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Archivio Istituzionale dei prodotti della ricerca

Morphological and phytochemical features in fruits of *Pimpinella anisoides* V. Brig. (Apiaceae)

This is the peer reviewed version of the following article:

*Original*

Morphological and phytochemical features in fruits of *Pimpinella anisoides* V. Brig. (Apiaceae) / Laface, V.L.A., Sicari, V., Musarella, C.M., Spampinato, G., Maruca, G., Giuffrè, A.M.. - In: PLANT BIOSYSTEMS. - ISSN 1724-5575. - 157:1(2023), pp. 210-220. [10.1080/11263504.2022.2160025]

*Availability:*

This version is available at: <https://hdl.handle.net/20.500.12318/133766> since: 2024-12-04T17:53:13Z

*Published*

DOI: <http://doi.org/10.1080/11263504.2022.2160025>

The final published version is available online at: <https://www.tandfonline.com/doi/full/10.1080/11263504>.

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(Article begins on next page)

# 1 **Morphological and Phytochemical Features in Fruits of *Pimpinella Anisoides* V.**

## 2 **Brig. (Apiaceae)**

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5  
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## 11 **Abstract**

12 In this study, the morphological characteristics, phytochemical composition of *Pimpinella anisoides*  
13 fruits (monocarps), an endemic plant of Italy was investigated.

14 The morphological analysis of the monocarps contributed to distinguish *P. anisoides* from other  
15 related *Pimpinella* species used for commercial purposes, in particular *P. anisetum* and *P. anisum*.

16 Our study showed that these fruits are different for their surface and shape: in particular, *P. anisoides*  
17 monocarps are glabrous, whereas the other two species are strigose.

18 Two main compounds were identified in the essential oil of the five studied accessions: Limonene  
19 (46.78-50.74%) and Anethole (38.50-42.77%) whose sum ranged between 85.28 and 93.51%.

20 Estragole and  $\beta$ -Phellandrene were other two representative compounds. All other volatiles accounted  
21 for less than 1% of the total volatile composition. One-way ANOVA analysis showed significant  
22 differences in almost all volatiles, whereas non-significant differences were found in the compounds  
23 detected in very low quantity. Hierarchical cluster analysis allowed to group the five accessions in  
24 three main clusters. Principal Components Analysis showed that the first two principal components  
25 explained 71.80 % of total variance.

52 Bartolucci et al. [19] reported 7 native species of *Pimpinella* and Galasso et al. [20] 1 alien. Following  
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subsequent updates, it is now possible to recognize 10 taxa in total, including 3 subspecies, of which 9 are native (N) and 1 alien (A) (Portal to the Flora of Italy, 2021). However, the interpretation of the Italian taxa belonging to the genus *Pimpinella* L. is controversial. Indeed, Stinca and Ricciardi [21,22] reports 12 Italian taxa of *Pimpinella*, including 9 species and 3 subspecies.

*Pimpinella anisoides* V. Brig. [Synonyms: *Apium anisoides* (Brign.) Caruel, *Apium gussonei* (C. Presl) Calest., *Pimpinella bubonoides* DC., *Tobion bubonoides* Raf.; Italian name “Tragoselino

## Introduction

The genus *Pimpinella* L. (Apiaceae Lindl., subfamily Apioideae, tribe Pimpinelleae) includes about 150-180 annual or perennial herbs distributed in the temperate and subtropical regions of Eurasia and Africa, but also in South America and in the western part of North America [1,2].

There have been numerous studies on this genus, but its taxonomy has not yet been fully clarified, representing one of the most complex genera of the Apiaceae family [3-7]. Fernández Prieto et al. [8] points out that the *Pimpinella* is a non-monophyletic group and that the *Pimpinella* taxa growing in Western Europe are part of phylogenetically independent groups that correspond to three different tribes of the Apioideae subfamily: Pimpinelleae (main group), Pyramidoptereae and Smyrnieae. The taxonomy of the genus *Pimpinella* but, in general, for different genera of Apiaceae family, is the fruit: shape, pubescence and anatomy [9-14] In particular, the anatomy of many species of this genus was studied [15,16,7,2] Another important character regarding this genus is its phytochemical composition. Tabanca et al. [17] studied the content of essential oils of 19 Turkish species of *Pimpinella* and established the phylogeny of *Pimpinella* species using gene sequence data and of essential oil analysis as a framework to ascertain whether there is a connection between the chemical and genetic profiles. Magee et al. [7] studied the phylogeny of 16 African and Malagasy species, including also 26 species from Eurasia. Fereidounfar et al. [18] analyzed 52 Southwest Asian species of *Pimpinella*. In the genus *Pimpinella*, the basic chromosome number is  $x = 8$  to 11 [18].

27 The study of monocarps morphological and phytochemical features showed how characterize and  
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28 recognize the ripe fruits of *P. anisoides* for distinguish this species from others to avoid commercial  
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529 sophistications and for a better conservation *in situ* and *ex situ*.  
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1031 **Keywords:** Black Anise; Calabria; essential oils; *ex situ* conservation; *in situ* conservation; Italy;  
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1232 sophistication.  
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1759 meridionale”; vernacular Calabrian name “aranzu”)] is an endemic species growing in some southern  
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1960 Italy regions, such as Lazio, Basilicata [23-25], Calabria [26,27], Campania [28], Sicily [29] , and  
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2261 Puglia [30,19,31,21,22], especially in the mountainous belt [27,25] occupied by mesophilic oak and  
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2462 chestnut woods. In Calabria, *P. anisoides* is reported for several locations. However, the practice of  
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2763 harvesting its fruits to flavor various traditional baked goods and liqueurs and as seasoning is known  
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2964 only for the Reventino Massif and Sila Catanzarese area [27].  
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3265 Although the species is currently considered LC (Least Concern) [32], it is collected for commercial  
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3466 purposes in several regions: this is compromising the reproductive potential of the species with  
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3667 negative effects on the size of populations [27-25].  
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3968 Some studies on the chemical composition of *P. anisoides* were carried out in the past. <sup>33</sup> Menichini  
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4169 et al. [33] studied acetylcholinesterase and butyrylcholinesterase inhibition of ethanolic extract and  
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4470 monoterpenes from *P. anisoides*, whereas Conforti et al. [34] described its chemical profile and in  
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4671 vitro properties, finding 23 major components in the ethanolic extract of its fruits.  
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4972 Distinguish *Pimpinella* species among them is not so easy [35]. For this purpose, morphometric  
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5173 studies are very important in discriminating botanical taxa [36,37,38]. In particular, in the genus  
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5374 *Pimpinella*, as well as in most of the Apiaceae, the fruits take on considerable diagnostic significance  
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5675 and are considered essential in taxonomic analysis [35]. According to Spjut [39], Apiaceae fruits are  
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5876 classified as polachenarium, i.e. a schizocarpic fruit, originating from an inferior ovary separating in  
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103 Fruits were analyzed and measured using a stereomicroscope equipped with an HD camera Invenio  
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1483 on *P. anisoides* fruits, to evaluate their characteristics for a proper valorization and protection of this  
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1784 endemic species.

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## 2286 **Materials and Methods**

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2487 The fruits (monocarps) were collected in five locations in the Sila Catanzarese, province of Catanzaro

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2688 (Central Calabria, S-Italy) (Fig. 1), at altitudes between 700 and 900 m above sea level, in mountain

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28 forests of chestnut or deciduous oaks which represent their habitats (Tab. 1). The fruits were harvested

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30 at the time of ripening between July 2017 and August 2018 by local farmers who also deal with their

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33 marketing.

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35 The fresh fruits were cleaned in the Geobotany laboratory of the Mediterranean University of Reggio

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37 Calabria (Italy), counted and analyzed for quality. To this purpose, 300 fruits were randomly sorted

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3993 into 6 groups of 50. In each group, the fruits were observed and distinguished by an optical

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4194 stereomicroscope Optech® into: “normal” (intact fruits, ripe, without evident macroscopic damages);

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43 “damaged” (for mechanical damages, malformations or with holes or injuries caused by insects);

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45 “aborted” (fruits that have not completed the ripening process correctly). Only “normal” fruits were

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47 used in the morphological and phytochemical analyses. Specimens of *P. anisoides* collected in the

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49 study area, are stored in the Herbarium of the Mediterranean University of Reggio Calabria

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5198 (REGGIO).

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### 56100 **2.1 Morphological analysis**

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77 single fruits (monocarps) from a longitudinal central axis (columella, or carpophore) often remaining  
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attached to a computer, where its DeltaPix inSight© software is installed. This software allowed  
In order to improve image quality, a multifocal system, to determine the exact measurements of the fruits  
at different magnifications and to replicate the measurements. Current research in General Catalan, Se  
Spain (Fig. 1) of botanical investigations have been launched on this important species. This work  
This is the first work obtained from a sample of 20 fruits for morphological investigations examined. In  
detail, the following dimensions were measured: monocarp width and length, length from base to  
maximum width of monocarp, stylopodium width and length (Fig. 2 and Tab. 1). Fruit terminology  
is in agreement with Yeşil et al. [35].

## 2.2 Essential oil extraction

Fruits were harvested and air dried at room temperature until constant weight. For each sample, a 30  
g aliquot was randomly taken out of 100 g. Fruits were powdered by a home grinder and blended with  
300 mL water. At this point, fruits were hydro-distilled in an all glass Clevenger-type apparatus for  
3 hours. The essential oil was separated from the water, placed in a glass vial and stored in a fridge  
in the dark at 4 °C before GC-MS analysis that was conducted within 48 h after extraction.

## GC-MS Analysis

It was applied for the qualitative analysis. The samples were analysed by gas chromatography with  
mass spectrometry (GC-MS) using a GCMS-QP2010 Ultra (Shimadzu, MI, Italy) operated by Lab  
solution software. The chromatographic separation was conducted using a fused capillary column  
(Mega SE52, 0.25 µm, 0.25mm, 30m). MS source temperature: 280 °C; interface temperature: 280  
°C; ionization energy: 70 eV; ionization current: 60A. The oil components were identified by  
computer matching against Nist library. The samples were analysed on three replicates

## GC-FID Analysis

153 The fruits are always glabrous in all their parts with 5 well evident dorsal ribs  $\pm$  prominent (Fig.3a).  
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1230 solution (Shimadzu, MI, Italy) equipped with flame ionization detectors (FID), and fused capillary  
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1531 column (Mega SE52, 0.25  $\mu$ m, 0.25 mm, 30 m). The oven temperature was programmed: isothermal  
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1732 40 °C for 3 min, from 40 to 220 °C (6 °C/min), from 220 °C to 250 °C (15 °C/min), and isothermal  
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1933 for 5min; injector temperature: 270 °C (injection mode: split 1/100); detector temperature: 270 °C;  
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11234 carrier gas: helium.

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1435 One  $\mu$ l of essential oil was directly injected. The relative proportions of the essential oil constituents  
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11736 were expressed as percentages obtained by peak-area normalization.

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2238 **Statistical analysis**

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21439 Means and standard deviations were calculated by Excel© 2010 software. Statistical differences were

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2740 calculated by one-way ANOVA and Tukey test for post hoc analysis at  $p < 0.05$  using the SPSS®

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21941 23.0 software (SPSS Inc., Chicago, IL, USA). Principal Component Analysis (PCA) were applied

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3142 using SPSS® software for Windows©, version 17.0 (Chicago, IL, USA). Then, a cluster analysis was

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3143 performed and a classification obtained through the mean link on the Euclidean distance squared

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144 similarity function.

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4146 **Results and Discussion**

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4447 **Morphology**

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41648 A preliminary morphological analysis shows that 50% of the fruits were 'normal', 30% 'aborted' and

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149 20% 'damaged'. The "normal" fruits are oblong-ovoid (length/width ratio 1.09) to oblong-cylindrical

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5150 (length/width ratio 2.05), on average 2.77 ( $\pm$  0.30) mm long and 1.33 ( $\pm$  0.14) mm wide; the monocarp

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151 length from base to maximum width is 1.12 ( $\pm$  0.14) mm. The stylopodium is on average 0.45 ( $\pm$

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51652 0.06) mm long and 0.33 ( $\pm$  0.09) mm wide (Tab.2).

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129 GC analysis was performed to quantify compounds using a GC2010 Plus Tracera operated with GC  
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1254 The monocarp is compressed dorsally. The ventral surface is flat, longitudinally is well visible the  
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155 columella that joins the two monocarps (Fig.3 b).

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1756 Examination of the fruit cross-section showed that *P. anisoides* monocarps are characterised by a  
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157 smooth, thin cuticle (Fig. 4). Five small vascular bundles (vb) are present in the lower half of the  
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1258 dorsal and lateral ribs.

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159 The vittae present in the mesocarp containing the essential oils in which the species is rich, are divided  
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170 into:

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1961 - vallecular vittae (vv): three between the central groove and the median groove and four in the  
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2262 median grooves closest to the commissural side. They are larger than the vascular bundles  
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2463 (vb);
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2764 - commissural vittae (cv): located on the commissural side are four, two larger and more  
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2965 obvious as typical feature of fruits of the genus *Pimpinella*, the other two smaller located near  
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3266 the lateral vascular bundles (vb).

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3567 The endosperm (e) occupies the largest part of the section (Fig. 4).The analysis of variance of results  
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3768 (Anova test) (Table 2) shows a high significance value of the data (\*\*\*) =  $p < 0.001$ .

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4069 The morphological analysis of the monocarps, allows to distinguish the fruits of *P. anisoides* from  
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4270 related *Pimpinella* species used for commercial purposes. In particular, the monocarps of *P.*  
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4471 *anisetum* Boiss. & Balansa are strigose with sharp, stiff, slanting hairs, hair-like scales or bristles  
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4772 which are appressed to the surface and ovoid in shape, whereas *P. anisum* L. has strigose, ovoid-  
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4973 subglobose fruits [35]. In *P. anisoides* the monocarps are completely glabrous and vary in form  
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5274 from oblong-ovoid to oblong-cylindrical (Table 3).

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5475 These distinctive characters are very important and necessary to know in order to prevent the sold  
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5776 material from being sophisticated with other types of fruits of inferior quality, which are often mixed  
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5977 with those of *P. anisoides*.

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205 5.67% and 5.54% in the essential oil extracted by a Clevenger-type apparatus with steam distillation  
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1280 Essential oils are an important discriminating factor between different taxa [40]. They can have  
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1581 several applications in preventing some diseases [41], but they are also a constituent of fragrances  
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1782 and flavours which are fundamental in a food or a beverage with regard to the approach of a consumer  
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1883 which: see, smell and taste a product before to eat or drink.  
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11  
11284 Little is known about the essential oil of the *Pimpinella* genus with very scarce findings for the *P.*  
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1485 *anisoides* and *P. anisetum* species and some information more for the *P. anisum* species.  
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11786 Thirty-two components were identified in the *P. anisoides* essential oil studied in this work (Table  
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1987 4). The four most preponderant compounds of the five studied accessions were: Limonene, Anethole,  
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2288 Estragole and  $\beta$ -Phellandrene, here listed in decreasing order for the highest quantity detected.  
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21489 The most representative was limonene ranging between 50-74% (Pa\_3) and 46.78% (Pa\_5) ( $p < 0.001$   
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2790 (Table 4). Limonene is a hydrocarbon monoterpene used to prepare flavourings, perfumes and in  
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21991 local medicine for its anaesthetic, antiseptic, expectorant, antiviral, antilithic and antimicrobial  
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3192 properties [42-45] even if Boughendjioua and Boughendjioua have found a lower antimicrobial  
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31493 activity of limonene than alcohols and phenols. Studies in aromatherapy have shown an anti-migraine  
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3694 activity [46,47]. Menichini et al [33] found a 13.5% limonene content in *P. anisoides* essential oil  
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3195 from Calabria region after fruits maceration in ethanol, this allows us to speculate that water used as  
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4196 a solvent facilitate the limonene extraction from fruits if compared with ethanol. *P. anisoides* was  
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4197 found to have a higher limonene content than *P. anisum*, in fact in the latter, the limonene content  
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4198 was: 2.06% in Iranian fruits [48]; 0.63 g/kg, 0.51 g/kg and 2.11 g/kg in Iranian fruits respectively  
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4999 obtained from plants grown in a soil without Zn addition and with 0.1% and 0.2% Zn addition (w/v)  
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5200 [49]; 0.06% in Serbian seeds [50]; 0.2% in Turkish seeds from Fadliya region, Mosul Province [51].  
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5201 Limonene is characteristic of the essential oil of *Citrus* genus and it was quantified for: 84.73% in  
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5202 lemon peel [52]; 39.74% in lemon flower [*Citrus ×limon* (L.) Osbeck] [53]; 60.8% in *Citrus medica*  
57  
5803 cv. ‘Diamante’ peel [54], 31.10% to 44.48% in bergamot (*Citrus bergamia* Risso) peel [55,56].  
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6204 Limonene is also contained in the essential oil of other genus and species but in lower quantity: 6.05%

## 179 **Phytochemical**

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2<sup>06</sup> respectively from flowers, fruits and leaves of wild fennel [*Foeniculum vulgare* Mill. subsp.  
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5<sup>07</sup> *piperitum* (Ucria) Bég.] [57], 3.80% to 10.11% in essential oil of needles of Calabrian pines (*Pinus*  
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7<sup>08</sup> *nigra* J.F. Arnold subsp. *laricio* Palib. ex Maire) [58].

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10<sup>09</sup> Anethole was the second major volatile in *P. anisoides* fruit essential oil and ranged between 42.77%  
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12<sup>10</sup> (Pa\_5) and 38.50% (Pa\_3) ( $p < 0.001$  (Table 2). Anethole is a phenylpropanoid. It is sweet (13 times  
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14<sup>11</sup> sweeter than sugar) and it is used in alcoholic beverages and in aromatised foods [59]. It is recognised  
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17<sup>12</sup> to have the following properties: antioxidant, anti-fungal and anti-microbial [60,25,61], spasmolytic  
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19<sup>13</sup> [62], gastro-protective [63,64], sedative [65] and in reducing the risk of Parkinson's disease [66].

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22<sup>14</sup> Studies in aromatherapy have shown that anethole might produce anti-migraine effects via the  
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24<sup>15</sup> inhibition of neurogenic inflammation through the MAPK and NF- $\kappa$ B pathways [67]. In the literature  
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27<sup>16</sup> it is reported as characteristic for *Pimpinella* genus. In a previous study conducted on *P. anisoides*  
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29<sup>17</sup> fruits collected in the region of Calabria (South Italy), anethole was found to be 54.5% of the total  
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32<sup>18</sup> volatiles, from essential oil extracted by maceration with ethanol [33]. Anethole was found to be the  
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34<sup>19</sup> predominant volatile in *P. anisetum* oil from Turkey obtained by a Clevenger apparatus (77.1% in  
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36<sup>20</sup> fruit oil and 55.2% in herb oil respectively) [68]. Anethole also predominated in *P. anisum*: 93.5% in  
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39<sup>21</sup> seed essential oil from Turkey obtained by hydrodistillation [51]; 74.40% in seed essential oil  
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41<sup>22</sup> obtained by a Sohlet extractor and distilled water as a solvent [48]; 88.49% in seed essential oil from  
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44<sup>23</sup> Serbia obtained by hydrodistillation and *n*-hexane as collecting solvent [50]. Anethole is also  
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46<sup>24</sup> contained (even if in lower quantity) in seed essential oil of fennel (*Foeniculum vulgare* Mill.) (49.76-  
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48<sup>25</sup> 81.21%) [69] and guarana [*Paullinia cupana* H.B.K. var. *sorbilis* (Mart.) Ducke] [70].

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51<sup>26</sup> Estragole (methyl chavicol) ranged between 5.76% (Pa\_5) and 6.67% (Pa\_2) ( $p < 0.001$ ) (Table 4).  
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54<sup>27</sup> Estragole is phenylpropanoid, more in detail an alkenylbenzene for which, if consumed in high  
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56<sup>28</sup> quantity, a carcinogenic and genotoxic activity was found (Scientific Committee on Food, 2001a,  
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58<sup>29</sup> 2001b, 2001c) even if estragole orally administered to mice for 10 mg/kg/day for 14 days showed no  
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61<sup>30</sup> toxicity [71].

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257 morphometric data have distinguished two main clades. Pa\_1 and Pa\_2 are at a distance of 1 and are  
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2<sup>2</sup>32 *anisoides* of the present work: 22.4% in fruit oil and 41.9% in herb oil. In studies conducted on the  
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2<sup>5</sup>33 essential oil of *P. anisum*, estragole was found to be: 2.4% in seeds submitted to hydrodistillation  
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2<sup>7</sup>34 with double-distilled water [72]; 1.39% and 0.63% from Algerian seeds respectively submitted to  
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2<sup>10</sup>35 hydrodistillation and to microwave assisted hydrodistillation [73]; 1.73% from seeds produced in  
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12<sup>13</sup>36 Turkey and submitted to hydrodistillation [51]; 1.87% from Iranian seeds and distilled water as a  
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2<sup>15</sup>37 solvent [48].

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17<sup>18</sup>38  $\beta$ -Phellandrene was detected in a range between 1.99% (Pa\_5) and 1.55% (Pa\_1) ( $p < 0.001$ ). It is a  
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19<sup>20</sup>39 cyclic monoterpene that was not listed as the major constituents of the *P. anisoides* essential oil in the  
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21  
2<sup>22</sup>40 only existing study conducted from fruits collected in the region of Calabria [33].  $\beta$ -Phellandrene is  
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24<sup>25</sup>41 one of the minor volatiles found in the peel essential oil some *Citrus* species: 0.40% in *Citrus*  
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2<sup>27</sup>42 *reticulata* Blanco grown in Algeria [74]; 0.19% and 0.07% respectively in *Citrus x aurantium* L. (sub  
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29<sup>30</sup>43 *Citrus sinensis*) and *Citrus x limon* (L.) Osbeck (sub *Citrus lemon*) taxa grown in Spain. Higher  
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32<sup>33</sup>44 quantity of  $\beta$ -phellandrene were found in the essential oil of wild populations of *Echinophora cinerea*  
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34<sup>35</sup>45 (Boiss.) Hedge & Lamond from Iran (13.11% - 10.29%) [75] and in *Artemisia dracuncululus* L.  
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37<sup>38</sup>46 growing in Tajikistan (3.2%) [76].

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39<sup>40</sup>47 Each one of all other volatiles of the five studied accessions of *P. anisoides* was detected for less than  
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41<sup>42</sup>48 0.50% (Table 4). No significant differences ( $p > 0.05$ ) were found for cis-Limonene-oxide (0.05%-  
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44<sup>45</sup>49 0.07%), Cyclohexene, trans-Carveol (0.03%-0.05%), 4-Terpenyl acetate (0.01%-0.03%), *p*-Acetonyl  
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46<sup>47</sup>50 anisole (0.03%-0.06%), Methyl eugenol (0.03%-0.05%), Farneseol (0.02%-0.03%), Limonene 1,2-  
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49<sup>50</sup>51 epoxide (0.01%-0.03%), Nerolidol (0.01%-0.03%),  $\alpha$ -Bisabolene (0.01%-0.02%), Cariophyllene  
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51<sup>52</sup>52 oxide (0.07%-0.10%) and Spatulanol (0.03%-0.05%) (Table 4).

## 52 53 54 55 56<sup>57</sup>54 **Cluster Analysis**

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58<sup>59</sup>55 The cluster analysis was conducted both on the basis of morphometric data and of the volatile  
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61<sup>62</sup>56 composition. Two different dendrograms were appreciated. The results obtained on the basis of the  
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231 Baser et al. [68] in a study on *P. anisetum* found estragole in a higher quantity if compared with *P.*  
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258 closely related to Pa\_3 at a distance of 3, whereas Pa\_4 and Pa\_5 are grouped in the second clade at  
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259 a distance of 16. This was due to the fruits shape which is oblong-ovoid in the Pa\_1, Pa\_2 and Pa\_3  
6  
2760 accessions, whereas is oblong-cylindrical in the Pa\_4 and Pa-5 accessions. More in detail, Pa\_1, Pa\_2  
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2761 and Pa\_3 show a fruit having a minor length/width ratio than Pa\_4 and Pa\_5 (Fig.5).

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1262 The results obtained on the basis of the volatile composition permit to appreciate three main clusters  
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2763 (Fig.6). The first cluster grouped Pa\_1, Pa\_2 and Pa\_4 at a distance of 6. Pa\_1 and Pa\_2 were at a  
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12764 distance of 1, this was due to the lowest  $\beta$ -Phellandrene content and a high limonene content. The  
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12965 second cluster distinguished Pa\_4 in relation with a significantly high limonene percentage and a  
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21  
2266 significantly low Anethole percentage. Finally, the third cluster distinguished the Pa\_3 essential oil  
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22467 which showed the lowest limonene and Estragole contents and the highest Anethole content (Fig.6).  
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26  
2768 In brief, for three out of five accessions (Pa\_3, Pa\_4 and Pa\_5) there was no relationship between  
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22969 morphometric data and volatile composition in contrast to Pa\_1 and Pa\_2 which were in both cases  
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31  
3270 grouped together at a distance of 1.

### 35 3672 **Principal Components Analysis (PCA)**

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32973 The analysis of chemical data by the technique of PCA permitted to group chemically the samples in  
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4274 three groups, in a way to express and evidence their similarities and differences. The significant factor  
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4475 loadings from the principal component analysis (PCA) of chemical constituent variables were  
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42976 obtained as suggested by D'Agostino et al. [77], after Varimax rotation. To explore the relationship  
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4977 between the samples from various areas and their relation to specific volatile compounds, the GC-  
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52178 MS data was subjected to PCA (Fig. 7). PCA results revealed that the first two principal components  
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5279 explained 71.80 % of total variance. The loadings of first and second principal components (PC1 and  
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5280 PC2) accounted for 44.08 and 27.72 % of the variance, respectively. The first component (PC1) is  
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5281 highly positively correlated with  $\alpha$ -Pinene,  $\beta$ -Phellandrene,  $\beta$ -Myrcene, Farnesol,  $\beta$ -Cariophyllene,  
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6282 Limonene 1,2 epoxide, Nerolidol. The second component (PC2) is characterized by the positive

308 8. Fernández Prieto JA, Sanna M, Bueno Sánchez Á, Molero-Mesa J, Llorens García L, Cires E.  
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284 Carveol, trans Carveol, Carvone D and Spatulenol. In the Figure 7, the biplot graph PC1 x PC2 of the  
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285 loadings and scores is presented, relating the data of chemical constituents of the essential oils of the  
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286 five collection areas with their essential oils, in order to correlate them, grouping them in 3 groups.  
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287 Thus, Anethole,  $\alpha$ -Bisabolene, Nerolidol,  $\alpha$ -Bisabolol,  $\beta$ -Myrcene,  $\beta$ -Santalol and Farnesol were the  
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1288 principal components that contributed to the clustering of the samples obtained of Pa\_4 and Pa\_5  
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1489 accessions. PCA statistical analyses indicate the existence of three groups. The first group of clustered  
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1290 accessions is formed by Pa\_4 and Pa\_5, the second by only Pa\_3 and the third by Pa\_1 and Pa\_2.  
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Tab. 1. Sources of *Pimpinella anisoides* V. Brig. fruits and their collection data in the province of Catanzaro (Calabria, S-Italy).

Accession Id	Location	Municipality	Month and year of collection	Collector Farm	Altitude (m a.s.l.m.)
Pa_1	Monticelli	Cicala	August 2018	Azienda Muraca	900 m
Pa_2	Cutura	Sorbo San Basile	August 2018	Azienda Muraca	780 m
Pa_3	Pietra di Fota	San Bernardo di Decollatura	July 2018	Azienda Talarico	840 m
Pa_4	Cutura	Cicala	July 2017	Azienda Talarico	700 m

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Table 2. Main characteristics of *Pimpinella anisoides* V.Brig. monocarps in the 5 locations reported with their Id accession. \*\*\* significance at  $p < 0.001$ ; \*\* significance at  $p < 0.01$ ;

	Pa_1	Pa_2	Pa_3	Pa_4	Pa_5	
	Mean±DS	Mean±DS	Mean±DS	Mean±DS	Mean±DS	Sig.
Monocarp width	1.58±0.12ab	1.64±0.17a	1.51±0.11b	0.84±0.14d	1.10±0.15c	***
Monocarp length	3.10±0.35a	3.10±0.34a	2.90±0.32a	2.25±0.30b	2.51±0.21b	***
Monocarp length from base to maximum width	1.23±0.14a	1.23±0.16a	1.22±0.16a	0.88±0.13c	1.05±0.12b	***
Stylopodium width	0.46±0.07ab	0.48±0.07a	0.46±0.06ab	0.41±0.05b	0.43±0.05b	**
Stylopodium length	0.39±0.09a	0.38±0.15ab	0.30±0.05c	0.31±0.08bc	0.28±0.06c	***
Length/width ratio	1.96±0.20c	1.90±0.22c	1.93±0.22c	2.72±0.35a	2.32±0.30b	***

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Table 3 - Monocarps measurements and features of three commercial *Pimpinella* L. species. The features of *P. anisoides* come from this study, those of *P. anisetum* and *P. anisum* from Yeşil et al. (2018).

Species	Fruit length and width (mm)	Length/width ratio	Indumentum	Shape of fruit
<i>P. anisoides</i> V. Brig.	2.53-3.84 x 1.44-2.07	(1.90) 2.2 (2.72)	glabrous	oblong-ovoid/ oblong-cylindrical
<i>P. anisetum</i> Boiss. & Balansa	1.5–1.57 × 1–1.08	1.5	strigose	ovoid
<i>P. anisum</i> L.	3.9–4 × 1.5–1.53	2.6	strigose	ovoid-subglobose

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Table 4. Fruit essential oil composition of the five accessions of *Pimpinella anisoides* collected in the Region of Calabria (South Italy). Values are expressed as percentage content ± Standard Deviation. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; n.s. =  $p > 0.05$ .

	Pa_1	Pa_2	Pa_3	Pa_4	Pa_5	
COMPOUND	Mean±DS	Mean±DS	Mean±DS	Mean±DS	Mean±DS	Sig.
$\alpha$ -Pinene	0.03±0.01ab	0.02±0.01b	0.03±0.01ab	0.05±0.01a	0.03±0.01ab	*
$\beta$ -Phellandrene	1.55±0.03c	1.59±0.03c	1.77±0.07b	1.83±0.03b	1.99±0.01a	***
$\beta$ -Myrcene	0.23±0.02c	0.22±0.02c	0.25±0.03c	0.34±0.02b	0.44±0.04a	***
Limonene	49.10±0.11b	49.11±0.07b	50.74±0.10a	48.00±0.05c	46.78±0.08d	***
<i>p</i> -Cresol	0.05±0.01ab	0.04±0.01ab	0.05±0.02ab	0.03±0.01b	0.06±0.01a	*
<i>trans-p</i> -Mentha-2,8 dienol	0.04±0.01c	0.05±0.01c	0.10±0.02b	0.14±0.01a	0.09±0.02b	***
<i>cis</i> -Limonene-oxide	0.07±0.01a	0.07±0.02a	0.07±0.02a	0.05±0.01a	0.07±0.01a	n.s.

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<i>cis-p</i> -Mentha-2,8 dienol	0.03±0.01b	0.04±0.02b	0.09±0.03a	0.05±0.01ab	0.06±0.01ab	*
<i>trans</i> -Limonene-oxide	0.03±0.01b	0.04±0.01b	0.09±0.02a	0.05±0.01b	0.05±0.01b	**
Cyclohexene	0.10±0.02a	0.10±0.02a	0.09±0.03a	0.09±0.01a	0.08±0.01a	n.s.
Geraniol	0.01±0.01b	0.02±0.01b	0.05±0.01a	0.02±0.01b	0.02±0.01b	***
Estragole	6.49±0.05b	6.67±0.04a	6.40±0.06b	6.19±0.03c	5.76±0.04d	***
<i>cis</i> -Carveol	0.04±0.01b	0.06±0.01b	0.13±0.03a	0.08±0.01b	0.07±0.01b	***
<i>trans</i> -Carveol	0.04±0.01a	0.03±0.01a	0.05±0.02a	0.05±0.01a	0.04±0.01a	n.s.
Carvone D	0.03±0.01c	0.05±0.02bc	0.13±0.03a	0.07±0.00bc	0.09±0.01ab	***
<i>p</i> -Anisaldehyde	0.39±0.02cd	0.45±0.02bc	0.49±0.05b	0.37±0.02d	0.66±0.02a	***
Anethole	40.98±0.05c	40.57±0.09d	38.50±0.07e	41.36±0.03b	42.77±0.09a	***
4-Terpinyl acetate	0.02±0.01ab	0.02±0.01ab	0.03±0.01a	0.01±0.01b	0.02±0.01ab	n.s.
<i>p</i> -Acetonyl anisole	0.03±0.01a	0.06±0.02a	0.04±0.01a	0.04±0.01a	0.03±0.01a	n.s.
Methyl eugenol	0.04±0.01a	0.04±0.01a	0.05±0.01a	0.04±0.01a	0.03±0.01a	n.s.
$\beta$ -Caryophyllene	0.38±0.03bc	0.38±0.03c	0.47±0.05b	0.64±0.03a	0.45±0.02bc	***
Humulene	0.03±0.01ab	0.03±0.01a	0.03±0.01a	0.03±0.01ab	0.01±0.01b	**
Farnesol	0.02±0.01a	0.02±0.01a	0.02±0.01a	0.03±0.01a	0.02±0.01a	n.s.
Limonene 1,2-epoxide	0.01±0.01a	0.01±0.01a	0.02±0.02a	0.03±0.01a	0.02±0.01a	n.s.
$\beta$ -Copaene	0.06±0.01ab	0.05±0.02b	0.10±0.03a	0.09±0.01a	0.07±0.01ab	**
$\beta$ -Santalol	0.02±0.01ab	0.02±0.01b	0.01±0.01b	0.04±0.01a	0.03±0.01a	**
Isohomogenol	0.04±0.01a	0.03±0.01ab	0.03±0.01ab	0.02±0.01b	0.03±0.01ab	*
Nerolidol	0.01±0.01a	0.01±0.01a	0.01±0.01a	0.03±0.01a	0.02±0.01a	n.s.
$\alpha$ -Bisabolene	0.02±0.01a	0.01±0.01a	0.01±0.01a	0.02±0.01a	0.01±0.01a	n.s.
Cariophyllene oxide	0.08±0.02a	0.10±0.02a	0.08±0.02a	0.10±0.01a	0.07±0.01a	n.s.
Spatulenol	0.04±0.01a	0.03±0.01a	0.05±0.01a	0.03±0.01a	0.03±0.01a	n.s.
$\alpha$ -Bisabolol	0.05±0.01bc	0.05±0.01bc	0.04±0.01c	0.07±0.01ab	0.09±0.01a	***

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Fig. 1. Map of *Pimpinella anisoides* V. Brig. fruits collection locations in the province of Catanzaro (CZ), Calabria region (S-Italy): 1. Monticelli, 2. Cutura, 3. Pietra di Fota, 4. Cutura, 5. Marignano.

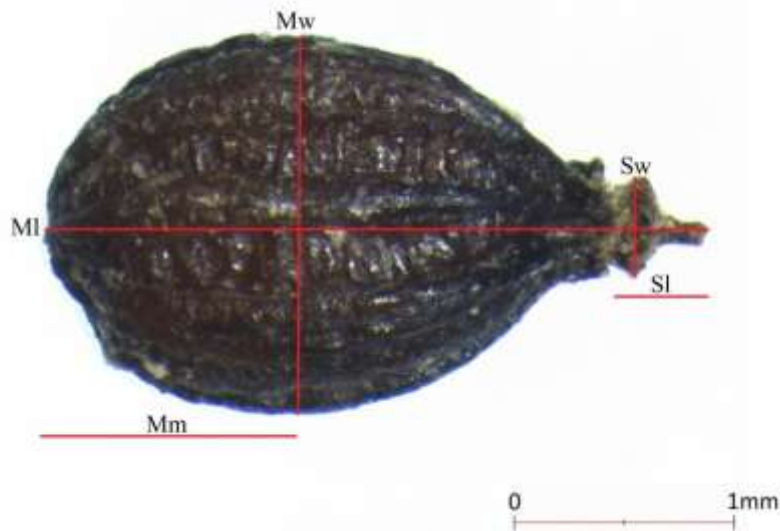


Figure 2 - Monocarp of *Pimpinella anisoides* V. Brig. photographed and measured under a stereomicroscope using the DeltaPix inSight software. Mw: Monocarp width; Ml: Monocarp length; Mm: Monocarp length from base to maximum width; Sw: Stylopodium width; Sl: Stylopodium length.



Figure 3 - Monocarp of *Pimpinella anisoides* V.Br. observed in dorsal (a) and ventral (b) view, with a stereomicroscope using the DeltaPix inSight software.

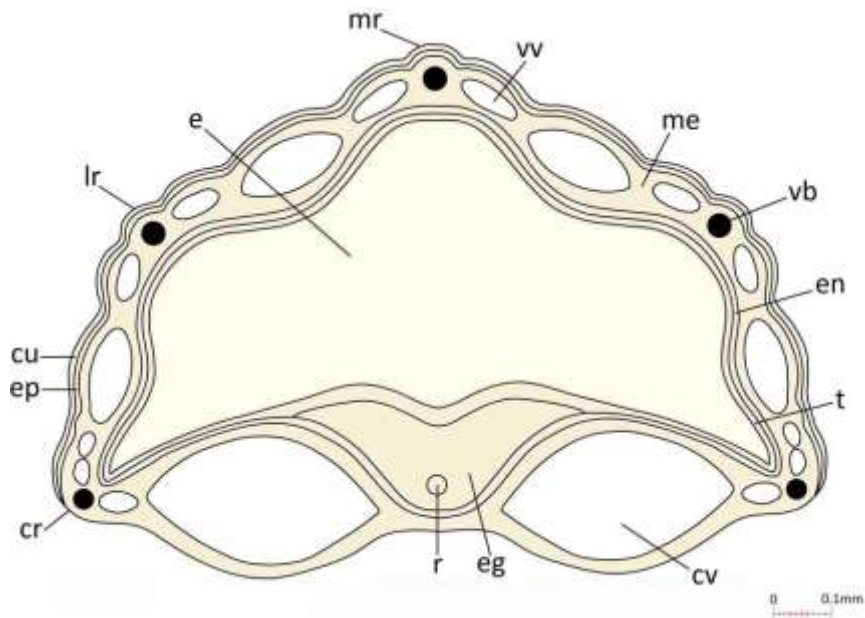
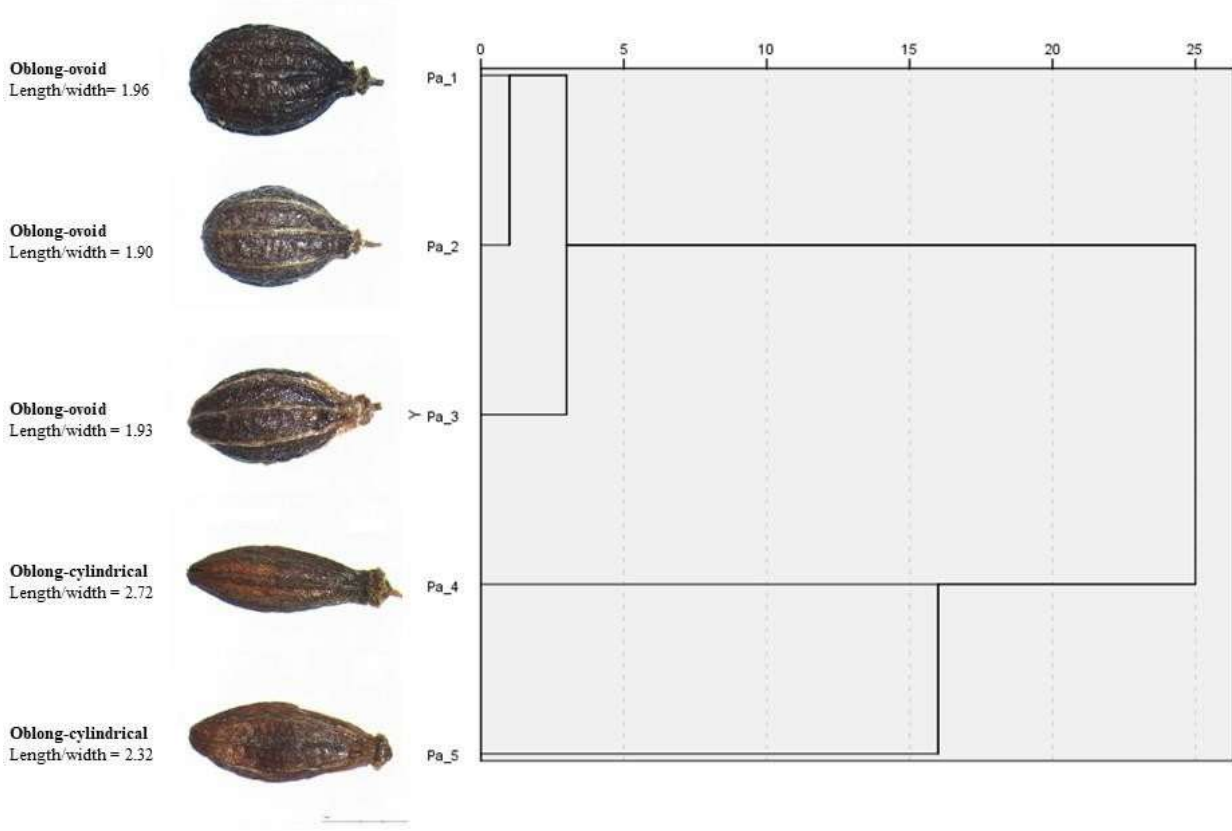


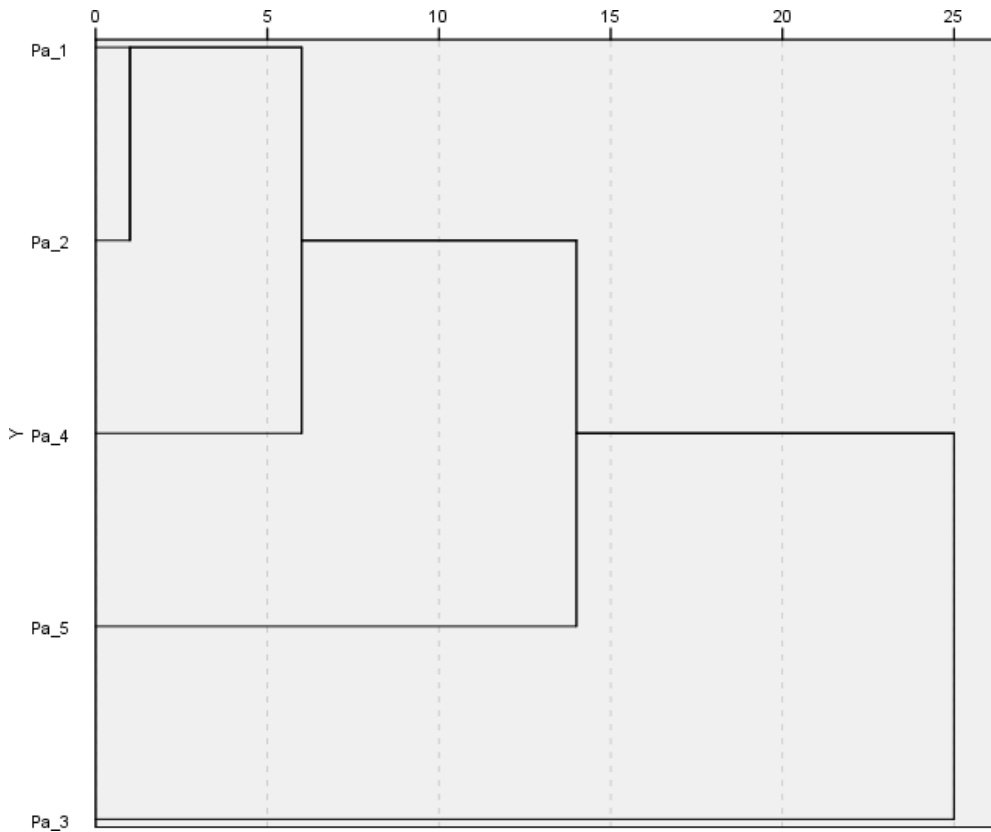
Fig. 4- Monocarp transverse section of *Pimpinella anisoides* V.Br. cr: commissural rib; cu: cuticula; cv: commissural vittae; eg: endosperm groove; en: endocarp; e: endosperm; ep: epiderm; lr: lateral rib; me: mesocarp; mr: median rib; r: raphe; vb: vascular bundle; vv: vallecular vittae; t: testa.



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Fig.5. Cluster analysis and corresponding monocarp shape of the different accessions of *Pimpinella anisoides* V. Brig.



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Fig.6. Cluster analysis and corresponding essential oil of the different accessions of *Pimpinella anisoides* V.Brig.

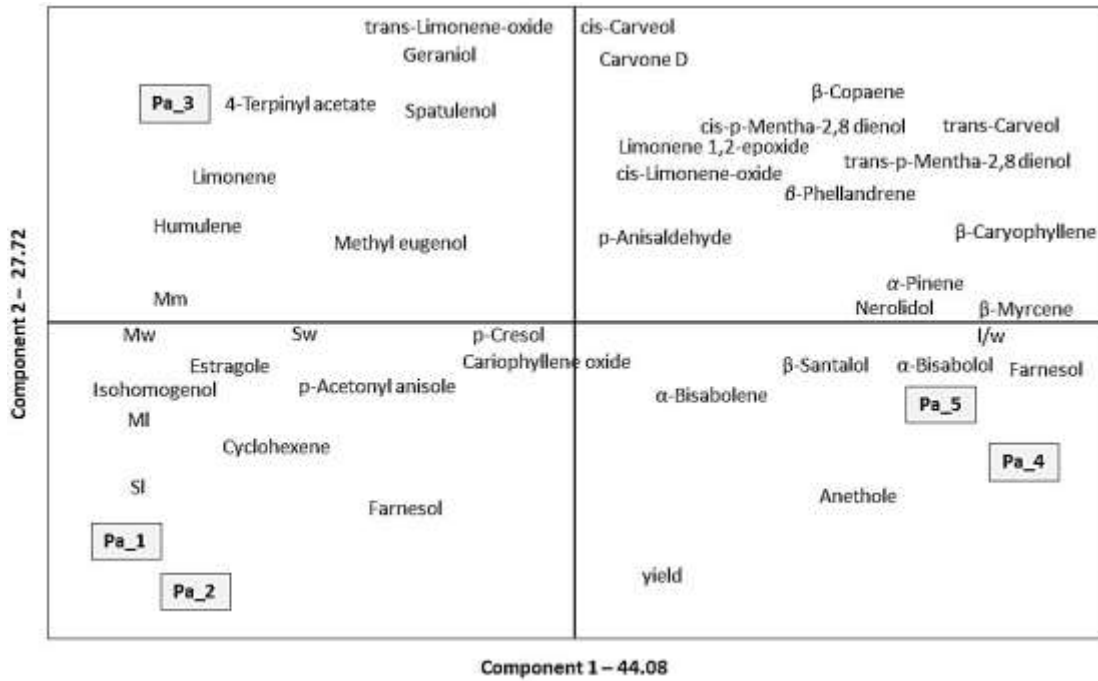


Figure 7 - Biplot graph, PC1 versus PC2, using loadings and scores for different essential oils of *Pimpinella anisoides* V. Brig. growing in different locations. Mm: Mericarp length from base to maximum width; Mw: Mericarp width; MI: Mericarp legth; Sw: Stylopodium width; SI: Stylopodium length; l/w: Length/width ratio.

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