

# Gas Chromatography–Mass Spectrometry-Based Classification of 12 Fennel (*Foeniculum vulgare* Miller) Varieties Based on Their Aroma Profiles and Estragole Levels as Analyzed Using Chemometric Tools

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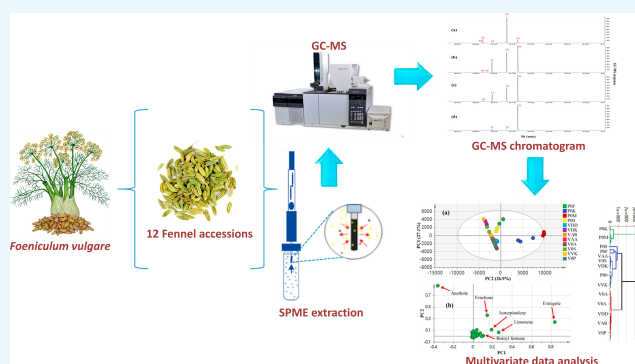


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**ABSTRACT:** Fennel (*Foeniculum vulgare* Miller) is a popular aromatic plant native to the Mediterranean basin and cultivated worldwide that is valued for the nutritional and health benefits of its fruits. Headspace solid-phase microextraction of 12 fennel accessions of cultivated (*F. vulgare* subsp. *vulgare*) and wild forms (*F. vulgare* subsp. *piperitum*) of different origins was carried out for assessing their volatile distribution. Fifty-four volatiles were identified, with ethers amounting for the major class at ca. 52–99% attributed to the abundance of (*E*)-anethole and estragole. Several subsp. *vulgare* accessions proved to be excellent sources of the chief aroma (*E*)-anethole (95.9–98.4%), whereas high levels of estragole at ca. 72% were observed in subsp. *piperitum* from Minia and Khartoum and must be considered in the safety assessment of fennel. Other volatile classes were detected including ketones, esters, aldehydes, alcohols, and hydrocarbons (monoterpenes, sesquiterpenes, and diterpenes). Fenchone exceeded 15% of the total volatiles in some fennel specimens, linked to a conspicuous bitter aftertaste. The members of subsp. *piperitum* were more enriched in monoterpene hydrocarbons with sabinene found exclusively in these, while subsp. *vulgare* comprised a higher content of ethers. Principle component analysis determined isoterpinolene as a special component in subsp. *piperitum*. In all specimens from the same group, estragole was the most distinguished volatile compound according to the findings from orthogonal partial least squares-discriminant analysis. The highest estimated estragole levels were detected in subsp. *piperitum* from Minia at 89.8 mg/g. This comparative study provides the first comprehensive insight into volatile profiling of 12 fennel fruit varieties.



## INTRODUCTION

Many culinary herbs and spices contain a variety of sensory metabolites that offer a wide range of aromatic, nutritional, and health-promoting effects.<sup>1</sup> The carrot family (Apiaceae, Umbelliferae) is one of the largest families of flowering plants, encompassing ca. 450 genera and 3100–3200 species with members mainly distributed in the northern hemisphere,<sup>2</sup> and it is well-known for its numerous aromatic species of commercial interest.<sup>3</sup> Owing to their essential oil content, many herbaceous Apiaceae species are used in medicine, cosmetics, beverages, and food industry as flavoring and antimicrobial agents.<sup>4,5</sup> Phenylpropanoids and terpenes are the major key aroma-imparting compounds in Apiaceae essential oils.<sup>6</sup>

Fennel (*Foeniculum vulgare* Miller) is one of the most popular aromatic plants within Apiaceae. The species is native to the Mediterranean basin, where it occurs as two commonly differentiated subspecies, subsp. *vulgare* and subsp. *piperitum*. The subspecies *piperitum* includes mostly wild forms.<sup>7</sup> It is therefore considered as the ancestral form of subsp. *vulgare*, which contains mostly domesticated varieties.<sup>8</sup> Fennel cultivars

are widely used as flavorings in bread, cheese, ice cream, pickles, pastries, beverages, meat, and fish cuisines.<sup>9,10</sup> While *F. vulgare* subsp. *vulgare* var. *dulce* is mainly used as a condiment, var. *azoricum* is a vegetable plant and var. *vulgare* is used as a source of (*E*)-anethole for pharmaceutical purposes.

While the oldest archaeological traces of fennel point to weedy forms in the Near Eastern Neolithic,<sup>11</sup> the oldest archaeological indications for its use by humans are from Ancient Egypt's Middle Kingdom, from 22nd to 18th century BCE,<sup>12,13</sup> followed by finds from Carthage.<sup>14</sup>

Today, fennel is naturalized globally, sometimes even as an invasive weed,<sup>15</sup> but mostly in its cultivated varieties. As an economic crop traded globally, fennel is commonly used in household remedies and as a culinary spice in many countries.

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Its traditional medicinal uses involve the treatment of gastrointestinal and upper respiratory complaints. It is highly recommended for patients suffering from catarrh, bronchitis, and chronic coughs as well as to prevent dyspeptic disorders (colic and flatulence) in nursing infants.<sup>16</sup> Fennel fruits have been also used to treat dysmenorrhea, vomiting, kidney stones, and diarrhea.<sup>17</sup> Other studies reported hepatoprotective, galactagogue, antimicrobial, anti-inflammatory, diuretic, anti-spasmodic, analgesic, and antioxidant effects of fennel.<sup>16,18</sup>

The main phytochemical components responsible for the therapeutic effects of fennel were phenolic and volatile compounds.<sup>19</sup> Providing a widely available source of natural antioxidants, the major phenolic compounds present in fennel were flavonoid glucosides (eriocitrin and quercetin-3-O-glucuronide) and hydroxycinnamic acids (chlorogenic and cryptochlorogenic acids).<sup>20</sup> In contrast, the prevalent volatile constituents in fennel were (*E*)-anethole, 1-octen-3-ol, estragole (methyl chavicol), and fenchone.<sup>16</sup> Meanwhile, *p*-cymene,  $\alpha$ -pinene,  $\beta$ -pinene, and limonene were detected as minor components.<sup>21</sup> Accounting for over 98% of fennel oil, 78 volatile compounds were reported including phenylpropanoids, monoterpene hydrocarbons, and oxygenated monoterpenes.<sup>22</sup> Based on the fennel's phenotype and origin, relative ratios of these compounds vary greatly.<sup>23</sup> The essential oil of subsp. *piperitum* was distinguished by relatively high levels of  $\alpha$ -phellandrene,  $\alpha$ -pentene, and fenchone, with low estragole and (*E*)-anethole concentrations.<sup>16</sup> Moreover, the essential oil composition, especially monoterpene components, was significantly affected by climatic conditions, namely, rainfall and temperature.<sup>24</sup> A recent study revealed the impact of roasting on fennel fruits and identified pyrans, furans, and pyrazines as newly occurring volatile classes upon roasting.<sup>25</sup>

Metabolomics is a relatively modern approach applied for profiling and/or fingerprinting of the overall chemical composition of plant-derived foods exposed to different conditions.<sup>26</sup> For volatile compound profiling, metabolomics mainly uses hyphenated tools such as solid-phase microextraction (SPME) followed by gas chromatography (GC) coupled with mass spectrometry (MS).<sup>27</sup> Headspace solid-phase microextraction (HS-SPME) is a very useful rapid and solvent-free technique to extract the less abundant volatiles, allowing their detection without the production of artifacts upon thermal decomposition.<sup>28</sup>

This study aimed to compare the aroma profiles of different fennel varieties collected at various localities to determine the metabolite heterogeneity among these varieties using multivariate data analyses and consequently for QC applications involving the detection of the authenticity and/or adulteration of fennel samples. Another goal was to determine the estragole levels in these accessions considering their relatively high toxicity.

## RESULTS AND DISCUSSION

Different varieties of fennel as indicated in Table 1 were subjected to comprehensive volatile profiling. Multivariate data analysis was further employed to help recognize the specimen markers and classification in an untargeted manner.

**Volatile Components and Their Contribution to the Aroma of Fennel Accessions Via SPME Analysis.** GC–MS analysis of volatiles (Figure 1) preceded by SPME resulted in the detection of 54 peaks belonging to ethers, ketones, esters, aldehydes, alcohols, monoterpenes, sesquiterpenes, and diterpenes as listed in Table 2 with ethers amounting for the

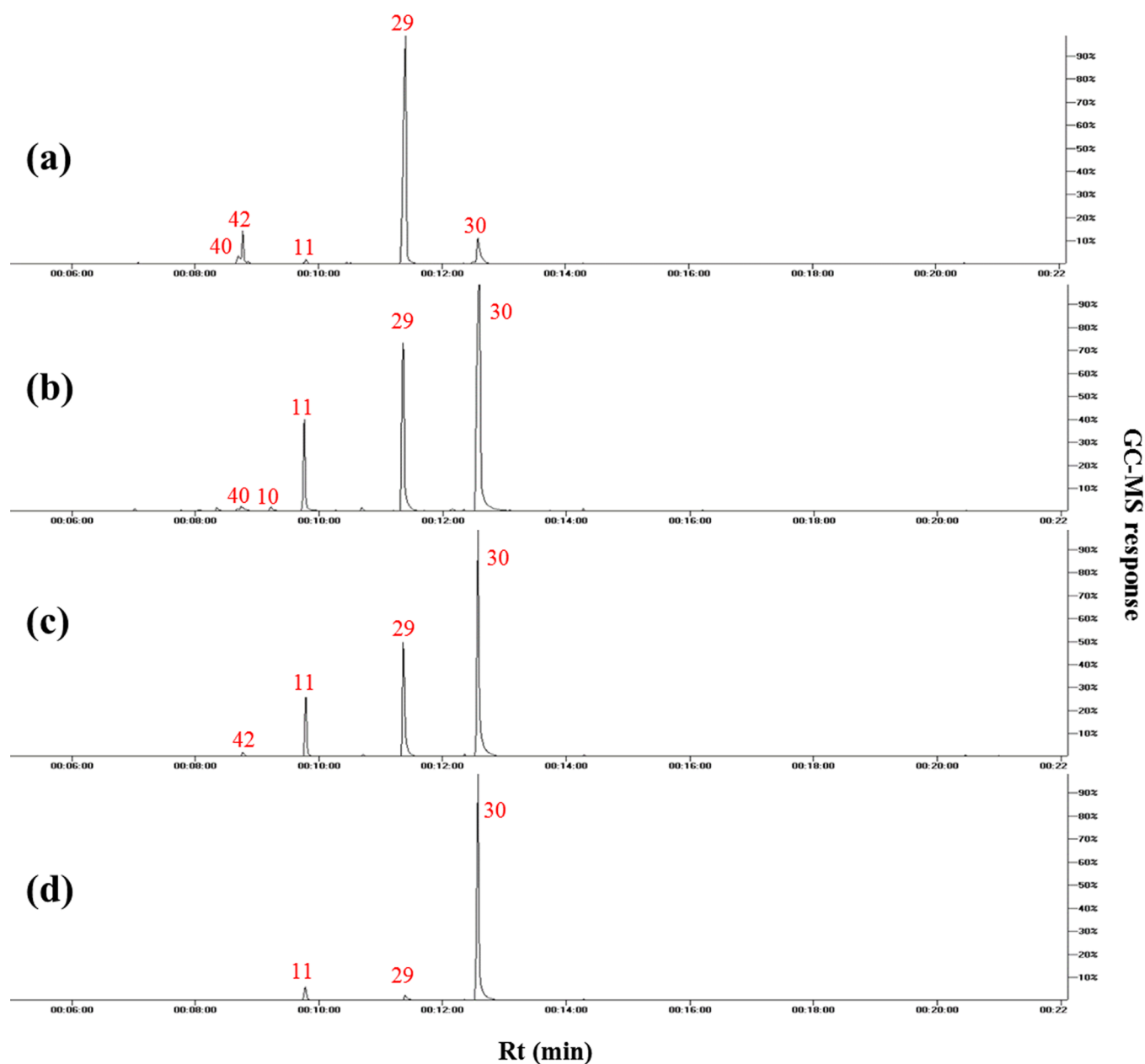
**Table 1. Origin of the Different *F. Mill.* (Fennel) Fruit Accessions Used in the Analysis<sup>a,b</sup>**

taxon and variety	origin	vendor and batch number/botanical garden and index seminum	internal sample code
<i>F. vulgare</i> Mill. subsp. <i>piperitum</i> (Ucria) Cout.			
ND	Egypt, Fayoum		P-01
ND	Egypt, Minia		P-02
ND	Sudan, Khartoum		P-03
ND	ND		P-04
<i>F. vulgare</i> Mill. subsp. <i>vulgare</i>			
var. <i>azoricum</i> (Mill.) Thell. "Deutscher Gemüesfenchel"	Austria	Arche Noah (FN014, 02–17)	V-A1
var. <i>azoricum</i> (Mill.) Thell. "Fino"	Austria	Reinsaat (Fe11 DBO 90)	V-A2
var. <i>dulce</i> (Mill.) P.FournThell.	Italy	Drogheria & Alimentari (L07315N)	V-D1
var. <i>dulce</i> (Mill.) P.FournThell.	Austria	Kotányi (L 170314 1012)	V-D2
var. <i>vulgare</i>	Austria	Kottas Pharma (W15205845)	V-V1
ND	Germany	HOH (IS 1990, No. 1431)	V-01
ND	Austria	Sonnentor (SOW15111904F04)	V-02
ND	India	P&B Foods (1G1211216)	V-03

<sup>a</sup>ND: not determined. <sup>b</sup>HOH: Hohenheimer Gärten (botanical garden and herbarium at the University of Hohenheim, Stuttgart, Germany).

major class in all fennel varieties. SPME is a relatively recent technique for aroma extraction without the use of solvents, in addition to its good sensitivity, low temperature used during extraction, and simplicity in revealing the true aroma of fennel fruits.<sup>29</sup>

**Ethers/Oxides.** Ethers/oxides were the most abundant class in all fennel varieties amounting for ca. 52.0–98.7% confirming their antioxidant and antimicrobial effects that make them as valuable food ingredients.<sup>30</sup> The results were in agreement with a previous study where ethers reached 76%.<sup>31</sup> Reaching 98.4% of the total volatiles in V-01, (*E*)-anethole (**30**) was the main volatile component in all fennel specimens except for subsp. *piperitum* from Minia and Khartoum (P-02 and P-03) where estragole (**29**) prevailed at 71.6–72.4%. (*E*)-anethole and its isomer estragole are phenylpropanoid derivatives widely distributed in other plants. (*E*)-anethole (**30**) constitutes the main volatile component in *Illicium anisatum* (star anise) and *Pimpinella anisum* (anise), while estragole (**29**) is abundant in *Ocimum basilicum* (sweet basil) and *Artemisia dracunculoides* (tarragon).<sup>32</sup> (*E*)-Anethole (**30**) is responsible for the sweet, distinct, aniselike flavor characterizing fennel fruits leading to its incorporation in cosmetics and food industry as a perfume and flavoring agent, respectively.<sup>33</sup> Conversely, estragole (**29**) has no significant impact on the overall aroma of fennel albeit estragole closely related alkenylbenzenes, that is, methyleugenol and safrole are recognized as carcinogens (IARC class 2B) that led the European Union (EU) to limit the use of estragole in beverages to 10 mg/kg.<sup>21</sup> Aside from its deep flavor, (*E*)-anethole (**30**) is reported to exhibit a broad range of pharmacological effects, namely, neuroprotective, immunomodulatory, antidiabetic, and anti-inflammatory effects.<sup>34</sup> In



**Figure 1.** Representative SPME–GC–MS total ion chromatograms of volatiles acquired from (a) P-03, (b) P-04, (c) V-D1, and (d) V-A2. For codes and peak numbers, refer to Tables 1 and 2.

contrast, estragole (29) has recently attracted attention for being genotoxic and a hepatocarcinogen.<sup>35</sup> These effects are attributed to the sulfuric ester of 1'-hydroxyestragole, a metabolite of estragole, forming an adduct with DNA.<sup>36</sup> Acknowledging these hazards, the European Medicine Agency recommended that administration of estragole should be minimized for nursing mothers, infants, and pregnant women.<sup>37</sup> In addition, although no authority has prevented the use of estragole-containing herbs, the EU Commission prohibited estragole as a food additive.<sup>35</sup> Therefore, the elevated levels of estragole (29) in P-03 and P-02 have to be considered in the assessment of whether these wild fennel varieties could represent hazards to consumers' health. Eugenol (31), a natural spicy fragrance characterizing clove buds and cinnamon barks,<sup>38</sup> was detected exclusively in P-01 samples suggestive to be a characterizing component for that accession.

**Ketones.** Ketones were the second dominant class of volatiles after ethers/oxides in all varieties of subsp. *vulgare* in addition to P-04 from subsp. *piperitum*. Ketones existed at comparable levels in P-04 and V-A2 at ca. 17.6% but were found at much lower levels in V-D1 at 0.5%, suggestive of the impact of origin and agricultural conditions, that is, soil, temperature, and water on fennel aroma. Our finding was in accordance with a previous study where ketones reached 19%.<sup>39</sup> Fenchone (11) was the only ketone detected in all fennel accessions and was reported to be another major volatile constituent of fennel reaching 16.9 and 15.7% in V-A2 and P-04, respectively. Accounting for the bitter aftertaste of fennel, fenchone (11) is used as a food flavor due to its camphorlike odor,<sup>40</sup> aside from its wound healing, acaricidal, and antifungal effects.<sup>41</sup> The European Pharmacopoeia stated that the volatile oil of bitter fennel encompasses at least 15% of fenchone.<sup>42</sup> Jasmine (14) and 4-methoxypropiphenone (16) were

Table 2. Relative Percentage of Volatile Metabolites in *F. vulgare* Fruit Accessions Analyzed Via SPME–GC–MS<sup>a</sup>

no.	name	Rt(min)	RI	P-01	P-03	P-02	P-04	V-D1	V-D2	V-A2	V-A1	V-01	V-02	V-V1	V-03
<b>alcohols</b>															
1	linalool	9.921	1076	0.20 ± 0.19	-	-	0.01 ± 0.02	-	-	-	-	-	-	-	-
2	4,8-dimethyl-1,7-nonadien-4-ol	10.139	1090	0.07 ± 0.09	-	-	-	-	-	-	-	-	-	-	-
3	fenchol	10.269	1098	0.14 ± 0.12	0.03 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.28 ± 0.27	0.03 ± 0.02	0.03 ± 0.02	-	-	-	-
4	2,6-dimethyl-2,7-octadiene-1,6-diol	10.513	1115	0.35 ± 0.21	0.36 ± 0.06	0.47 ± 0.10	0.02 ± 0.00	0.04 ± 0.05	0.22 ± 0.26	0.04 ± 0.01	0.02 ± 0.01	-	0.04 ± 0.02	-	-
5	(Z)-β-terpineol	11.153	1161	0.05 ± 0.03	0.04 ± 0.01	0.05 ± 0.01	0.01 ± 0.01	-	-	0.01 ± 0.01	-	-	-	-	-
6	(Z)-carveol	11.828	1209	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.01	-	-	-	-	-	-	-	-	-
7	nerolidol	15.443	1497	0.14 ± 0.07	-	-	-	-	-	-	-	-	-	-	-
8	1,14-tetradecanediol	20.587	1794	0.07 ± 0.04	-	0.01 ± 0.02	-	-	-	0.01 ± 0.01	-	-	-	-	-
9	isophytol	21.703	1863	1.06 ± 0.68	0.05 ± 0.01	0.07 ± 0.00	-	-	0.03 ± 0.04	0.12 ± 0.10	0.01 ± 0.01	-	-	-	-
	total alcohols	-	-	2.17	0.57	0.75	0.09	0.06	0.53	0.22	0.06	-	0.04	-	-
<b>ketones</b>															
10	fenchone isomer	9.633	1059	0.07 ± 0.10	-	0.09 ± 0.13	1.58 ± 2.23	-	0.22 ± 0.31	-	-	-	-	-	-
11	fenchone	9.781	1068	4.02 ± 2.89	5.91 ± 2.06	1.38 ± 0.21	15.74 ± 4.24	0.51 ± 0.07	6.23 ± 2.02	16.90 ± 2.54	3.20 ± 0.30	1.05 ± 0.31	1.58 ± 0.35	1.93 ± 0.25	2.12 ± 0.31
12	camphor	10.711	1130	0.05 ± 0.04	0.08 ± 0.00	0.02 ± 0.00	0.26 ± 0.06	-	0.03 ± 0.02	0.17 ± 0.02	0.02 ± 0.00	-	-	-	-
13	carvone	12.145	1233	0.14 ± 0.07	0.26 ± 0.08	0.27 ± 0.03	0.05 ± 0.01	-	0.08 ± 0.05	0.29 ± 0.05	0.93 ± 0.02	-	-	-	-
14	jasnone	13.837	1366	8.22 ± 4.34	-	-	-	-	-	-	-	-	-	-	-
15	anisylacetone	14.052	1382	0.15 ± 0.09	0.18 ± 0.08	0.40 ± 0.07	-	-	-	0.25 ± 0.35	0.31 ± 0.13	-	-	0.06 ± 0.05	-
16	4-methoxypropionophenone	14.675	1434	2.32 ± 0.75	-	-	-	-	-	-	-	-	-	-	-
	total ketones	-	-	14.98	6.43	2.17	17.63	0.51	6.56	17.61	4.45	1.05	1.58	2.00	2.12
<b>aldehydes</b>															
17	p-anisaldehyde	12.35	1248	1.43 ± 0.28	0.08 ± 0.02	0.19 ± 0.11	0.25 ± 0.08	0.32 ± 0.10	0.67 ± 0.26	0.67 ± 0.19	0.25 ± 0.03	0.04 ± 0.02	0.90 ± 0.96	0.09 ± 0.08	0.12 ± 0.10
18	methoxycinnamaldehyde	16.547	1573	0.27 ± 0.09	-	-	-	-	-	-	-	-	-	-	-
	total aldehydes	-	-	1.70	0.08	0.19	0.25	0.32	0.67	0.67	0.25	0.04	0.90	0.09	0.12
<b>esters</b>															
19	benzyl acetate	10.936	1145	1.35 ± 0.88	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	-	-	-	-	-	-	-	-
20	sabinyl acetate	11.488	1189	0.02 ± 0.00	3.47 ± 4.86	0.02 ± 0.01	0.14 ± 0.14	-	-	0.02 ± 0.01	-	-	-	-	-

Table 2. continued

no.	name	Rt(min)	RI	P-01	P-03	P-02	P-04	V-D1	V-D2	V-A2	V-A1	V-01	V-02	V-V1	V-03
<b>esters</b>															
21	fenchyl acetate	11.758	1203	0.21 ± 0.03	0.22 ± 0.01	0.16 ± 0.05	0.13 ± 0.04	0.04 ± 0.03	0.29 ± 0.33	0.15 ± 0.06	0.06 ± 0.01	-	0.01 ± 0.01	-	0.01 ± 0.01
22	bornyl formate	12.488	1257	0.98 ± 0.90	1.01 ± 0.24	1.64 ± 0.36	0.54 ± 0.38	-	0.80 ± 1.14	0.52 ± 0.37	0.06 ± 0.03	-	-	-	-
23	(Z)-3-hexenyl benzoate	16.061	1541	0.60 ± 0.67	-	-	-	-	-	-	-	-	-	-	-
24	methyl jasmonate	17.125	1611	0.13 ± 0.11	-	-	-	-	-	-	-	-	-	-	-
25	benzyl benzoate	19.794	1752	3.33 ± 3.61	-	-	-	-	-	-	-	-	-	-	-
26	methyl ester of palmitic acid	21.504	1850	0.40 ± 0.37	0.03 ± 0.02	0.06 ± 0.02	-	-	-	-	-	-	-	-	-
<b>total esters</b>															
<b>ethers/oxides</b>															
27	cineole	8.849	1008	0.36 ± 0.20	0.38 ± 0.13	0.33 ± 0.10	0.12 ± 0.04	0.06 ± 0.03	0.59 ± 0.80	0.10 ± 0.01	0.03 ± 0.01	-	0.06 ± 0.05	-	-
28	limonene oxide	10.436	1110	0.28 ± 0.16	0.22 ± 0.03	0.34 ± 0.05	0.06 ± 0.01	0.08 ± 0.05	0.26 ± 0.28	0.10 ± 0.01	0.02 ± 0.00	-	0.04 ± 0.02	-	-
29	estrangle	11.387	1177	12.31 ± 8.22	72.38 ± 1.41	71.63 ± 0.59	26.81 ± 6.57	0.74 ± 0.15	2.10 ± 0.54	20.98 ± 1.97	1.34 ± 0.06	0.30 ± 0.02	0.68 ± 0.31	0.18 ± 0.01	0.45 ± 0.02
30	(E)-anethole	12.597	1267	38.84 ± 6.17	0.15 ± 0.02	5.04 ± 2.76	49.55 ± 11.52	97.78 ± 0.63	84.35 ± 5.27	56.69 ± 1.34	92.93 ± 0.15	98.43 ± 0.29	95.90 ± 1.24	97.57 ± 0.24	96.94 ± 0.02
31	eugenol	13.596	1343	0.23 ± 0.13	-	-	-	-	-	-	-	-	-	-	-
<b>total ethers/oxides</b>															
<b>monoterpene hydrocarbons</b>															
32	unknown	6.84	893	0.05 ± 0.06	0.12 ± 0.16	0.17 ± 0.10	0.03 ± 0.04	-	-	-	-	-	-	-	-
33	$\alpha$ -thujene	6.963	899	0.02 ± 0.02	0.18 ± 0.11	0.19 ± 0.13	0.07 ± 0.05	-	-	-	-	-	-	-	-
34	$\alpha$ -pinene	7.071	905	0.55 ± 0.27	0.79 ± 0.24	0.32 ± 0.16	0.26 ± 0.09	-	-	0.04 ± 0.02	-	-	-	-	-
35	sabinene	7.789	946	0.10 ± 0.07	0.15 ± 0.05	0.11 ± 0.05	0.07 ± 0.04	-	-	-	-	-	-	-	-
36	$\beta$ -pinene	7.889	952	0.07 ± 0.03	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.01 ± 0.01	0.26 ± 0.20	0.03 ± 0.02	0.04 ± 0.01	-	-	-	-
37	myrcene	8.072	962	0.09 ± 0.01	0.12 ± 0.06	0.15 ± 0.07	0.33 ± 0.17	0.04 ± 0.03	0.60 ± 0.65	0.19 ± 0.04	0.05 ± 0.02	-	-	-	-
38	$\alpha$ -phellandrene	8.381	980	2.85 ± 4.03	-	1.52 ± 2.14	-	-	-	-	-	-	-	-	-
39	limonene isomer	8.461	987	0.24 ± 0.33	-	0.84 ± 1.18	-	-	-	-	-	-	-	-	-
40	isoterpinolene	8.675	997	4.17 ± 2.96	5.88 ± 2.63	3.04 ± 2.28	0.87 ± 0.46	-	0.20 ± 0.28	0.44 ± 0.55	-	-	-	-	-
41	<i>p</i> -cymene	8.698	998	0.39 ± 0.27	0.31 ± 0.09	0.20 ± 0.08	0.96 ± 0.89	-	0.04 ± 0.06	0.06 ± 0.01	-	-	-	-	-
42	limonene	8.765	1003	3.73 ± 2.64	6.90 ± 0.45	10.13 ± 5.60	0.83 ± 0.40	-	0.27 ± 0.24	1.14 ± 0.28	0.19 ± 0.05	-	-	-	-

Table 2. continued

no.	name	Rt(min)	RI	P-01	P-03	P-02	P-04	V-D1	V-D2	V-A2	V-A1	V-01	V-02	V-V1	V-03
<b>monoterpene hydrocarbons</b>															
43	$\tau$ -terpinene	9.239	1033	0.14 ± 0.10	0.23 ± 0.13	0.07 ± 0.03	0.73 ± 0.36	-	-	0.08 ± 0.03	-	-	-	-	-
44	$\beta$ -terpinene	9.553	1053	4.02 ± 5.68	-	0.25 ± 0.35	-	0.59 ± 0.83	-	-	-	-	-	-	-
45	isoterpinolene isomer	9.667	1060	-	-	-	0.12 ± 0.07	-	-	-	-	-	-	-	-
46	fenchene	10.339	1104	0.29 ± 0.28	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.08 ± 0.05	1.08 ± 1.46	0.10 ± 0.05	0.02 ± 0.00	-	0.04 ± 0.02	-	-
	total monoterpene hydrocarbons	-	-	16.70	14.77	17.08	4.34	0.13	3.04	2.08	0.31	-	0.04	-	-
<b>sesquiterpene hydrocarbons</b>															
47	bergamotene	14.282	1402	0.13 ± 0.10	0.08 ± 0.02	0.14 ± 0.07	0.25 ± 0.04	0.29 ± 0.11	0.58 ± 0.24	0.43 ± 0.13	0.22 ± 0.02	0.13 ± 0.05	0.75 ± 0.75	0.13 ± 0.08	0.33 ± 0.21
48	$\beta$ -farnesene	14.296	1410	0.03 ± 0.01	-	0.01 ± 0.01	0.03 ± 0.03	-	-	-	0.02 ± 0.01	-	-	-	-
49	$\alpha$ -farnesene	14.842	1448	0.06 ± 0.07	-	-	-	-	-	-	0.09 ± 0.01	-	-	-	-
50	$\alpha$ -curcumene	14.887	1452	0.06 ± 0.05	0.05 ± 0.01	0.10 ± 0.02	-	-	-	0.02 ± 0.02	0.01 ± 0.02	-	-	-	-
51	$\alpha$ -farnesene isomer	14.989	1460	0.44 ± 0.52	0.03 ± 0.01	0.07 ± 0.04	0.04 ± 0.03	-	0.22 ± 0.13	0.04 ± 0.02	0.08 ± 0.04	0.04 ± 0.01	-	0.02 ± 0.02	0.02 ± 0.03
52	unknown	20.758	1803	0.78 ± 0.99	0.02 ± 0.01	0.05 ± 0.04	-	-	-	0.03 ± 0.02	-	-	-	-	-
	total sesquiterpene hydrocarbons	-	-	1.51	0.18	0.36	0.32	0.29	0.80	0.52	0.42	0.16	0.75	0.15	0.36
<b>diterpene hydrocarbons</b>															
53	neophytadiene isomer	20.44	1786	2.91 ± 3.81	0.07 ± 0.04	0.15 ± 0.07	0.02 ± 0.01	-	0.01 ± 0.01	0.30 ± 0.12	0.05 ± 0.04	-	-	-	-
54	neophytadiene	20.985	1818	1.02 ± 1.28	0.03 ± 0.01	0.07 ± 0.03	-	-	-	0.04 ± 0.03	-	-	-	-	-
	total diterpene hydrocarbons	-	-	3.93	0.10	0.22	0.02	-	0.01	0.34	0.05	-	-	-	-

<sup>a</sup>n = 3 and results represent average ± std. deviation. For code explanation, refer to Table 1.

present exclusively in subsp. *piperitum* from Fayoum (P-01) at 8.2 and 2.3%, respectively, suggestive to be distinguishing components for that accession. 4-Methoxypropiophenone has a fruity, acetone-like flavor,<sup>43</sup> while jasmone (14) is used widely in the manufacturing of perfumes owing to its warm fruity smell.<sup>44</sup>

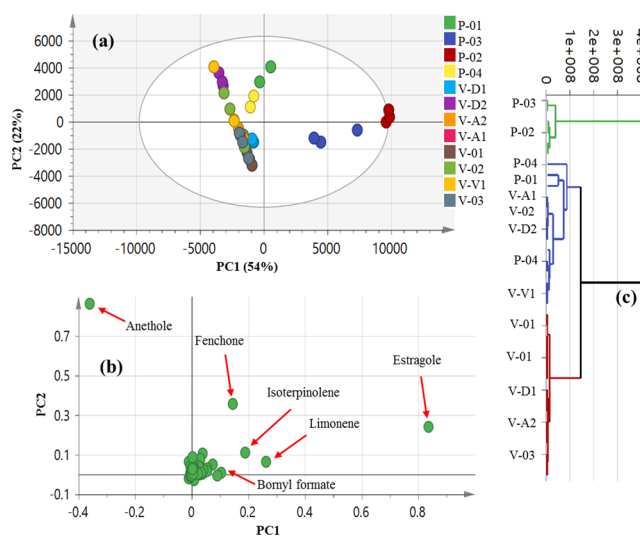
**Mono-/Sesqui-/Diterpene Hydrocarbons.** Compared to ketones, monoterpene hydrocarbons were the second abundant class after ethers/oxides in subsp. *piperitum* from Egypt and Sudan (P-01, P-02, and P-03) at ca. 14.8–17.1% and to a lesser extent in P-04 in addition to V-D2 and V-A2, two different varieties from subsp. *vulgare* as indicated in Table 1 (2.1–4.3%), and reached almost 0% in the rest of the accessions. This was in accordance with a previous study where hydrocarbons reached 7%.<sup>39</sup> The major identified monoterpenes included limonene (39, 42) and isoterpinolene (40, 45) detected at their highest levels in P-02 (10.1%) and P-03 (5.9%), respectively. Limonene (39, 42), a major constituent of citrus fruits, is added to many food products for its lemonlike flavor aside from its protective effect against multimodal intestinal inflammation.<sup>45</sup> Comparable quantities of isoterpinolene (40, 45) and its precursor  $\alpha$ -pinene (34)<sup>46</sup> were detected, accounting for the positive correlation between their levels in the studied samples.  $\alpha$ -Phellandrene (38) was detected only in P-01 and P-02 at 2.9 and 1.5%, respectively, with a minty, herbal, spicy note,<sup>47</sup> suggestive to be a distinguishing component for these fennel accessions.

P-01, a subsp. *piperitum* from Fayoum, encompassed the highest levels of sesqui- and diterpene hydrocarbons at 1.5 and 3.9%, respectively. Neophytadiene (53, 54) was the only detected diterpene. This branched hydrocarbon is a predominant component in tobacco leaves<sup>48</sup> and exhibits a myriad of effects, that is, antioxidant, anti-inflammatory, antimicrobial, and analgesic effects.<sup>49</sup>

**Alcohols/Aldehydes/Esters.** Contributing to the antioxidant and antimicrobial effects of various essential oils,<sup>50</sup> alcohols were most abundant in P-01 at 2.1% as exemplified by isophytol (9). Isophytol (9) is used as a flavoring agent due to its fine floral balsamic smell.<sup>51</sup> Aldehydes included less diverse number of volatiles than alcohols with methoxycinnamaldehyde (18) detected exclusively in P-01. In contrast, *p*-anisaldehyde (17) was present in all fennel accessions and had been previously reported as a component of fennel.<sup>31</sup> *p*-Anisaldehyde (17) has a strong, sweet, aniselike fragrance and is formed from (*E*)-anethole (30) upon oxidation.<sup>52</sup> Esters were more abundant in subsp. *piperitum* from Egypt and Sudan (P-01, P-02, and P-03) ranging from 1.9 to 7.0% compared to the other specimens. Sabinyl acetate (20) was present at a much higher level in P-03 (3.5%) concurrent with the highest levels of its precursor sabinene (35). Sabinyl acetate (20) detected also in V-A2 (a ssp. *vulgare* var. *azoricum*) has a fresh spicy, fruity note, while fenchyl acetate (21) is characterized by a camphoraceous, herbal flavor.<sup>53</sup> Having a balsamic, herbal aroma, benzyl benzoate (25) was found only in subsp. *piperitum* from Fayoum P-01 (Table 1) at 3.3%. Benzyl acetate (19) was detected at higher levels (1.4%) in P-01 with a floral, fresh sweet note.<sup>54</sup>

**Multivariate Data Analysis of Fennel Fruit Aroma Profile.** Recognizing the considerable number of detected peaks in 12 fennel accessions, each comprising of three replicates, we tried to categorize them holistically using chemometric tools. Multivariate data analyses including principal component analysis (PCA) and hierarchical cluster-

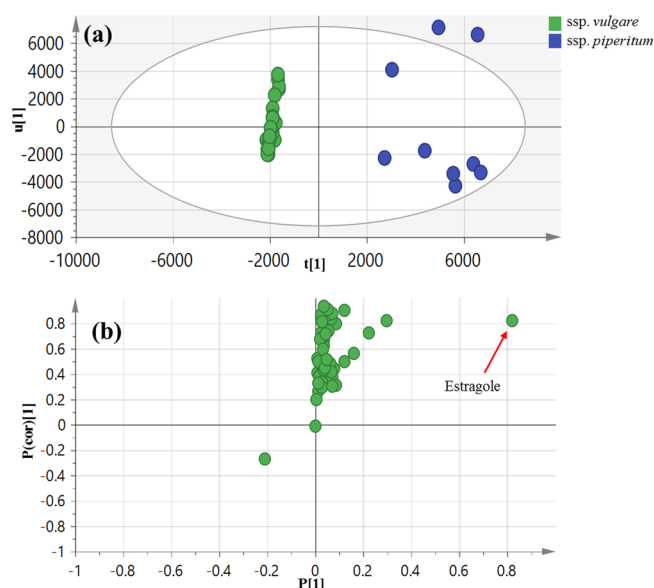
ing analysis (HCA) as unsupervised pattern recognition models were applied to the volatile metabolite GC–MS abundance data set (Figure 2). The PCA score plot (Figure



**Figure 2.** Unsupervised multivariate data of the studied *F. vulgare* fruit accessions derived from modeling of volatile profiles analyzed via GC–MS ( $n = 3$ ). (a) PCA score plot of PC1 vs PC2 scores. (b) Respective loading plot for PC1 and PC2 providing their assignments. (c) HCA plot. The metabolome clusters are placed in a two-dimensional space at distinct locations defined by two vectors of principal components PC1 = 54% and PC2 = 22%. For codes, refer to Table 1.

2a) showed 54% of the total variance among the samples prescribed by PC1 and PC2. It also revealed good segregation of the two clusters, subsp. *piperitum* from Khartoum and Minia (P-03 and P-02), at the right side of PC1, in addition to a part from subsp. *piperitum* from Fayoum P-01 samples. Conversely, all other specimens including subsp. *vulgare* were positioned collectively at the left side of PC1 albeit distributed along PC2. Examination of the corresponding loading plot (Figure 2b) revealed that estragole (29) was the discriminatory volatile component for P-02, rationalizing for its allocation at the far side of PC1 as shown in Figure 2a. In contrast, limonene (42), isoterpinolene (40), and bornyl formate (22) were identified as markers for subsp. *piperitum* from Egypt and Sudan (P-01, P-02, and P-03) and confirmed their enrichment in monoterpene hydrocarbons. Oxygenated monoterpenes, that is, ketones were represented by fenhone (11) in P-04 and V-A2, whereas (*E*)-anethole (30) was more enriched in V-V1, ssp. *vulgare* var. *vulgare*, and V-02 leading to higher levels of ethers/oxides. Similar findings with consistent clustering of both subsp. *piperitum* samples from Khartoum and Minia (P-03 and P-02) at one subgroup were observed, whereas overlapping of the other samples was observed in the HCA-derived dendrogram at the other subgroup (Figure 2c).

Another PCA model (Figure S1) was adopted to help identify the differences among subsp. *vulgare* that was clustered in the first PCA model (Figure 2). However, this second model was not successful in discriminating subsp. *vulgare* of different origins. Subsequently, OPLS-DA (Figure 3) was further employed to model subsp. *vulgare* against subsp. *piperitum* accessions to reveal the volatile characteristics of each fennel subspecies with a  $p$  value less than 0.001. The clear discrimination between the two subspecies in the OPLS-DA



**Figure 3.** HS-SPME GC-MS-based OPLS-DA score plot (a) derived from modeling volatile metabolites of subsp. *vulgare* against subsp. *piperitum* ( $n = 3$ ). The respective loading S-plots (b) showing the covariance  $p[1]$  against the correlation  $p(\text{cor})[1]$  of the variables of the discriminating component of the OPLS-DA model. Cutoff values of  $p < 0.001$  were used. Designated variables are highlighted and identifications are discussed in the text.

score plot (Figure 3a), in addition to the respective model parameters  $R^2$  and  $Q^2$  at 0.72 and 0.67, respectively, indicated good model fitness and predictability. The OPLS-DA-derived S-plot (Figure 3b) revealed that estragole (29) was more enriched in subsp. *piperitum* with no markers for subsp. *vulgare*. This result suggested that absolute quantification of estragole in subsp. *piperitum* should now follow to assess the safety of regular use of fennel.

**Absolute Determination of Estragole Levels in Fennel Accessions.** Being a potential genotoxic carcinogen, estragole detected at levels higher than 70% in some fennel fruits necessitated its accurate determination.<sup>55</sup> The mechanism of its carcinogenicity was previously explained owing to the release of 1'-hydroxyestragole.<sup>36</sup> HS-SPME coupled with GC-MS was applied as a simple method for estragole quantification using standard estragole over a concentration range of 1–500  $\mu\text{g}/\text{mL}$ . Quantitative differences were observed in estragole levels among fennel fruit accessions (Table 3) ranging from 0.03 to 89.86 mg/g. P-02 subsp. *piperitum* from Minia contained the highest levels at 89.86 mg/g of estragole indicating higher levels than that usually reported. In general, subsp. *piperitum* encompassed much higher amounts of estragole at ca. 17.4–89.9 mg/g compared with subsp. *vulgare* (0.03–4.5 mg/g) as indicated by HCA modeling of fennel accessions based on its estragole level only (Figure S2). Considering that 5 g of P-02 is used to make fennel tea per day, the average estragole exposure for infants would be at ca. 35 mg/kg body weight compared to 0.02 mg/kg reported from another study.<sup>56</sup> However, 33 mg/kg body weight of estragole induced hepatocellular carcinoma in female mice exposed for 12 months.<sup>57</sup> Further assessment is required for exposure of estragole in sensitive groups including infants, young children, nursing mothers, and pregnant women.

**Table 3.** Absolute Determination of Estragole Levels in Different Fennel Varieties<sup>a</sup>

fennel accession	estragole level mg/g
P-01	17.36 $\pm$ 2.41
P-03	54.25 $\pm$ 12.57
P-02	89.86 $\pm$ 1.05
P-04	24.59 $\pm$ 21.88
V-D1	4.50 $\pm$ 0.44
V-D2	0.63 $\pm$ 0.06
V-A2	0.32 $\pm$ 0.27
V-A1	0.05 $\pm$ 0.03
V-01	0.03 $\pm$ 0.03
V-02	0.04 $\pm$ 0.03
V-V1	0.08 $\pm$ 0.04
V-03	0.04 $\pm$ 0.03

<sup>a</sup>For codes, refer to Table 1. ( $n = 3$ ) and results represent average  $\pm$  std. deviation.

## CONCLUSIONS

The present study provides the most comprehensive aroma profiling amongst *F. vulgare* fruit accessions of various origins. Moreover, the significant discriminatory capacity of subsp. *vulgare* and subsp. *piperitum* has been revealed. Within the identified 54 volatiles, ethers, ketones, esters, and monoterpene hydrocarbons were the major classes that mediate the medicinal and culinary attributes of fennel. Ethers expectedly were the most abundant class and their enriched levels can be observed in subsp. *vulgare* due to their elevated (*E*)-anethole level. Fenchone, as a source of bitterness in fennel, was detected in at 16.9%, implying a more bitter taste compared to other accessions. In contrast, subsp. *vulgare* var. *dulce* from Italy likely exhibited the sweetest taste, being the lowest in fenchone (0.5%) and most enriched in (*E*)-anethole (97.8%). Subsp. *piperitum* from Fayoum, Egypt appeared to be the most diverse in volatiles with 53 volatile metabolites with jasnone, 4-methoxy-propiofenone, methoxycinnamaldehyde, 3-hexenyl benzoate, methyl jasmonate, and benzyl benzoate present exclusively in this accession. Among the main identified volatiles, estragole reached maximal levels in mostly *F. vulgare* Mill. subsp. *piperitum* accessions with maximal levels in specimens from Khartoum and Minia. Absolute quantification of estragole was determined to enable the estimation of dietary exposures of fennel varieties, with subsp. *piperitum* encompassing much higher levels of estragole (17.4–89.9 mg/g) compared with subsp. *vulgare* (0.03–4.5 mg/g) indicating a potential risk for the former subspecies. These results suggest that the volatile biosynthesis of subsp. *piperitum* is more activated toward the production of estragole compared to subsp. *vulgare* though such hypothesis needs to be confirmed by analyzing accessions from other origins. The exposure of estragole in infants and young children needs extensive investigation and clear dietary and pharmaceutical regulatory guidelines regarding the nonspecific label information found on most fennel products should be mandated to specify estragole levels in the formulation as in other typical food hazards, that is, trans fat.

Estragole cannot be identified as a chemomarker for *piperitum* subspecies, owing to its presence in subsp. *vulgare* as well. Profiling of secondary metabolites in both subspecies, that is, flavonoids should be considered in the future in the search for further more unique markers. Development of simple colorimetric assays specific for estragole detection



should also allow for the rapid online screening of fennel fruits at an industrial level.

Our adopted technique can certainly be used as a powerful tool for analyzing fennel from other sources to assess the impact of agricultural practice, storage, growth stage, and seasonal variation on the metabolic makeup. Liquid chromatography coupled to MS (LC–MS) can also be performed in a wider prospect to pinpoint the heterogeneity of metabolites among fennel accessions and more related to their health effects, that is, antioxidants.

## ■ EXPERIMENTAL SECTION

**Plant Material.** *F. vulgare* Miller fruits were obtained from different sources with sample information presented in Table 1. After cleaning with water, fruits from each specimen were separately homogenized with a mortar and a pestle under liquid nitrogen and then stored in tight glass containers at  $-20\text{ }^{\circ}\text{C}$  till further analysis. Vouchers of fennel specimens were deposited at the College of Pharmacy Herbarium, Cairo University, Egypt.

**Chemicals and Fibers.** For SPME sampling, fibers of polydimethylsiloxane (PDMS) or divinylbenzene–carboxen–polydimethylsiloxane (DVB–CAR–PDMS) of 1 cm length and 50/30  $\mu\text{m}$  were obtained from Supelco (Bellefonte, PA, USA). All standards, chemicals, and solvents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

**SPME–GC–MS Volatile Analysis.** Volatile analysis using HS–SPME was carried out as reported by Farag et al.<sup>58</sup> The finely pulverized fennel fruits, 20 mg spiked with 10  $\mu\text{g}$  of (Z)-3-hexenyl acetate, were accurately weighed in 1.5 mL tightly sealed glass vials with an SPME fiber implanted manually and volatile compounds were extracted at  $50\text{ }^{\circ}\text{C}$  for 30 min. Then, the SPME fiber was subsequently removed and placed manually in the injection port of the GC–MS. The analysis of volatile compounds was conducted using an Agilent 5977B GC/MSD equipped with a DB-5 column (30 m length, 0.25 mm i.d., and 0.25  $\mu\text{m}$  film thickness, Supelco) and interfaced with a quadrupole mass spectrometer. The injector and MS interface temperatures were both held at  $220\text{ }^{\circ}\text{C}$ . The analysis conditions were as follows: the constant flow of helium in the column was kept at 0.9 mL/min, the oven temperature was held at  $40\text{ }^{\circ}\text{C}$  for 3 min, then raised at a rate of  $12\text{ }^{\circ}\text{C}/\text{min}$  to  $180\text{ }^{\circ}\text{C}$ , maintained at  $180\text{ }^{\circ}\text{C}$  for 5 min, and finally increased to  $240\text{ }^{\circ}\text{C}$  at a rate of  $40\text{ }^{\circ}\text{C}/\text{min}$  and kept for 5 min. SPME fibers were cleaned for the next analysis by maintaining in the injection port at  $220\text{ }^{\circ}\text{C}$  for 2 min. Blank GC–MS runs were performed during sample analyses. For evaluation of biological replicates, under the same conditions mentioned above, three separate samples were analyzed for each fennel specimen. The quadrupole mass spectrometer was operated at 70 eV using the electron impact mode with a scan range of 40–500  $m/z$ . Peak abundance was extracted using a default parameter using the MET-IDEA tool.<sup>59</sup> The relative percentile was calculated by dividing each peak area to the total area of the identified peaks multiplied by 100 within each specimen.

Absolute quantification of estragole levels in fruits was carried out using a standard calibration curve recorded under the same SPME conditions with serial dilutions of estragole dissolved in ethanol at 1, 10, 100, and 500  $\mu\text{g}/\text{mL}$ . A volume of 2  $\mu\text{l}$  was aliquoted from each concentration level and placed in a 1.5 mL vial and subjected to SPME volatile analysis. Each fennel sample (20 mg) was placed in triplicate inside the same 1.5 mL vial and subjected to the same volatile extraction

conditions as standards. The standard calibration curve for estragole showed excellent linearity following the equation  $y = 18,002 \times +195.33$  with 0.999 as the correlation coefficient. Limit of quantification and limit of detection were 0.82 and 0.02  $\mu\text{g}/\text{mL}$ , respectively.<sup>25</sup>

**Metabolite Identification and Multivariate Data Analyses.** Volatile components were identified by comparing their Kovats indices relative to C6–C20 *n*-alkane series as well as by matching the mass spectra obtained with the NIST and WILEY libraries and with standards when available. Before mass spectral matching, peaks were first deconvoluted using AMDIS software ([www.amdis.net](http://www.amdis.net)), and their abundance data were extracted using the MET-IDEA tool.<sup>59</sup> The data table was then normalized to a spiked internal standard before exporting to PCA, HCA, and OPLS-DA using the SIMCA-P version 13.0 software package (Umetrics, Umeå, Sweden). Subsequently, markers were determined by analyzing the S-plot, which revealed covariance (p) and correlation (pcor). All variables were Pareto-scaled and mean-centered. Validation of models was conducted by computing the diagnostic indices, that is, Q<sup>2</sup> and R<sup>2</sup> values, and by permutation testing.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c06188>.

Unsupervised multivariate data of the studied *F. vulgare* fruits derived from modeling of volatile profiles of subsp. *vulgare* analyzed via GC–MS and HCA of estragole alone in fennel varieties (PDF).

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### Notes

The authors declare no competing financial interest.

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