Essential oil composition of berries of *Juniperus oxycedrus* ssp. *oxycedrus* according their ripening stage

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Some important essential oils (EOs) can be extracted from berries of different species and their composition depends on their maturation level. The main objective of this work was to relate the EO composition of *Juniperus oxycedrus* L ssp. *oxycedrus* berries with their ripening degree. For this purpose, a classification method based on the CIEL*a*b* colour measurement was applied. Once the samples were classified according their ripening stage, they were subjected to simultaneous distillation extraction (SDE) and gas chromatography (GC/FID and GC/MS). Among the total of 71 compounds identified, hydrocarbon monoterpenes constituted the most important fraction (42.8-89.8 %), mainly represented by α-pinene (30.1-66.4 %) and myrcene (6.1-34.8 %). Hydrocarbon sesquiterpenes accounted for 4.0-26.4 %, with germacrene-D (0.2-16.9 %) as the major component. The results of discriminant analysis proved that CIEL*a*b* implementation was useful to objectively classify the ripening stage. Regarding the EO chemical composition, statistically significant differences were observed in the proportion of myrcene and hydrocarbon monoterpenes, as well as the grouped oxygenated monoterpene, sesquiterpene and diterpene compounds, over the maturation process. In general, an increase in the proportion of oxygenated compounds was noted as maturation progressed. As for oil yield, the maximum was observed in the intermediate stages of ripening.Keywords: word; another word; lower case except names

Keywords: *Juniperus oxycedrus* L, berries, essential oils, ripening stage, colour

Introduction

Juniperus oxycedrus L. ssp. *oxycedrus* (*Cupressaceae*) is an extended heliophilic and xerophilic shrub or small tree growing wild in stony areas from Mediterranean and Near East countries. In addition to its traditional use as food and beverages flavouring (1-4), many applications have been reported owing to its biological activity. The essential oil (EO) of berries of *J. oxycedrus* shows antioxidant activity (4-6), which explains its well-known use as food preservative (7). Antifungal and antimicrobial activity have been also reported (6, 8- 10). Other researches deal with healthcare field: wounds healing (11), activity against protozoan parasites of the genus *Leishmania* (12) or antiproliferative activity in leukemia cells (13), as well as insecticidal activity like that reported against *Sitophilus oryzae* (14) or the larvicidal action against *Culex pipiens* (15).

With regard to EO chemical profile of berries, it is relatively homogeneous from a qualitative point of view. As referred by Boti et al. (16) in a chemotaxonomic study with samples from Corsica, these oils can be grouped according to their relative amounts of α-pinene, myrcene and germacrene-D. The major fraction is constituted by hydrocarbon monoterpenic compounds in which α-pinene is usually the major compound, reaching up to 70.63 % in ripe berries (9). This compound, together with myrcene, limonene and germacrene-D, are cited as major compounds in numerous samples from different geographical origins: Croatia (1), Sardinia (Italy) (9), Corsica (France) (16), Lebanon (5), Macedonia, (10) and Turkey (17, 18). Indeed, the bacteriostatic activity of this EO can be largely attributed to its α-pinene content (19). Within these similar profiles, it is worth highlighting the predominance of myrcene in samples from Greece (14), Abruzzo (Italy) (20), and Kosovo (21).

As for seasonal variations, significant differences were found between ripe and unripe berries affecting α-pinene and myrcene amounts (9). In the same way, noticeable changes affecting mainly α-pinene and germacrene-D were found by Salido et al. (22) comparing unripe and ripe berries in *Juniperus oxycedrus* ssp. *badia*. Bakkour et al. (23) have referred very significant changes between ripe and unripe berries of *Juniperus excelsa* affecting the amount of α-pinene (major component), which shows a noteworthy increase in ripening berries and, in parallel, a decrease in oxygenated sesquiterpenic fraction ((*E*)-nerolidol and (*Z* ,*E*)-farnesol, mainly).

Given that many EOs are extracted from fruits and seeds of different plant taxa, the relevance of possible changes in chemical composition due to ripening stage should be emphasized. Some representative examples belong to *Juniperus* and *Myrtus* genus, but others are also worth to mention as *Illicium verum* (star anise) or *Elettaria cardamomum* (cardamom). These changes, based on the simple visual appreciation of colour associated to seasonal development, have been reported for *Myrtus communis* L. (24-26). Nevertheless, since the maturation period lasts beyond one year in berries of *Juniperus* species, some ripening stages may co-occur not only in the same populations, but in the same individuals. This fact may contribute to hindering the possibility of taking the seasonal development as criterion for defining the ripening stages.

In order to use these EOs as source of bioactive molecules, their changes over maturation period should be well-known. For this purpose, they should be defined by means of measurable parameters, unlike the usual distinction between ripe and unripe berries exclusively established from a visual point of view. The aim of this work was to propose a colorimetric based methodology for an objective classification of *Juniperus oxycedrus* berries according their ripening stage, in such a way that it can be correlated with composition and yield of the EOs

Material and methods

Plant material

Plant material was collected in December 2016 – January 2017 from three locations in Comunitat Valenciana (Spain): Segart, Lliria and Ahín. Three individuals were selected in each location in such a way that all the ripening range was represented. The coordinates of these locations are the following: Segart (39º 40’ 46’’ N, 0º 22’35’’ W, 303 m a.s.l.), Lliria (39º 37’38’’ N, 0º 40’46’’ W, 183 m a.s.l.), Ahín (39º 53’38’’ N, 0º 21’26’’ W, 765 m a.s.l.). Voucher specimens were lodged in the herbarium at the Mediterranean Agroforestry Institute (VALA) (Universitat Politècnica de València, Spain).

From the bioclimatic point of view, Segart and Lliria can be considered as thermo mediterranean zones (27), whereas Ahín as meso mediterranean zone (28).

Selection of samples

A first visual classification of ripening stages of berries was carried out according the following criteria (Figure 1):

A. Berries showing homogeneously green colour on its whole surface

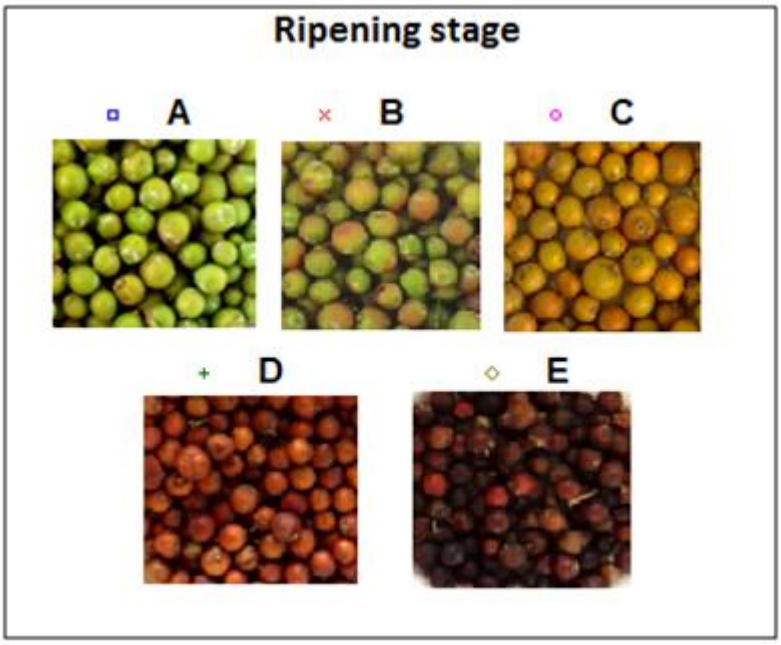
B. Berries showing a mixed half-and-half green-brownish colour

C. Brown yellowish colour.

D. Berries homogenously brown-reddish colour on its whole surface.

E. Berries showing dark red -black colour.

Figure 1. Visual ripening stages



Five samples were separated from each ripening stage in order to carry out the CIE L*a*b* colour measurement (25 samples in total) (29, 30). This colorimetric method has been usually applied for classifying fruits and vegetables according their ripening level (31-33).

Ten measures of the variables: L* (lightness), a* (green-red) and b* (yellow-blue) were registered by means of a colour reader CR-10 Plus (Konica Minolta®). A one-way ANOVA analysis was applied to these measures in order to test the significance of differences in their values according the previously defined ripening stages.

Once the results from ANOVA analysis were considered, mean values for each sample were subjected to discriminant analysis in order to validate the visual grading and obtain the classification functions for applying them to possible new samples.

Determination of moisture content

In order to evaluate the yield as referred to dry material, the moisture content was

determined in triplicate by drying 3 g samples for each of the five ripening stages up to constant weight (34), employing a vacuum oven (Vaciotem-T, JP Selecta, Spain) set at 30ºC.

Essential Oil extraction

10 g of berries belonging to each one of the 25 samples were crushed with a domestic mill just before being subjected for 3 h to simultaneous extraction distillation using a Likens-Nickerson type apparatus (35). The extracts were then dried with anhydrous sodium sulphate and evaporated under reduced pressure at room temperature. After adding 1.5 mL of dichloromethane, the extracts were kept in sealed chromatographic vials and stored at -18ºC until further analysis. The EO yield was determined based on dry weight of samples by addition of 1 or 2 μL of tetradecane (Sigma-Aldrich®) as internal standard, according to the weight of samples.

GC and GC/MS Analysis

The analysis of samples was carried out by GC-FID and GC-MS. A Clarus 500 GC (Perkin-Elmer Inc., Wellesley, PA, USA) chromatograph equipped with a flame ionization detector (FID) and capillary column ZB-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex Inc, Torrance, CA, USA) was used for quantitative analysis. The injection volume was 1 μL. The GC oven temperature was programmed from 50°C to 250°C at a rate of 3°C min−1. Helium was the carrier gas (1.2 mL min−1). Injector and detector temperatures were set at 250°C. The percentage composition of the EO was computed from GC peak areas without correction factors by means of the software Total Chrom 6.2 (Perkin-Elmer Inc., Wellesley, PA, USA).

Analysis by GC-MS was performed using a Clarus 500 GC-MS apparatus equipped with the same capillary column, carrier and operating conditions described

above for GC analysis. Ionization source temperature was set at 200°C and 70 eV electron impact mode was employed. MS spectra were obtained by means of total ion scan (TIC) mode (mass range m/z 45-500 uma). The total ion chromatograms and mass spectra were processed with the Turbomass 5.4 software (Perkin-Elmer Inc.). Retention indices were determined by injection of C8–C25 n-alkanes standard (Supelco, Bellefonte, PE, USA) under the same conditions. The EO components were identified by comparison of calculated retention indices, high probability matches according to mass spectra computer library search (NIST MS 2.0) and available data from literature (36). Identification of the following compounds was also confirmed by comparison of their experimental lineal retention index (LRI) with those of authentic reference standards (Sigma-Aldrich, Madrid, Spain): α-pinene, β-pinene, camphene, myrcene, limonene, (*Z*)-β-ocimene, camphor, terpinolene and terpinen-4-ol.

Statistical processing of data

One-way ANOVA analysis followed by multiple range test were applied to determine the significance of differences in colour parameters L*, a* and b* among samples, as well as in the yield and composition among ripening stages by means of Statgraphics Centurion XVI® (Statpoint Technologies, Inc.). Tukey's HSD (honest significant difference) test (at $P < 0.05$) was applied in order to evaluate the significance of the differences. Original percentage data were subjected to arcsin [square root (%/100)] transformation in order to fulfill the homocedasticity requirement.

To validate the classification of samples according their ripening stage based on colour, the mean values of variables for the 25 samples were subjected to a stepwise discriminant analysis based on Fisher’s linear functions by means of Statgraphics

Centurion XVI ® software. This statistical tool allows understanding what differentiates the groups and predicting group belonging for unclassified objects.

Results

*Definition of ripening stages by CIEL*a*b* method*

The results of the one-way ANOVA applied to the measured values of L*, a* and b* are displayed in table 1.

Table 1. Values expressed as mean ± standard deviation of L*, a* and b* according to the ripening stage (Values in the same row with different letters differ significantly for Tukey's test at $p<0.05$)

Table 1. Values expressed as mean ± standard deviation of L*, a* and b* according the ripening stage (Values in the same row with different letters differ significantly for Tukey's test at $p<0.05$)

	Ripening stage				
Variable	A	B	C	D	E
L*	-39.43 ± 4.27 a	-41.68 ± 5.24 ab	-42.17 ± 3.69 b	-43.79 ± 13.26 b	-49.67 ± 2.2 c
a*	-5.34 ± 1.48 a	-1.31 ± 2.84 b	3.25 ± 2.13 c	8.77 ± 3.16 d	3.04 ± 2.26 c
b*	23.11 ± 3.72 a	22.58 ± 3.76 a	20.84 ± 2.86 b	14.82 ± 2.84 c	11.06 ± 0.92 d

The parameter b* marks a clear distinction between the first two ripening stages (green berries and beginning of ripening with brown tones) and the rest (where the berries evolve from a yellowish brown to a dark red, almost black). Parameter a* provides complementary information when distinguishing the first two stages of maturation. On the other hand, the parameter L * marks a clear difference between the initial stage (A) and the last three ones (C, D and E), when berries have totally lost their green colour.

The result of discriminant analysis of the mean values of L*, a* and b* of samples is displayed in Fig. 2 The two discriminant functions were statistically significant ($P < 0.05$) with λ (Wilks) values: 0.0063 and 0.1601, respectively, accounting for 99.57 % of total variance. The 100 % of cases were well classified.

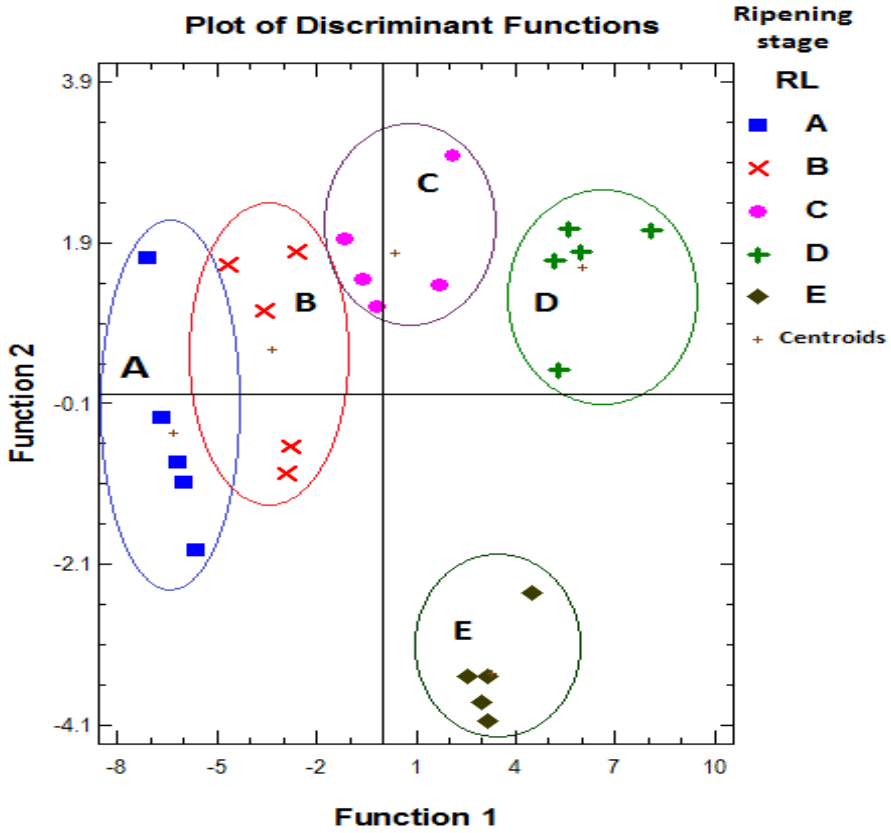
F1 and F2 were defined as follows:

$F1 = -0.0582\ L^* + 0.8340\ a^* - 0.5687\ b^*$

$F2 = 0.6125\ L^* + 0.5367\ a^* + 0.7026\ b^*$

As displayed in Fig. 2, F1 described the colour grading of stages A, B, C and D. The difference between these stages and E was explained by F2, in which this stage showed the lowest value.

Figure 2. Scatterplot of Discriminant Functions for ripening stages according the variables defining the colour.



Yield and composition of EO according the ripening stage.

The composition of the EOs analysed is similar to the most extended profile reported in the literature. Seventy-one compounds were identified accounting for 95.0 – 99.8 % of the total chromatogram. α-pinene (30.1-66.5 %), myrcene (6.1-34.3 %) and germacrene D (0.8-16.9 %) were the major components. Some samples belonging to the last ripening stages also showed appreciable amounts of camphor (up to 6.4 %). Small, but not negligible (0.1-2.7 %), was the contribution of the diterpenic fraction. Total ion chromatogram of a representative sample in which the main compounds are pointed out is displayed in Figure 3. The whole EO composition is shown in table 2.

Table 2. Yield and composition of essential oil from berries of Juniperus oxycedrus ssp. Oxycedrus, according to their ripening stage.

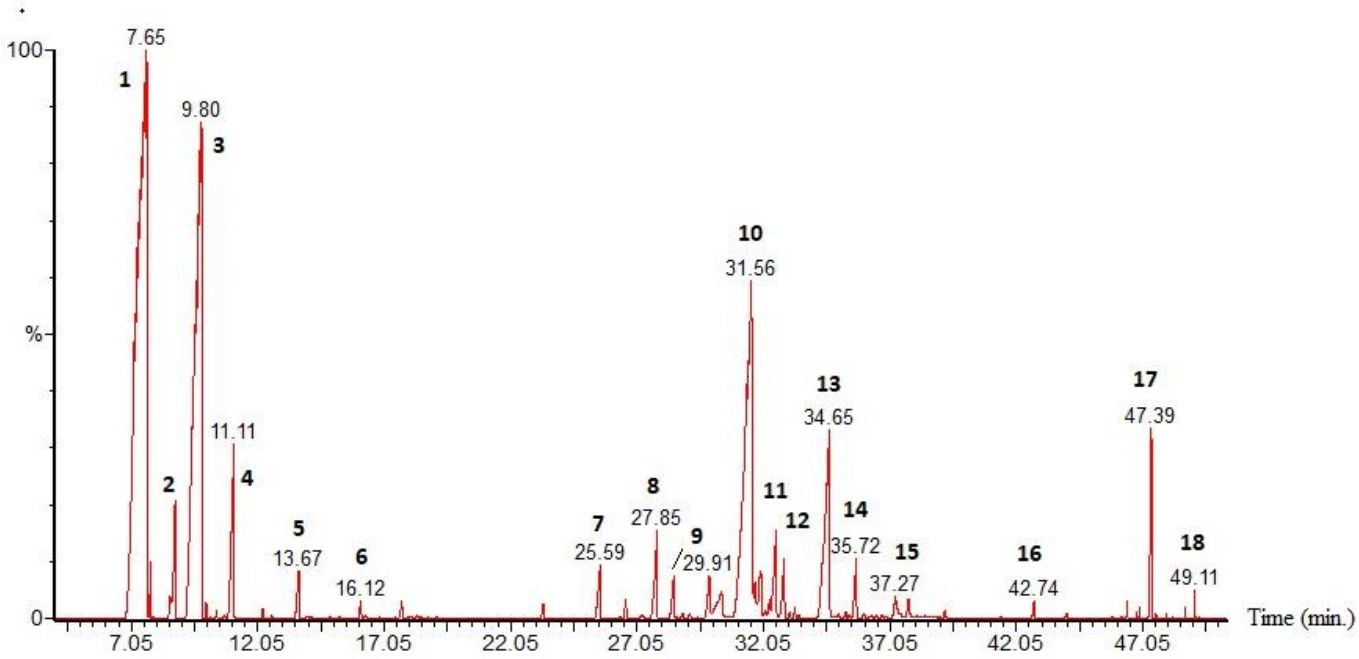
			Ripening stages																							
			A					B					C					D					E			
Compound[1]	**LRI[2]**	**LRI (lit)[3]**	**A1**	**A2**	**A3**	**A4**	**A5**	**B1**	**B2**	**B3**	**B4**	**B5**	**C1**	**C2**	**C3**	**C4**	**C5**	**D1**	**D2**	**D3**	**D4**	**D5**	**E1**	**E2**	**E3**	**E4**	**E5**
Tricyclene	922	921	0.2	0.1	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.3	0.1	0.1	0.31	0.2	0.3	0.2	0.1	0.2
α-Thujene	926	924	0.1	0.1	0.1	t[4]	0.1	0.1	t	t	[5]-	0.1	t	0.1	t	0.1	0.1	t	0.1	t	-	-	-	-	t	-	t
α-Pinene	931	932	66.6	49.5	54.6	51.6	42.5	34.7	40.3	46.6	39.1	41.9	42.1	50.7	50.6	48.1	52.1	42.1	60.4	46.8	30.1	52.7	38.6	50.4	51.7	41.0	59.5
Camphene	952	946	0.5	0.3	1.1	0.5	0.7	0.3	0.3	0.4	0.4	0.3	0.3	0.4	0.3	0.5	0.5	1.0	0.8	0.3	0.3	0.56	0.5	1.2	1.0	0.7	1.1
Thuja-2,4(10)-diene	955	953	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.1	t	-	0.03	0.6	0.6	0.4	0.5	0.5
Sabinene	972	969	0.5	0.7	0.7	0.4	0.5	0.5	0.4	0.3	0.6	0.5	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.1	0.28	0.1	0.1	0.1	-	0.1
β-Pinene	977	974	1.8	1.1	2.5	2.1	1.6	1.5	1.5	1.9	1.9	1.0	2.1	1.7	1.4	2.4	2.4	2.1	2.5	1.0	1.0	2.12	1.9	2.1	1.6	1.2	1.7
Myrcene	1003	988	15.5	34.3	23.2	24.6	22.2	30.6	17.4	17.4	23.3	29.7	27.7	20.6	16.4	24.5	25.7	15.4	11.0	24.9	9.2	26.2	21.7	8.7	9.8	7.5	6.1
α-Phellandrene	1007	1002	t	-	-	0.1	0.1	0.1	-	0.1	0.1	-	-	0.1	t	0.1	0.1	0.2	t	-	-	0.07	t	0.2	0.1	0.1	0.2
α-Terpinene	1016	1014	t	0.0	0.1	t	0.1	0.1	t	-	0.1	t	0.1	0.1	t	0.1	t	0.1	t	t	-	0.09	t	-	-	-	-
p-Cymene	1022	1020	t	-	-	t	-	t	t	0.1	t	-	0.1	0.1	0.1	0.1	0.1	0.4	0.3	0.1	0.1	0.14	0.2	0.5	0.5	0.4	0.5
Limonene	1030	1024	2.3	2.9	2.6	2.8	2.6	3.5	2.1	2.5	3.0	2.5	3.1	2.7	2.2	3.8	0.2	3.0	2.7	2.4	1.5	3.32	3.3	2.4	2.5	2.0	2.1
(*E*)-β-Ocimene	1036	1032	0.0	-	-	-	-	t	-	-	-	-	-	-	-	t	3.8	-	0.1	-	-	-	-	-	-	-	-
γ-Terpinene	1055	1054	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.3	t	0.1	0.1	0.1	-	0.17	0.1	0.1	-	-	-
Terpinolene	1086	1086	0.8	0.7	0.7	0.7	0.9	1.1	0.6	0.7	0.9	t	0.7	1.0	0.7	1.3	1.1	0.7	0.6	0.4	0.5	0.75	0.5	0.3	0.2	0.2	0.1
Perillene	1097	1102	t	t	t	t	0.1	t	t	-	0.1	0.8	-	-	t	t	0.1	-	-	-	-	-	-	0.1	t	-	t
Hydrocarbon monoterpenes			88.3	89.8	85.7	83.1	71.6	72.6	62.7	70.0	69.7	77.0	76.6	78.0	72.1	81.7	86.6	65.8	79.1	76.3	42.8	86.7	67.7	66.9	68.2	53.7	72.1
(*Z*)-Sabinene hydrate	1063	1065	t	0.1	0.1	t	0.1	0.1	0.1	t	0.1	-	-	-	-	-	-	0.1	0.1	-	-	-	-	0.1	-	-	-
(*Z*)-Thujone	1100	1101	-	-	-	-	-	-	-	-	-	-	-	-	-	t	t	0.6	0.4	0.4	0.4	0.4	0.2	0.1	-	-	-
(*E*)-Thujone	1111	1112	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	t	0.0	-	-	t	-	-	-	-	-
α-Campholenal	1121	1122	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	t	0.1	0.1	1.4	0.8	0.1	0.2	0.1	0.3	2.0	1.2	1.2	1.9
Camphor	1138	1141	0.4	0.1	0.5	0.4	0.3	0.2	0.1	0.6	0.3	-	0.2	0.2	0.1	0.2	0.2	4.6	2.6	0.7	1.0	1.0	1.0	6.4	2.8	2.9	3.4
Camphene hydrate	1143	1145	0.1	-	t	t	t	-	t	0.1	t	-	-	t	-	-	t	0.8	0.1	-	-	t	0.1	1.0	0.5	0.5	0.5
(Z)-Pinocamphone	1154	1158	-	-	-	-	-	-	-	-	-	-	t	0.1	0.2	0.1	0.1	t	0.1	-	-	-	0.4	0.9	0.6	0.6	0.7
Pinocarvone	1156	1160	-	-	-	-	-	-	-	-	-	0.1	-	-	-	0.0	0.1	1.3	1.3	0.5	0.6	0.6	-	-	-	-	-

(*E*)-Pinocamphone	1167	1172	0.2	0.1	0.1	0.1	0.1	0.4	0.1	0.1	0.1	-	0.1	0.1	0.1	0.1	0.1	2.5	1.6	0.3	0.4	0.3	0.6	2.9	1.3	1.7	1.7
Terpinen-4-ol	1173	1174	-	-	t	t	-	-	-	-	-	0.1	0.1	t	t	t	t	-	-	-	-	-	-	-	-	-	-
p-Cymen-8-ol	1181	1179	0.2	0.1	0.3	0.2	0.2	0.3	0.2	0.3	0.2	-	0.2	0.3	0.2	0.6	0.6	0.4	0.6	0.3	0.2	0.2	0.3	0.7	0.3	0.4	0.4
α-Terpineol	1186	1186	0.1	-	t	t	0.1	0.1	t	0.1	t	-	0.2	0.0	0.1	0.2	0.3	2.0	0.5	0.2	0.4	t	1.2	2.1	1.2	1.6	1.3
γ-Terpineol	1199	1199	t	t	-	0.1	t	t	-	0.1	t	-	0.2	t	-	0.1	0.1	2.0	0.4	t	0.2	t	0.4	2.1	0.7	1.0	0.8
Carvone	1238	1239	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.4	0.1	-	-	-	-	-	0.1	-	-	0.0
Oxigenated monoterpenes			1.8	1.4	1.7	1.6	1.2	1.3	0.9	1.9	1.1	0.9	1.4	1.4	1.6	1.6	1.6	16.8	9.5	3.0	5.3	3.1	5.1	20.1	10.2	11.7	13.0
α–Cubenene	1347	1345	0.4	0.4	0.2	2.5	0.9	0.2	3.2	2.6	1.2	1.9	0.7	1.0	1.2	0.6	0.5	0.4	1.2	0.6	2.9	0.5	1.7	0.3	1.9	2.3	1.6
α-Copaene	1371	1374	0.1	0.1	t	0.5	0.2	0.1	0.7	0.5	0.3	0.4	0.2	0.2	0.2	0.2	0.1	0.1	0.3	0.2	0.7	0.1	0.6	0.1	0.6	0.8	0.6
β-Elemene	1386	1389	-	-	-	t	t	t	0.1	0.1	t	0.1	t	t	-	t	t	-	-	t	-	-	0.2	-	-	-	-
β-Caryophyllene	1415	1417	0.3	0.2	0.3	0.4	0.7	1.0	0.8	0.5	0.8	0.6	0.8	0.6	0.6	0.7	0.6	0.6	0.4	0.9	1.5	0.4	1.3	0.2	0.4	0.7	0.2
β-Copaene	1424	1430	-	-	-	-	0.0	0.1	t	-	0.0	0.1	t	t	-	t	t	-	-	0.0	-	-	-	-	-	-	-
Bergamotene-α-<trans->	1431	1432	-	-	-	0.1	0.1	0.1	0.2	-	0.1	-	0.1	t	-	t	t	-	-	0.1	0.1	t	0.1	-	0.1	0.1	-
Aromadendrene	1438	1439	-	-	-	-	t	-	t	-	-	-	-	-	-	-	-	0.5	0.3	0.7	1.0	0.3	-	-	-	-	-
(*Z*)-Muurola-3,5-diene	1450	1448	0.3	0.2	0.3	0.3	0.7	-	0.8	0.5	-	0.6	-	-	-	0.7	0.6	-	-	-	-	-	-	-	-	-	-
α-Humulene	1451	1452	0.1	0.1	t	0.6	1.1	2.2	2.0	0.8	1.9	0.5	1.4	1.1	1.2	0.3	0.2	0.3	0.1	0.7	1.5	0.3	1.9	0.3	0.9	1.5	0.3
Sesquisabinene	1462	1457	0.1	t	0.1	0.1	0.1	-	0.2	0.1	0.1	0.1	-	0.2	0.3	0.1	0.1	0.1	-	-	0.3	-	t	0.1	0.2	0.4	0.1
γ-Gurgujene	1472	1475	0.1	0.1	0.1	0.2	0.2	0.1	-	-	t	0.1	0.1	0.1	0.2	0.3	0.3	-	0.2	-	0.6	-	1.7	0.4	0.5	0.8	0.3
Germacrene-D	1490	1484	4.2	3.2	5.7	5.6	14.4	16.9	15.5	7.0	14.5	9.5	10.6	9.2	8.5	9.3	6.6	4.9	2.8	8.2	8.6	3.5	7.0	0.8	1.1	1.8	0.2
γ-Amorphene	1494	1495	0.1	0.1	0.1	0.2	0.3	0.2	0.8	0.4	0.4	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.4	0.7	0.1	0.3	0.2	0.4	0.7	0.3
α-Muurolene	1499	1500	0.3	0.4	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.5	t	t	0.1	0.1	0.1	0.6	0.1	0.9	0.2	-	-	-
γ-Cadinene	1515	1513	0.2	0.1	0.2	0.6	1.0	0.9	1.4	0.9	1.4	0.4	1.2	0.7	0.9	0.9	0.8	1.1	-	0.7	2.1	0.4	0.2	0.9	1.8	3.0	1.6
δ-Cadinene	1523	1522	0.1	0.2	0.3	0.2	0.5	0.6	0.5	0.5	0.6	0.4	0.5	0.5	0.3	0.4	0.3	0.5	0.3	0.7	1.1	0.3	1.3	0.5	0.4	0.7	0.2
Cadina-1,4-diene-<trans->	1529	1533	-	-	-	-	t	-	-	-	-	-	-	-	-	-	-	-	-	t	-	-	2.1	-	-	-	-
α–Cadinene	1534	1537	-	-	-	t	0.1	0.1	0.1	-	0.1	-	0.1	t	-	t	t	t	-	t	-	-	0.1	-	-	0.2	-
α-calacorene	1538	1544	-	-	-	-	t	-	t	-	t	-	-	-	-	-	-	-	-	0.1	-	-	0.1	-	-	-	-
Hydrocarbon sesquiterpenes			6.3	4.9	7.8	11.4	20.5	22.5	26.2	14.0	21.3	14.9	16.0	13.9	14.1	13.7	10.3	8.8	5.9	13.4	21.7	6.1	19.5	4.0	8.4	12.9	5.4
(*E*)-neronidol	1569	1561	0.3	0.2	1.2	3.8	1.1	2.1	5.2	4.2	1.6	0.4	3.4	7.5	1.6	0.9	1.5	1.1	1.8	5.9	0.9	0.3	1.7	5.4	7.6	1.5	1.5
Caryophyllene oxyde	1578	1582	-	-	t	t	0.1	t	0.1	0.1	0.1	0.1	t	0.1	t	t	0.8	0.2	0.7	1.3	0.3	1.1	1.0	0.8	1.4	0.4	0.4
Humulene epoxide (II)	1605	1608	-	-	t	0.1	0.1	-	0.1	0.1	t	0.2	t	0.1	t	-	0.8	0.2	0.4	1.7	0.2	0.7	0.9	0.7	1.3	0.3	0.3

Cubenol <1- epi->	1624	1627	-	-	-	-	-	-	-	-	0.1	-	-	0.4	-	-	-	-	0.1	0.4	t	t	-	-	0.2	-	-
Cadinol-epi-α	1638	1638	t	0.1	0.1	0.2	0.1	0.3	0.3	0.2	t	0.5	0.1	0.1	0.1	0.1	0.3	0.1	0.3	1.8	0.1	0.1	0.2	0.3	0.6	0.1	0.1
Muurolol <-epi-α>	1641	1640	-	-	-	t	t	0.1	0.1	0.1	0.2	0.1	t	t	t	t	0.3	0.1	0.2	0.9	0.1	0.2	0.3	0.3	0.4	0.1	0.1
Selin-11-en-4α-ol	1653	1658	0.1	0.1	t	0.2	0.3	0.1	0.4	0.3	-	0.3	0.2	0.2	-	-	0.8	0.1	0.7	3.5	0.4	0.9	0.4	0.6	1.1	0.3	0.3
Bisabonol-<epi-β>	1667	1670	-	-	-	t	-	-	-	t	-	t	t	0.1	-	-	t	-	0.3	0.1	t	0.3	t	-	-	-	-
Bisabonol-<epi-α>	1681	1683	-	-	-	t	t	-	0.1	0.1	0.1	0.1	t	t	-	-	0.5	0.1	0.2	1.3	0.2	0.8	0.5	0.4	0.9	0.2	0.2
Oxigenated sesquiterpenes			0.4	0.3	1.2	4.2	1.6	2.5	6.4	5.0	2.0	1.6	3.7	8.4	-	-	5.0	1.8	4.6	17.0	2.2	4.5	4.9	8.4	13.6	2.8	2.8
Rosa-5,15-diene -ent-	1928	1933	-	0.1	t	0.2	0.1	0.3	0.2	0.1	-	0.1	0.1	0.1	t	t	0.1	-	-	0.2	-	t	-	t	0.1	-	-
Sandaracopimara-8(14),15-diene	1962	1968	0.1	t	0.1	0.1	t	0.1	0.2	0.1	0.4	0.1	0.0	0.1	t	t	0.1	0.1	0.1	0.4	0.1	0.1	0.1	0.2	0.3	0.3	0.3
Abietatriene	2056	2055	-	t	-	t	-	-	-	-	-	-	-	-	-	-	t	-	t	-	t	-	t	-	t	-	-
Hydrocarbon diterpenes			0.1	0.1	0.1	0.3	0.1	0.4	0.4	0.2	0.4	0.2	0.1	0.2	0.0	t	0.1	0.1	0.1	0.6	0.1	0.1	0.1	0.2	0.4	0.3	0.3
Geranyl linanol <Z,Z>	1953	1960	-	-	-	0.0	0.0	-	-	t	0.1	0.1	t	-	-	-	t	-	-	0.1	-	0.1	0.1	t	0.1	t	t
Manoyl oxide	1994	1987	0.1	0.6	0.2	0.8	0.8	1.6	1.6	1.4	t	1.8	0.1	0.1	0.1	t	2.0	0.8	0.5	2.1	0.3	0.2	1.7	1.2	2.0	1.1	1.1
Sandaracopimarinal	2197	2187	t	t	-	t	t	t	t	-	-	-	t	-	-	-	t	-	t	-	-	-	t	-	t	-	-
Oxigenated diterpenes			0.1	0.6	0.2	0.8	0.9	1.6	1.6	1.4	0.1	1.9	0.1	0.1	0.1	t	2.0	0.8	0.5	2.1	0.3	0.3	1.7	1.2	2.0	1.1	1.1
2-Nonanone	1090	1087	-	-	-	-	-	-	-	-	-	-	-	-	-	t	0.3	0.1	-	-	-	0.1	0.4	0.3	0.2	0.3	0.3
Tridecane	1298	1300	1.1	0.7	0.7	0.3	0.3	0.4	0.6	0.3	0.6	0.5	0.7	0.8	0.1	0.1	0.4	0.8	0.5	1.8	0.4	0.4	0.6	1.0	1.0	1.5	1.5
Hexadecane	1597	1600	2.3	1.5	1.4	0.8	0.6	1.0	1.5	0.6	1.4	1.2	1.5	2.0	t	t	0.1	1.9	1.3	4.2	1.0	1.1	1.8	2.5	3.0	3.4	3.4
Heptadecane	1692	1700	0.2	0.1	0.1	0.1	t	0.1	0.1	t	0.3	0.1	0.1	0.1	-	-	-	0.1	0.1	0.3	0.1	-	0.1	0.2	t	0.3	0.3
Octadecane	1792	1800	0.6	0.4	0.4	0.2	0.2	0.2	0.3	0.1	-	0.3	0.4	0.5	-	-	0.2	0.4	0.3	1.0	0.2	0.2	0.3	0.5	0.5	0.8	0.8
Nonadecano	1894	1900	t	-	t	-	-	-	-	-	0.1	-	t	-	-	-	-	t	-	-	-	-	-	-	-	0.1	0.1
Other			3.1	2.0	1.9	1.00	0.8	1.3	1.9	0.7	1.8	1.5	2.1	2.6	0.0	0.0	0.3	2.5	1.6	5.5	1.3	1.3	2.2	3.1	3.5	4.6	6.4
Total identified			99.8	99.8	98.4	99.8	99.8	99.7	95.8	96.2	99.7	97.2	99.3	99.7	99.3	99.3	99.3	98.6	99.6	99.7	95.0	99.9	97.9	99.6	99.5	97.3	98.7

[1] Constituents listed in order of increasing linear retention indices (LRI) for each group of compounds. Unidentified components less than 0.5% are not reported.
[2] Temperature-programmed LRI referred to n -alkanes, determined on a DB5 capillary column.
[3] LRI Values coming from R.P. Adams (35)
[4] Percentage values less than 0.1% are denoted as t (traces).
[5]No detected.
Method of identification: All the reported compounds have been identified by comparison of GC-MS data of NIST computer mass library and LRI with those reported by R. P. Adams (35). α-pinene, β-pinene, myrcene, α-terpinene, *p*-Cymene, limonene, terpinolene, (*Z*)-thujone, (*E*)-thujone, camphor and terpinen-4-ol were identified by comparison of LRI with those of authentic samples.

Figure 3. MS total ion chromatogram from a representative sample of berries of *Juniperus oxycedrus* ssp. *oxycedrus* EO



1 α–pinene; 2 myrcene; 3 β-pinene; 4 limonene; 5 terpinolene; 6 camphor; 7 α-cubebene ; 8 tetradecane; 9 β-caryophyllene; 10 germacrene-D; 11 γ-cadinene; 12 δ-cadinene; 13 (*E*)-nerolidol; 14 hexadecane; 15 muurolol <epi-α >; 16 octadecane 17 manoyl oxide; 18 sandaracopiraminal.

Mean values ± standard deviation and significant differences for the main compounds and grouped compounds are displayed in table 3. The optimum yield (regarding dry weight of crushed berries) was achieved at the beginning of ripening (when berries are changing from green to brown), showing balanced results for A, C and D stages. On the contrary, a noticeable decrease is observed in the dark berries (E).

Table 3. Yield and EO composition throughout the ripening process.

	Ripening stage				
	A	B	C	D	E
Yield	1,6 ± 0,4 a	2,3 ± 0,6 b	1,8 ± 0,6 ab	1,6 ± 0,4 a	0,8 ± 0,2 c
α-pinene	53.0 ± 8.8 a	40.5 ± 4.3 b	48.7 ± 4.0 ab	46.4 ± 11.4 ab	48.2 ± 8.5 ab
Myrcene	24.0 ± 6.7 a	23.7 ± 6.4 a	23.0 ± 4.5 a	17.3 ± 7.8 ab	10.8 ± 6.3 b
Limonene	2.6 ± 0.2 a	2.7 ± 0.6 a	2.4 ± 1.4 a	2.6 ± 0.7 a	2.5 ± 0.5 a
Hydrocarbon monoterpenes	83.8 ± 7.2 a	70.5 ± 5.2 bc	79.0 ± 5.4 ab	70.2 ± 17.0 bc	65.8 ± 7.0 c
Oxigenated monoterpenes	1.5 ± 0.3 a	1.2 ± 0.4 a	1.5 ± 0.1 a	7.5 ± 5.8 b	12.0 ± 5.4 c
Germacrene-D	6.6 ± 4.5 a	12.7 ± 4.2 b	8.8 ± 1.5 ab	5.6 ± 2.7 ac	2.7 ± 2.9 c
Hydrocarbon sesquiterpenes	7.7 ± 5.0 a	15.5 ± 5.6 b	10.6 ± 1.7 ab	7.6 ± 4.0 a	5.4 ± 4.1 a
Oxigenated sesquiterpenes	1.3 ± 1.8 a	3.5 ± 2.1 abc	4.7 ± 3.5 ab	6.1 ± 6.2 bc	6.9 ± 4.3 c
Hydrocarbon diterpenes	0.2 ± 0.1 a	0.3 ± 0.2 a	0.1 ± 0.1 a	0.2 ± 0.2 a	0.2 ± 0.1 a
Oxigenated diterpenes	0.4 ± 0.3 ab	1.1 ± 0.7 ab	0.4 ± 0.8 a	1.2 ± 0.9 b	1.3 ± 0.7 b
No oxigenated compounds	94.5 ± 3.3 a	88.0 ± 5.8 ab	91.4 ± 3.1 a	81.0 ± 12.1 bc	75.2 ± 6.8 c
Oxigenated compounds	3.3 ± 1.8 a	5.9 ± 2.8 a	4.8 ± 3.5 a	14.9 ± 8.9 b	20.4 ± 7.4 b

Values with different letter for the same row are significantly different (Tukey HSD, $P \leq 0.05$)

Discussion

As for the hydrocarbon monoterpene fraction, α-pinene shows its highest proportion in the green berries, with a significant decrease at the beginning of ripening (Table 3). Further ripening stages maintain stable values for this compound. These changes agree with those reports by Angioni et al. (9) if unripe/ripe is related to stages B and C, respectively. This comment can be extended to hydrocarbon monoterpene fraction as a whole. However, myrcene showed a different profile, with a progressive decrease becoming significant at the last two ripening stages (Table 3). This decrease is also reported for myrcene in the aforementioned research from unripe to ripe stages.

Germacrene-D is by far the most important component of hydrocarbon sesquiterpene fraction. It achieves its highest proportion when berries begin to ripe (stage B), showing a significant decrease at the end (Table 3). The most regular change is observed in the oxygenated sesquiterpene compounds, showing a significantly progressive increase from the first to the last ripening stage.

In spite of the high variability in the measurement of the colour parameters due to the small size of the berries, the parameters L * and, mainly, a* and b*, can be useful to define objectively a colour rank associated to the ripening process.

The ripening process in *J. oxycedrus* ssp. *oxycedrus* implies a progressive increase in the proportion of oxygenated compounds, both monoterpenic and sesquiterpenic ones. The most influencing compound in this tendency is myrcene, showing a noticeable decrease at the end of ripening process. On the other hand, the yield data show a maximum value at the beginning of maturation, whereas only the extremely mature berries have a very poor yield.

These results evidence that an intuitive classification based on purely visual criteria is not enough to clearly define the ripening stages and correlate them with the yield and chemical composition of berry EOs. Neither can the sampling time be adopted as a criterion, since given the duration of the maturation process, berries with different degrees of maturity can coexist in the same individual.

An objective definition of maturation stages based on the measurement of physical parameters, such as colour, can help optimize yield and composition of this type of EOs according to the type of biological activity implied. On the other hand, from the chemotaxonomic point of view, as it happens relating with the rest of variability sources, the correct definition of chemotypes requires controlling the degree of ripening, since the variations observed in major compounds could be, in that respect, relevant.

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