the elution of the analytes using normal syringe. Following the elution of the analytes, 20 µL of eluent was injected into the HPLC-UV system and the peak area was utilized in order to examine the extraction efficiency of the SPDE-PTh/MWCNTs-disk procedure.

Results and Discussion

The PTh/MWCNTs nanocomposite was characterized through the use of FT-IR and SEM instruments. The highest extraction efficiency of the SPDE-PTh/MWCNTs-disk-HPLC-UV method was simply achieved by the optimization of experimental parameters, including the type of elution solvent, pH, ionic strength and extraction time, whose influence on the response for separate and combined parts were studied and optimized by central composite design (CCD).

Characterization of the PTh/MWCNTs Nanocomposite

The morphology of the PTh/MWCNTs nanocomposite was investigated experimentally based on the scanning electron microscopy (SEM). SEM images of the PTh (Figure 2a, 2b) and PTh/MWCNTs (Figure 2c, 2d) nanocomposite with different magnifications exhibit porous structures (Figure 2a-2d) that make its surface area larger and suitable for a better sorption of polar and semi-polar analytes. Nonetheless, the severe porosity of the PTh/MWCNTs nanocomposite is a guarantee for its vast surface area as well as rapid mass transfer process, and consequently causes high adsorption capacity and extraction efficiency for thymol and carvacrol. On the other hand, the platinization of the substrate promotes the porosity and adherence of the needle bed, and improves its interaction with nanocomposite coating. Such a proper cohesion between the nanocomposite film and the needle substrate causes a greater mechanical strength and chemical resistance, and also induces more durability and extends the lifetime of the constructed sorbent. The investigation of the functional groups of MWCNT, PTh and PTh/MWCNTs nanocomposite by FT-IR spectra (Figure 5) indicate vibrational modes of ~3470 ~1743 and ~1647 cm^{-1} in the MWCNTs structure that indicate the O-H, C=O and C=C bonds, respectively (Figure 3). The transmission infrared spectrum of the electrodeposited PTh film show aromatic stretching bands at 1649, 1548, 1317, and 1201cm^{-1}. The main absorption bands of PTh film is composed of two bands that are highly strong and are

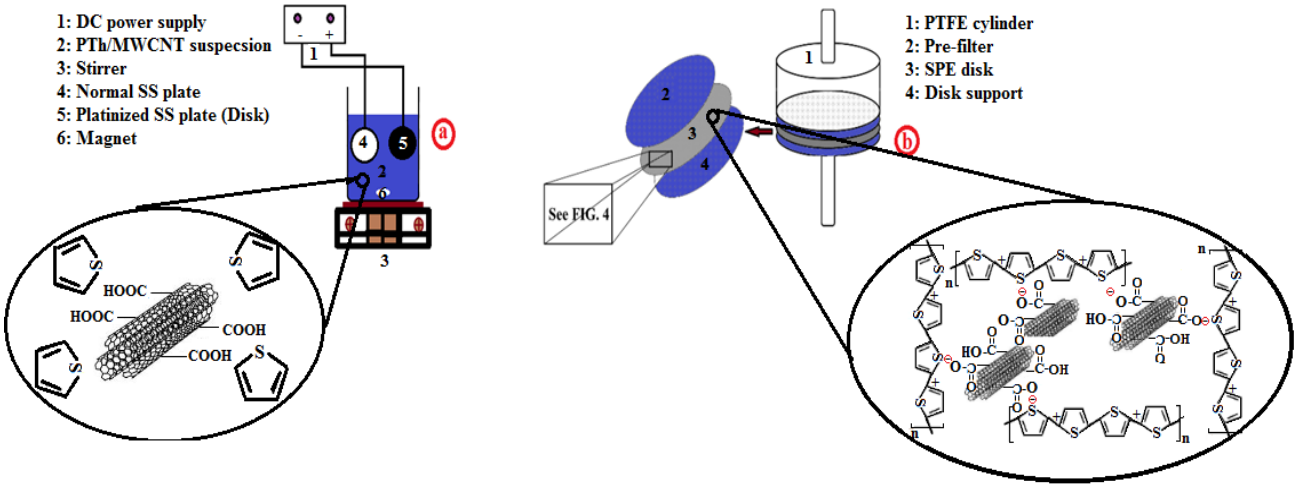

Figure 1. The Schematic of the Preparation of the SPE Disk Coated by PTh/MWCNTs Nanocomposite (a), Schematic of the SPE Column (b).

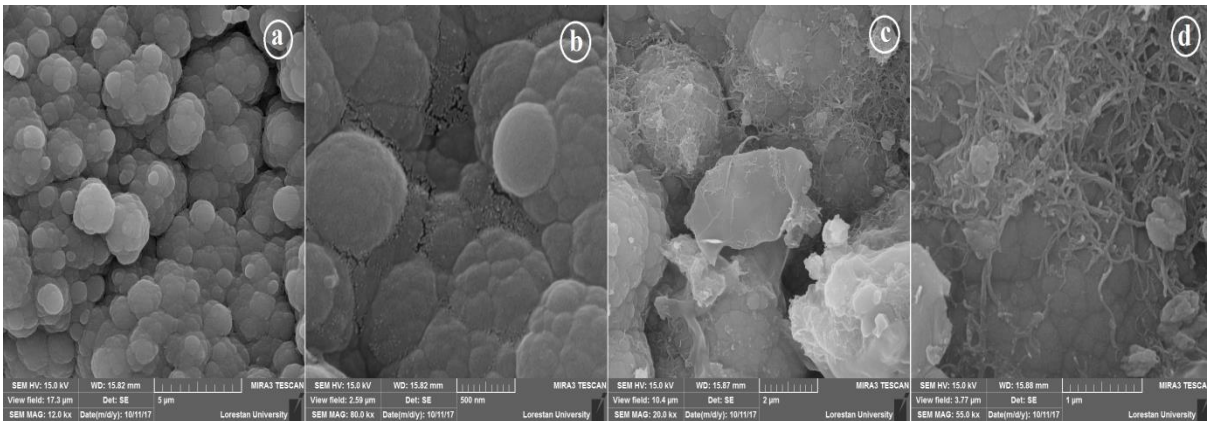

Figure 2. SEM Images of the PTh (a, b), PTh/MWCNTs (c, d) Nanocomposite.

attributed to the C-C vibration at 1122 cm^{-1}. A highly strong absorption at 786 cm^{-1} was assigned to the C-H out of the plane vibration of the 2, 5-disubstituted thiophene ring (due to α–α′ polymerization). The extremely weak band at 724 cm^{-1} is ascribed to the C-H out of the plane vibration of the mono substituted thiophene ring that indicates a high level of polymerization, while PTh C-S stretching vibration band appeared at 629 cm^{-1}. In PTh/MWCNTs structure, peaks appeared at 1740, 3397 and 1630 cm^{-1} is attributed to the MWCNTs. The comparison of the FT-IR spectra of PTh and PTh/MWCNTs indicates the reduction of the intensity of several peaks such as 786, 724, and 1122 cm^{-1} that in turn reveals the efficient formation of the PTh/MWCNTs nanocomposite on the SS needle by EPD method.

Effect of the Eluent Solvent

The type of elution solvent was investigated firstly based on certain criteria, including its ability for efficient and quantitative elution with the least volume of sat, while the disk was not destroyed, and accordingly all experiments were undertaken. Elution of the extracted analytes was carried out using a conical micro-vial in a dynamic mode. The SPDE disk was eluted with 70 µL of four different eluents, namely water (H_2O), acetonitrile (ACN), methanol (MeOH), and ethanol (EtOH) in the SPDE-PTh/MWCNTs-disk-HPLC-UV procedure. The respective results have been indicated in Fig. S-2. The highest extraction efficiency was achieved in the presence of ACN. It is a polar solvent, which is

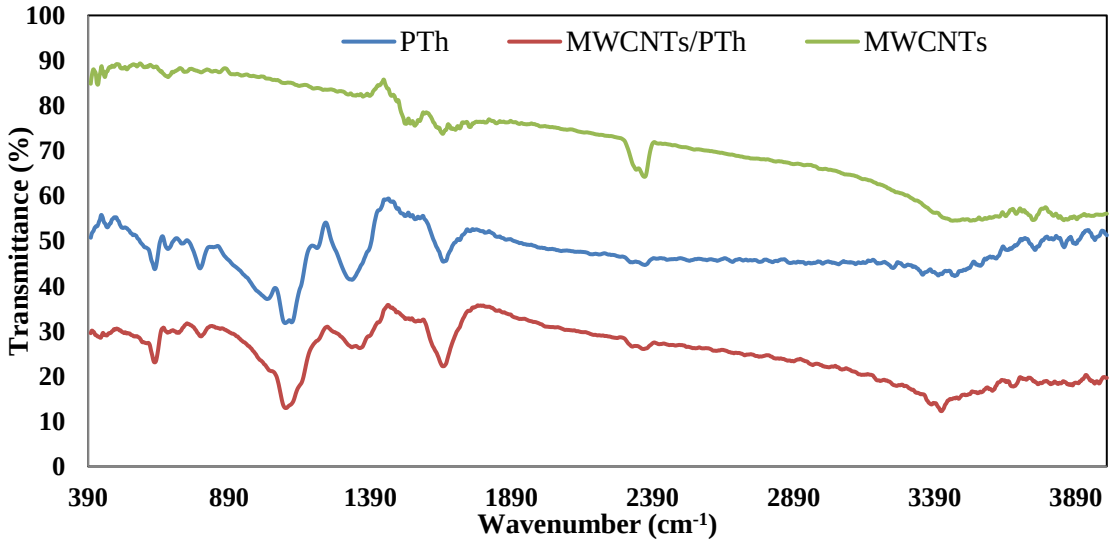

Figure 3. The FT-IR Spectra of the PTh, MWCNTs, PTh/MWCNTs Nanocomposite Sorbent.

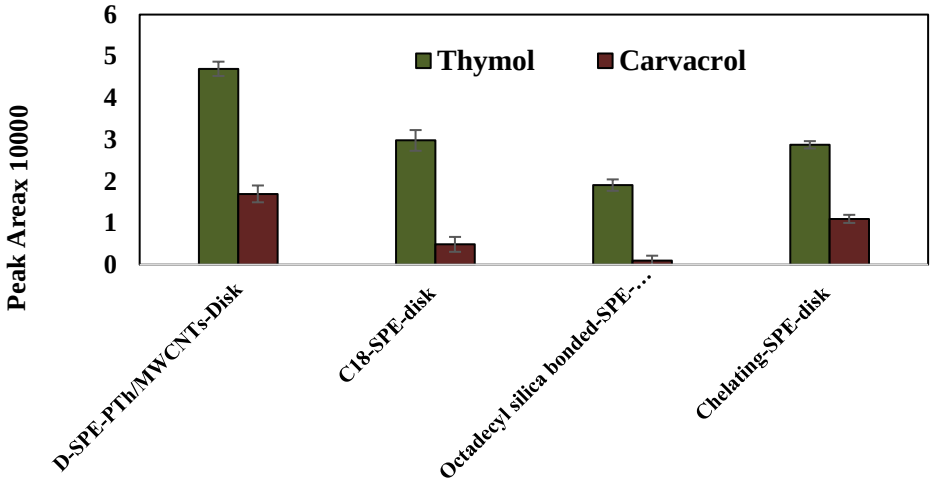

Figure 4. A Comparison of the Suggested PTh/MWCNTs-Disk with Other Commercial SPE Disks for the Extraction of Thymol and Carvacrol by the SPDE-Disk-HPLC-UV Method.

suitable for extracting polar analytes such as thymol, and carvacrol that is completely compatible with the HPLC-UV system. Hence, ACN was suitable for eluting thymol and carvacrol, and was selected as the optimum eluent.

Optimization of Variables by CCD

CCD was used to investigate the effects of critical factors and their interactions and model efficiency via analyzing variance (ANOVA) at 95% confidence interval following the conduction of minimum number of runs and minimizing the cost and errors. Three were independent variables (pH 1.5-7.5, X_1), ionic strength (0-4 w/v, X_2) and extraction time (10-50 min, X_3) with coded value (-1, 0, +1) and the star points of +2 and -2 for +α and -α, respectively. In this model, 15 random experiments were selected to reduce the effects of uncontrolled variables, and then their responses were presented in Table S-1. The calculated results have been indicated in Table S-2. As it could be inferred from Table 2, an analysis of the results using CCD indicated that factors A, B and C were the most effective factors on the measurement of thymol and carvacrol, and were significant factors (for all three variables P-value ≤ 0.05 and F-value ≥ 0.05) in

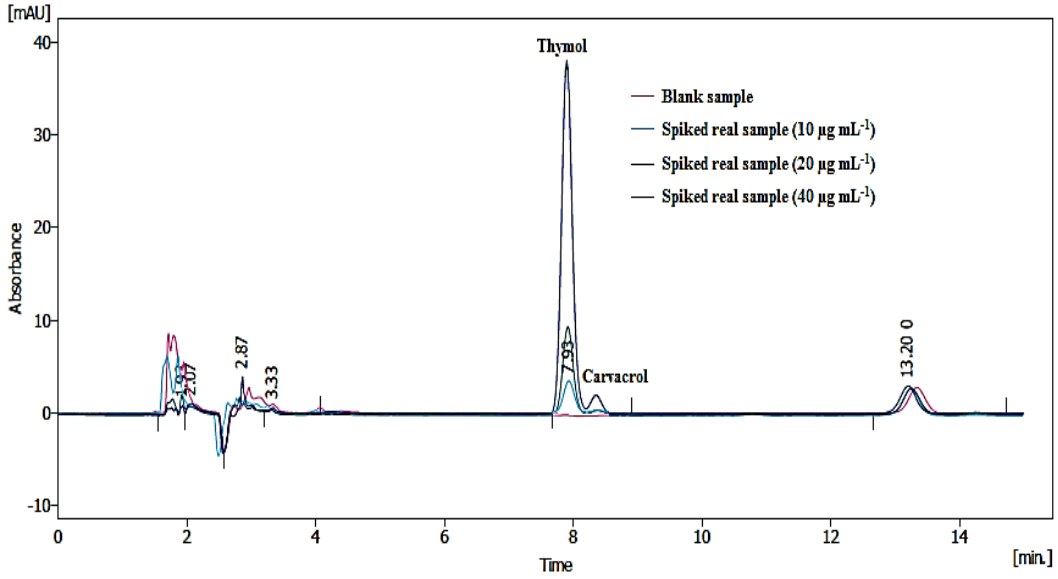

Figure 5. The HPLC-UV Chromatogram Obtained after the SPDE-PTh/MWCNTs-Disk Method Extraction of Thyme Sample.

the studied range. Based on the results of the performed experiments, the second order polynomial equation was obtained, as shown in the following equation, for thymol and carvacrol (Eq 1, 2):

$$Y\,(\text{thymol}) = 37376.35 - 6134.75 * A - 2970.0 * B - 775.0 * C + 4737.75 * AB - 864.37 * A^2 - 906.99 * B^2 - 1409.49 * C^2 \quad Eq.1$$

$$Y\,(\text{carvacrol}) = 11555.23 - 2034.5 * A - 2124.75 * B - 454.75 * C + 1819.0 * AB - 324.04 * A^2 - 759.16 * B^2 - 1591.91 * C^2 \quad Eq.2$$

Where, Y = peak area of thymol and carvacrol; A, B and C represent the pH, ionic strength and extraction time. The quadratic model was highly suitable for the evaluation of experimental data based on their high F value (> 0.05) and highly significant p-value <0.0001 (Table S-2). The propriety and sufficiency of the selected model were further explicated via ANOVA indicated in Table S-2. The statistical parameters value, sum of square, degree of freedom, mean of square, F-value and P-value for equation terms were calculated. P-value less than 0.050 indicates the significant model terms, while P-values greater than this critical value indicates their insignificance. The main interaction and quadratic effects investigation suggest the effect of A, B and C, and confirm that the interaction between the two variables of A and B (AB) are significant. The "Lack of Fit F-value" of 2.593E-004 and 0.014 for thymol and carvacrol respectively implies its non-significant relative to the pure error. The non-significant lack-of-fit (LOF) P-value of 0.9879 and 0.9121 (more than 0.05) for the recovery of thymol and carvacrol respectively was indicative of

Table 1: SPDE-PTh/MWCNTs-Disk-HPLC-UV Method Validation Factors for the Preconcentration of Thymol and Carvacrol in Thyme Essential Oil.

Parameters	**Results**	
	Thymol	**Carvacrol**
Eluent type	Acetonitrile	
Eluent volume (µL)	70	
pH	3	
Ionic strength (w/v)	1	
Sampling flow rate (mL min^{-1})	12	
Extraction time (min)	20	
LOD & LOQ (µg mL^{-1})	0.005 & 0.09	0.07 & 0.01
Regression equation	Y=2345X+249.57,	Y=1654.1X+22.76,
Linearity (R^2)	0.9997	0.9998
LR (µg mL^{-1})	0.09-100	0.01-100
Enrichment factor	87	54
Preconcentration factor	142.8	
ER %	60.9	48.3
Intra-day precision 10, 20, 40% (n = 5, RSD %)	4.1, 3.8, 6.8	4.3, 4.7, 5.1
Inter-day precision 10, 20, 40% (n = 5, RSD %)	9.9, 10.2, 11.4	8.3, 10.5, 12.4

Table 2: The Recovery and Determination of Thymol and Carvacrol in Thyme Samples Using the D-SPE-PTh/MWCNTs-disk-HPLC-UV and the Validated HD-GC-MS Methods.

Sample	Added (µg mL^{-1})	Analytes were determined (µg mL^{-1}) using			
		SPDE-PTh/MWCNTs-disk-HPLC-UV		HD-GC-MS[25]	
		Thymol	Carvacrol	Thymol	Carvacrol
Thyme	0	75.7 (9.4)*	20.3 (3.6)	69.5 (3.5)	19.5 (3.6)
	10	86.4 (6.8)	29.5 (2.9)	78.3(4.6)	31.1(2.1)
	R%	107	92	88	116
	20	93.1(4.1)	42.7 (6.8)	91.9 (1.9)	37.9 (6.0)
	R%	87	112	112	92
	40	119.3(8.1)	59.6 (2.1)	107.9 (5.3)	58.8 (3.9)
	R%	109	98.2	96	98.2

*The numbers in parentheses refer to the RSD obtained by three replicated analysis.

the adequate predictability of the model. The coefficient of the determination R-squared (0.9988, 0.9949), Adj-R-squared (0.9966, 0.9858) and Pred-R-squared (0.9981, 0.9895) accounts for the percentages of variability revealed by the predicted model, and thus greater values indicate the better model. The "Pred-R^2" exhibited a reasonable agreement with the "Adj-R^2". "Adequate Precision (AP)" indicated the ratio of signal to noise. Meanwhile, the value higher than 4 was considered desirable. The ratios of 78.482 and 30.280 justify the adequacy of the signal and confirm the capability of this model to navigate the design space. Moreover, the values of the coefficient of variation (C.V. =1.24, 4.61%), standard deviation (SD=430.46, 434.20) and predicted residual sum of squares (PRESS= 1.432E+006, 1.960E+006) were relatively low that demonstrate acceptable punctuality and trustworthy experiments of the model. Fig. 7 indicates the actual data that had been predicted from the CCD experiment. As it was observed, the majority of the points were quite close to their predicted values which support that the model was robust and precise. An examination of the findings using response surface methodology (RSM) for plotting peak area versus significant variables was carried out. The results have been shown in Fig. 8. The three-dimensional (3D) response surface revealed the reduction of thymol and carvacrol peak area with the increasing pH from 3.0 to 6.0. This fact is rooted in the presence of -COOH groups on the PTh/MWCNTs sorbent that was ionized at high pH. Such polar acidic groups are indeed in neutral form at low pH. They are suitable for the absorption of polar analytes, including thymol and carvacrol. The highest response was achieved at pH acidic (3), and indicated high dependency of thymol and carvacrol recovery to ionic strength that was subsequently examined adding different values of $NaNO_3$ (0–4% w/v) to sample solutions. The response increases with adding $NaNO_3$ from 0 to 1% and subsequently diminishes by raising to 4% w/v. This phenomenon is due to alteration in the physical conditions of the sorbent and Nernst diffusion layer (29). Otherwise, the adding of salt to the aqueous sample often reduces the extraction efficacy because of decreasing the diffusion coefficients of analytes (particularly for polar analytes, including thymol and carvacrol, due to compensation for the salting-out impact (28)) via mutating the physical characteristics of the Nernst diffusion layer (29). The extraction efficiency increased by raising time from 10 to 20 min, and subsequently at higher time (20 to 50 min) led to significant signal reduction which is related to the replacement of adsorbed analytes with other species excited in the sample solution. The results of ANOVA (Table S-2) suggest that the interaction between the

Table 3: A Comparison of the Analytical Figures of Merit of the Proposed SPDE-PTh/MWCNTs-Disk-HPLC-UV Method with Certain Reported Procedures for the Determination of Thymol and Carvacrol in Thyme.

Extraction method	Detection system	LOD (mg L^{-1})	LR (mg L^{-1})	RSD (%)	EF	Ref.
HD-HSME	GC-FID	1.87, 0.23	6.25-81.25, 1.25-87.50	6.37, 11.8	*N.R	[4]
Dissolving-HPLC	HPLC-FLD	0.0017, 0.00156	0.008-0.2	2.9, 2.5	N.R	[26]
UAME-NMSPD	HPLC-UV	0.00023, 0.00021	0.005-2	2.14, 4.9	N.R	[13]
UAE-DLLME	GC-FID	---, 0.0002	0.001-21, ---	< 11	N.R	[6]
VASEDLLME	HPLC-UV	0.0016	0.005-4	< 4.7	N.R	[14]
HPLC	HPLC-UV	0.6, 2.8	2-9, 15-90	0.8, 1.9	N.R	[27]
DI-SPME	HPLC-UV	0. 6, 0. 8	1-80	6.8, 12.7	N.R	[12]
SPDE-Hand-made-Disk	HPLC-UV	0.005, 0.007	0.01-100	6.8, 5.7	80, 50	Present work

* Not reported

two variables of A and B (pH and ionic strength) is significant.

A Comparison of Hand-Made PTh/MWCNTs-Disk with Other Commercial SPE Disks

To ensure the reliability and superiority of the prepared sorbent in comparison with other widely used sorbents, it was compared with Octadecyl silica bonded-SPE-disk, Chelating-SPE-disk and C_{18}-SPE-disk commercial SPE disk as well as a handmade nanostructured PTh coated disk. To this end, all the disks were applied under the optimized experimental conditions. The results (Figure 4) indicate that the PTh/MWCNTs sorbent possesses higher extraction efficiency compared to other examined disks. On the other hand, the prepared disk does not have the problems corresponded to the commercial disks such as low capacity, high-cost, swelling in organic solvents and fragility.

Analytical Figures of Merit

To assess the analytical data correspond to the optimized method, the eluent type of acetonitrile, eluent volume of 70 µL, pH of 3.0, ionic strength of 1 (w/v), sampling flow rate of 12 mL min^{-1}, and the extraction time of 20 min were examined for the extraction and determination of thymol and carvacrol. Under the optimized conditions of the SPDE-PTh/MWCNTs-disk-HPLC-UV method, limits of detection (LODs) were 0.005 and 0.007 µg mL^{-1} for thymol and carvacrol respectively. Linear dynamic range (LDR) for the calibration curves of both analytes were 0.01-100 µg mL^{-1}, while their respective relative standard deviation (RSD%, n=6) were lower than 6.8 and 5.7 for thymol and carvacrol respectively. The enrichment factor (EF) values were calculated as the ratio of the target analytes concentration in the final extract to respective primary sample solution (Eq.3). C and C_0 are the target analytes concentration in the final extract and in the primary sample solution, respectively. The preconcentration factor values were calculated as the ratio of eluent volume to volume of sample solution (Eq.3). Moreover, extraction recovery (ER) was calculated as the ratio of enrichment factor

to preconcentration factor (Eq.3). EF was 87 for thymol and 54 for carvacrol, while their preconcentration factor was 142.8. The summary of the quantitative analysis of the SPDE-disk-HPLC-UV method has been presented in Table 1.

$$EF = \frac{C}{C_0}, PF = \frac{V}{V_0}, ER = \frac{EF}{PE} * 100 \qquad Eq.3$$

Real Sample Analysis

The proposed SPDE-PTh/MWCNTs-disk-HPLC-UV strategy was applied for the extraction of thymol and carvacrol in thyme sample solutions. Hence, distinct aqueous solutions of thyme were prepared and spiked with 10, 20 and 40 µg $mL^{-1}$of both thymol and carvacrol. Subsequently, the blank, spiked and non-spiked samples were evaluated using SPDE-PTh/MWCNTs-disk with the proposed disk (Table 2). To carry out the extraction, a certain volume of aqueous sample containing thymol and carvacrol was transferred to extraction vial, and the analytes were extracted dynamically on the disk. In the next step, the analytes were washed with 500 µL acetonitrile (as eluting solvent) from the disk, and 20 µL of eluting solvent was injected to HPLC loop for the quantification of thymol and carvacrol whose quantities were analyzed through the HD-GC-MS procedure as the validated method (25). The results were compared with the proposed SPDE-PTh/MWCNTs-disk-HPLC-UV procedure. These findings indicated that the PTh/MWCNTs-disk could be successfully used to concentrate and purify thymol and carvacrol in thyme by the proposed SPDE-PTh/MWCNTs-disk-HPLC-UV strategy.

A Comparison of the SPDE-PTh/MWCNTs-Disk Method with Reported Procedures

To provide a more comprehensive explication of the reliability and applicability of the developed SPDE-PTh/MWCNTs-disk-HPLC-UV strategy for the separation and quantification of thymol and carvacrol in thyme, the most significant items of its figures of merit (LOD, LDR, RSD, EF, extraction method and detection system) were compared with those of published reports, and the results have been summarized in Table 3. As it was found, in the majority of cases, the analytical figures of merit corresponded to the SPDE-PTh/MWCNTs-disk-HPLC-UV strategy that are comparable or better than the published reports. These results confirm that this setup use a simple, low-cost, repeatable and available detection system (HPLC-UV) with a high enrichment factor which is easily available and applicable in most regular laboratories. Otherwise, it is an authentic procedure for the accurate and precise investigation of solid samples without any sample preparation step.

Conclusion

A flexible, porous, unbreakable and low-cost SPDE-PTh/MWCNTs-disk device was fabricated and evaluated for the determination of thymol and carvacrol in thyme. The disk that was described had a higher extraction efficiency compared to other commercial disks in the SPDE method. Moreover, it was simple, low-cost, repeatable and available. Furthermore, since the sorbent was chemically disposed on a SS plate bed, there was no possibility of sorbent leakage from the disk and the header on the adsorbent. Likewise, the need for an adhesive that could cause adsorption interfaces and blockage of adsorbent pores was eliminated. Due to the use of SS plate as a disk bed, the SPDE-PTh/MWCNTs-disk was in accordance with green chemistry. It was very inexpensive, non-toxic and environmentally friendly. The unique extraction power of the dick that was described could be attributed to the inherent porosity of the metallic bed and the presence of functional groups that acted as sorbents, a very high surface area, the existence of highly ordered cavities in porous polythiophene and its chemical modification with MWCNTs attributed to its efficiency for the extraction of the phenolic compounds of thyme.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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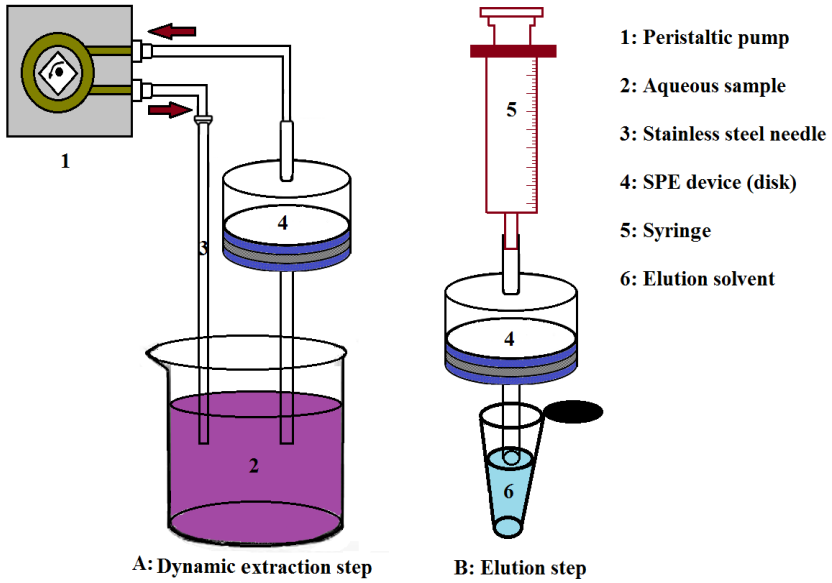

Figure S-1. The schematic of the SPDE-PTh/MWCNTs-disk procedure. A: dynamic extraction step, B: elution step.

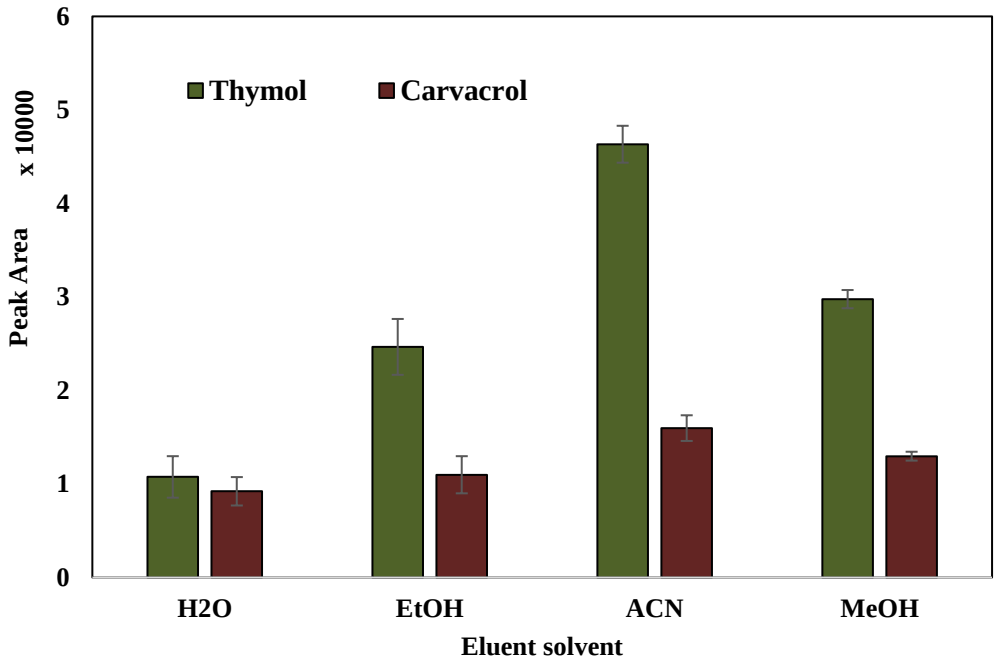

Figure S-2. Effect of type of eluent solvent on the extraction efficiency of thymol and carvacrol using the SPDE-PTh/MWCNTs-disk-HPLC-UV method.

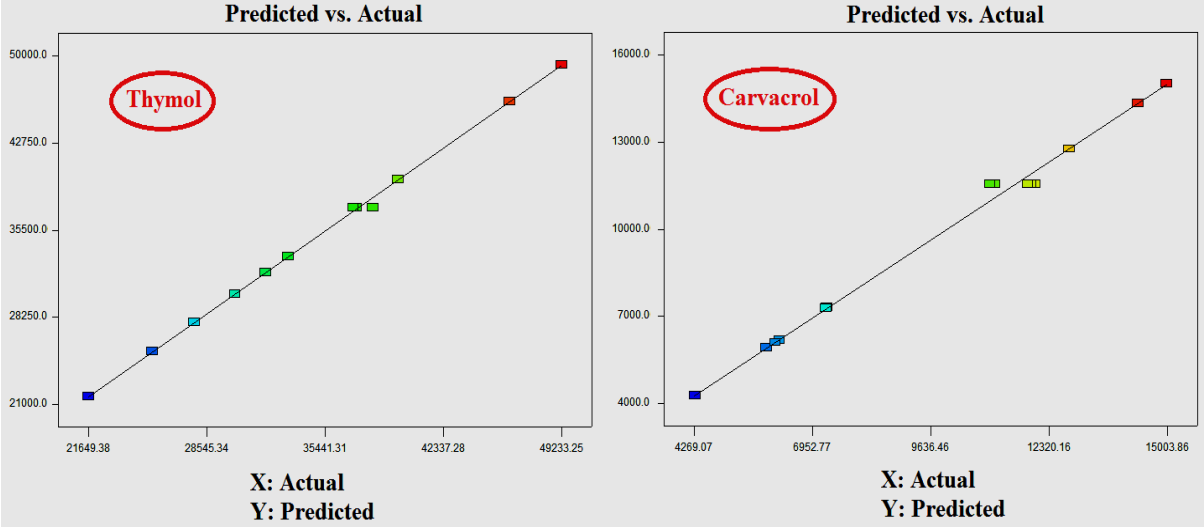

Figure S-3. Correlation of actual and predicted determination of thymol (a) and carvacrol (b).

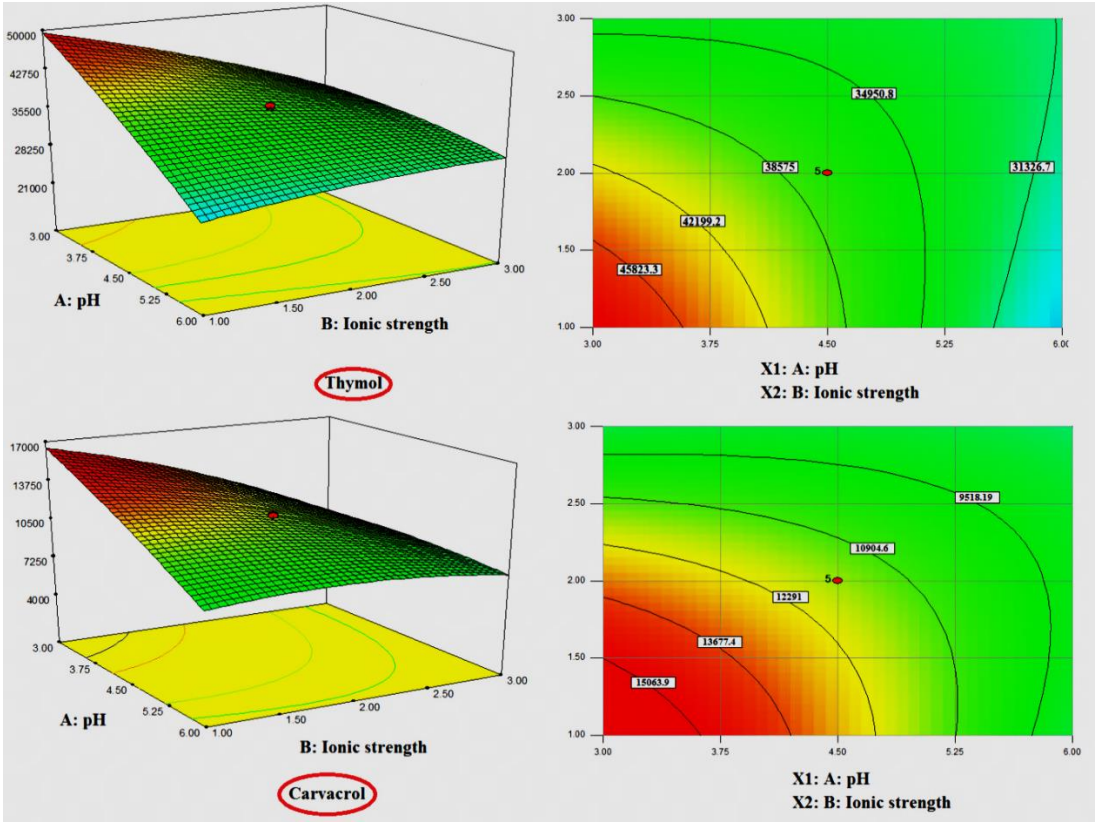

Figure S-4. Three-dimensional response surface and contour diagrams showing the effects of the mutual interactions between two independent variables pH and ionic strength.

Table S-1: Levels of the factors (X_n) and CCD with experimental results.

Factors			**Levels**		**Star points**	
			Low (-1)	**Center (0)**	**High (+1)**	**-α**	**+α**
(X_1) pH			3.0	4.5	6.0	1.5	7.5
(X_2) Ionic strength (w/v)			1.0	2.0	3.0	0.0	4.0
(X_3) extraction time (min)			20.0	30.0	40.0	10.0	50.0
		Factor				**Response (peak area)**	
Run	**A: pH**	**B: Ionic strength(w/v)**		**C: extraction time (min)**		**Thymol**	**Carvacrol**
1	4.5	2.0		30.0		37218	11090 (Center)
2	4.5	4.0		30.0		27810	4281
3	3.0	1.0		20.0		49230	14980
4	6.0	1.0		40.0		25379	7256
5	7.5	2.0		30.0		21651	6202
6	1.5	2.0		30.0		46190	14340
7	4.5	2.0		10.0		33290	6109
8	6.0	3.0		20.0		30180	7280
9	4.5	0.0		30.0		39690	12780
10	4.5	2.0		30.0		37090	10980 (Center)
11	4.5	2.0		30.0		38230	11900 (Center)
12	3.0	3.0		40.0		31980	5909
13	4.5	2.0		50.0		30190	4290
14	4.5	2.0		30.0		37109	12000 (Center)
15	4.5	2.0		30.0		37238	11830 (Center)

Table S-2: ANOVA results for CCD of thymol and carvacrol and model summary statistics.

Source	**Degree of freedom**	**Sum of squares**		**Mean square**		**F-value**		**P-value**	
	Thy $ Car	**Thy**	**Car**	**Thy**	**Car**	**Thy**	**Car**	**Thy**	**Car**
Model	9	7.584E+008	1.849E+008	8.427E+007	2.055E+007	454.78	108.99	< 0.0001	< 0.0001
A	1	3.011E+008	3.311E+007	3.011E+008	3.311E+007	1624.89	175.64	< 0.0001	< 0.0001
B	1	7.057E+007	3.612E+007	7.057E+007	3.612E+007	380.84	191.57	< 0.0001	< 0.0001
C	1	4.805E+006	1.654E+006	4.805E+006	1.654E+006	25.93	8.78	0.0038	0.0314
AB	1	5.986E+007	8.823E+006	5.986E+007	8.823E+006	323.04	46.80	< 0.0001	0.0010
AC	1	53960.17	50050.67	53960.17	50050.67	0.29	0.27	0.6126	0.6283
BC	1	2.061E+005	5.310E+005	2.061E+005	5.310E+005	1.11	2.82	0.3399	0.1541
A^2	1	1.747E+007	2.455E+006	1.747E+007	2.455E+006	94.29	13.02	0.0002	0.0154
B^2	1	1.924E+007	1.348E+007	1.924E+007	1.348E+007	103.82	71.49	0.0002	0.0004
C^2	1	4.646E+007	5.926E+007	4.646E+007	5.926E+007	250.73	314.33	< 0.0001	< 0.0001
Residual	5	9.265E+005	9.426E+005	1.853E+005	1.885E+005				
Lack of fit	1	60.04	3244.91	60.04	3244.91	2.593E-004	0.014	0.9879	0.9121
Pure error	4	9.264E+005	9.394E+005	2.316E+005	2.348E+005				
Total error	14	7.593E+008	1.859E+008						
Model summary statistics		**Std. Dev.[a]**	**Mean**	**C.V. %[b]**	**PRESS[c]**	**R-Squared**	**Adj R-Squared**	**Pred R-Squared**	**AP[d]**
Thymol		430.46	34831.67	1.24	1.432E+006	0.9988	0.9966	0.9981	78.482
Carvacrol		434.20	9415.13	4.61	1.960E+006	0.9949	0.9858	0.9895	30.280

a: Standard deviation
b: Coefficient of variation
c: Predicted residual sum of squares
d: Adequate precision