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Simultaneous Determination of Fenchone and *Trans*-Anethole in Essential Oils and Methanolic Extracts of *Foeniculum vulgare* Mill. Fruits Obtained from Different Geographical Regions Using GC-MS Approach

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Abstract: The gas chromatography–mass spectrometry (GC-MS) approach is established for the simultaneous determination of fenchone (FCO) and *trans*-anethole (TOH) in the essential oils and methanolic extracts of fennel (*Foeniculum vulgare* Mill.) fruits obtained from India (IND), Pakistan (PAK), and Saudi Arabia (SA). The simultaneous determination of FCO and TOH was performed via Agilent 190914S HP–5MS fused-silica capillary column (30 m × 250 µm ID, 0.25 µm film thickness). The proposed GC-MS approach was linear in the range of 0.10–50 µg/g for FCO and TOH. FCO's detection (LOD) and quantification (LOQ) limits were calculated to be 0.04 and 0.12 µg/g, respectively. The LOD and LOQ values for TOH, on the other hand, were calculated to be 0.05 and 0.15 µg/g, respectively. In addition, the proposed GC-MS approach was accurate and precise for the simultaneous determination of FCO and TOH. The amount of FCO in essential oils of *F. vulgare* was computed as 0.021, 0.034, and 0.029 mg/g in the samples obtained from IND, PAK, and SA, respectively. The amount of TOH in the essential oils of *F. vulgare* was computed as 7.40, 14.8, and 10.2 mg/g in the samples obtained from IND, PAK, and SA, respectively. However, the amount of FCO in the methanolic extract of *F. vulgare* was estimated as 0.031, 0.021, and 0.057 mg/g in the samples obtained from IND, PAK, and SA, respectively. On the other hand, the amount of TOH in the methanolic extract of *F. vulgare* was estimated as 0.440, 0.498, and 1.74 mg/g in the samples obtained from IND, PAK, and SA, respectively. These findings suggested that the proposed GC-MS approach might be used to simultaneously determine the FCO and TOH in a variety of essential oils and plant extracts.

Keywords: essential oils; fenchone; *Foeniculum vulgare*; GC-MS; plant extract; *trans*-anethole; simultaneous determination

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1. Introduction

Foeniculum vulgare Mill. (commonly known as fennel; family: Apiaceae) is a biennial or perennial medicinal crop [1]. The main phytoconstituents of fennel fruits are essential oils, which are used in several industries as the flavoring agents [1–3]. The phytoconstituents of essential oils of fennel fruits have been reported extensively using a gas chromatography–mass spectrometry (GC-MS) approach [1,3–11]. Fenchone (FCO; chemical structure: Figure 1A) is one of the biomarker compounds of essential oils of fennel fruits [1]. The Chiral GC approach was applied for the determination of FCO in the essential oils of fennel [12]. FCO in fennel extract and eight different commercial formulations of FCO were also determined using the GC-MS approach [13].

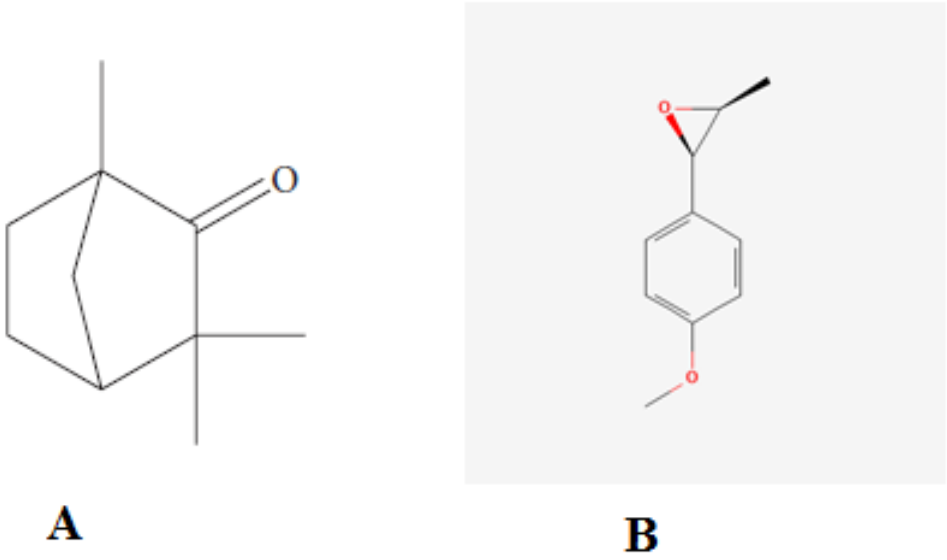

Figure 1. Molecular structures of (**A**) fenchone (FCO) and (**B**) *trans*-anethole (TOH).

Trans-anethole (TOH; chemical structure: Figure 1B) is a monoterpene present in the essential oils of a variety of plants, including fennel fruits [14]. The most prevalent component in the essential oils of fennel fruits, according to a GC-MS analysis [13], is TOH. For the determination of TOH in various plant extracts, essential oils, and biological samples, various analytical approaches such as high-performance liquid chromatography (HPLC) [15–19], GC-MS [20–26], nuclear magnetic resonance spectroscopy [23], liquid chromatography–mass spectrometry (LC-MS) [27], differential pulse voltammetry [28], and high-performance thin-layer chromatography (HPTLC) [29] have been used. Recently, a more environmentally friendly HPTLC method was used to determine the TOH in methanolic extract, essential oils, and several commercial fennel formulations [30].

FCO and TOH are present in several commercially available polyherbal formulations. As a results, their qualitative and quantitative standardization is essential to evaluate the quality of the products. Simultaneous determination of FCO and TOH is poorly reported in the literature. As a result, the goal of this study was to develop and validate a sensitive GC-MS method for determining FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* obtained from different geographical regions, including India (IND), Pakistan (PAK), and Saudi Arabia (SA). Following "The International Council for Harmonization (ICH)" Q2 (R1) recommendations, the proposed GC-MS approach for the simultaneous assessment of FCO and TOH was validated for linearity range, accuracy, precision, and sensitivity [31].

2. Materials and Methods

2.1. Materials

The standard FCO and TOH were obtained from "Sigma Aldrich (St. Louis, MO, USA)". HPLC grade methanol and other analytical grade reagents were procured from "E-Merck (Darmstadt, Germany)". *F. vulgare* fruits were collected from three separate geographical regions: IND, PAK, and SA. The fruits were checked against a voucher specimen at the "Medicinal, Aromatic, and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, King Saud University, Riyadh, Saudi Arabia".

2.2. GC-MS Analysis

2.2.1. SCAN Method

The GC-MS analysis was performed using an "Agilent 7890 system (Santa Clara, CA, USA)" gas chromatograph equipped with a split–splitless injector (split ratio of 20:1) and a 5975 TAD mass detector running in the emission ionization (EI) mode (70 eV) with a 3 min filament delay. The GC column was Agilent 190914 S HP–5MS fused-silica capillary column (30 m × 250 µm ID, 0.25 µm film thickness) consisting of Crossbond (5% diphenyl, 95% dimethyl polysiloxane). At a flow rate of 1.0 mL/min, helium (He) was used as the carrier gas. To separate the phytocomponents, a linear temperature program was used: initial 50 °C (hold time: 1 min), then ramped to 100 °C at a rate of 20 °C/min (hold time: 1 min), and then increased to 300 °C at a rate of 20 °C/min (hold time: 1 min). The injector base, transfer line, and ionization source were all kept at 280 °C, 230 °C, and 150 °C, respectively. The mass range was set at 40 to 600 amu, and the entire run time was 15.5 min.

2.2.2. Single Ion Current (SIM) Method

In SIM method, most of the chromatographic conditions were similar to SCAN method. The total run time was 12 min in this case. For the subsequent selective and more sensitive analysis of TOH (calibration graphs and sample assay), the chromatograms were reconstructed at the SIM mode (at 148.1 amu, the base and molecular peak of TOH) (Figure 2). For FCO analysis, 152.2 amu was selected (Figure 3).

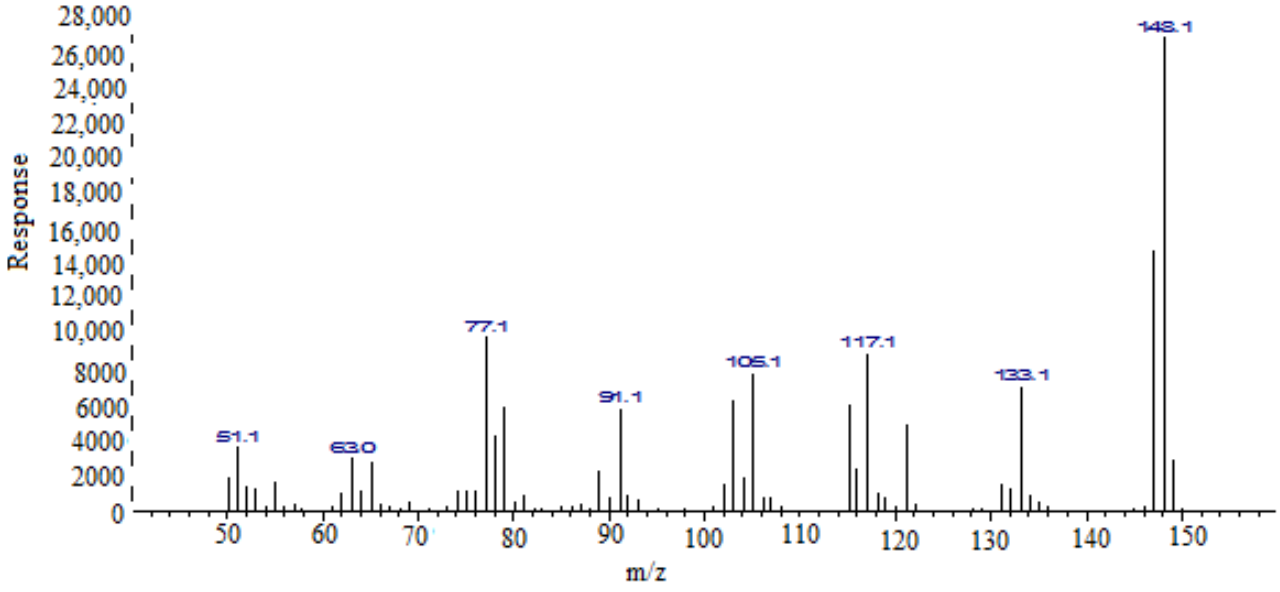

Figure 2. Mass spectra (m/z) of standard TOH.

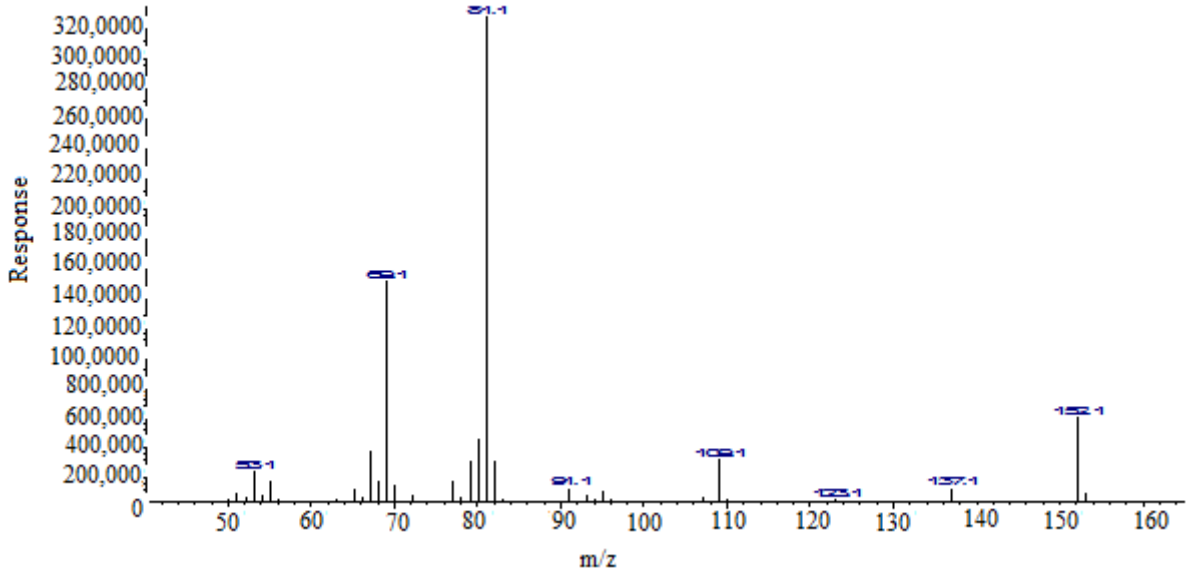

Figure 3. Mass spectra (*m*/*z*) of standard FCO.

2.3. Calibration Curves of FCO and TOH

FCO and TOH stock solutions were prepared separately by dissolving the prescribed amounts of FCO and TOH in the requisite volume of methanol to provide a final solution of 100 µg/g for both compounds. After that, serial dilutions of this solution were created by diluting with methanol with different volumes of FCO or TOH solution to obtain concentrations in the range of 0.10–50 µg/g for both substances. Approximately 2 µL of each concentration of FCO and TOH was injected into GC-MS system, and GC-MS response for each concentration of FCO and TOH was recorded. The calibration curve for FCO and TOH was constructed by plotting the concentrations of each compound against measured GC-MS response in three replicates (n = 3).

2.4. Extraction Procedure for F. vulgare Obtained from IND, PAK, and SA

Approximately 10 g *F. vulgare* fruits obtained from IND, PAK, and SA were taken and triturated using a pestle and mortar to make the fine powder. The powder of *F. vulagre* obtained from different geographical regions was refluxed in a water bath for one hour with 100 mL of methanol. The resulting extracts were filtered, and the remaining marc was refluxed for roughly an hour in 70 mL of methanol before being filtered again. A rotary vacuum evaporator was used to evaporate the solvent. The residue was then reconstituted in 50 mL methanol, and the operation was repeated three times (n = 3) [30]. This solution was utilized as a test for solution for simultaneously determining FCO and TOH in methanolic extracts of *F. vulgare* obtained from IND, PAK, and SA using the proposed GC-MS approach.

2.5. Isolation of the Essential Oil from F. vulgare Fruits Obtained from IND, PAK, and SA

The essential oils of *F. vulgare* fruits obtained from IND, PAK, and SA were isolated using hydro-distillation approach, as per the protocol summarized in Egyptian Pharmacopoeia. In brief, approximately 200 g fruits of *F. vulgare* from IND, PAK, and SA regions were placed into Clevenger trap apparatus separately for the isolation of essential oils. *F. vulgare* fruits from IND, PAK, and SA were combined with 1000 mL of water and distilled separately for around eight hours. The oily layer and water separation from each sample were strapped using dichloromethane (3 × 50 mL). Finally, using a rotating vacuum evaporator, the organic layer was condensed to obtain pure essential oil from each sample. [30]. This essential oil from different geographical regions was used as a test material for the simultaneous determination of FCO and TOH using the proposed GC-MS approach.

2.6. Method Validation

Following the ICH-Q2-R1 recommendations, the proposed GC-MS approach for the simultaneous assessment of FCO and TOH was validated for linearity range, accuracy, precision, and sensitivity [31]. The linearity range for FCO and TOH was assessed by plotting the concentrations of FCO and TOH against measured GC-MS response. The linearity for FCO and TOH was assessed using seven different concentrations, including 0.1, 1, 2, 5, 10, 20, and 50 µg/g (range 0.10–50 µg/g) for both of the compounds in triplicates (n = 3). The accuracy for the proposed GC-MS approach for the simultaneous determination of FCO and TOH was determined as the percent recovery. For FCO and TOH, the accuracy was tested using low-quantity control (LQC; 5 µg/g), middle-quantity control (MQC; 10 µg/g), and high-quantity control (HQC; 50 µg/g) samples. The accuracy was determined in standard compounds without spiking with essential oils. The percent recovery was calculated for each FCO and TOH quality level (n = 3).

Intra/inter-assay precision was used to test the precision of the suggested GC-MS approach for the simultaneous measurement of FCO and TOH. Quantitation of freshly created FCO and TOH solutions at LQC, MQC, and HQC on the same day was used to determine intraday precision for FCO and TOH in triplicates (n = 3). However, the quantification of newly generated solutions at LQC, MQC, and HQC on three consecutive days was used to examine the interday precision for the simultaneous determination of FCO and TOH in triplicates (n = 3).

Using a standard deviation technique, the sensitivity of the proposed GC-MS approach for the simultaneous assessment of FCO and TOH was evaluated in terms of "detection (LOD) and quantification (LOQ) limits". FCO and TOH "LOD and LOQ" values were determined using established equations as stated in literature (n = 3) [31].

2.7. Application of Proposed GC-MS Approach in the Simultaneous Determination of FCO and TOH in Methanolic Extracts and Essential Oils of F. vulgare Obtained from IND, PAK, and SA

The obtained solutions of methanolic extracts and essential oils of *F. vulgare* obtained from IND, PAK, and SA were injected in GC-MS, and their GC-MS responses were recorded using the same experimental procedures utilized for the simultaneous determination of standard FCO and EOH (n = 3). The amounts of FCO and TOH in all investigated solutions were computed utilizing the calibration curves of FCO and TOH.

3. Results and Discussion

3.1. GC-MS Method Validation

Various parameters for the validation of the proposed GC-MS approach for the simultaneous determination of FCO and TOH were computed by following the ICH-Q2-R1 recommendations [31]. Table 1 summarizes the findings of the linear regression analysis of calibration curves of FCO and TOH.

The FCO and TOH calibration curves were found to be linear in the range of 0.10–50 µg/g for both substances. The representative calibration curves for FCO and TOH are presented in Figure 4. The representative GC-MS chromatogram for FCO and TOH is shown in Figure 5. The retention time (R_t) for FCO and TOH was recorded as 6.091 and 7.889 min, respectively, with a total run time of 12 min. The GC-MS chromatographic parameters were reliable for the simultaneous determination of FCO and TOH. Figure 6 shows the superimposed GC-MS spectra of different calibration concentrations of FCO and TOH, which exhibited identical R_t for all the concentrations investigated. The results of the linear regression analysis revealed a strong linear association between the FCO and TOH concentrations and the measured GC-MS responses. For FHO and TOH, the determination coefficient (R^2) was calculated to be 0.9938 and 0.9957, respectively. In addition, the values of regression coefficient (R) for FHO and TOH were computed as 0.9968 and 0.9978, respectively. For FCO and TOH, the R^2 and R values were extremely significant ($p < 0.05$). These findings showed that the suggested GC-MS approach was suitable for the simultaneous detection

of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* obtained from various geographical regions.

Table 1. Results for linear regression analysis for the simultaneous determination of fenchone (FCO) and *trans*-anethole (TOH) using gas chromatography–mass spectrometry (GC-MS) approach (mean ± SD; n = 3).

Parameters	FCO	TOH
Linearity range (µg/g)	0.1–50	0.1–50
R^2	0.9938	0.9957
R	0.9968	0.9978
Slope ± SD	96,653 ± 115	13,574 ± 38
Intercept ± SD	115,884 ± 985	20,084 ± 442
Standard error of slope	66.69	21.93
Standard error of intercept	568.70	255.19
95% confidence interval of slope	96,367–96,938	13,479–13,668
95% confidence interval of intercept	113,436–118,331	18,958–21,182
LOD ± SD (µg/g)	0.04 ± 0.00	0.05 ± 0.00
LOQ ± SD (µg/g)	0.12 ± 0.0	0.15 ± 0.00

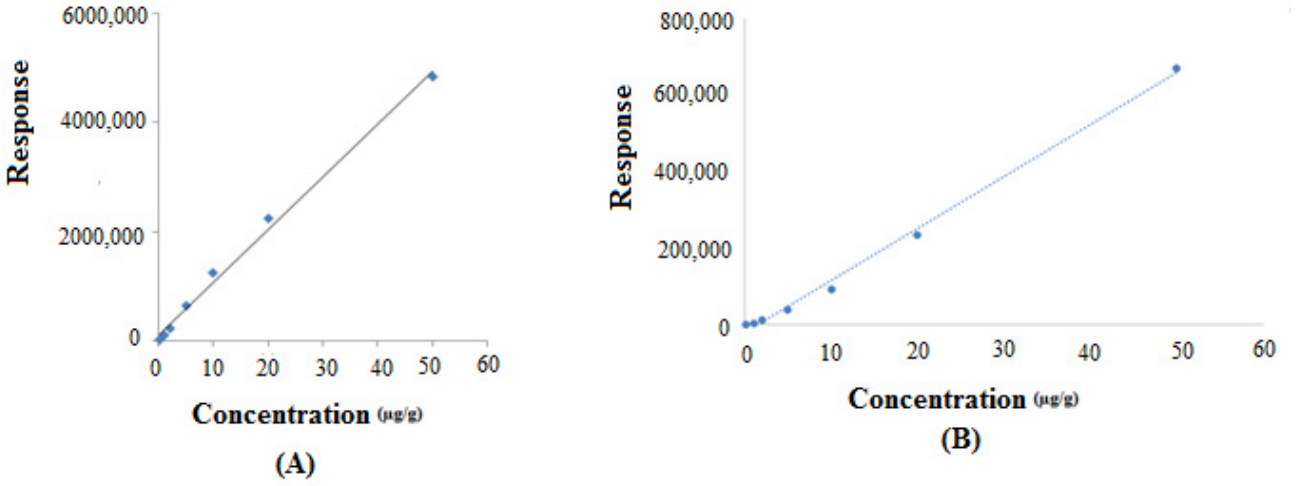

Figure 4. Calibration curves of (**A**) FCO and (**B**) TOH.

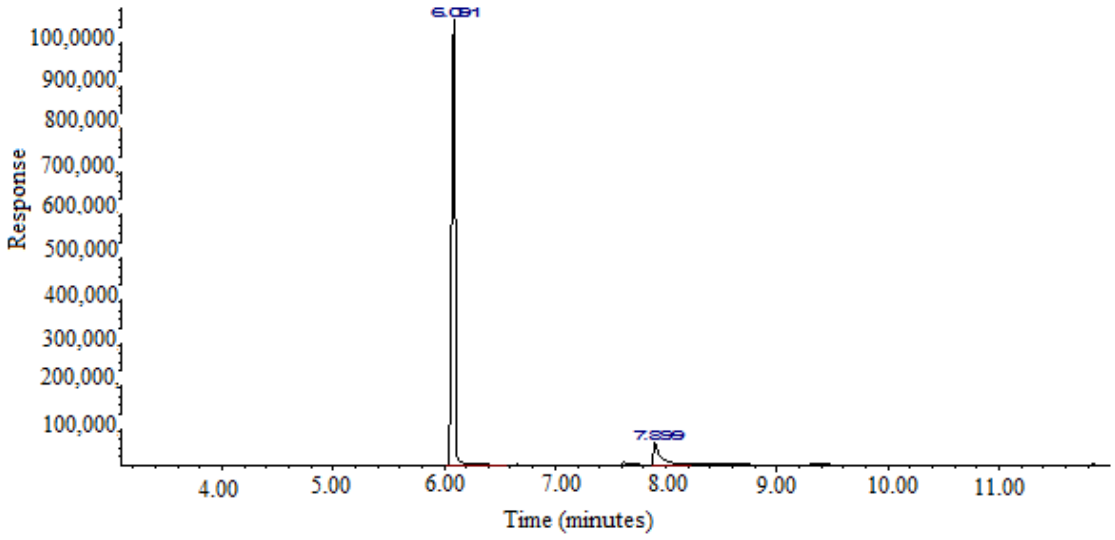

Figure 5. Gas chromatography–mass spectrometry (GC-MS) peaks of standard FCO (R_t = 6.091 min) and TOH (R_t = 7.889 min).

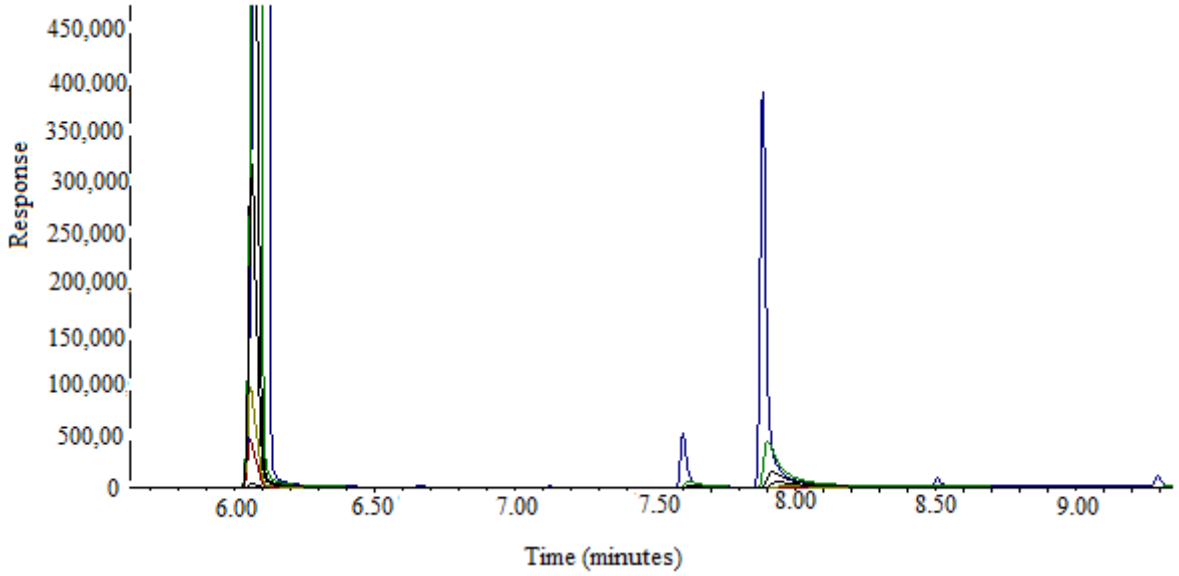

Figure 6. Overlaid GC-MS spectra of standard FCO and TOH at different concentrations.

The accuracy of the proposed GC-MS approach for the simultaneous determination of FCO and TOH was computed as the percent of recovery, and findings are summarized in Table 2.

Table 2. Determination of the accuracy of FCO and TOH for GC-MS approach (mean ± SD; *n* = 3).

Conc. (µg/g)	Conc. Found (µg/g) ± SD	Recovery (%)	CV (%)
	FCO		
5	5.07 ± 0.10	101.40	1.97
10	9.84 ± 0.14	98.40	1.42
50	49.38 ± 0.32	98.76	0.64
	TOH		
5	4.97 ± 0.06	99.40	1.20
10	10.18 ± 0.10	101.80	0.98
50	49.92 ± 0.18	99.84	0.36

The percent recoveries of FCO and TOH at LQC, MQC, and HQC were computed as 98.76–101.40 and 99.40–101.80 percent, respectively, utilizing the proposed GC-MS approach. The obtained values of the percent recoveries for FCO and TOH showed the accuracy of the suggested GC-MS approach for the simultaneous determination of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* obtained from different geographical regions.

The suggested GC-MS approach for determining the FCO and TOH simultaneously was evaluated in terms of intra/inter-assay precision, with results expressed as a percentage of the coefficient of variation. Table 3 summarizes the results of the precision analysis for the simultaneous determination of FCO and TOH using the suggested GC-MS approach.

For intra-assay variation, the percent CVs of FCO and TOH were calculated as 0.54–1.40 and 0.43–1.01 percent, respectively. For inter-assay variance, the percent CVs of FCO and TOH were calculated as 0.88–1.76 and 1.02–1.59 percent, respectively. These results revealed that the proposed GC-MS approach for determining FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* collected from various geographical regions was precise.

Table 3. Determination of intra/interday precision of FCO and TOH for GC-MS approach (mean ± SD; *n* = 3).

Conc. (µg/g)	Intraday Precision			Interday Precision		
	Conc. (µg/g) ± SD	Standard Error	CV (%)	Conc. (µg/g) ± SD	Standard Error	CV (%)
			FCO			
5	4.97 ± 0.07	0.04	1.40	5.09 ± 0.09	0.05	1.76
10	10.12 ± 0.12	0.06	1.18	9.63 ± 0.14	0.08	1.45
50	51.08 ± 0.28	0.16	0.54	48.86 ± 0.43	0.24	0.88
			TOH			
5	4.92 ± 0.05	0.05	1.01	5.03 ± 0.08	0.04	1.59
10	10.14 ± 0.09	0.09	0.88	10.13 ± 0.12	0.06	1.18
50	50.78 ± 0.22	0.22	0.43	49.76 ± 0.51	0.29	1.02

The suggested GC-MS approach's sensitivity for the simultaneous determination of FCO and TOH was evaluated as "LOD and LOQ", and the results are given in Table 1. FCO's "LOD and "LOQ" values were calculated as 0.04 ± 0.00 and 0.12 ± 0.00 µg/g, respectively. The "LOD and LOQ" values for TOH, on the other hand, were calculated as 0.05 ± 0.00 and 0.15 ± 0.00 µg/g, respectively. These findings demonstrated the sensitivity of the proposed GC-MS approach for the simultaneous measurement of FCO and TOH in the methanolic extracts and essential oils of F. vulgare obtained from various geographical regions.

3.2. Application of Proposed GC-MS Approach in the Simultaneous Determination of FCO and TOH in Methanolic Extracts and Essential Oils of F. vulgare Obtained from IND, PAK, and SA

The utility of the proposed GC-MS approach was verified in the simultaneous determination of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* obtained from different geographical regions. The GC-MS chromatograms of FCO and TOH from the methanolic extracts and essential oils of *F. vulgare* were identified by comparing the R_t values of FCO and TOH with those of standard FCO and TOH. The representative GC-MS chromatograms of FCO and TOH in the methanolic extracts of *F. vulgare* obtained from IND, PAK, and SA are presented in Figure 7. The representative GC-MS chromatograms of FCO and TOH in the essential oils of *F. vulgare* obtained from IND, PAK, and SA are presented in Figure 8. The R_t values of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* obtained from IND, PAK, and SA were found to be identical with those of standard FCO and TOH. These findings suggested the specificity of the proposed GC-MS approach for the simultaneous determination of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* obtained from different geographical regions.

Using the FCO and EOH calibration curves, the quantities of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* derived from IND, PAK, and SA were calculated, and the results are given in Table 4.

Table 4. Application of the proposed GC-MS approach in simultaneous determination of FCO and TOH in methanolic extracts and essential oils of *F. vulgare* obtained from different geographical regions (mean ± SD; *n* = 3).

Samples	Amount of FCO (mg/g)	Amount of TOH (mg/g)
F. vulgare extract (IND)	0.031 ± 0.002	0.440 ± 0.010
F. vulgare extract (PAK)	0.021 ± 0.001	0.498 ± 0.012
F. vulgare extract (SA)	0.057 ± 0.008	1.74 ± 0.018
F. vulgare oil (IND)	0.021 ± 0.002	7.40 ± 0.342
F. vulgare oil (PAK)	0.034 ± 0.004	14.8 ± 0.621
F. vulgare oil (SA)	0.029 ± 0.005	10.2 ± 0.464

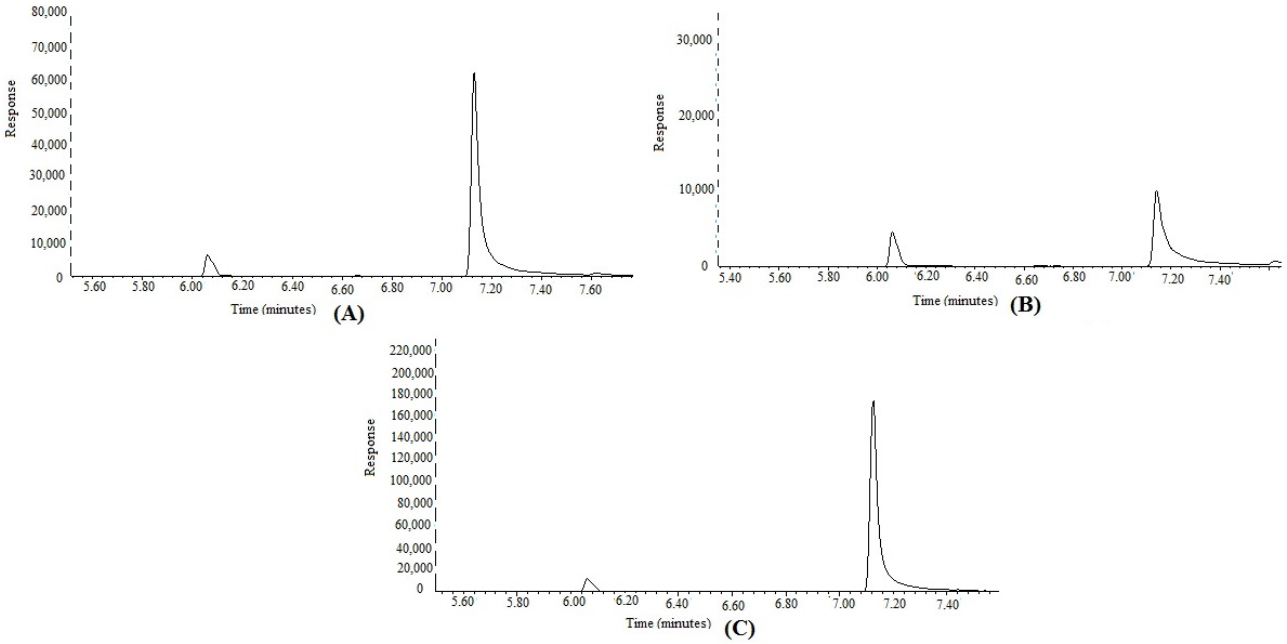

Figure 7. Representative GC-MS peaks of FCO and TOH in methanolic extracts of *F. vulgare* obtained from (**A**) IND, (**B**) PAK, and (**C**) SA.

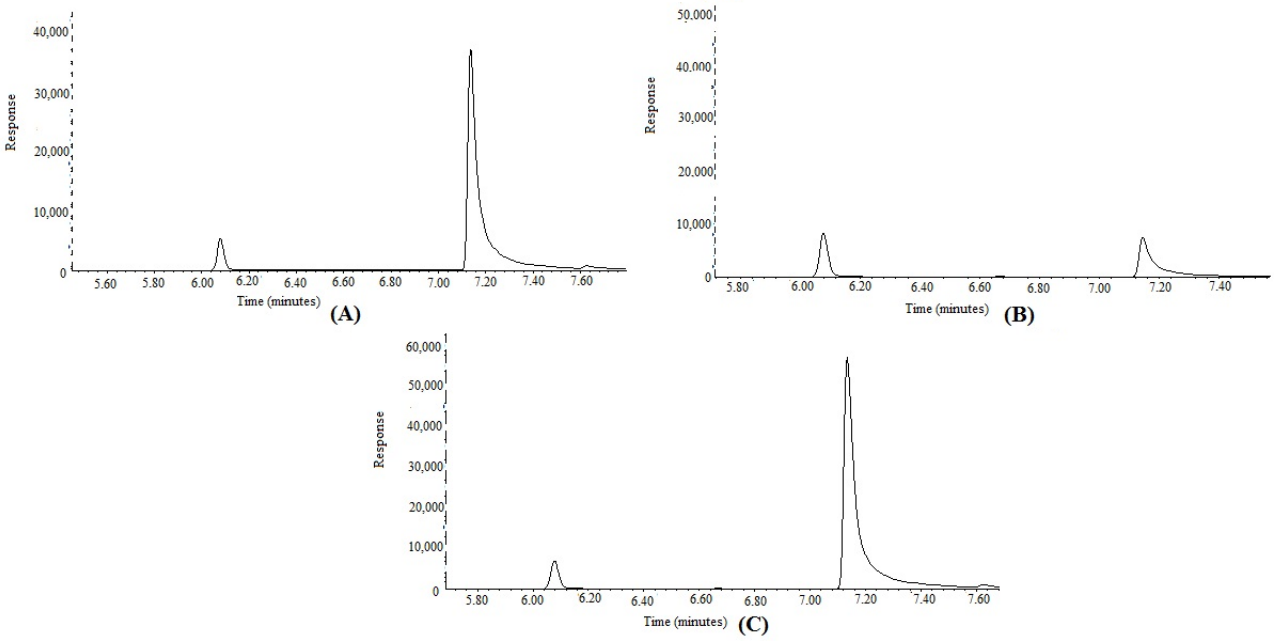

Figure 8. Representative GC-MS peaks of FCO and TOH in essential oils of *F. vulgare* obtained from (**A**) IND, (**B**) PAK, and (**C**) SA.

The amounts of FCO in the essential oils of *F. vulgare* were computed as the maximum in the PAK sample (0.034 ± 0.004 mg/g) followed by the SA (0.029 ± 0.005 mg/g) and IND samples (0.021 ± 0.002 mg/g). However, the amounts of FCO in the methanolic extracts of *F. vulgare* were computed as the maximum in the SA sample (0.057 ± 0.008 mg/g) followed by the IND (0.031 ± 0.002 mg/g) and PAK samples (0.021 ± 0.001 mg/g). The amounts of TOH in the essential oils of *F. vulgare* were computed as the maximum in the PAK sample (14.8 ± 0.621 mg/g) followed by the SA (10.2 ± 0.464 mg/g) and IND samples (7.40 ± 0.342 mg/g). On the other hand, the amounts of TOH in the methanolic extracts of *F. vulgare* were computed as the maximum in the SA sample (1.74 ± 0.018 mg/g) followed

by the PAK (0.498 ± 0.012 mg/g) and IND samples (0.440 ± 0.010 mg/g). Using a GC-MS approach, the amount of FCO in the commercial extract of *F. vulgare* and various commercial formulations containing *F. vulgare* was recorded in the range of 0.002–9.78 mg/g in previous studies [13]. Using an HPTLC approach, the amount of TOH in the essential oils of *F. vulgare* was recorded as 8.82 mg/g, which was lower than the PAK and SA samples but higher than the IND sample [30]. Using an HPTLC approach, the amount of TOH in the traditional methanolic extract of *F. vulgare* was recorded as 6.44 mg/g, which was much higher than the amount recorded in the present case [30]. The amounts of active constituents in plants may vary from time to time. These contents may vary due to various factors, such as temperature, humidity, seasonal variations, and environmental factors. The amounts of TOH were significantly higher in the essential oils of *F. vulagre* compared to its methanolic extracts ($p < 0.05$). The same observation was also seen in previous studies using an HPTLC approach [30]. Hence, the contents of the TOH in *F. vulgare* were in accordance with the literature [30]. These findings suggested the reliability of the proposed GC-MS approach in the simultaneous determination of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare*.

4. Conclusions

The simultaneous determination of FCO and TOH in the essential oils and methanolic extracts of *F. vulgare* derived from IND, PAK, and SA is accomplished using a GC-MS approach that has been established and validated. For the simultaneous determination of FCO and TOH, the suggested GC-MS approach is simple, rapid, accurate, precise, and very sensitive. The quantities of TOH and EOH in the essential oils of *F. vulgare* were found to be substantially greater than in the methanolic extracts. For the simultaneous determination of FCO and TOH in the methanolic extracts and essential oils of *F. vulgare* collected from IND, PAK, and SA, the proposed GC-MS approach has been confirmed to be reliable. These findings show that the proposed GC-MS approach can be used to simultaneously determine the FCO and TOH in a variety of materials, including plant-based pharmaceuticals and commercial formulations containing FCO and TOH as active medicinal components.

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