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Article

Preliminary Investigation of Medicinal Herb Adulteration Using Comprehensive Two-Dimensional Gas Chromatography and Chemometric Analysis

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Anise is frequently found among the ingredients described in the label of cosmetic products. However, the use of fennel instead of anise may occur due to the lower price of fennel. The main differences in volatile profile of anise and fennel fruits were, for the first time, evaluated using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/TOFMS) and chemometric analysis. Aproximately 950 peaks were found in chromatograms of each sample and Fisher ratio appointed 31 volatile analytes as the most discriminating between anise and fennel samples. These 31 compounds were used in principal component analysis and canonical discriminant analysis that designated seven compounds (estragole, methyl eugenol, 4,5-dehydro-isolongifolene, calamenene, linalool, β-ocimene and fenchol) as the most important to verify the use of fennel or anise in personal care products. Among these seven compounds, thrree coeluted with other compounds and were correctly identified only with the use of GC×GC/TOFMS.

Keywords: *Foeniculum vulgare, Pimpinella anisum*, GC×GC, estragole, adulteration

Introduction

Fennel (*Foeniculum vulgare* Mill.) and anise (*Pimpinella anisum* L.) belong to Apiaceae family (also known as Umbelliferae) and their fruits, mistakenly called "seeds", have been extensively used in perfumes, food and also in the pharmaceutical industry, especially in personal care products due its pleasant aroma.[1] These fruits are widely used to make tea and present similar morphological aspect from the general public perspective (non-experts). This similarity may cause confusion among consumers who purchase in bulk whenever they need to choose between both fruits. Similar taste and aroma profiles also corroborate to create confusion between both seed fruits. In Brazil, fennel fruits of some commercial brands are sometimes designated as "national anise", which makes the choice of the consumer even more difficult. A very important aspect in this context is that anise is the herb usually found in the descriptions of ingredients in the packaging of cosmetic products. The price of anise fruits is higher than the price of fennel (roughly, $ 18 for anise compared to $ 7 for fennel *per* pound or *per* 450 g) in Amazon's web page[2] and such a difference may stimulate the practice of adulteration, as the similar taste and flavor of both plant fruits also help to cover up the real composition of these cosmetic products. In addition to flavoring properties and to the extensive use of these fruits in cosmetics, the fruits themselves, their essential oils or the aqueous extracts of fennel and anise have showed important therapeutic applications due to their antioxidant[1] and antimicrobial properties.[3] According to popular knowledge, both products have been utilized in gastrointestinal treatment and fennel infusion is indicated in the treatment of flatulence and baby colic, whereas, anise has been used to increase the amount of milk in lactating women.[4]

Some authors have found anethole as the major constituent of both essential oils and fruits of anise[5-7] and fennel.[6,8-10] This compound was identified as the main contributor to the characteristic taste and smell of these products.[3] Anethole has similar chemical structure to that of

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dopamine and this characteristic may explain the relaxing effect on the intestine caused by fennel and anise.[4]

Despite the beneficial property of anethole, another component called estragole (also called *p*-allylanisole or methyl chavicol, an isomer of anethole) has been identified as genotoxic and carcinogenic[11,12] and its addition as a flavoring substance to foodstuffs has been banned by the regulatory authorities of the European Union.[13] Almeida *et al.*[6] found estragole in essential oil of fennel fruits, but this compound was not detected in the essential oil of anise fruits grown in Salerno (Italy). Fennel from China showed higher chromatographic area percentage of estragole, (2.98% of normalized peak area) when compared to anise (0.97%).[8]

According to our knowledge, the characterization of fruits and essential oils obtained from fruits of fennel and anise has already been done using one one-dimensional gas chromatography with a mass spectrometric detector (1D-GC/MS).[6-8] A detailed verification at these 1D-GC data shows that there are many unresolved peaks, due to the high complexity of these samples. Two or more co-eluting compounds may prevent the achievement of a correct identification of these volatile compounds and this is especially cumbersome when traces of volatile compounds are hidden by other co-eluting compounds. This means that important information on volatile composition may be missing and consequently misidentification of target components may occur.[13]

Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry detection (GC×GC/TOFMS) is an excellent choice for the investigation of the composition of complex samples, as it allows the analysis of the whole sample in the same analysis time required for a normal 1D-GC run, providing the selectivity of two different stationary phases, along with superior sensitivity and structurally organized elution of components in the 2D plots.[14] GC×GC/TOFMS has already been employed to verify distinctions among more expensive herbs and false and cheaper herbs,[15] although in this case the interpretation of color plots was performed through manual inspection, which is a time consuming and tedious task. The use of GC×GC/TOFMS along with chemometric tools have helped to design a more inclusive and fast approach where all the 2D data is employed for the preliminary analysis of the most discriminant compounds among classes. This strategy has already been successfully applied by this research group to verify distinctions among the volatile profile of wines according to different grape variety[16] and also to investigate which compounds differentiate sparkling wines from base wines.[17] Investigation of adulteration in sesame and peanut oils[18] and differentiation of two clam species[19] have also been performed using a similar strategy, however, it has not yet been used to investigate consistent differences between real and false herbs that may be subjected to adulteration.

The purpose of this study is, for the first time; investigate the main differences in volatile profiles of fennel and anise fruits, as well as in personal care products based on these two medicinal herbs, using GC×GC/TOFMS data and chemometric tools in order to verify the possibility of adulteration of personal care products with fennel, which is the less expensive between the two herbs under study.

Experimental

Sample preparation analytical reagents, and supplies

Fruits of fennel and anise were purchased from different suppliers in four different markets in Porto Alegre city (state of Rio Grande do Sul, Brazil) and were kept in the dark prior to analysis. The fruits were sampled from plants cultivated in Rio Grande do Sul and the botanical identity of each purchased sample was verified according to morphological characteristics with the supervision of a botanist. Five samples were collected from each commercial brand of fennel and anise fruits and placed inside glass vials in order to provide five replicates of extraction and analyses for each product. Fruits were analyzed as a whole, without any pre-treatment. The five cosmetic products evaluated were shampoo, hair conditioner, liquid soap, deodorant, skin-clearing lotion.

The dibasic sodium phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Synth (São Paulo, Brazil). Standard compounds 1-hexanol, 3-methyl butanol, α-pinene, eucalyptol, benzyl alcohol, linalool, phenylethylalcohol, 4-terpineol and γ-terpinene were obtained from Sigma (Steinheim, Germany) and prepared in hexane at a concentration of 5 mg L^{-1}.

Headspace solid phase micro extraction of volatile compounds

The volatiles and semi-volatiles were extracted by one 50/30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA) in headspace mode (HS-SPME). Prior to use, the fiber was conditioned according to manufacturer's recommendation. The HS-SPME extraction conditions for fennel and anise fruit samples were 0.125 g of sample and 2 mL of buffer solution in 20 mL headspace vials with magnetic screw caps and silicone septa (Supelco). In the case of cosmetic products, 0.04 g of the samples was employed for volatiles extraction, in the same conditions described for fennel

and anise fruits. The buffer solution was a 0.025 mol L^{-1} solution of sodium phosphate dibasic and potassium dihydrogenphosphate (Na_2HPO_4/KH_2PO_4). The fiber was exposed to the sample headspace during a period of 20 min at 30 °C. After sampling, the fiber was thermally desorbed in a GC×GC injection port at 230 °C for 15 min in splitless mode. In order to avoid carryover, the fiber was reconditioned for 5 min at 260 °C prior to each analysis.

Chromatographic determination of volatile compounds

A GC×GC/TOFMS Pegasus-4D system equipped with a liquid nitrogen quad-jet modulator and a CTC Combipal autosampler was used. The GC×GC system consisted of an Agilent 6890N (Agilent Technologies, Palo Alto, CA, USA) equipped with a Pegasus time-of-flight mass spectrometry detector (Leco Corporation, St. Joseph, MI, USA). The column set consisted of a polyethylene glycol phase (DB-Wax) in the first dimension (^{1}D) of 30 m, 0.25 mm i.d. and 0.25 μm film thickness (d_f), coupled to a dimethylpolysiloxane phase (DB-1) column (1.7 m length × 0.1 mm i.d. × 0.1 μm d_f) in the second dimension (^{2}D). Both columns were purchased from Agilent Technologies (Santa Clara, California, USA). The injector and detector port temperature were kept at 230 °C and the primary oven temperature was programmed to start at 60 °C, where it was kept for 0.20 min, being raised to 190 °C at 3 °C min^{-1}. The secondary oven offset was 40 °C and the interface temperature was 200 °C. The modulation period and the hot pulse duration were 4 s and 1.6 s, respectively. The MS parameters were as follows: electron ionization of 70 eV, detector voltage at −1500 V, acquisition rate of 100 Hz and mass range (*m*/*z*) from 40 to 400. The maximum number of peaks before Chroma TOF data treatment was set to 1000. Linear temperature programmed retention index (LTPRI)[20] was calculated 1D-GC/MS for the purpose of tentative identification of volatile compounds. The LTPRI was experimentally determined for a DB-5 column (5% diphenyl-95% dimethyl polysiloxane, 30 m × 0.25 mm × 0.25 μm, J&W Scientific Inc., Folsom, CA) and for a DB Wax (polyethylene glycol, 30 m × 0.25 mm × 0.25 mm, J&W Scientific Inc.) column. Experimental conditions of 1D-GC were: injector and detector temperature at 250 °C for DB-5 and 220 °C for DBWax. Helium flow rate was 1.0 mL min^{-1}. Oven temperature program was initially 60 °C and reached 250 °C at 3 °C min^{-1} for DB-5 column and 200 °C for DBWax column. Quadrupole mass spectrometric detector was operated in the electronic impact mode at 70 eV, and mass/charge range was 40 to 450. Electron multiplier was at 1250 V.

Data processing

LECO ChromaTOF version 4.22 software was used for all acquisition control, data processing and Fisher ratio calculations. Automated peak find and spectral deconvolution with a baseline offset of 0.5 and signal-to-noise (S/N) of three were employed during data treatment for peak detection. Tentative identification of volatile compounds was achieved by comparing 1D-GC experimental LTPRI with retention indices reported in the scientific literature. Nine compounds (listed in the Experimental section and indicated in Table 1) were positively identified through comparison of retention and mass spectra data of unknown compounds with those of authentic standards. Retention data obtained with a series of *n*-alkanes (C9 to C24), under the same experimental conditions employed for the chromatographic analysis of volatiles of herbs and personal care products were used to determine experimental LTPRI of volatile compounds. Mass spectrometric information of each chromatographic peak was compared to NIST mass spectra library version 2005, considering a minimum similarity value of 80%. Chromatographic analyses of all samples were made in a short period of time, continuously, in order to assure comparable performance of the mass spectrometric detector response for all samples.

The area percentage of each compound was obtained considering the sum of the areas of all compounds present in the samples, which represents 100%. Chromatographic peaks related to column bleeding and to compounds that have shown spectral similarity below 80% were not taken into consideration when the area percentage of a specific compound was calculated. In a general view, the chromatograms of anise samples were very similar; the same was observed for fennel samples. Then, one chromatogram of each type of sample (anise and fennel) was used to calculate a rough estimate of the area percentage of every compound in each type of herb. Linear response of the mass spectrometric detector was verified using compounds considered as representative of the several classes of compounds present in the headspace of samples (Figure S1 and Table S1, in the Supplementary Information (SI) section).

Statistical analysis

Tools used in the statistical treatment of the data generated by GC×GC/TOFMS were Fisher ratio, principal component analysis (PCA), multivariate analysis of variance (MANOVA) and canonical discriminant analysis (CDA).[16,17]

The Fisher ratio is a supervised method applied when the classes of samples are previously known. The whole set of GC×GC/TOFMS data was employed for the initial Fisher ratio determination, including column and SPME bleed and chromatographic peaks that showed low spectral similarity with the information found in the mass spectral library. The idea behind this procedure is to perform a simplified data treatment that has not demanded a previous manual analysis of the data before statistical treatment. In fact, calculation of Fisher ratio allowed a simplification of GC×GC data, as it showed the compounds that had the greatest changes in their chromatographic peak areas through comparison of the three classes under study: fennel, anise and personal care products. Compounds with Fisher ratios higher than 2590 were used in the second stage of the statistical analysis (PCA) as these components showed signal-to-noise ratio (S/N) value at least two times higher in class 1 (fennel) than in class 2 (anise) or *vice versa*. S/N ratio was calculated using ChromaTOF software.

Data obtained by ChromaTOF software (retention data, chromatographic areas and mass spectra) were exported to Predictive Analytics Software Statistics 18, release 18.0.0 for PCA, MANOVA and CDA that are unsupervised methods.

PCA was employed to transform the set of variables selected by Fisher ratio into another set of fewer synthetic variables denominated factors. The association between the variables was investigated and the dimension of variables was defined by eigenvalues higher than one.

MANOVA was used to compare the differences between the mean vectors of fennel, anise and personal care products. The significant compounds appointed by MANOVA were used in CDA, which is a supervised method applied for classification purposes. CDA classification model was constructed through the application of a stepwise variable selection procedure. Wilk's lambda was employed as selection criteria to help choosing the most significant variables and F-statistic factor was used to determine the significance of changes in lambda whenever the influence of a new variable was evaluated. The variables (most discriminant compounds) were included in the model one by one, choosing at each step the variable that made the most significant additional contribution to the discrimination (i.e., with the largest F-value). Whenever a different variable was tested in the model, the resulting classification of samples was compared to the previously proposed classification to check their agreement. The Wilk's lambda selection algorithm is a measure of discrimination between groups. The larger the dispersion among groups, the lower the Wilk's lambda value and the greater the significance of that compound for the classification method.[21] The prediction capacity of the discriminant models was studied by a "leave-one-out" cross validation. The strategy of this method of validation consists of discarding one observation of the reference group and estimating the classification model with the other observations. The observation left out is then used to assess the performance of the classifier method and the whole process is repeated for all the other samples, until each one has been left out once.[22]

Results and Discussion

On average, over 950 peaks were detected *per* sample and they included spurious components (column and SPME bleeding, compounds with spectral similarity below 80%, etc.) and compounds of several chemical classes mainly terpenic compounds, esters, acids, aldehydes, ketones, alcohols, phenols, and others. After taking out the spurious chromatographic peaks, roughly 175 compounds remained. The sum of the areas of approximately 175 chromatographic peaks was considered as 100% of the compounds for the purpose of area percentage calculation. Figure 1 shows color plots obtained after HS-SPME-GC×GC/TOFMS analyses of fennel and anise fruits and personal care products based on these fruits, where sample complexity and co-elutions in ^{1}D are easily observed, as well the high number of volatile compounds detected.

The major compound of anise and fennel fruits is anethole (approximately 68% and 56%, respectively). This compound was also found as the major component of fennel essential oils grown in Italy (76%),[6] China (68%),[8] Egypt (66%),[9] Turkey (84%)[10] and Brazil (78%).[23] Moreover, high proportions of anethole has been also found in anise from Serbia (88%),[5] Italy (97%),[6] and from various European countries including France, Greece, Scotland, Spain and Germany (around 94%).[7] Anethole is the most studied compound of anise and fennel. The interest of pharmaceutical industry for isolated constituents of natural materials has increased and scientific research has demonstrated the inhibitory effect of anethole in nonimmune inflammation,[24] its inducing property of apoptosis in human breast cancer[25] and its anti-inflammatory effect in lung injury.[26]

GC×GC/TOFMS data were used to calculate Fisher ratios and 31 compounds with Fisher ratio value above a threshold of 2590 were used in this work, because the volatile compounds contribution for class differentiation was small below this value (see criteria selection in the Experimental part, in the Statistical analysis section). The higher the Fisher ratio value, the greater the variance among classes of samples for a particular compound.

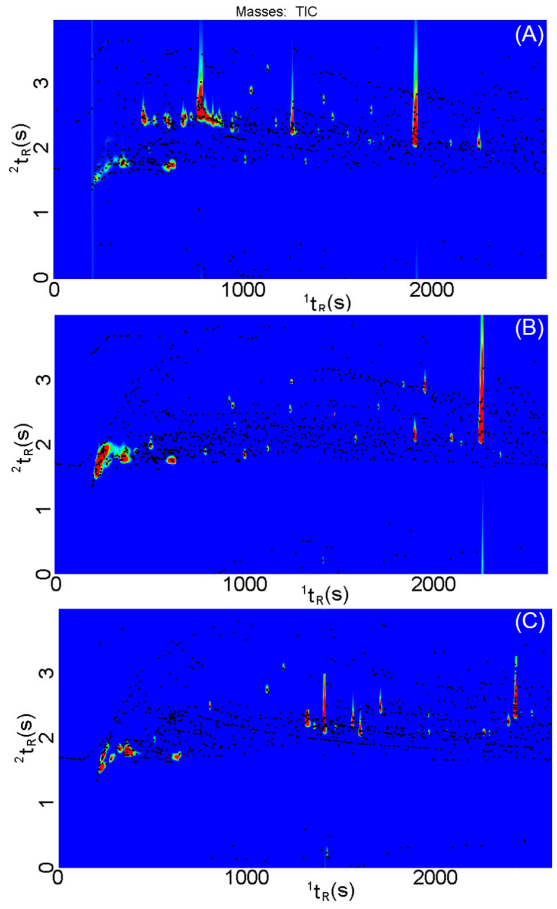


Figure 1. GC×GC/TOFMS total ion current chromatograms (TIC) presented as color plots of volatile compounds of the headspace of (A) fennel fruits, (B) anise fruits and (C) personal care products. Retention time in seconds in the first dimension [^1t_R(s)] and in the second dimension [^2t_R(s)] are represented in X-axis and Y-axis, respectively. The color gradient reflects the intensity of the TOFMS signal (Z-axis) from low (blue) to high (red). Some trace volatile compounds are not visible in this chromatogram due to the higher chromatographic area of some components such as anethole, estragole, benzaldehyde, methyleugenol, borneol, and 3-methyl-1-butanol. Each black point in the chromatogram indicates a compound detected by GC×GC/TOFMS. Chromatographic conditions are described in the Experimental section (Chromatographic determination of volatile compounds).

Taking into account that only 31 compounds presented a major contribution for differentiation between fennel and anise fruits, it is possible to conclude that volatile profiles of both fruits are rather similar.

The tentatively identified compounds, their respective CAS number, classification of chemical class, spectral similarity between mass spectra of sample compounds and NIST library, Fisher ratio value, as well as experimentally determined LTPRI and literature LTPRI for each compound are shown in Table 1. Compounds are sorted in descending order according to their Fisher ratio values. Differences between experimentally determined LTPRI and literature LTPRI for tentatively identified compounds were less than 16 units.

Considering all 31 compounds chosen by the Fisher ratio criteria as a 100%, sesquiterpenes were the predominant chemical class (35.5%). Other compounds tentatively identified among this group belong to monoterpenes hydrocarbons (22.6%), oxygenated monoterpenes (22.6%), alcohols (12.9%) and phenylpropanoids (6.4%) (Table 1).

PCA was carried out in the second step of chemometric analysis and included all the 31 compounds of Table 1. This approach selected two factors with eigenvalues greater than one, which included 11 volatile compounds and explained the 87.7% of the total variance (Table 2). Eigenvalues correspond to the variance of each principal component and the number of significant eigenvalues was determined by the Kaiser rule, which considers only the components with eigenvalue greater than one. Variables with higher loading values are the ones that significantly contributed to explain the factors and they are marked in bold letters in Table 2. Variables related to components 1 and 2 are positioned according to factor loadings in Figure 2.

The following statistical tools were applied in an attempt to validate a model to differentiate fennel and anise fruits based on their volatile profiles: MANOVA and CDA. This model will be employed to identify the compounds that might be used to verify which one of these herb fruits were employed in the manufacture of different personal care products that are designated as anise products. MANOVA was applied in the third stage of statistical analysis and the results are shown in Table 3. The distribution of the samples in the plan defined by the first two components was obtained by PCA (Figure 3). Volatile compounds related to the first factor (Table 3) that differentiate anise, fennel and personal care products were 4,5-dehydro-isolongifolene, methyleugenol, calamenene, α-pinene, 4-terpineol, alloaromadendrene and estragole. Furthermore, these compounds are responsible for the similarity between personal care products and fennel. These results indicate that the anise (as stated on the product label) may have been replaced by fennel in elaboration of personal care products. Volatiles associated with the second factor (Table 3) that differentiate the three types of samples analyzed in this work were β-ocimene, sabinene, fenchol and linalool (Figure 3).

Chromatographic areas of β-ocimene, sabinene and fenchol were higher in the headspace of anise fruits than those found in fennel fruits, and linalool was found only in fennel as observed in a closer view of the chromatograms of fennel and anise (results not shown). However, these compounds had not been found in personal care products, probably due to chemical reactions that might occur during the preparation/storage of these products. β-Ocimene, for

Table 1. Tentatively identified compounds appointed by Fisher ratio as the most important analytes to differentiate fennel and anise fruits through HS-SPME and GC×GC/TOFMS analyses. The compounds were sorted in descending order according to their Fisher ratio values

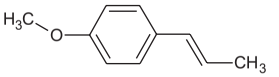
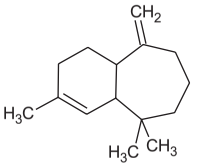
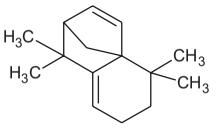
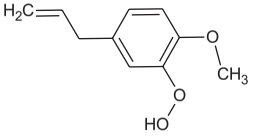
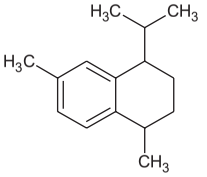
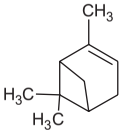
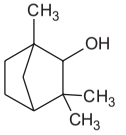
No.	Compound	CAS	Chemical class	S[a]	LTPRI $DB\text{-}5_{exp}$[b]	LTPRI $DB\text{-}5_{lit}$[c]	LTPRI wax_{exp}[d]	LTPRI wax_{lit}[e]	Fisher ratio	Structural formula	Area / %[f] Fennel	Area / %[f] Anise
1	1-Methoxy-4-(1-propenyl) benzene; anethole	104-46-1	Phenyl propanoid[g]	900	1295	1289[27]	1820	1808[28]	15296		56.00	68.00
2	3,5,5-Trimethyl-9-methylidene-2,4a,6,7,8,9a-hexahydro-1H-benzo(7)annulene; α-himachalene	3853-83-6	Sesquiterpene	889	1448	1447[29]	1600	1610[30]	8343		0.05	0.42
3	4,5-Dehydro-isolongifolene	156747-45-4	Sesquiterpene	927	1514	1500[31]	1903	1913[32]	7610		0.75	0.89
4	1,2-dimethoxy-4-(2-propenyl)-benzene; methyleugenol[h]	93-15-2	Phenyl propanoid[g]	884	1405	1401[29]	2017	2007[33]	6661		4.40	[h]
5	1,6-Dimethyl-4-(propan-2-yl)-1,2,3,4-tetrahydronaphthalene; calamenene	483-77-2 483-77-2	Sesquiterpene	855	Nf[j]	1540[29]	1834	1826[34]	6347		0.08	0.10
6	4,7,7-Trimethylbicyclohept-3-ene; α-pinene[i]	80-56-8	Monoterpene hydrocarbon	897	935	939[29]	1046	1034[35]	6246		0.07	1.28
7	1,3,3-Trimethylbicycloheptan-2-ol; fenchol	1632-73-1	Oxygenated monoterpene	881	Nf[j]	1117[29]	1370	1371[36]	5344		0.10	0.90

Table 1. Tentatively identified compounds appointed by Fisher ratio as the most important analytes to differentiate fennel and anise fruits through HS-SPME and GC×GC/TOFMS analyses. The compounds were sorted in descending order according to their Fisher ratio values (cont.)

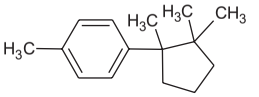
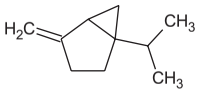
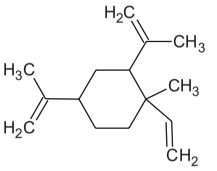
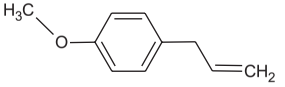
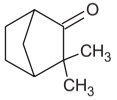
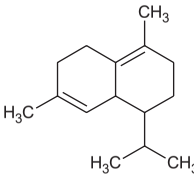
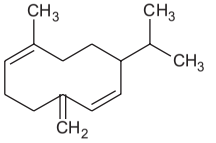
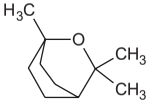
No.	Compound	CAS	Chemical class	S[a]	LTPRI DB-5$_{exp}$[b]	LTPRI DB-5$_{lit}$[c]	LTPRI wax$_{exp}$[d]	LTPRI wax$_{lit}$[e]	Fisher ratio	Structural formula	Area / %[f] Fennel	Anise
8	1-Methyl-4-(1,2,2-trimethylcyclopentyl)-benzene; cuparene	16982-00-6	Sesquiterpene	873	Nf[j]	1502[29]	1791	1803[37]	4920		0.09	0.24
9	4-Methylidene-1-propan-2-ylbicyclohexane; sabinene	3387-41-5	Monoterpene hydrocarbon	905	979	976[29]	1122	1134[33]	4908		0.08	0.17
10	1-Ethenyl-1-methyl-2,4-di(prop-1-en-2-yl) cyclohexane; β-elemene	515-13-9	Sesquiterpene	845	Nf[j]	1375[29]	1590	1595[35]	4335		0.33	0.11
11	1-Methoxy-4-prop-2-enylbenzene; estragole[h]	140-67-0	Phenyl propanoid[g]	889	1199	1195[29]	1670	1661[33]	4214		14.00	[h]
12	6,6-Dimethyl-5-methylidenebicycloheptane; camphene	79-92-5	Monoterpene hydrocarbon	912	Nf[j]	953[29]	1070	1077[35]	4095		0.02	0.15
13	4,7-Dimethyl-1-propan-2-yl-1,2,3,5,6,8a-hexahydronaphthalene; δ-cadinene	483-76-1	Sesquiterpene	882	1512	1524[29]	1735	1729[38]	2602		0.11	0.25
14	1-Methyl-5-methylene-8-(1-methyethyl)-1,6-cyclodecadiene; germacrene D	23986-74-5	Sesquiterpene	932	Nf[j]	1480[29]	1711	1722[35]	4024		0.08	0.01
15	4,7,7-Trimethyl-8-oxabicyclo[2.2.2]octane; 1,8-cineole; eucalyptol[i]	470-82-6	Oxygenated monoterpene	917	1030	1033[29]	1229	1224[39]	3704		0.38	6.84

Table 1. Tentatively identified compounds appointed by Fisher ratio as the most important analytes to differentiate fennel and anise fruits through HS-SPME and GC×GC/TOFMS analyses. The compounds were sorted in descending order according to their Fisher ratio values (cont.)

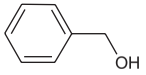
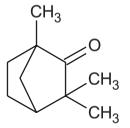
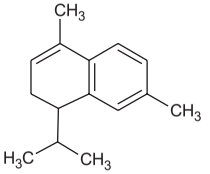
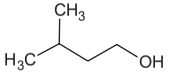
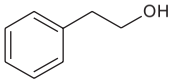
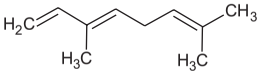
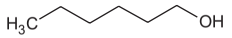
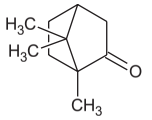
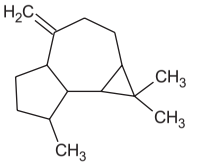
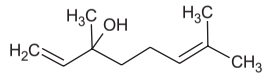
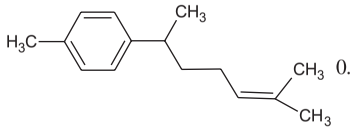
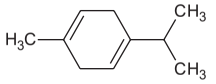
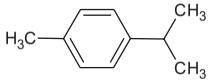
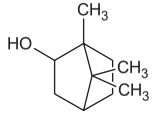
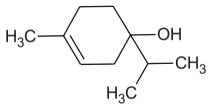
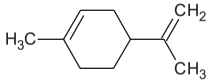
No.	Compound	CAS	Chemical class	S[a]	LTPRI DB-5$_{exp}$[b]	LTPRI DB-5$_{lit}$[c]	LTPRI wax$_{exp}$[d]	LTPRI wax$_{lit}$[e]	Fisher ratio	Structural formula	Area / %[f] Fennel	Anise
16	Benzylalcohol[i]	100-51-6	Alcohol	926	1040	1032[29]	1891	1882[40]	3681		0.02	1.72
17	1,3,3-Trimethylbicycloheptan-2-one; fenchone	1195-79-5	Oxygenated monoterpene	908	1091	1094[41]	1401	1402[41]	3519		0.03	0.95
18	4,7-Dimethyl-1-propan-2-yl-1,2-dihydronaphthalene; α-calacorene	21391-99-1	Sesquiterpene	911	Nf[j]	1548[42]	1912	1901[43]	3491		0.26	0.09
19	3-Methyl-1-butanol[i]	123-51-3	Alcohol	947	Nf[j]	734[29]	1228	1230[44]	3264		0.03	5.45
20	Phenylethylalcohol[i]	60-12-8	Alcohol	924	1126	1110[29]	1924	1931[44]	3133		5.05	0.03
21	3,7-Dimethyl-1,3,6-octatriene; β-ocimene	3779-61-1	Monoterpene hydrocarbon	906	Nf[j]	1040[29]	1245	1245[35]	2859		0.04	0.16
22	1-Hexanol[i]	111-27-3	Alcohol	897	Nf[j]	867[29]	1362	1360[39]	2714		1.01	0.85
23	1,7,7-Trimethylbicycloheptan-2-one; camphor	76-22-2	Oxygenated monoterpene	900	1148	1143[29]	1510	1498[45]	2663		0.08	0.05
24	Decahydro-1,1,7-trimethyl-4-methylene-cyclopropazulene; allo aromadendrene	25246-27-9	Sesquiterpene	888	1457	1461[29]	1662	1650[46]	2641		0.03	0.07

Table 1. Tentatively identified compounds appointed by Fisher ratio as the most important analytes to differentiate fennel and anise fruits through HS-SPME and GC×GC/TOFMS analyses. The compounds were sorted in descending order according to their Fisher ratio values (cont.)

No.	Compound	CAS	Chemical class	S[a]	LTPRI $DB\text{-}5_{exp}$[b]	LTPRI $DB\text{-}5_{lit}$[c]	LTPRI wax_{exp}[d]	LTPRI wax_{lit}[e]	Fisher ratio	Structural formula	Area / %[f] Fennel	Anise
25	3,7-Dimethyl-1,6-octadien-3-ol; linalool[i,h]	78-70-6	Oxygenated monoterpene	937	1102	1098[29]	1558	1554[35]	2639		0.06	[h]
26	1-(1,5-Dimethyl-4-hexenyl)-4-methyl benzene; α-curcumene	644-30-4	Sesquiterpene	860	1480	1483[29]	1779	1764[34]	2635		0.02	0.27
27	1-Methyl-4-(1-methylehtylidene) cyclohexene; γ-terpinene[i]	99-85-4	Monoterpene hydrocarbon	892	1058	1062[29]	Nf	1262[35]	2622		0.09	0.01
28	3,7-Dimethyl-1,3,7-octatriene; *p*-cymene	99-87-6	Monoterpene hydrocarbon	854	1027	1026[29]	1274	1282[35]	2610		0.07	0.10
29	1,7,7-Trimethyl-bicyclo[2.2.1]heptan-2-ol; borneol	507-70-0	Oxygenated monoterpene	939	Nf[j]	1165[29]	1650	1642[46]	2605		2.94	[h]
30	2-(4-Methyl-1-cyclohex-3-enyl)propan-2-ol; 4-terpineol[i]	562-74-3	Oxygenated monoterpene	901	1181	1177[29]	1608	1616[35]	4092		0.33	0.01
31	1-Methyl-4-prop-1-en-2-ylcyclohexene; limonene	5989-27-5	Monoterpene hydrocarbon	933	1030	1031[29]	1201	1212[39]	2590		0.05	0.01

[a]Similarity=S (S means similarity of the mass spectrum of a wine compound with the spectra of standard compounds in the NIST05); [b]Linear temperature programmed retention index (LTPRI) calculated using n-alkanes (C9-C24) in apolar column (DB-5, 5% polyethyleneglycol); Conditions for the chromatographic analysis are described in Chromatographic determination of volatile compounds, in Experimental section. LTPRI were calculated based on first dimensional separation retention data; [c]LTPRI: literature LTPRI on a DB-5 columns or equivalent stationary phase; [d]Linear temperature programmed retention index (LTPRI) calculated using n-alkanes (C9-C24) in polar column (DB-Wax, 100% polyethyleneglycol); [e]LTPRI: literature LTPRI on a DB-WAX columns or equivalent stationary phase; [f]The area percentage of each compound was obtained considering the sum of the areas of all compounds present in the samples, which represent 100%. Chromatographic peaks resulting from column or SPME bleeding or compounds that showed spectral similarity below 80 % when compared to commercial library mass spectra were not considered in this determination; [g]Terpene derivative; [h]Compound found only in fennel samples; [i]Compound positively identified with standard compound; [j]Nf:compound not found in the headspace of fruits of anise, neither in fennel.

Table 2. Factor loadings obtained in principal component analysis of volatile compounds tentatively identified in the headspace of fennel and anise fruits after determination of Fisher ratio used to find the more important compounds to differentiate these two groups of samples. The variables with higher loadings values are the ones that contributed most significantly to explain that specific factor and they are marked in bold letters

No. in Table 1	Compound	Factor 1	Factor 2
	Eigenvalue	7.180	3.346
	Cumulative variance / %	59.83	87.73
3	4,5-Dehydro-isolongifolene	**0.994**	–
30	4-Terpineol	**0.993**	–
4	Methyleugenol	**0.993**	–
6	α-Pinene	**0.983**	–
5	Calamenene	**0.957**	–
9	Estragole	**0.921**	–
24	Allo aromadendrene	**0.874**	–
7	Fenchol	–	**0.957**
25	Linalool	–0.112	**0.919**
21	β-Ocimene	0.115	**0.889**
11	Sabinene	0.174	**0.884**

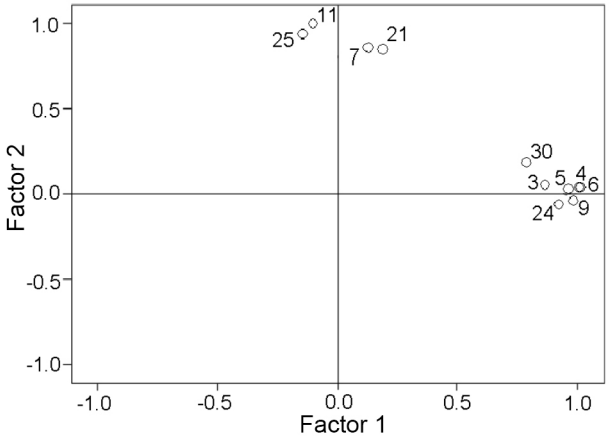


Figure 2. Distribution of volatile compounds in a plane defined by the first two factors obtained in PCA. Numbers refer to the tentatively identified compounds that are indicated in Table 1: (3) 4,5-dehydro-isolongifolene; (4) methyleugenol; (5) calamenene; (6) α-pinene; (7) fenchol; (9) sabinene; (11) estragole; (21) β-ocimene; (24) allo aromadendrene; (25) linalool; (30) 4-terpineol.

example, has been reported to promptly undergo oxidation within a short exposure to air.[47] Similarly, sabinene and fenchol may be degraded when exposed to high temperature (38 °C).[48] Due to the fact that these monoterpenes may rapidly undergo chemical changes and be absent in products derived from anise, they are not appropriate as markers to verify authenticity of products made with anise.

Table 3. Results of multivariate analysis of variance (MANOVA) used to check for significant differences between fennel, anise and personal care products in relation to each volatile compound selected by factor analysis

	No. in Table 1	Compound	Sample	Mean[a]
Factor 1	3	4,5-Dehydro-isolongifolene	Anise	15.124a
			Fennel	6.847b
			Products	6.847b
	4	Methyleugenol	Anise	17.826a
			Fennel	6.847b
			Products	6.847b
	5	Calamanene	Anise	16.822a
			Fennel	6.972b
			Products	6.847b
	6	α-Pinene	Anise	14.053a
			Fennel	7.284b
			Products	6.847b
	9	Estragole	Anise	16.337a
			Fennel	7.649b
			Products	6.847b
	30	4-Terpineol	Anise	17.507a
			Fennel	6.847b
			Products	6.847b
	24	Allo aromadendrene	Anise	12.474a
			Fennel	6.847b
			Products	6.847b
Factor 2	21	β-Ocimene	Anise	15.856a
			Fennel	10.160b
			Products	19.265c
	11	Sabinene	Anise	11.850a
			Fennel	21.841b
			Products	6.850c
	7	Fenchol	Anise	16.699a
			Fennel	9.202b
			Products	22.930c
	25	Linalool	Anise	13.210a
			Fennel	6.847b
			Products	19.544c

[a]Means followed by the same letter are not significantly different from each other ($p > 0.05$).

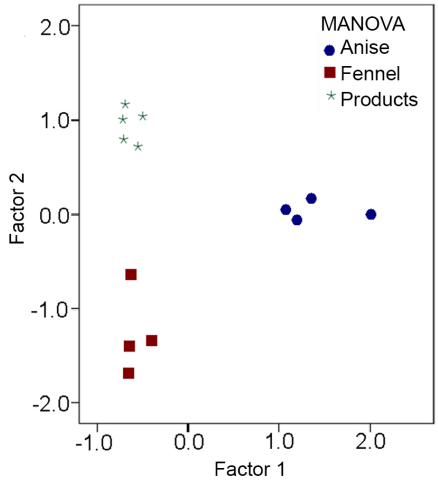


Figure 3. Three distinct groups of samples (fennel, anise and personal care products) positioned on the first two components set-up according to the variables that demonstrated significant differences by multivariate analysis of variance (MANOVA).

Table 4. Canonical discriminant characteristics of selection test of the variables used to obtain a model of classification of fennel, anise and personal care products that contain one of these fruits

F[a]	Eigenvalue	Variance / %[b]	Canonical correlation	Wilk's lambda	Chi-square	Df[c]	Signif.[d]
1	9076.544	99.8	1.000	0.000	84.896	14	0.000
2	19.356	100	0.975	0.049	21.094	6	0.002

[a]Function; [b]cumulative variance; [c]degrees of freedom; [d]significance (values < 0.05).

CDA was applied in the fourth step of statistical analysis to obtain a classification model for fennel, anise and personal care products that contain one of these fruits. The results are shown in Table 4. Eigenvalues indicate that the first discriminant function has the maximum canonical correlation (1.000) and explains almost the total of variance (99.8%). According to these values, the first two functions are responsible for discrimination because they provide the contribution to the total discrimination (100%). The statistical significance of each discriminant function was evaluated on the basis of the Wilk's lambda factor. This parameter ranges from 1.0 (no discriminatory power) to 0.0 (perfect discriminatory power).[22] The Wilk's lambda values of the first two discriminant functions are 0.000 and 0.049, respectively (Table 4), which indicates a very good discriminant power of the model. The significance values less than 0.002 (values lower than 0.05 were considered significant) and chi-square test indicates that there is a highly significant difference between the groups' centroids. Centroids of each group of samples, which correspond to the average value of a discriminating factor, are viewed in Figure 4 and the higher the value of chi-square, the more the function contributes to the discrimination between groups of samples (Table 4). Figure 4 shows the separation among sample groups by plotting the first and second discriminant functions. This separation occurred due to the differences in volatile profile of fennel and anise, which have been discussed along the text.

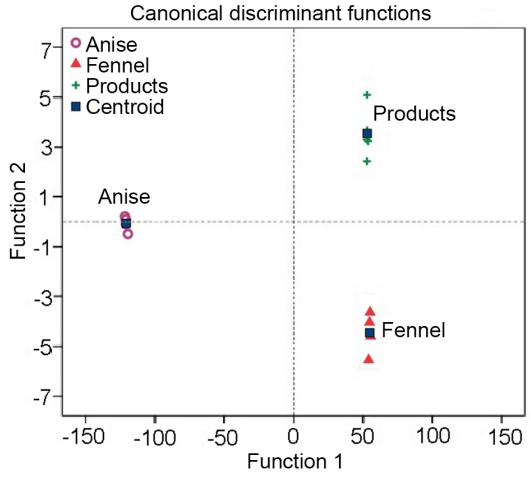


Figure 4. Plot of samples on a plane defined by two canonical discriminant functions related to the volatile compounds of fennel, anise and personal care products containing fennel and/or anise. Centroids correspond to the average discriminating score for each group.

The structure matrix coefficients of canonical discrimination were used to verify the correlations of each variable in the model in relation to the two main discriminant functions (Table 5). The first discriminant function is correlated to estragole, methyleugenol, 4,5-dehydro-isolongifolene and calamenene. This can be seen through the highest values of coefficients of canonical discrimination in function 1 of Table 5. In the same way, the second discriminant functions are related to linalool, β-ocimene and fenchol, which present the highest values of coefficients of canonical discrimination in function 2.

Table 5. Structure matrix coefficients of canonical discrimination related to fennel, anise and personal care products used to verify the correlations of each variable in the model in relation to the two main discriminant functions

No. in Table 1	Compound	Function 1	Function 2
9	Estragole	–0.315	0.015
4	Methyl eugenol	–0.057	–0.09
3	4,5-Dehydro-isolongifolene	–0.041	–0.07
5	Calamenene	–0.020	–0.08
25	Linalool	0.001	0.531
21	β-Ocimene	–0.002	0.416
7	Fenchol	0.245	0.281

The canonical discriminant functions appeared to have a good classification power with 100% of the original group cases being correctly classified. In order to determine the model stability, a "leave-one-out" cross validation was done and 100% of the cases were correctly classified (Table 6). These results confirm that the model is suitable to verify the presence of fennel or anise in personal care products.

Among the seven terpenic compounds appointed by canonical discriminant model as the most important to differentiate fennel, anise and personal care products, three of these compounds, including estragole, methyleugenol and calamenene, co-eluted with other compounds in the first dimension (^{1}D) of GC×GC. These co-elutions in

Table 6. Classification and cross-validation results of samples of fennel and anise according to volatile compounds related to the two first canonical discriminant functions

	Case[a]	Actual group	Original	Cross-validated
			Predicted group	
Fennel	1-Fa	Fennel	Fennel	Fennel
	2-Fb	Fennel	Fennel	Fennel
	3-Fc	Fennel	Fennel	Fennel
	4-Fd	Fennel	Fennel	Fennel
Anise	5-Aa	Anise	Anise	Anise
	6-Ab	Anise	Anise	Anise
	7-Ac	Anise	Anise	Anise
	8-Ad	Anise	Anise	Anise

[a]The fennel and anise samples were identified by fennel (F) and anise (A) followed by letters a, b, c and d that correspond to the different samples purchased from different suppliers.

^{1}D indicate the difficulties that may arise whenever only 1D-GC/MS is employed, possibly resulting in insufficient chromatographic separation and consequently, incorrect identification. Figures 5a, 5d and 5g present an expansion of part of the GC×GC color plot of Figure 1, showing the usefulness of the ^{2}D column for resolution of selected volatile compounds of anise and fennel. After using GC×GC to have a detailed idea of the composition of the complex samples, it might be possible to define the target volatile analytes that are important for discriminating between anise and fennel. A subsequent step might be choosing a stationary phase that separates these specific compounds in 1D-GC. Such an approach would imply in simplification of this analytical procedure for routine quality control. However, GC×GC would be important as a preliminary step, as it reveals the co-elutions present in complex samples with a faster and more accurate approach. Another important aspect to improve the present approach is a higher number of samples and the use of a more strict quantitative treatment[49] to validate the results obtained in the present approach.

Separation of co-eluted compounds in ^{2}D is shown in Figures 5b, 5e and 5h. Estragole was found only in fennel samples and co-eluted with cubebene. This co-elution would probably prevent its correct identification in 1D-GC. Similarly, estragole was not either found in Serbian[5] neither in Italian[6] anise. The correct identification of estragole is important due to its ability to give rise to DNA adducts that characterize its genotoxic potential. The carcinogenic effects of estragole have been extensively discussed and were revised by Gori *et al*.[50] Most studies deal with the toxicological profile of this sole molecule and do not provide a comprehensive risk profile of the whole complex phytochemical mixture of fennel constituents, where other bioactive compounds may occur and impart their own health effects or may present synergistic or antagonistic actions with other components present in this herb. In any case, using fennel instead of anise in personal care products implies in exposure of the population to health risks. Cubebene has also been recently studied due to its neuroprotective effect, having been suggested for the treatment for neurodegenerative disease.[51] This sesquiterpene has not been found in anise samples of Italy[6] and Serbia.[5]

Another compound identified by CDA as important to differentiate fennel, anise and personal care products was methyleugenol, which co-eluted with anethole and 8,9-dehydro-neoisolongifolene in ^{1}D of GC×GC. Methyleugenol was not found in anise samples, but was identified in the headspace of fennel fruits and of personal care products. Methyleugenol has been appointed as an initiating agent of hepatocellular carcinoma in rats because this compound may form adducts with DNA after its biotransformation that occurs mainly in liver.[52] In view of this genotoxic effects, methyleugenol was classified as a possible carcinogenic to humans (group 2B).[53] The beneficial properties of anethole were formerly presented in this manuscript. In relation to biological properties of 8,9-dehydro-neoisolongifolene, no information was found in scientific literature and this compound has not yet been reported in other fennel or anise samples.[5,6,8,10]

Calamenene coeluted with *cis*-carveol in ^{1}D of GC×GC. Properties of these terpenic compounds have been poorly investigated. Takei *et al.*[54] found that calamenene showed good results regarding induction of immune response to cancer. *Cis*-carveol is a fragrance ingredient used in cosmetics and no toxic or beneficial effects have been reported about it.[55]

Conclusions

The use of HS-SPME-GC×GC/TOFMS and chemometric analysis, including Fisher ratio, PCA, MANOVA and CDA, allowed the differentiation of fennel and anise fruits based on their volatile profile. Furthermore, these tools provided a comprehensive and interesting preliminary approach to verify which of these herbs might have been used in adulteration of formulations of personal care products. Seven compounds were appointed by statistical analysis as the most important to differentiate fennel and anise fruits: estragole, methyl eugenol, 4,5-dehydro-isolongifolene, calamenene, linalool, β-ocimene and fenchol. However, a higher number of samples will be necessary to confirm the potential markers of adulteration of products of anise with

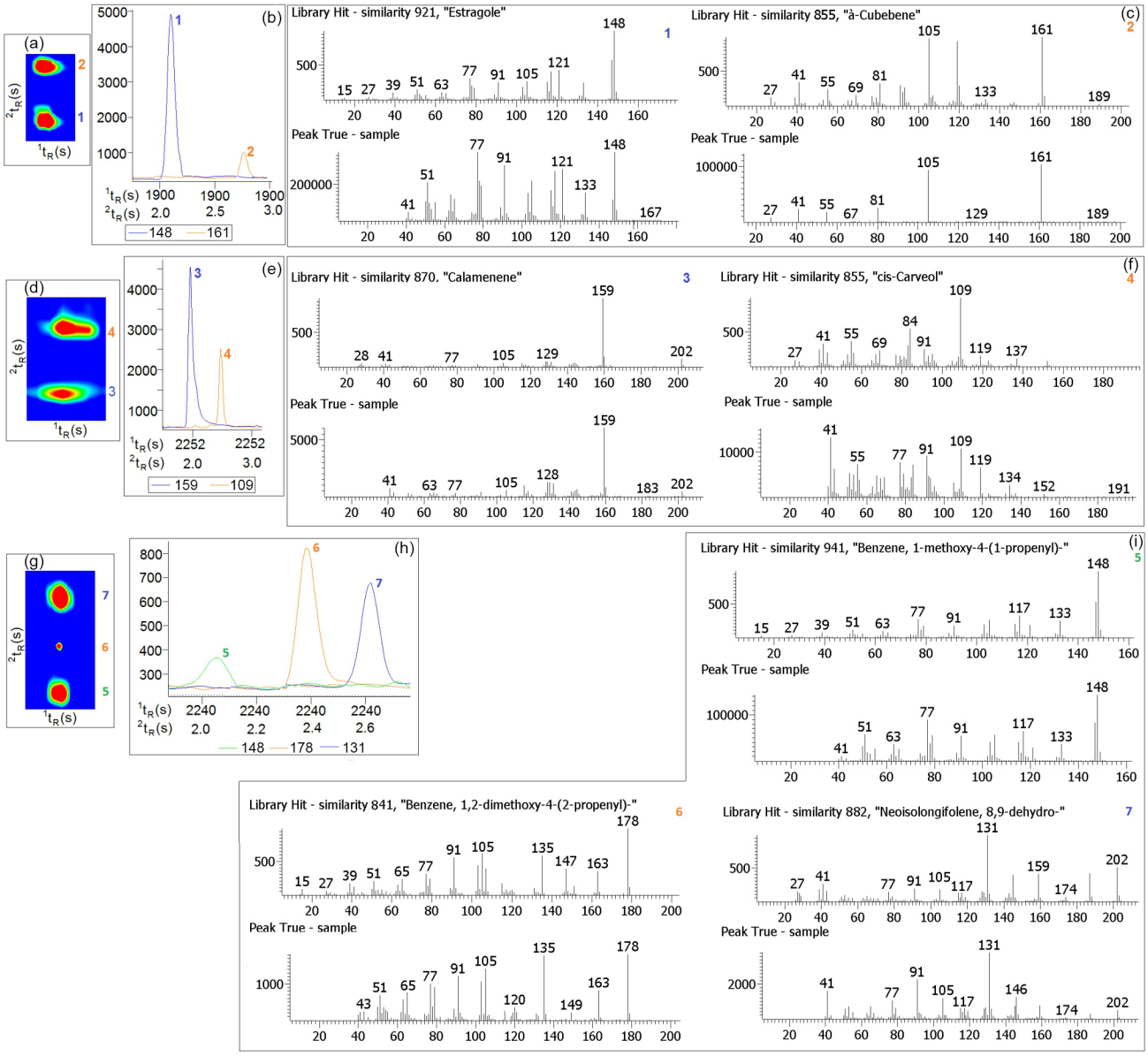


Figure 5. Example that demonstrates the separation of co-eluted volatile compounds in ^{1}D due to selectivity in ^{2}D: regions (a), (d) and (g) of the color plot show co-eluted peaks in^{1}D. Parts (b), (e) and (h) of the chromatogram of modulated peaks of the volatile compounds that co-eluted in ^{1}D and were separated in ^{2}D: (1) estragole; (2) α-cubebene; (3) calamanene; (4) *cis*-carveol; (5) anethole [or benzene, 1-methoxy-4-(1-propenyl) as in MS library hit]; (6) methyleugenol [or benzene, 1,2-dimethoxy-4-(2-propenyl) as in MS library hit] and (7) 8,9-dehydro-neoisolongifolene. (c), (f) and (i) show the deconvoluted mass spectra of peaks tentatively identified as estragole (base peak *m/z* 148), α-cubebene (base peak *m/z* 161), calamanene (base peak *m/z* 159), *cis*-carveol (base peak *m/z* 109), anethole (base peak *m/z* 148), methyleugenol (base peak *m/z* 178), 8,9-dehydro-neoisolongifolene (base peak *m/z* 131) and the corresponding mass spectra from NIST library 2005.

fennel, as indicated in this preliminary approach, as well as a more strict quantitative treatment in order to validate these results. The resolution power of GC×GC/TOFMS was demonstrated through the separation of three compounds that were among the seven components appointed by chemometric tools as the most discriminant ones for anise and fennel fruits (estragole, calamenene and methyl eugenol), which were separated from other four components due to the aditional selectivity obtained with the second chromatographic dimension. The strategy proposed in this manuscript to find out differences among products of two herbs through their volatile profiles might be beneficial if considered for different cases of adulteration involving diverse products, such as personal care products, food and beverage, traditional and herbal medicines, etc.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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