Chemical Diversity of Essential Oils from Pinus Nigra Arn. Subspecies Growing in Tunisia

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Abstract

Background: The aim of this study was the determination of chemical diversity of *Pinus nigra* Arn essential oils. The work was conducted on needles collected from eighteen provenances of Black pine grown in common garden located in West-Northern Tunisia and belonging to four different subspecies (*P. nigra* subsp. *nigra*, *P. nigra* subsp. *salzmannii*, *P. nigra* subsp. *pallasiana* and *P. nigra* subsp. *laricio*). The GC analysis was carried out with a Gas Chromatograph coupled to a mass spectrometry.

Results: Oil yields ranged from 0.19% to 0.68%. Twenty-three constituents accounting about 98% of total oil composition were identified. The essential oil compositions appeared to be very different in the different provenances. There appear to be five basic essential oil chemotypes in *P. nigra* plants investigated: caryophllene oxide, camphene, β caryophyllene, α amorphene and Germacrene D. Oils from *nigra* subsp. were the richest in α-pinene.

Conclusions: this study showed that the analyzed oils belonged to five different chemotypes. Caryophyllene oxide was the main component of almost studied oils.

Background

Pinus nigra Arnold (Black pine) is circum-Mediterranean species belonging to *Pinaceae* family. The species is divided into six subspecies: *P. nigra* subsp. *nigra* (Arn.), *P. nigra* subsp. *salzmannii* (Dun.), *P. nigra* subsp. *dalmatica* (Vis.), *P. nigra* subsp. *pallasiana* (Lamb.), *P. nigra* subsp. *mauretanica* (Mair. & Pey.) and *P. nigra* subsp. *laricio* (Poir.) (Quézel and Medail 2003).

This species is discontinuously distributed from Southwest Europe to Asia Minor, extending to the Crimea and is also found in North Africa (Morocco and Algeria).

Black pine is characterized by a genetic, morphological, phenotypic and biochemical diversity. The chemical composition and the intraspecific variation of *Pinus nigra* volatiles have been the subject of numerous studies (Arbez et al. 1974; Gerber 1989; Paci et al.1990; Roussis et al. 1995; Zara et al. 1996; Rezzi 2001; Macchioni et al. 2003, Sezik et al. 2010).

These investigations indicated a significant variability in chemical composition of Black pine essential oils between provenances and subspecies.

In Tunisia, four subspecies from nineteen provenances of *P. nigra* were introduced in a common garden in the north-west of the country since 1966. For the best of our knowledge, no study addressed the variability of terpene profile of these provenances.

Thus, this work was conducted to study, for the first time, the variability in essential oils obtained from the needles of 18 provenances of *P. nigra* growing under humid biolimate in Northwest of Tunisia. This study could be helpful to highlight the adaptation of the plant in local pedoclimatic conditions at the southern limit of its range.

Methods

Plant material

Eighteen samples of *P. nigra* needles were collected from Souiniet common garden located in the Khroumirie region in West-Northern Tunisia (8 ° 48 'E, 35 ° 54' N, 492 m) characterized by a cold and humid Mediterranean bioclimate with temperate winters. The eighteen samples correspond to eighteen provenances from different geographic origins (Table 1), which have been planted since 1966 in provenances trials experimental site.

Table 1
Geographic origin of the eighteen provenances of *Pinus nigra*

Subspecies	Code	Provenances	Country of origin	Altitude (m)	Latitude (degré)	Longitude (degré)
salzmannii	P1	Brouzet-lès-Alès	France	-	44°07 N	4°05 E
	P12	St Guilhem	France	350–400	43°41 N	3°35E
	P16	Cazorla	Spain	1500	37°50N	3°00O
	P18	Olette (Pyr-Orient)	France	-	42° 36 N	2°14E
laricio	P2	Trenta	Italy	1050	39°25N	16°35E
	P3	Les Barres	France	150	47°50N	2°45E
	P4	Cosenza	Italy	1300	39°15N	16°17E
	P10	Cantanzaro	Italy	-	38°54N	16°34E
	P14	Grancia	Italy	850	39°41N	16°58E
	P17	Tavola	Italy	950	39°25N	16°35E
	P19	les Barres	France	150	47°50N	2°45E
	P5	Bois Frerot (Ardennes)	France	100	-	-
	P11	les Barres (leint)	France	150	47°50N	2°45E
	P20	Marghese (Corse du sud)	France	1100	41°39N	9°12E
nigra	P6	Puget Théniers	France	1600	33°52N	4°04E
	P8	Kustendil	Bulgaria	-	43°57N	6°53E
pallasiana	P9	Alaçam	Turkey	800–1000	39°35N	28°35E
	P13	Crimée	Russia	500	44°33N	34°17E

Essential Oil Extraction

The essential oil of all air-dried needles was extracted by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (British Pharmacopoeia 1988). The essential oils were stored in tightly closed dark vials at 4 °C until analysis.

Oil Yield

The results were expressed as the percentage (%) of grams of essential oil per gram of dry needles.

Gas Chromatography

The GC analysis was carried out with an Agilent 7890B Gas Chromatograph coupled to a mass spectrometry (Agilent, USA). The column used was an HP-5 30 m × 0.32 mm × 0.25 µm. Helium was the carrier gas at 1.0 mL min^{-1}. Oven temperature was programmed as follows: initial oven temperature was set at 40 °C (held for 5 min), raised to 250 °C at 2 °C min^{-1} and held for 5 min and finally increased at 275 °C at 5 °C min^{-1}. Injector and detector temperatures were set at 250 °C and 280 °C, respectively. Diluted samples (1/100 in n-hexane, v/v) of 1.0 µL were injected in the split mode (split ratio 1:100).

Statistical analysis

Data were analyzed using the GLM procedure (General Linear Models) of the SAS (9.0) program. An analysis of variance of the studied parameters was performed. All values are the mean of three replications. Principal component analysis was evaluated with R (version 3.1.1) program.

Results

Oil yield

Statistical analysis showed a high variability between the five studied subspecies ($p < 0.0001$). The highest yield was recorded by needles of *nigra* subspecies (Table 2). The lowest values were reached by both *calabrica* and *salzmannii* subspecies. Furthermore, a significant variability was recorded between the nineteen provenances. The most important oil yield (0.68%) was reached by needles from P6 (*Pinus nigra austriaca*; Puget-Théniers-France). P2 (*Pinus nigra calabrica*; Trenta-Italy) showed the lowest oil yield with 0.17%.

Table 2
Essential oil yield of *Pinus nigra* needles

Provenance	Oil yield (%)
P1	$0.24^{j} \pm 0.01$
P2	$0.19^{k} \pm 0.01$
P3	$0.30^{g} \pm 0.02$
P4	$0.37^{f} \pm 0.01$
P5	$0.41^{e} \pm 0.03$
P6	$0.68^{a} \pm 0.05$
P8	$0.38^{f} \pm 0.01$
P9	$0.41^{e} \pm 0.02$
P10	$0.51^{c} \pm 0.05$
P11	$0.26^{i} \pm 0.04$
P12	$0.47^{d} \pm 0.08$
P13	$0.41^{e} \pm 0.01$
P14	$0.46^{d} \pm 0.03$
P16	$0.30^{g} \pm 0.01$
P17	$0.66^{b} \pm 0.02$
P18	$0.40^{e} \pm 0.01$
P19	$0.27^{h} \pm 0.02$
P20	$0.37^{f} \pm 0.01$

Chemical Composition

The results of identified compounds by GC–MS are shown in Table 3. Twenty-three constituents accounting about 98% of total oil composition were identified.

Table 3
Chemical composition of essential oils from P. nigra

	p 1	p 2	p 3	p 4	p 5	p 6	p 8	p 9	p 10	p 11	p 12	p 13	p 14	p 16	p 17
α-pinene	-	-	-	-	-	19.34	-	-	-	-	2.16	2.12	-	-	-
Verbenol	0.40	2.17	0.81	-	-	-	0.66	-	-	0.62	-	-	0.80	-	0.15
Myrcenol	4.19	3.56	1.86	-	0.80	-	-	-	-	1.41	-	-	1.60	1.05	-
3-udecyne	0.23	0.77	0.88	-	-	-	0.98	-	-	0.30	-	-	0.36	-	0.23
geranyl acetate	3.51	1.20	0.73	0.87	1.20	-	-	-	-	1.13	-	-	0.84	-	-
camphenol acetate	0.95	2.20	0.60	1.39	-	-	0.95	-	0.40	0.53	-	-	1.70	-	0.31
Camphene	19.95	8.40	6.70	9.17	3.25	-	-	0.73	38.07	16.77	0.42	-	5.08	5.61	0.15
5,9 tetradecane	0.53	-	0.85	-	-	-	-	-	-	-	0.59	0.50	-	-	-
β-caryophyllene	13.72	4.75	1.27	-	42.82	15.65	-	0.80	23.28	12.89	32.16	30.29	32.93	0.79	-
Limonene	1.82	1.49	0.91	-	2.53	0.47	-	-	1.33	1.71	2.96	1.64	2.85	-	-
α-caryophyllene	1.59	1.52	5.29	1.92	1.98	-	1.79	0.64	-	1.58	8.84	9.44	1.74	1.11	1.20
α-amorphene	6.69	7.38	0.59	-	11.22	-	-	-	2.60	26.04	0.52	2.46	8.30	-	-
β-phenethyl butyrate	2.81	2.33	0.53	-	0.89	23.17	-	0.48	8.32	5.39	2.61	0.83	4.82	-	0.35
germacrene D	3.28	3.09	2.46	2.51	0.77	27.13	-	0.76	10.89	0.41	1.08	0.86	0.44	7.11	0.16
bicyclogermacrene	0.82	3.14	5.24	-	1.44	0.21	1.05	0.65	1.21	2.20	3.33	3.80	0.72	1.25	0.57
γ-muurolene	2.92	1.36	2.07	0.98	3.82	1.92	-	0.90	2.16	4.02	1.49	1.44	2.23	-	-
γ-cadinene	0.29	2.28	3.85	-	0.64	-	-	0.94	-	1.22	-	0.46	1.01	-	0.20
caryophyllene oxide	16.10	22.86	41.53	80.54	8.14	1.22	88.51	75.11	8.33	10.33	38.71	41.85	23.75	66.49	90.05
3-decyne	0.89	2.17	8.19	-	0.52	0.29	1.74	9.72	0.37	1.14	1.17	1.82	0.85	5.39	3.42
limonene oxide	14.21	8.14	6.88	-	4.18	0.19	0.84	1.87	0.40	7.09	0.75	0.49	4.15	5.38	0.87
α-cadinol	2.05	5.23	2.80	0.62	13.23	8.41	1.02	2.34	0.64	1.72	0.77	-	2.08	3.82	0.18
farnesene epoxide	0.59	8.13	1.34	-	-	-	-	1.17	-	0.45	-	-	-	-	-
Linalool	0.45	5.83	2.97	-	0.58	-	0.46	1.08	-	1.06	0.43	-	0.74	-	0.16
Monoterpenes % :	47,81	33,88	23,32	12,55	13,31	47,13	1,96	4,44	50,69	30,2	7,8	5,11	16,5	19,15	1,49
Sesquiterpenes % :	44,77	56,65	63,98	84,06	83,29	27,41	92,37	82,55	38,22	60,45	85,82	89,74	72,76	73,46	92,2

The essential oil compositions appeared to be very different in the different provenances. The major essential oil components were especially variable in occurrence and concentration among the different provenances, ranging from almost absent in some samples to more than 90% of the total essential oil composition in others.

There appear to be five basic essential oil chemotypes in *P. nigra* plants investigated (Fig. 1): (a) caryophllene oxide as the major component (provenances Trenta (22.86%), Les Barres (41.53%), Cosenza (80,54%), Kustendil (88.51%), Alaçam (75.11%), Crimée (41.85%), St Guilhem (38.71%), Cazorla (66.49%), Olette (87.74%), Tavola (90.05%) and Marghese (90.85%), (b) camphene as the major compound (provenances Brouzet-lès-Alès (19.95%) and Cantanzaro (38.07%)), (c) β caryophyllene (provenances Bois Frerot (42.82%), Grancia (32.93%) and les Barres (51.10%)), (d) α amorphene (les Barres (leint) (26.04%)) and (e) Germacrene D (Puget Théniers (27.13%)).

Only the essential oils from Brouzet-lès-Alès (P1), Puget Théniers (P6) and Cantanzaro (P10) provenances were more rich in monoterpenes than sesquitepenes, while the oils from the other provenances (P2, P3, P4, P5, P8, P9, P11, P12, P13, P14, P16, P17, P18, P19 and P20) had more sesquiterpenes than monoterpenes.

The results of principal component analysis showed that β-caryophyllene, caryophyllene oxide, linalool, myrcenol and γ-muurolene were the most significant variables for classification of the *P. nigra* essential oils. These parameters were considerably loaded into the two major principal components (Dim1 and Dim 2) explaining more than 50% of the variance. According to the analysis, five different groups were revealed (Fig. 2). The first group contained P6, P19 and P10 samples which had the main concentrations of β-caryophyllene and the lowest rate of caryophyllene oxide. The second group contained only P12 and P13 samples which showed the highest amount of both β-caryophyllene and caryophyllene oxide. The third group regrouped P1, P5, P11 and P14 which showed the most important amount of γ-muurolene. The fourth group contained P2 and P3 oils characterized by the highest amount of linalool. The fifth group regrouped all the other samples studied which showed the highest rate of caryophyllene oxide.

When considering the variability between the four studied subspecies, statistical results showed that oils from *nigra* subsp. were the richest in α-pinene. This richness is related to the high amount found in P6 (Puget Théniers) oil (19.34%).

The results of principal component analysis showed the presence of three groups (Fig. 3); the first group regrouped *laricio* and *salzmannii* subspecies which showed the highest rate of camphene and limonene, the second group contained *pallasiana* subsp. which showed the most important amount of caryophyllene oxide and the third one enclosed only *nigra* subsp representing the highest amount of α-pinene.

Discussion

Various terpenoid compounds, which are characteristic constituents of conifer, have been reported in *P. nigra* needles. Several studies that reported the chemical composition of essential oils extracted from needles of *Pinus* species growing in Tunisia (*P. halepensis, P. pinea* and *P. pinaster*); showed that β-caryophyllene, amorphene, limonene and germacrene D were the major components in all essential oils of pine needles (Amri et al. 2012; Hamrouni et al. 2015; Fkiri et al. 2019). These results were supported by our study.

In our study, caryophllene oxide was the major compound in oils of *pallasiana* subsp. from Turkey. These results was not similar to those found by Dogan and Bagci (2018) that demonstrated that the main compounds of oils of *pallasiana* subsp. from Turkey were α-pinene, limonene and β-caryophyllene. In the same context, Sezik et al. (2010) reported that α-pinene and β – pinene were the main constituents of *P. nigra* essential oils from Turkey.

Several studies investigated the chemical composition of Italian oils. Macchioni et al. (2003) indicated that α-pinene was the principal constituent of oils. In the same context, Beder et al. (2000) indicated that oils from *laricio* subsp. were rich in α-pinene, β-caryophyllene and germacrene-D. According to our results, the main compounds in Italian oils were β-caryophyllene, comphene and caryophyllene oxide.

P. nigra subsp. *laricio* essential oils from Corsica were investigated by Rezzi et al. (2001) which demonstrated that α-pinene, manoyl oxide and germacrene-D were found to be the main constituents. These findings are different from ours. In our case, oils from *laricio* subsp. of Corsica showed three main compounds which are β-caryophyllene, α-amorphene and caryophyllene oxide.

According to Jurc et al. (1999), the terpene profile of *P. nigra* subsp. *salzmannii* from France was characterized by high amount in **γ**-cadinene and δ-cadinene. Our results differ considerably from those of Jurc et al. (1999). In this study oils from *salzmannii* subsp., France was characterized by important rate of camphene and caryophyllene oxide.

In almost previous literature focused on *P. nigra* essential oils composition, α-pinene was mentioned as the major compound, while in our study this compound was absent in the almost studied provenances excepting *nigra* subsp. austriaca which was mainly composed by Germacrene and α-pinene. This finding was supported by Jurc et al. (1999).

These comparisons with earlier studies indicates that, when planted in common garden in Tunisia, where the pedoclimatic conditions were constant, *P. nigra* from four subspecies and deriving from eighteen provenances has undergone a significant change in its chemotypes. Plants of black pine seem to be adapted to local climate and soil conditions.

This was supported by Amri et al. (2017) which mentioned that there is a great variation in chemical composition of the essential oil from *P. nigra* subsp. *laricio* grown in Tunisia and those from other countries.

It has long been known that pedoclimatic conditions influence the volatile oil content as well as its chemical composition. Ormeño and Fernandez (2012) mentioned that both biotic and abiotic conditions influence terpenoid production in plants, especially light and temperature. Staudt and Lhoutellier (2011) determined the effect of these two environmental factors on monoterpene and sesquiterpene leaf emissions. In addition, water availability is known to be one of the most important environmental factors controlling volatile organic compounds from plants (Ormeño et al. 2018).

Under the same pedoclimatic conditions of the studied experimental site, *P. nigra* plants showed a significant chemical variability. This could be explained by an eventual genetic variability between the eighteen provenances.

Conclusions

From our study, we could conclude that the analyzed oils belonged to five different chemotypes and the results obtained showed differences in the quantitative and qualitative composition. Caryophyllene oxide was the main component of almost studied oils. Hence, these natural products may be used for pharmaceutical and therapeutically purposes.

Declarations

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: SF and FM contributed equally to laboratory analysis, result interpretation and manuscript preparation. All authors read and approved the final manuscript.

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References

1. Arbez M, Bemard-Dagan C, Fillon CH (1974) Variabilité intraspécifique des monoterpènes de *Pinus nigra* Arn. Ann Sci Fi 31:57–70
2. Amri I, Gargouri S, Hamrouni L et al (2012) Chemical composition, phytotoxic and antifungal activities of *Pinus pinea* essential oil. J Pest Sci DOI. 10.1007/s10340-012-0419-0
3. Amri I, Hanana M, Jamoussi B et al (2017) Essential oils of *Pinus nigra* Arnold subsp. *laricio* Maire: Chemical composition and study of their herbicidal potential. Ar J Chem 10:3877–3882
4. Bader A, Flamini G, Cioni PL et al (2000) Composition of the Essential Oils from Leaves, Branches and Cones of *Pinus laricio* Poiret Collected in Sicily, Italy. J Essent Oil Res 12: 672–674
5. Dogan G, Bagci E (2018) Chemical Composition of Essential Oil of *Pinus nigra* subsp. *pallasiana* (*Pinaceae*) Twigs, From Different Regions of Turkey. TEOP 21:511–519
6. Fkiri S, Ghazghazi H, Rigane G (2019) chemical compositions and biological activities essential oil from the needles of North African *Pinus pinaster* VAR. Rev Roum Chim 64:511–517
7. Gerber S (1989) Chimiotaxinomie et hybridation inter-raciale chez les pins noirs. Dissertation, National Institute of Agronomy Paris Grignon
8. Hamrouni L, Hanana M, Amri I el al (2015) Allelopathic effects of essential oils of *Pinus halepensis* Miller: chemical composition and study of their antifungal and herbicidal activities. Arch Phytopath Plant Prot 48:145–158
9. Jurc D, Bojovic S, Jurc M (1999) Influence of endogenous terpenes on growth of three endophytic fungi from the needles of *Pinus nigra* Arnold. - Phyton (Horn Austria) 39:225–229
10. Macchioni F, Cioni PL, Flamini G et al (2003) Chemical composition of essential oils from needles, branches and cones of *Pinus pinea*, *P. halepensis*, *P. pinaster* and *P. nigra* from central Italy Essential Oils of *Pinus* Spp. FlaV Frag J 18: 139–143
11. Ormeño E, Fernandez C (2012) Current Bioactive Compounds Effect of Soil Nutrient on Production and Diversity of Volatile Terpenoids from Plants. Curr Bio Comp 8:71–79
12. Ormeno E, Mevy JP, Vila B et al (2007) Water deficit stress induces different monoterpene and sesquiterpene emission changes in Mediterranean species. Relationship between terpene emissions and plant water potential. Chemosphere 67:276–284
13. Paci M, Michelozzi M, Vilrich V (1990) Contentuto di monoterpeni di oteoresine corticali in provenienze diverse di *Pinus nigra* Arn. Ac It Sci Fore 38:223–231
14. Quézel P, Médail F (2003) Ecologie et biogéographie des forêts du bassin méditerranéen. Collection Environnement, Elsevier, France
15. Roussis V, Petrakis PV, Ortiz AN et al (1995) Volatile constituents of needles of five P*inus* species grown in Greece. Phytochem 39:57–361
16. Rezzi S, Bighelli A, Mouillot D et al (2001) Composition and chemical variability of the needle essential oil of *Pinus nigra* subsp. *laricio* from Corsica. Flavour Frag J 16: 379–383
17. Rezzi S, Bighelli A, Mouillot D et al (2001) Composition and chemical variability of the needle essential oil of *Pinus nigra* subsp. *laricio* from Corsica. Flav Frag J 16:379–383
18. Sezik A, Ustun O, Demirci B et al (2010) Composition of the essential oils of *Pinus nigra* Arnold from Turkey. Turk J Chem 34:313–325
19. Sezik E, Ustun O, Demirci B et al (2010) Composition of the essential oils of *Pinus nigra* Arnold from Turkey. Turk J Chem 34:313–325
20. Staudt M, Lhoutellier L (2011) Monoterpene and sesquiterpene emissions from *Quercus coccifera* exhibit interacting responses to light and temperature. Biogeosc 8:2757–2771
21. Zara AR, Dodd RS, Zavarin E (1996) Genetic Diversity in Foliar Terpenoids Among Natural Populations of European Black Pine. Biochem Sys Ecol 24:325–339

Figures

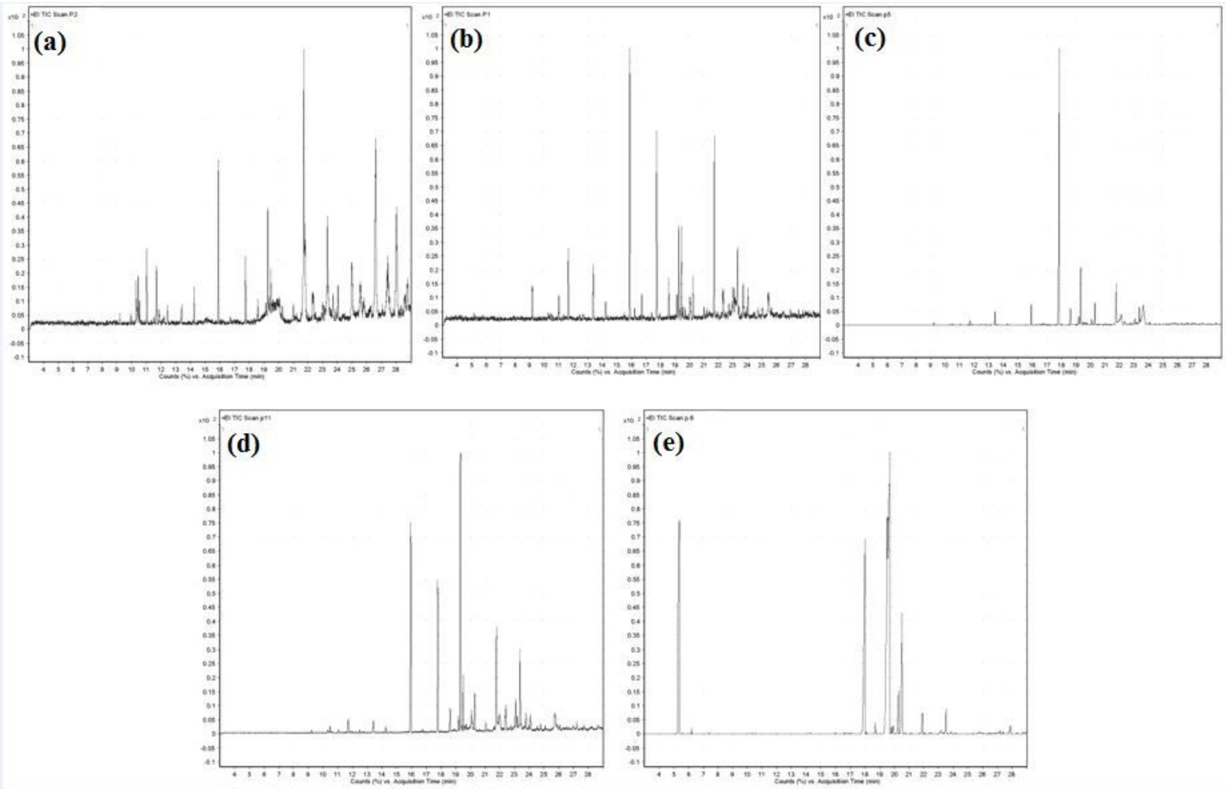

Figure 1

Chromatograms of the five chemotypes of P. nigra essential oils; (a) caryophllene oxide, (b) camphene, (c) β caryophyllene, (d) α amorphene, (e) Germacrene D.

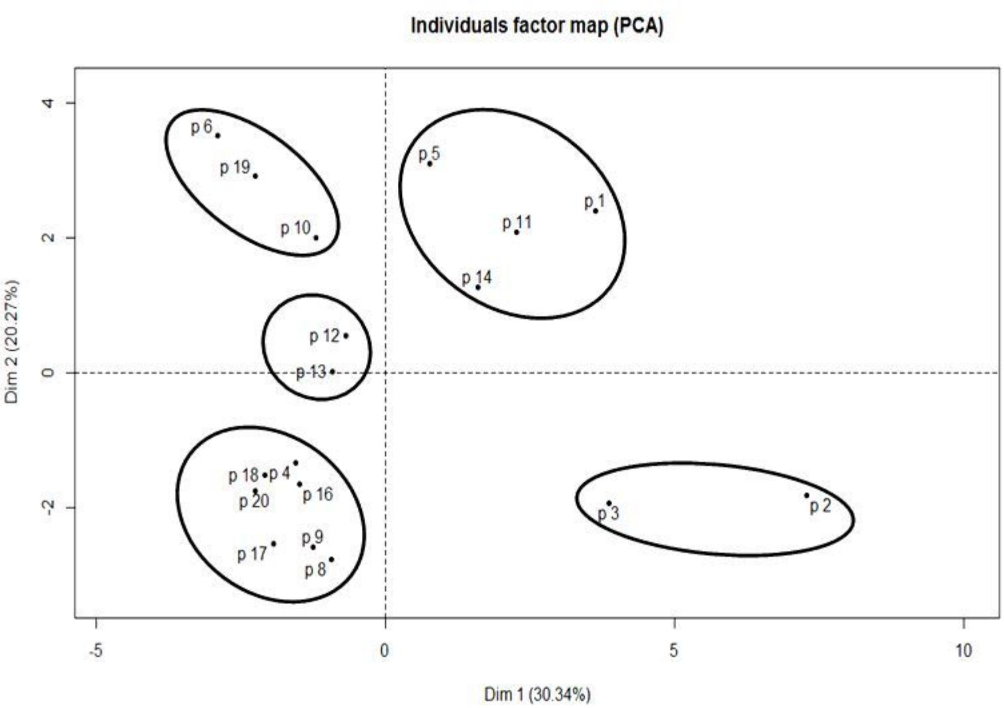

Figure 2

Individual factor map obtained from the PCA of data about the composition of P. nigra essential oils from 18 provenances.

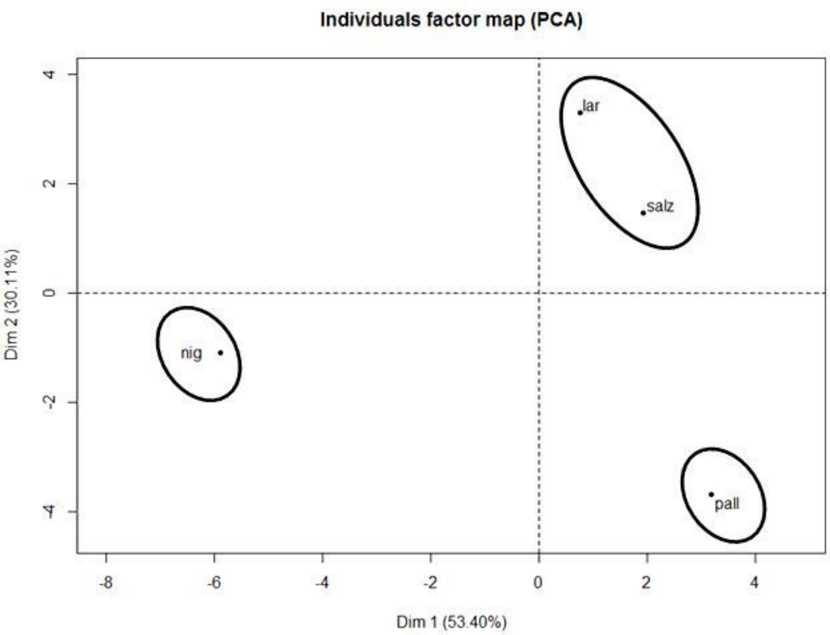

Figure 3

Individual factor map obtained from the PCA of data about the composition of P. nigra essential oils from four subspecies; lar: laricio, nig: nigra, salz: salzmannii, pall: pallasiana.