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Use of Nuclear Magnetic Resonance Spectroscopy in Analysis of Fennel Essential Oil

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Abstract – A simple and rapid method based on proton nuclear magnetic resonance spectroscopy was developed for determination of *trans*-anethole content in fennel essential oil. Spectra of pure *trans*-anethole, of the pure essential oil of fennel, and of the pure oil of fennel with thymol internal standard were recorded. The signal of H-1′ was used for quantification of *trans*-anethole. This proton signal is well separated in the proton magnetic resonance spectrum of the compound. No reference compound is needed and cheap internal standard was used. The results obtained from spectroscopic analysis were compared with those obtained by gas chromatography. Additionally, the developed method was used for determination of the type of vegetable oil used as a carrier in commercial products, which cannot be quantified as such by gas chromatography. This study demonstrates the application of proton nuclear magnetic resonance spectroscopy as a quality control method for estimation of essential oil components.

Keywords – Fennel, Essential oil, *trans*-Anethole, ^{1}H NMR spectroscopy, Carrier oil

Introduction

Fennel (*Foeniculum vulgare* Mill.) is a perennial plant belonging to the family Apiaceae.[1] This plant is well-known for its essential oil which has been widely used for many years as a flavoring agent in food products and as a constituent in many pharmaceutical products. It is a main constituent in "Gripe Water" given to infants in case of colic and gastrointestinal discomfort.[2] Essential oil of fennel has carminative, insecticidal, antioxidant and anti-microbial effects.[3-8] According to the British Pharmacopoeia, two varieties are known for this plant species; bitter and sweet fennel.[9] Bitter fennel, variety *vulgare*, contains not less than 40 ml/kg of essential oil. Sweet fennel, variety *dulce*, contains not less than 20 ml/kg of essential oil. Essential oil of sweet fennel is used in aromatherapy, while essential oil of bitter fennel should not be applied to the skin. Sweet fennel essential oil should be avoided by breast-feeding women, pregnant women and women with endometriosis. This is mainly due to its high content of *trans*-anethole, which shows estrogenic activity at high concentration.[10] Bitter fennel oil contains not less than 60.0 per cent of *trans*-anethole and sweet fennel oil contains not less than 80.0 per cent of *trans*-anethole.[9]

It is important to develop simple and rapid analytical methods for determination of essential oil components in food and pharmaceutical products. Gas chromatography (GC) is the mostly used analytical technique for qualitative and quantitative determination of the marker components in essential oils. It is used for analysis of commercial samples of fennel essential oil depending on *trans*-anethole content.[9] It is also used to determine concentration of estragole in commercial fennel herbal teas to reduce its exposure in infants and breast-feeding women.[11] Although GC is characterized by high sensitivity, it suffers from several drawbacks such as time consumption, need of column selection, problems associated with chromatographic peaks, and need of calibration curve using authentic reference compounds.

In this report, a simple, rapid and easy method based on proton nuclear magnetic resonance spectroscopy was developed for determination of *trans*-anethole content in essential oil of fennel. The result obtained was compared to that obtained by GC. The developed method was used for determination of the type of vegetable oil used as a carrier for the essential oil in commercial products.

Experimental

Chemicals – Deuterated chloroform (99.8%) and thymol

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were obtained from SIGMA-ALDRICH. The reference compound, *trans*-anethole and pure essential oil sample of fennel were generously provided by Dr. Alaa Abdel sattar, Elrazy Pharmaceuticals Co., Cairo, Egypt. Commercial essential oil sample of fennel were obtained from the local market, HARRAZ® (Cairo, Egypt).

^{1}H NMR preparation – ^{1}H NMR samples of *trans*-anethole and pure essential oil sample of fennel were prepared by precisely weighing the specified amounts followed by addition of $CDCl_3$. The internal standard was added to the fennel pure essential oil and the mixture was reconstituted in deuterated chloroform for nuclear magnetic resonance measurements. In case of commercial products of fennel essential oil, the sample preparation was carried out by dilution of the oil with deuterated chloroform.

^{1}H NMR analysis – The NMR spectrometer used was Bruker model AVANCE III HD (Fälladen, Switzerland) and Bruker 400 AEON Nitrogen-Free Magnet, and operating at the basic frequency of 400.13 MHz for ^{1}H. The spectrometer is equipped with direct detection broadband observe (BBO) probe. All NMR measurements were acquired at 298 K (25 ºC). Data is analyzed using Topspin 3.1 software. Chemical shifts (δ) are expressed in ppm with reference to the residual solvent signals. Scalar coupling constants (*J*) are given in Hertz. The following conditions were used for recording of ^{1}H NMR spectra: 30° pulse experiment; acquisition time of 4.1 seconds; relaxation delay 1.0 second; sweep width 15.1 ppm (8012 Hz); data points 65536 and dummy scan 2. The data were processed using line broadening 0.1 Hz. For each sample, 16 scans were recorded. Free induction decays were Fourier transformed with line broadening factor set at 0.1 Hz for resolution enhancement. The spectra were subjected to manual phase adjustment. Peak integration was used for quantitative analysis. All experiments were based on at least triplicate measurements. The content of *trans*-anethole in fennel essential oil was calculated using the equation:

$$W_X = (G_X/G_{IS})\ (M_X/M_{IS})W_{IS}$$

where W_X is the weight of *trans*-anethole, W_{IS} is the weight of internal standard, G_X is the integration value for *trans*-anethole, G_{IS} is the integration value for internal standard, MX is the molecular weight of *trans*-anethole, M_{IS} is the molecular weight of internal standard, and W_{IS} is the weight of internal standard.

Gas chromatography analysis – Gas chromatography analysis was carried out by an Agilent 6890 N Network gas chromatograph system (Agilent Technologies, USA). An Agilent 19097J-413 (30 m × 0.32 mm) capillary column

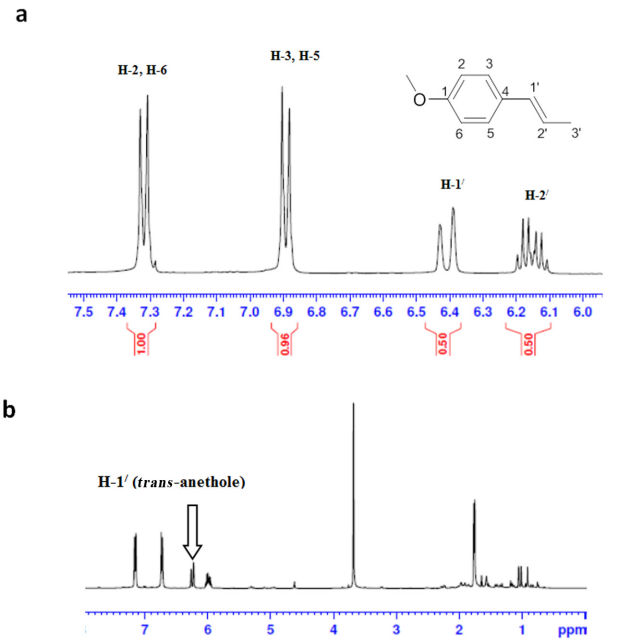

Fig. 1. ^{1}H NMR spectra of *trans*-anethole (a) and of pure essential oil of fennel (b).

with a 0.25 µm film thickness was used with nitrogen as the carrier gas at a flow rate of 30 ml/min. The GC oven temperature was programmed at an initial temperature of 70 ºC for 2 minutes, then heated up to 190 ºC at 4 ºC/min and held at 140 ºC for 10 minutes. Injector temperature was set at 250 ºC and detector temperature (flame ionization detector) was set at 280 ºC using hydrogen gas flowing at 30 ml/min and air flowing at 300 ml/min. The identification of *trans*-anethole peak was determined by their GC retention time compared with authentic reference compound.

Result and Discussion

Quantitative nuclear magnetic resonance (qNMR) bears a great potential in quantitative analysis of natural products.[12]As an example, *trans*-anthole content in fennel essential oil, which is typically de termined by gas chromatography, was analyzed using qNMR. The ^{1}H NMR spectrum of *trans*-anetholere ference standard is shown in Fig. 1(a). The spectrum shows two sets of two chemically equivalent aromatic protons, predicted from the integration values, resonating at δ 7.32 and 6.89 ppm. The two protons H-2 and H-6, close to the inductive electron withdrawing effect of the oxygen atom were assigned to signal at δ 7.32 (2H, d, J = 8.8, H-2 & H-6). The other two aromatic protons were assigned to signal at δ 6.89 (2H, d, J = 8.8, H-3 & H-5). The two olefinic protons of

Table 1. ^{1}H NMR spectral data of *trans*-anethole in $CDCl_3$ (δ ppm)

Position	δ (multiplicity, *J* in Hz)
2,6	7.32 (2H, d, *J* = 8.8)
3,5	6.89 (2H, d, *J* = 8.8)
1-OCH_3	3.84 (3H, s)
1'	6.41 (1H, d, *J* = 15.6)
2'	6.15 (1H, qd, *J* = 6.8, 15.6)
3'	1.92 (3H, dd, *J* = 1.6, 6.8)

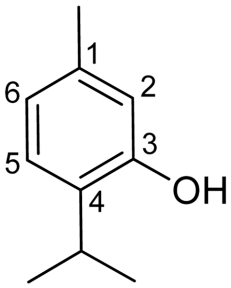

Fig. 2. Chemical structure of thymol.

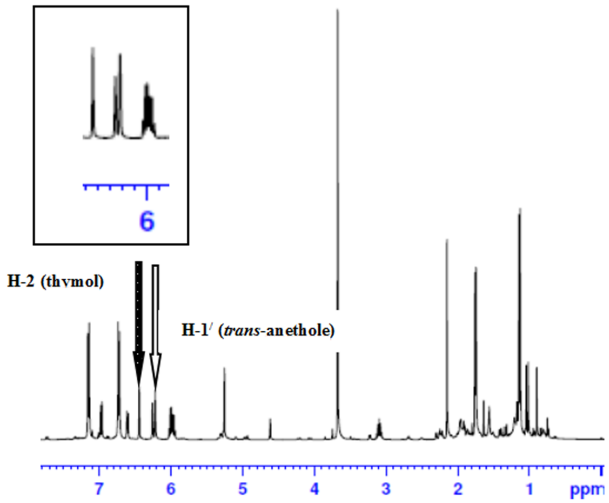

Fig. 3. ^{1}H NMR spectrum of pure essential oil of fennel and thymol added as an internal standard. The inset shows the H-2 of thymol used as a reference peak for analysis.

the propenyl moiety resonated at δ 6.41 (1H, d, *J* = 15.6, H-1') and δ 6.15 (1H, qd, *J* = 6.8, 15.6, H-2'). Table 1 shows chemical shift and coupling constants of ^{1}H NMR signals assigned to its correspon- ding positions in the structure of *trans*-anethole. The signal of H-1' was selected as a target peak for quantitative analysis. This signal is in a region where there is no interference with other signals in the ^{1}H NMR spectrum of fennel essential oil (Fig. 1(b)). Signals of *trans*-anethole can be easily distinguished in the spectrum of the oil because it represents the major component of the oil.

Thymol was used as an internal standard (Fig. 2). H-2 of thymol was detected as a singlet peak at δ 6.44. It is well separated from signals of the other compounds. This signal was used as a reference peak of the internal standard for analysis (Fig. 3). Samples of pure essential oil of fennel (36 - 54 mg) were analyzed for the *trans*-anethole content by ^{1}H NMR using thymol as an internal standard. The content of *trans*-anethole in pure essential oil of fennel using the developed ^{1}H NMR method was 63.80 ± 5.58 per cent. This complies with the standard of the British Pharmacopoeia for bitter fennel.[9] The result obtained was compared to that obtained by GC analysis. Fig. 4 shows the GC chromatogram of pure essential oil of fennel. The chromatogram revealed the presence of 28 components in the oil. The peak of *trans*-anethole (t_R = 19.4 min) was identified by comparison with chromatogram of reference standard of this compound. *trans*-anethole accounted for 64.41 ± 4.55 percent in the pure essential oil of fennel. This result is comparable to that obtained by the developed ^{1}H NMR method. The developed spectroscopic method can be used for quality control purpose specifically in determination of the plant variety from which the oil was extracted. No reference compound is needed and cheap internal standard was used.

There are many studies reporting the use of different spectroscopic methods in analysis of essential oil for many aromatic plants. For example, Raman spectroscopy was used to differentiate essential oils of the sweet and bitter fennel.[13] The Raman spectra of the dominant substances *trans*-anethole and fenchone were used for comparison. Infrared spectroscopy was employed in assessment of essential oil components and identification of individual essential oils of *Cinnamomum zeylanicum*, *Cinnamomum camphora*, *Lippia multiflora*, *Ravensara aromatica*, and *Syzygium aromaticum*.[14] ^{13}C NMR was used as a complementary tool in identification of essential oil constituents.[15] Analysis of essential oils using ^{13}C NMR was used to obtain direct information about the molecular structure and functional groups of the constituents without preliminary separation.[16] The essential oil of *Chenopodium ambrosioides* was analysed by GC, GC-MS and ^{13}C NMR.[17] This allowed estimation of the actual content of ascaridole which undergoes thermal degradation to isoascaridole and therefore underestimated when analyzed by GC. ^{1}H- and ^{13}C NMR spectroscopy were used in analysis of chemical composition of essential oil of pine needles grown in different regions of Belarus.[18] Moreover; qNMR was also used for characterization of standard pure compounds isolated from essential oils.[19]

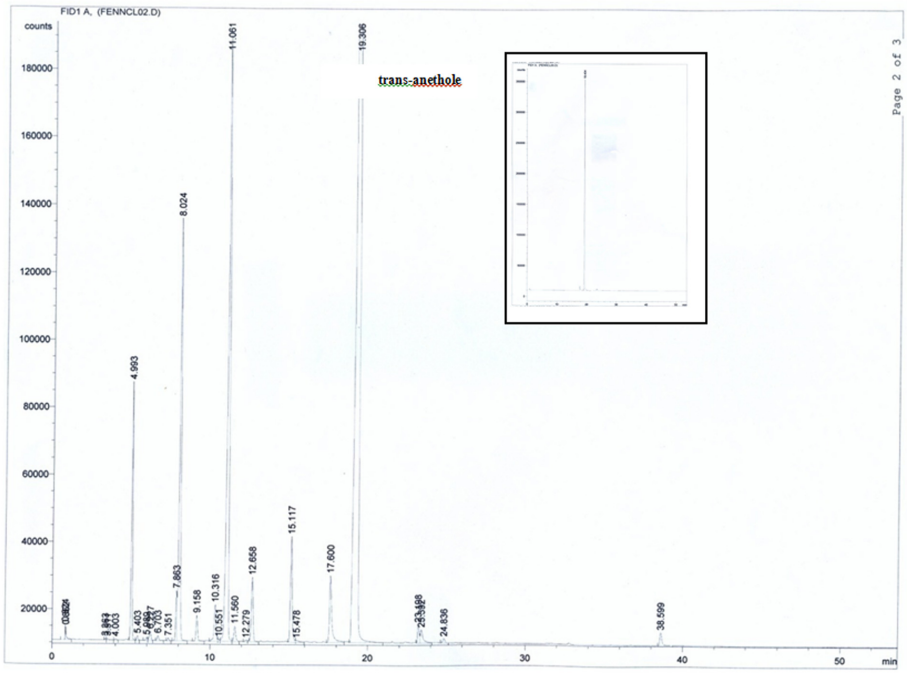

Fig. 4. GC chromatogram of the essential oil of fennel. The inset shows analysis of authentic reference standard of *trans*-anethole.

Essential oils intended for external use are usually diluted with carrier or base oils. The carrier oils are fixed oils such as almond, olive, sunflower or sesame oils. They mainly help in spreading of the essential oil on the surface of skin and prevent exposure of the skin to high concentration of the essential oil constituents. Fixed oils are mainly composed of esters of fatty acids with glycerol. Since these oils are not volatile; they can't be analyzed by GC without derivatization. Other techniques including chromatographic, spectroscopic and isotopic techniques have been used for analysis of fixed oils. For example, ^{1}H- and ^{13}C NMR have been used in authentication of olive oil.[20-21] The ^{1}H NMR spectrum of olive oil can be explained as clusters of signals due to chemical similarity of different triglyceride esters in the oil. The technique was used for investigating adulteration of olive oil with sunflower oil or hazelnut oil.[22]

In case of essential oil of fennel, commercially available products in Egypt are usually diluted with sunflower oil (personal communication). Sunflower oil is distinguished from other fixed oils by a very high content of linoleic acid.[23] Moreover, it is characterized by absence or low content of linolenic acid. ^{1}H NMR spectrum of a commercial sample of fennel essential oil was recorded (Fig. 5). Tentative elucidation of the spectrum showed that signals due to the carrier oil (fixed oil) dominated the spectra. Signals of the essential oil components including the target analyte *trans*-anethole appear as minor ones.

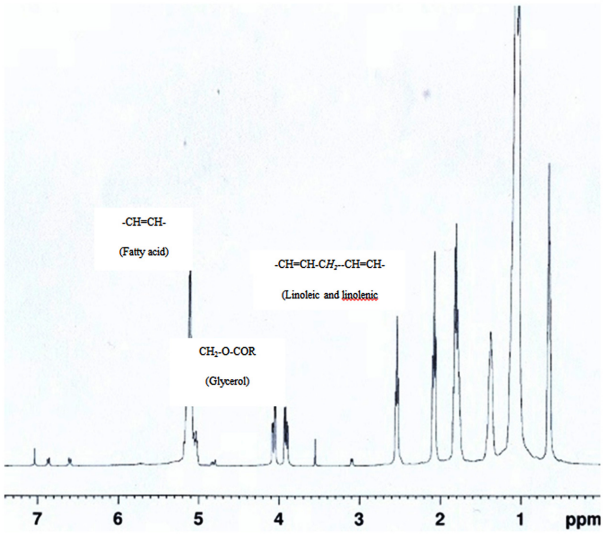

Fig. 5. ^{1}H NMR spectrum of a commercial sample of essential oil of fennel.

The same results were obtained with other commercial oil samples analyzed using the developed spectroscopic method even after using high concentration of the oil (ca. 50 mg dissolved in the deuterated solvent). Qualitative characterization of the carrier oil was possible, as ^{1}H NMR spectrum of the commercial sample showed that sunflower oil was used for dilution of the essential oil. This was clearly observed by the high intensity signals at δ 5.15 and 2.54 ppm due to linoleic acid. In addition, the spectra showed absence or minor triplet signal due to

linolenic acid that usually appear at δ 0.95 ppm.[22]

In conclusion, a simple and rapid method was developed for determination of the amount of *trans*-anethole in the essential oil of fennel based on ^{1}H NMR spectroscopy. The method would be useful in quality control procedure of fennel essential oil. In particular, it can be used for determination of source of fennel fruits from which the oil was extracted. The developed method allowed for the characterization of the carrier oil in commercial fennel essential oils.

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