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Determination of Estragole in Pharmaceutical Products, Herbal Teas and Herbal Extracts Using GC-FID

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ABSTRACT

Recent concerns about the potential carcinogenicity of estragole necessitate accurate determination of estragole in different products and extracts. GC-MS method has been used for characterization of Fennel extract; Estragole was found to constitute more than 65% of the extract. Accurate and simple method for the determination of estragole in pharmaceutical products, herbal teas and herbal extracts was developed using GC-FID technique and p-anisaldehyde as an internal standard. Target analyte was extracted from different matrices by applying various extraction procedures such as hydro-distillation and ultrasound-assisted extraction. The hydro-distillation technique provided higher amounts of extracted estragole compared to ultrasound-assisted extraction. The calibration curve showed excellent linearity over a concentration range of 0.1–10 mg/ml with a correlation coefficient of 0.9997. Accuracy of back calculated calibration standards were within ±7.3 %. Precision and accuracy of quality control samples were within ± 9.0 %. Estragole levels were accurately measured in Fennel fruits, Chinese and Japanese Star Anise, Sekem® teabags for cough, Baby Calm® teabags, Balsam®, Guava® syrup for cough and Aqua ream® syrup for diarrhea. Accurate concentrations of estragole should replace the non-specific label information found on most of the tested products.

INTRODUCTION

Accurate measurement of active constituents in herbal medicine and pharmaceutical products that contain medicinal plants is a mandatory requirement in the field of pharmaceutical industry (Kunle *et al.,* 2012). However, the chemical composition of herbal medicines is complicated and active components are infrequently identified. On the other hand the amounts of active components in herbal medicines are variable according to environmental conditions, harvest time, storage condition and processing methods, also the extraction procedures used to isolate the active components strongly affect the quantities of in the final extracts (Chun *et al.*, 2011). Essential oils are widely used in food, cosmetics, fragrances, herbicides, insecticide and also in traditional medicine as digestives, diuretics, expectorants and sedatives. They are available as infusions, tablets and syrups and related to many biological effects such as antioxidant, anti-inflammatory, antiviral, antibacterial and CNS stimulants (Fornari *et al.*, 2012).

Estragole (4-allyl anisole,1-methoxy-4-enylbenzene) is a naturally occurring compound which can be extracted from Anise, Star Anise, Fennel and Basil. Flavors and fragrances containing estragole are used in food products, perfumes, soaps and detergents. The Flavor and Extract Manufacturers Association (FEMA) noted that the estimated daily exposure of estragole in USA was found to be 70 µg/ capita consumption (McDonald 1999). Anise and Star Anise (also known as Anise seed, Sweet Cumin Illicium, Chinese Anise, Chinese Star Anise) contain about 1-4% volatile oil, estragole constitutes the main component. Anise and Star Anise oils are used in pharmaceuticals and cosmetic products, they also used as stimulants and expectorants. Star Anise is used in Chinese Medicine for the same biological properties.

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Fennel (*Foeniculum vulgare Mill. Var. Vulgare. Apiaceace*) contains about 2-6 % volatile oil which contains trans-anethole and estragole, it is used in flavor foods, perfumes and at home to relieve gastrointestinal symptoms, flatulence, colic spasm and respiratory tract symptoms. It is widely used for making herbal liqueurs by distillation process (Rodríguez-Solana *et al.,* 2014) Several methods have been developed for determination of estragole; Stir Bar Sorptive Extraction (SBSE) and GC/MS method have been reported for quntification of estragole in commercial fennel herbal teas (Raffo *et al.,* 2011). Soxhlet and accelerated solvent extraction (ASE) have been used for extraction of essential oil from Fennel (Rodríguez-Solana *et al.,* 2014). Solvent extraction using dichloromethane has been reported for extraction of estragole in Fennel tea followed by GC-MS analysis (Zeller and Rychlik 2006). The effect of using different internal standards on the analytical method performance for estragole has been studied, results have concluded that using p-propyl anisole as an internal standard provided accurate results comparable to those obtained from other methods using stable isotopically labeled internal standard (Zeller *et al.,* 2009). HPLC method using different microextraction procedures have been applied for extraction of estragole and other bioactive compounds from different plant extracts and human urine (Rajabi *et al.,* 2014). GC-MS method has been developed and validated for determination of estragole and other flavoring compounds in semi-solid, dry solid, fatty solid and liquid food (Lopez *et al.,* 2015). GC-FID method has been applied for the determination of estragole, safrole and eugenol methyl ether in food products using a simultaneous distillation extraction (SDE) technique (Siano *et al.,* 2003). Estragole has been demonstrated to be genotoxic and carcinogenic in experimental animals; reductions in exposure and restrictions in use levels as a flavoring substance have been recommended by the Scientific Committee on Food as there is no safe exposure limit has been established (Raffo *et al.,* 2011). Addition of estragole as flavoring agent to food products has been prohibited in the recent European Regulation on Flavoring, the maximum levels of estragole has been established in certain naturally occurs compound (Raffo *et al.,* 2011). Estragole was listed as generally recognized as safe (GRAS) by Expert Panel of the Flavor and Extract Manufacturers' Association (Hall and Oser 1965). In 2000, the Committee of Experts on Flavoring Substances (CEFS) of the Council of Europe described estragole as a naturally occurring genotoxic carcinogen in experimental animals after either chronic exposure or few repeated doses (Demyttenaere 2015). The estragole metabolite carcinogen (1'-Hydroxyestragole) has been found also in the urine of men dosed with 1 µg/kg (Sangster *et al.,* 1987). Estragole is considered a rodent liver carcinogen which requires bio-activation. The mechanism of its carcinogenicity in rat has been reported (Ding *et al.,* 2015). The level of estragole varied according to the origin, harvesting and processing method; estragole was found to constitute 12.36 % of the wild fennel extract. However, the estragole content in the cultivated one was found to be 65.3% (Abd-Allah *et al.,* 2015). An UHPLC method and a safety assessment using the Margin of Exposure (MOE) approach have been reported for the analysis of estragole in dry fennel preparations and in infusions. The obtained MOEs have indicated a priority for risk management for children (van den Berg *et al.,* 2014). It was noted that some of the widely used pharmaceutical products and tea bags containing Fennel and Anise extracts have un-specific label information about the estragole content. The aim of this work is to develop a simple and accurate method for determination of estragole in different materials such as dried fruits, tea bags and pharmaceutical products. Chemical structures of estragole and p-anisldehyde are shown in Figure 1

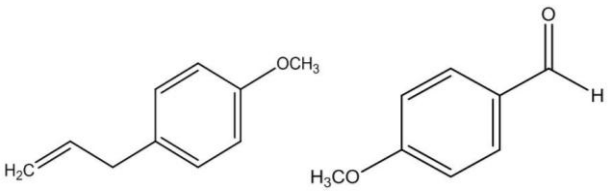

Fig. 1: Chemical structures of (a) Estragole, (b) P-anisaldehdye.

EXPERIMENTAL

Materials and reagents

Estragole (4-allyl anisole,1-methoxy-4-enylbenzene; 98.9%) and P-anisldehyde (4-methoxybenzaldehyde; 99%) were purchased from Acros Organics Chemical, part of Thermo Fisher Scientific (Pittsburgh, PA, USA). Dried Fennel Fruits (*F.Vulgare Miller*) and dried Star Anise Fruits (*Illicium Verum Hook. Fil)* (Chinese) and *(Illicium Anisatum Hook Fil*) (Japanese) were purchased from the local market. Sekem® teabags for cough which labeled to contain (Thyme, Fennel, Salvia, Anise, Liquorice, Melissa and Guava), Baby Calm® teabags which labeled to contain (0.225 g Chamomile, 0.825 g Fennel, 0.225 g Anise and 0.225 g Caraway), Balasm® syrup for children which labeled to contain (41.70 mg Guava leaf extract, 83.33 mg Thyme leaf extract, 83.33 mg Tilia leaf extract, 400 mg Honey and 0.33 mg Fennel oil), Guava® syrup which labeled to contain (Extracts of Guava leaves, Tilia Flowers and Fennel oil) and Aqua ream® syrup which labeled to contain (1 mg Caraway oil, 2.2 mg Dill oil, 1.5 mg Fennel oil and 0.5 mg Ginger oil) were obtained from the local pharmacy. n-hexane, methanol, di-ethyl ether and methylene chloride (HPLC grad) were purchased from Thermo Fisher Scientific (Waltham, MA USA).

Preparation of stock solutions, calibration standards and quality control samples

A 50 mg/ml stock solution of estragole (4-allyl anisole) was prepared in n-hexane and stored in an amber colored glass bottle at 4 °C. Internal standard stock solution of p-anisaldehyde was prepared at a concentration of 1.0 mg/ml in n-hexane and stored in an amber colored glass bottle at 4 °C. The calibration standards were prepared by appropriate dilution of stock solution at concentrations of (0.1, 1.0, 2.0, 4.0, 7.0 and 10 mg/ml) in n-hexane. Quality control (QC) samples were prepared by appropriate dilution of stock solution at concentrations of (0.1, 2.0, and 10 mg/ml) in n-hexane.

Sample Preparation

Ultrasound assisted extraction

The Ultrasound assisted extraction procedure was carried out as described before (Gursale *et al.*, 2010). A 10 ml of extracting solvent (n-hexane, di-ethyl ether, methylene chloride or methanol) was added to 1 gm dried powdered fennel fruit in a stoppered tube and the contents of the tube were sonicated in an ultrasonic bath for 15 min at room temperature and were filtered through a Whatman® filter paper (pore size, 44µm). For Sekem® and Baby Calm® teabags; the contents of 10 teabags of each brand were mixed well and a quantity equivalent to one teabag was extracted as described above.

Hydro-distillation using Clevenger apparatus

Clevenger apparatus (Clevenger 1928) set up was as shown in Figure 2. A 500 gm of each dried powdered fruit (Fennel, Chinese or Japanese Star Anise fruits) was added into the round flask and completed up to half by distilled water; the distillation flask was resettled on heating mantle then heated slowly. Volatile oil was obtained after about 3 hours of distillation; oil was separated and dried on anhydrous sodium sulfate and was kept in an amber glass bottle at 4 °C. For Sekem® and Baby Calm® teabags; the contents of 10 packages of each brand were mixed well and extracted as described above. For Guava®, Aqua ream® and Balsam® syrups; the contents of 10 bottles of each brand were mixed well and extracted as described in section above; a 5 ml n-hexane was added to facilitate the oil separation. Oil extracts were dried under nitrogen gas to remove n-hexane.

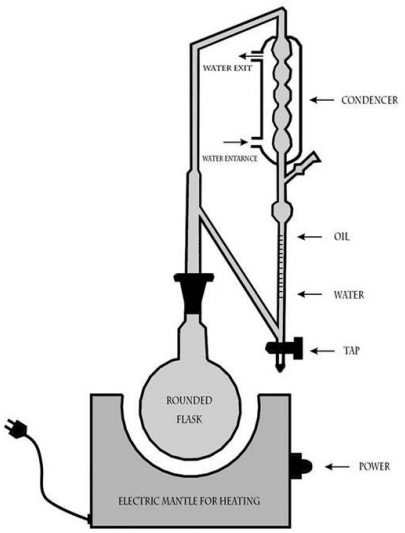

Fig. 2: Schematic diagram of Clevenger apparatus set up used for hydro-distillation.

GC-MS analysis

GC-MS analysis was carried out on an Agilent Gas Chromatograph equipped with an Agilent Mass Spectrometer (Agilent Technologies Inc. Palo Alto, CA, USA), with a direct capillary interface and fused silica capillary column PAS-5ms (30m x 0.32mm x 0.25µm film thickness) (Agilent Technologies Inc. Palo Alto, CA, USA). Samples were injected under the following conditions; Helium was used as carrier gas at approximately 1ml/min, pulsed splitless mode. The solvent delay was 3 min and the injection size was 1.0 µl. The mass spectrometer was operated in electron impact ionization mode at 70 e.V; scanning from m/z 50 to 500. The ion source temperature was 230 °C and the quadrupole temperature was 150 °C. The electron multiplier voltage (EM voltage) was maintained at 1250 V above auto tune. The instrument was manually tuned using Perfluorotributylamine (PFTBA). The GC temperature program was started at 40 °C for 2 min then elevated to 280 °C at a rate of 8 °C /min and 10 min hold at 280 °C. The detector and injector temperatures were set at 280 and 250 °C, respectively. Wiley and NIST 05 mass spectral data base was used in the identification of the separated peaks.

GC-FID Analysis

GC-FID analysis was performed on a Thermo Scientific TRACE™ GC Ultra Gas Chromatograph equipped with FID detector (Thermo Scientific, Waltham, MA, USA) and TR-WAXMS capillary column (30 m x 0.25 mm x .025 µm film thickness) (Thermo Scientific, Waltham, MA, USA) working with the following temperature program: 40 °C for 5 min, rising at a rate of 10 °C /min to 240 °C then hold for 2 min at 240 °C, injector and detector temperatures were 280 °C, carrier gas helium (He) with splitless and hydrogen (H_2) gas for flame FID, The injection volume was 1µl and the run time was 27 minute.

Quantification of estragole using GC-FID

Calibration curve was constructed at six calibration curve levels (0.1, 1.0, 2.0, 4.0, 7.0 and 10 mg/ml). Peak area ratios of analyte to internal standard were plotted vs. the concentrations. Linearity was assessed through evaluation of correlation coefficient (r). The back calculated concentrations should be within ± 15% of the nominal concentrations for all levels and within ± 20 % at the lower limit of quantitation (LLOQ) level. The precision and accuracy were evaluated at three levels; low, mid and high QC (0.1, 2.0, and 10 mg/ml). Each sample was analyzed six times; precision and accuracy should be within ± 15 % for all levels and within ± 20 % at the low QC level. LLOQ was considered the lowest concentration that can be measure with acceptable preciosn and accuracy, and the Limit of detection (LOD) was measured as the lowest concentration that can be detected at signal to noise ration of 3:1. Selectivity is established by the ability of the method to accurately determine the target analyte and internal stanadrd in the presence of different matrices components.

RESULTS AND DISCUSSION

Characterization of fennel extract using GC-MS

GC/MS analysis of fennel extract showed more than 45 isolated components along with the percentage of each component in the extract, estragole was found to constitute more than 65% of

the isolated components; retention times and percentages of the main isolated components are shown in table 1. It has been reported that the level of estragole varied according to the origin, harvesting and processing method; estragole has been found to constitute only 12.36 % of the wild fennel oil extract in comparison to 65.3% of the cultivated one (Abd-Allah *et al.*, 2015).

Table 1: Characterization of Fennel fruits extract using GC-MS.

R.T	Compound	%
5.48	Alpha-pinene	1.25
5.72	Alpha-pinene	1.59
7.48	dl-limonene	7.10
8.48	Alpha-fenchone	2.67
11.18	**Estragole**	**65.29**
12.41	Carvone	1.43
12.81	Trans-anthole	15.51
13.75	Isoeugenol	0.13
13.9	Alpha-copaene	0.13
14.16	Methyl eugenol	0.57
14.56	Trans-caryophellene	0.28
15.44	Cubebene Beta	0.43
15.54	Zingiberene	0.15
15.96	Myristicine	0.65
	Other compounds	2.82

GC-FID Method development

GC temperature program has been optimized to obtain excellent peak shape for both estragole and internal standard in a 27 min run time Figure 3. Retention times and peak shape were consistent and reproducible through the study; estragole was eluted at 20.62 ± 0.04 min (%RSD 0.2%). Ultrasound assisted extraction is known to be cheap, simple and efficient method in addition to; producing high extraction yield (Shah andMinal 2013), different extracting solvents such as methylene chloride, diethyl ether, methanol and n- hexane have been tested and compared in terms of peak area ratios of analyte to internal standard.

Estragole has been successfully extracted from fennel fruits. However, the yield of the extraction was very low in comparison to the hydro-distillation procedure; 5.2 mg/gm of dried powdered fennel fruit was obtained using n-hexane as an extracting solvent. Hydro-distillation is one of the most recommended methods for the extraction of volatile oil from plant sources. It is considered a simple form of steam distillation which is often used to isolate insoluble, low and high boiling point natural products, the target components can be distilled at a temperature lower than 100 ºC (Clevenger 1928).

Although, this method is considered a time consuming procedure in comparison to other simple extraction procedure, however, high yield enable scientist to accurately measure the target analyte. Hydro-distillation method using Clevenger apparatus (Figure 2) has been used for extraction of estragole from different matrices and has been successfully used to isolate estragole from teabags and pharmaceutical syrups; it showed approximately three folds higher yield than ultrasound assisted extraction. Different extraction parameters such as sample weight, water volume, distillation time and temperature have been optimized to maximize the extraction recovery.

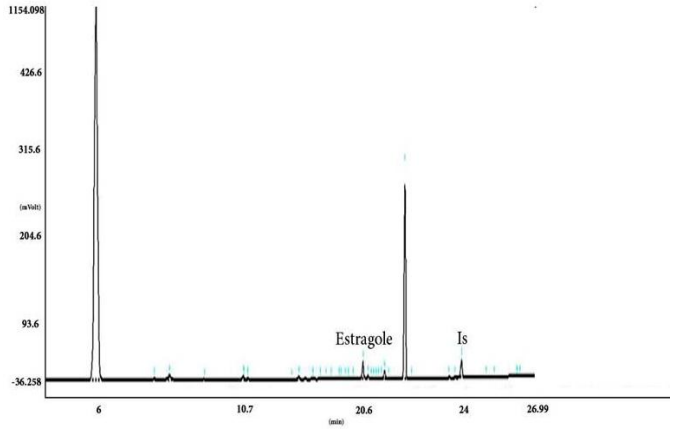

Fig. 3: GC-FID chromatogram of estragole and P-anisaldehdye in Gauva syrup.

Quantification of estragole using GC-FID

Linearity

Excellent linearity was obtained over a concentration range of 0.1-10 mg/mL with a correlation coeffecient of 0.9997 (Figure 4). The accuracy was reperesented as percent differennce from nominal values (%DFN) and precision represented as percent realtive standard deviation (%RSD). The accuracy of back calculated calibration standards (%DFN) was within ±7.3% (Table 2). LOQ and LOD were 0.1 and 0.03 mg/ml, respectively. Precision and Accuracy of quality control samples (n=6) were within ± 8.9 % and ± 3.4%, respectively.

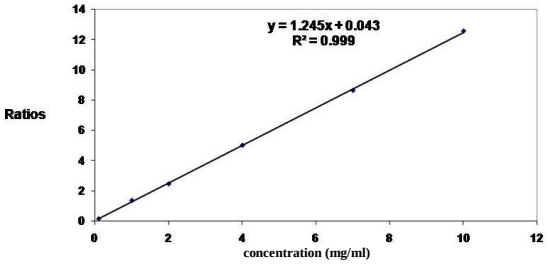

Fig. 4: Calibration curve of standard estragole.

Table 2: Calibration parameters for estragole.

Parameters	Estragole
Linearity range (mg/ml)	0.1-10
Slope	1.245
Intercept	0.0436
Correlation coefficient (r^2)	0.9997
% DFN	≤ 7.3 %

Selectivity

Selectivity of the analytical method is established by the ability of the method to accurately determine the target analyte in the presence of other matrix components. The proposed method was applied for determination of estragole in different matrices. Estragole and internal standard peaks were chromatographically resolved from all other observed peaks. No interference or co-elution was observed from different matrices.

Table 3: Estragole content in tested samples (n=3).

Sample	Determined concentration Mean ± SD	Label information
Fennel fruits	16.74 ± 0.42 mg/g	------
Chinese Star Anise fruits	0.166 ± 0.0001 mg/g	------
Japanese Star Anise fruits	0.0074 ± 0.0002 mg/g	------
Sekem® teabags for cough	0.052 ± 0.005 mg/teabag	Thyme, Fennel, Salvia, Anise, Liquorice, Melissa, Guava
Baby Calm® teabags	0.093 ± 0.004 mg/teabag	0.225 g Chamomile, 0.825 g Fennel, 0.225 g Anise, 0.225 g Caraway
Guava® syrup	0.0167 ± 0.0007 mg/5 ml	Extracts of Guava leaves, Tilia Flowers, Fennel oil.
Aqua ream® syrup	0.0117 ± 0.0005 mg/5 ml	1 mg Caraway oil, 2.2 mg Dill oil, 1.5 mg Fennel oil, 0.5 mg Ginger oil
Balsam® for children syrup	0.121 ± 0.0026 mg/5 ml	41.70 mg Guava leaf extract, 83.33 mg Thyme leaf extract, 83.33 mg Tilia leaf extract, 400 mg Honey, 0.33 mg Fennel oil

Applications

The optimized GC-FID method has been applied for quantification of estragole extracted from plant sources, teabags and pharmaceutical products using P-ansialdyhde as an internal standard.

Fennel fruits, Chinese and Japanese Star Anise fruits

Home-made infusions using dried plant sources such as Fennel fruits and Star Anise are very popular worldwide and are considered healthy and caffeine free drinks. However, presence of estragole in these plant sources necessitates accurate assessment of using theses drinks at home; especially for new born, infant and children. On the other hand; variations in the preparation techniques such as using powdered of intact fruits, boiling time, quantity of dried fruits per water cup and number of cubs per day will produce various quantities of estragole. In addition to, age and weight of consumers. Table 3 shows the quantities of estragole isolated from Fennel, Chinese and Japanese Star Anise fruits after extraction with the optimized hydro-distillation procedure; each one tablespoonful (~1 gm) contains 16.74, 0.166, 0.0074 mg of estragole, respectively, the level of estragole in Chinese Star Anise was about 100 times higher than that in the Japanese one, Figure 5 shows the determined estragole in Japanse Star Anise, excellent peak shape was observed in all tested plant source samples; target analyte was chromatographically resolved from all other matrices peaks.

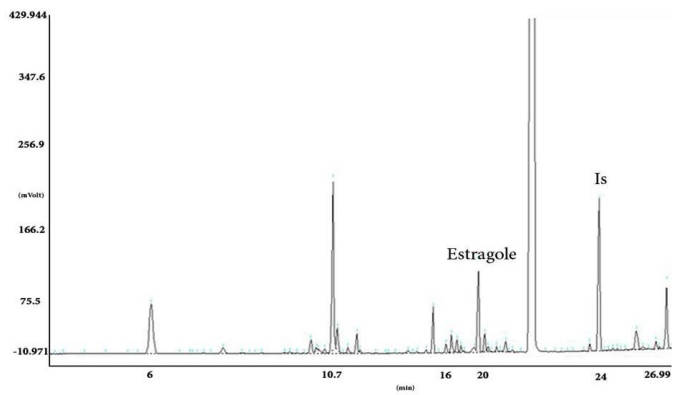

Fig. 5: GC-FID chromatogram of estragole and P-anisaldehdye in Japanse star anise.

The high quantities of estragole which has been isolated from Fennel fruits indicated that; increasing the public awareness of benefits and risks of using Fennel fruits in food, home-made infusion and other natural products recipes is mandatory.

Sekem® teabags for cough and Baby Calm® teabags

Sekem® teabags are widely used in the Egyptian market for the treatment of cough in adults, each bag contains (Thyme, Fennel, Salvia, Anise, Liquorice, Melissa and Guava), the manufacturer label information are non-specific and do not indicate either the quantity of each component or the estragole content. By applying the proposed GC-FID method; each Sekem® teabag was found to contain 0.052 mg of estragole. Baby Calm® teabags are used for the treatment of GIT symptoms in newborn and infant, each bag contains (0.225 g Chamomile, 0.825 g Fennel, 0.225 g Anise and 0.225 g Caraway); as shown in the manufacturer label; it describes the quantity of each component, however, this information still incomplete due to the lack of the accurate or even estimated estragole content; Baby Calm® teabag was found to contain 0.093 mg of estragole. As shown in Table 3. The results of the proposed GC-FID method showed more specific and accurate description of the estragole content per each teabag which could be used as a guide for specify the recommended dose (teabag/day) for both adults and children and Figure 6 shows the determined estragole in sekem teabags; no interferences or co-elution were observed in both Baby Calm® and Sekem® extracted teabags samples

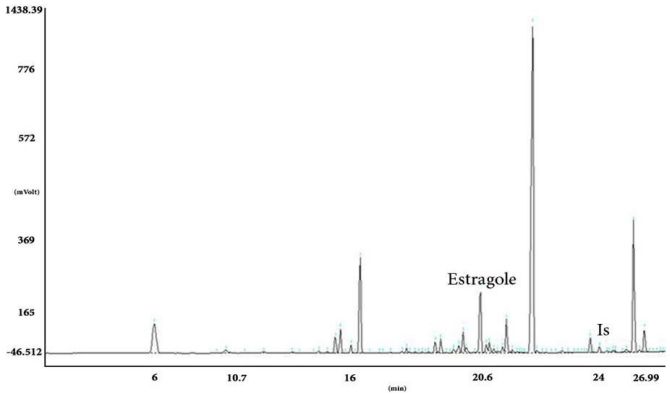

Fig . 6: GC-FID chromatogram of estragole and P-anisaldehdye in Sekem teabags.

Guava®, Aqua ream®, Balsam® for children syrup

Many of pharmaceutical products contain active constituents from plant sources have non-specific labeling as well.

Accurate determination of estragole quantities in pharmaceutical preparations is mandatory and more critical than dried plant sources or teabags, as the content of each pharmaceutical product dose will be introduced to the human body in full and inaccurate estimation of the estragole contents may lead to exceeding the Margin of Exposure (MOE).

Balasm® syrup for children contains (41.70 mg Guava leaf extract, 83.33 mg Thyme leaf extract, 83.33 mg Tilia leaf extract, 400 mg Honey and 0.33 mg Fennel oil) is used as antitussive and expectorant for children, the recommended dose according to the manufacturer is 5-10 ml three times daily, estragole content was found to be 0.121 mg/5ml. Guava® syrup contains (Extracts of Guava leaves, Tilia Flowers and Fennel oil) is a cough suppressant, used for both adult and children; the recommended dose according to the manufacturer is 2.5-5 ml/2-3 times daily for children and 5-10 ml/2-3 times daily for adults. The label information of Guava® syrup is also non-specific, the determined concentration of estragole was 0.0167 mg/5ml. Aqua ream® syrup contains (1 mg Caraway oil, 2.2 mg Dill oil, 1.5 mg Fennel oil and 0.5 mg Ginger oil) is prescribed for infant and children in case of acute and persistent diarrhea, the recommended dose according to the manufacturer is 2.5-5 ml three times daily. The determined estargole concentration was 0.0117 mg/5 ml. Table 3 shows the determined concentrations of estragole in different tested samples. Figure 7 shows the estragole in Aqua ream syrup excellent peak shape was observed in all samples; no interferences from either other components or placebo were observed.

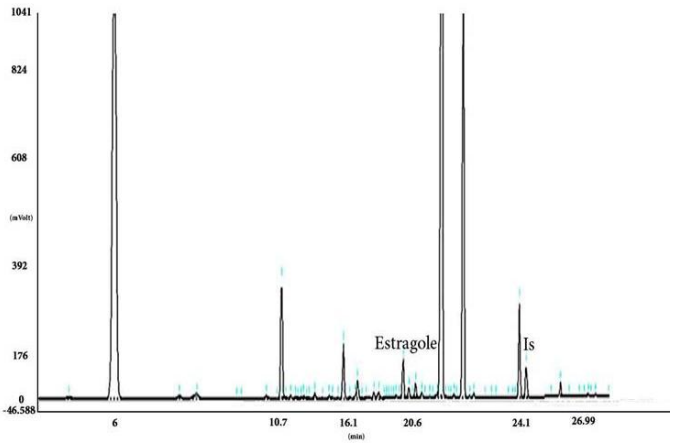

Fig. 7: GC-FID chromatogram of estragole and P-anisaldehdye in Aqua ream syrup.

CONCLUSIONS

A simple GC-FID method for determination of estragole in different matrices and in pharmaceutical products has been developed. Ultrasound assisted extraction using different extracting solvents have been tested for isolation of estragole from dried fruits and teabags. However, Hydro-distillation method showed higher yields and has been used for extracting estragole form different matrices. The quantities of estragole in widely used products with (non-specific manufacturer label information) have been accurately measured and reported. GC/MS analysis of fennel extract showed that estragole constituted more than 65% of the isolated components. The exposure of estragole in infant, young children, pregnant and breastfeeding women need more assessment. Labeling and consumption of products that contain naturally occurring compounds such as estragole need extensive investigation and clear dietary and pharmaceutical regulatory guidelines.

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Conflict of Interests: There are no conflicts of interest.

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