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Odor-active constituents of *Cedrus atlantica* wood essential oil

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ABSTRACT

The main odorant constituents of *Cedrus atlantica* essential oil were characterized by GC-Olfactometry (GC-O), using the Aroma Extract Dilution Analysis (AEDA) methodology with 12 panelists. The two most potent odor-active constituents were vestitenone and 4-acetyl-1-methylcyclohexene. The identification of the odorants was realized by a detailed fractionation of the essential oil by liquid-liquid basic extraction, distillation and column chromatography, followed by the GC-MS and GC-O analyses of some fractions, and the synthesis of some non-commercial reference constituents.

1. Introduction

The Atlas cedar, native to the Atlas mountains of Algeria and Morocco, is often considered as a unique species (*Cedrus atlantica* (Endlicher) Carrière) separated from the two other main groups of Mediterranean cedars located in Cyprus and Northeastern Mediterranea, since they show slight morphological differences (M'Hirit and Blérot, 1999; M'Hirit, 1994). However, some authors prefer to consider these three populations as subspecies of *Cedrus libani*: *C. libani* subsp. *atlantica* (Endlicher) from North Africa, *C. libani* subsp. *brevifolia* (J. Hooker) in Cyprus and *C. libani* A. Richard subsp. *libani* (cedar of Lebanon) formerly widespread in Syria and Lebanon, and now present mainly in Southern Turkey (Eckenwalder, 2009). This latter subspecies was overexploited for thousands of years by several ancient civilizations since the durability and the rot-resistant character of its aromatic wood was very useful to build ships, houses and temples, the most famous example being the King Solomon's temple in Jerusalem. Today, *C. atlantica* is the largest remaining population, and the most commonly cultivated subspecies. It covers about 160 000 ha, mostly in the Rif and Atlas mountains of Morocco. The trees can reach a height up to 40 m and a trunk diameter of 2 m, and their wood is still highly appreciated for construction. Indeed, *C. atlantica* is the main species of Moroccan forests used for timber production, and the sawdust produced during wood processing is often valorised by hydrodistillation to afford an essential oil displaying the very typical odor of the wood. This oil is entirely different (chemically and olfactorily) from the most common Texas and Virginia cedarwood oils obtained from *Juniperus* species. Its very peculiar odor has been described as slightly camphoraceous-cresylic, with a sweet and tenacious, woody undertone, reminiscent of cassie and mimosa (Arctander, 1960). The composition of *Cedrus atlantica* essential oil is generally dominated by α- and β-himachalenes **1** and **2** (Aberchane et al., 2004; Chalchat et al., 1994; Dahoun et al., 1993; Derriche et al., 1996; Plattier and Teisseire, 1972) and/or himachalol **3** (Boudarene et al., 2004; Paoli et al., 2011; Satrani et al., 2006), with significant amounts of bisabolane sesquiterpenic ketones such as α-atlantone **4** (Aberchane et al., 2004; Boudarene et al., 2004; Chalchat et al., 1994; Dahoun et al., 1993; Derriche et al., 1996; Paoli et al., 2011; Plattier and Teisseire, 1972; Satrani et al., 2006; Pfau and Plattner, 1934), γ-atlantone **5** (Aberchane et al., 2004; Boudarene et al., 2004; Chalchat et al., 1994; Derriche et al., 1996; Plattier and Teisseire, 1972; Pfau and Plattner, 1934), and deodarone **6** (Adams et al., 1974; Nam et al., 2015) which are besides commonly found in the related himalayan *Cedrus deodara* species (Adams et al., 1974).

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Despite the large number of publications dealing with the composition of Atlas cedarwood oils, accurate descriptions of the odor-active constituents of this material are scarce. As soon as 1902, Grimal reported that the distillation of Atlas Cedarwood oil furnished a fraction "which possessed exactly the odor of the original essence" containing a ketone of the formula $C_9H_{14}O$ (Grimal, 1902). However, Pfau and Plattner claimed later that the mixture of atlantones **4** and **5** is "the true aromatic principle of the oils" (Pfau and Plattner, 1934). Thereafter, the odor of vestitenone **7** has been described as characteristic of the wood (Shankaranarayan et al., 1977), and recent secondary sources mentioned that the odor of Atlas cedarwood was due to "materials such as α-atlantone and deodarone (**4** and **6**)" (Sell, 2003, 2006). However, the experimental data supporting the olfactory importance of these components were never accurately reported. Actually, the relative odor contribution of **4**–**7** is still unknown, as well as the qualitative and quantitative olfactory differences between each of the eight α-, β- and γ-atlantone isomers (**4**, **8**, **5**).

To better understand the olfactory contribution of the volatile constituents of Atlas cedarwood, we performed a detailed investigation on a sample of Moroccan essential oil. We first carried out a series of fractionations by acido-basic liquid-liquid extraction, distillation and flash chromatography, followed by GC-MS analysis. Subsequently, GC-O analysis was applied to the whole essential oil and its fractions to characterize the main odor-active constituents, and to guide to other fractionations in order to identify additional odor impact compounds.

2. Results and discussion

The chemical composition of the sample of Atlas Cedarwood essential oil investigated in this work is reported in Table 1, and is consistent with most of the studies published in the literature for this material (Aberchane et al., 2004; Chalchat et al., 1994; Dahoun et al., 1993; Derriche et al., 1996; Plattier and Teisseire, 1972). The GC-O investigation of the whole essential oil permitted to characterize several odorant zones, and a careful fractionation of the oil combined with the GC-O analyses of the fractions and of pure reference compounds helped us to attribute some odor-active components to their corresponding zones. The AEDA methodology (Ullrich and Grosch, 1987) was applied to characterize the most important olfactory contributors of our sample. Although GC-O is nowadays recognized as the most reliable tool for such task, it should be kept in mind that it has some limitations as it does not take into account the synergies between the odorants (Barkat et al., 2012; Zou and Buck, 2006). Moreover, several authors have criticized the Dilution to Threshold methodologies such as AEDA since they are based on the false assumption that the odor intensity increases linearly with the concentration. In fact, different odorants are not necessarily characterized by the same slope in their psychophysiological function (Neuner-Jehle and Etzweiler, 1991), and for this reason a ranking of the odorants of a mixture cannot be based solely on their evaluation in the mixture near their detection threshold. Indeed, as pointed out by Ferreira (Ferreira et al., 2002), it should be considered that initially, AEDA experiments were used only as a rough guide for aroma reconstruction experiments. Hence, the results reported in our study should be interpreted carefully, as a simple attempt to bring some precisions to the apparent controversies of the literature concerning the odor-active constituents of Atlas Cedarwood. The AEDA method was selected since it does not require well-trained panelists, on the contrary to the more difficult Direct and Posterior Intensity methods (Casimir and Whitfield, 1978; Delahunty et al., 1996; Etievant et al., 1999; Guichard et al., 1995; Van Ruth and O'Connor, 2001). It is also more informative than the Detection Frequency methods (Linssen et al., 1993) to distinguish the olfactory contributors on the basis of their relative importance. One of the drawbacks of the AEDA is that it is time-consuming and not always compatible with the participation of a large number of judges. However, Ferreira mentioned that a good compromise between experimental time and precision of the results can be achieved with a dilution rate of 4–20 (Ferreira et al., 2002), thus significantly lowering the number of required experiments in comparison with AEDA studies based on a dilution rate of 2. In our study, 12 panelists performed GC-O analyses with a dilution rate of 4, corresponding then to a total of about a dozen of 25 minutes sessions per panelist. They detected a total of 44 different odor zones, but only 6 were perceived unanimously by all of the panelists. The individual olfactory descriptions of the 20 zones perceived by more than half of the panelists are reported in Table 2, together with each individual FD value. This presentation of the results without any summarization of the data is deliberate, as it illustrates the large differences of sensitivity among the panelists and the complexity of the raw data obtained with a large panel of judges of various cultural backgrounds and olfactory educations. Indeed, our objective was to identify the most characteristic cedarwood-like odorants of our sample, recognized as such by an average population. Then, the only common training to which was submitted our panel was the memorization of the odor of the whole essential oil, for the panelists not yet familiar with the odor of this material. The variability of the qualitative descriptors used by the panelists in this study can then be due to real differences in the perceptions, but is also probably the result of the large variations in the olfactory cultures of non-trained panelists, unable to define olfactory sensations with a common standardized vocabulary.

Despite such interindividual variability, interesting information can be easily extracted from the whole set of data, in order to characterize the main contributors of the typical odor of Atlas Cedarwood oil. All of the panelists agreed that the contribution of the atlantones and deodarone **4**–**6** and **8** was insignificant in comparison with that of several more volatile constituents. Indeed, only one odor zone at a Linear Retention Index ($LRI_{GC\text{-}O}$) of 1748 seemed to match with the signal of one of these ketones, (*Z*)-**4**, and was perceived by half of the panel with a rather low mean $\log_4$(FD). Only one judge reported a cedarwood-like odor, while the 11 other panelists described it as mushroom-like/woody, not particularly typical of cedarwood. In contrast, two constituents showing the highest mean FD value of all odorants were detected by all the panelists at $LRI_{GC\text{-}O} = 1152$ and 1473. The former was almost unanimously recognized as "typically cedarwood" (11 panelists out of 12), and 7 panelists out of 12 described the latter as reminiscent of cedarwood, while the other sniffers attributed less typical descriptors, such as "lemon-like". A first survey of our mass spectral and LRI databases led us to the conclusion that these two constituents could be respectively 4-acetyl-1-methylcyclohexene **9** and vestitenone **7**, contained in respective percentages of 1 and 0.45% in the essential oil. **9** was probably the $C_9H_{14}O$ ketone that Grimal had mentioned earlier, without being able to determine the structure (Grimal, 1902) and was besides described several times in the following old phytochemical studies on Atlas Cedarwood essential oil (Plattier and Teisseire, 1972; Pfau and Plattner, 1934) but surprisingly much less in several more recent studies (see Fig. 1).

In view of the potential importance of **7** and **9** in our olfactory study, we had to confirm unambiguously their identification and their contribution to the odor of the essential oil. Even if the chromatograms of both constituents showed that their GC-O and GC-MS signals matched perfectly, one cannot exclude the possibility that the perceived odor is actually due to a coeluting minor constituent. This situation is indeed one of the main difficulties of GC-O experiments, which can be overcome only by the evaluation of pure

Table 1
Constituents of *Cedrus atlantica* wood essential oil.

LRI_{HP-5}[a]	LRI_{Litt}[b]	Identification	%[c]
<800	771	toluene	0.18
831	828	furfural	tr
963	957	5-methylfurfural	tr
936	932	α-pinene	0.03
992	988	myrcene	0.01
1026	1020	p-cymene	tr
1030	1024	limonene	tr
1033	1026	1,8-cineole	tr
1048	1044	(*E*)-β-ocimene	tr
1049	1054	γ-terpinene	tr
1073	1071	*p*-cresol **18**[d,e]	tr
1076	–	6-methyl-3,5-heptadien-2-one	tr
1079	1086	α-terpinolene	tr
1091	1087	guaiacol[d,e]	tr
1092	1089	dehydro-*p*-cymene	0.02
1125	1118	endo-fenchol	tr
1134	–	4-acetyl-1-methylcyclohexene **9**[e]	1.02
1148	1135	trans-pinocarveol	tr
1150	–	1-(4-methyl-3-cyclohexen-1-yl)ethanol **12** (diastereomer 1)[e]	0.07
1153	–	1-(4-methyl-3-cyclohexen-1-yl)ethanol **12** (diastereomer 2)[e]	0.06
1166		4-ethylphenol[d,e]	tr
1171	1165	borneol[e]	tr
1181	1174	terpineol-4	tr
1187	1179	p-methylacetophenone **15**[e]	0.15
1194	1186	α-terpineol[e]	0.02
1195	1190	decan-2-one	0.01
1213	1204	verbenone	0.01
1219	–	1-(4-methyl-3-cyclohexen-1-yl)propanal **11** (diastereomer 1)[e]	tr
1221	–	1-(4-methyl-3-cyclohexen-1-yl)propanal **11** (diastereomer 2)[e]	tr
1200	1200	dodecane	tr
1207		1-(4-methylphenyl)ethanol[f]	0.04
1295	1293	undecan-2-one **16**[e]	0.83
1298	–	1-(4-methyl-3-cyclohexen-1-yl)propanol **10** (diastereomer 1)[e]	tr
1299	–	1-(4-methyl-3-cyclohexen-1-yl)propanol **10** (diastereomer 2)[e]	tr
1359	1350	α-longipinene	0.06
1390	1383	β-damascenone **17**[g]	tr
1395	–	Unknown[h]	>1
1415	1407	longifolene[f]	0.52
1436	–	himachala-2,4-diene[f]	0.47
1450	1444	vestitenone **7**[e]	0.45
1461	1449	α-himachalene **1**	12.74
1489	1481	γ-himachalene	8.31
1492	1485	himachala-1,4-diene	2.20
1513	1500	β-himachalene **2**	33.45
1523	1516	α-dehydro-ar-himachalene	3.11
1532	1522	δ-cadinene	2.78
1536	–	(8,9)-dehydro-(neo)isolongifolene[f]	1.42
1548	–	Unknown[i]	1.61
1553	1544	α-calacorene	1.17
1586	1578	himachalene epoxide	0.66
1610	1599	longiborneol	0.45
1625	1615	β-himachalene oxide	1.02
1632	–	Unknown (oxygenated sesquiterpenoid)[j]	1.06
1639	1628	1-epicubenol	1.12
1661	1652	himachalol **3**	0.96
1674	1661	allo-himachalol	1.93
1677	1668	dihydroatlantone	0.13
1686	1675	cadalene	0.06
1700	1694	(*Z*)-γ-atlantone (*Z*)-**5**	1.04
1707	1698	deodarone **6**	2.61
1712	1706	(*E*)-γ-atlantone (*E*)-**5**	1.28
1722	1717	(*Z*)-α-atlantone (*Z*)-**4**	1.45
1783	1777	(*E*)-α-atlantone (*E*)-**4**	6.23

[a] LRI_{HP-5}: linear retention index in GC-MS on HP-5 column.
[b] LRI_{Litt}: linear retention index on HP-5 column reported in: Adams, R. P., 2007. Identification of Essential Oil Components by GC/MS, 4th ed., Allured Publishing Corp., Carol Stream, USA.
[c] Massic percentage calculated from predicted response factors according to Tissot et al. (2012). tr: trace (<0.01%).
[d] Identification in the acidic fraction.
[e] Identification confirmed by coinjection of commercial or synthesized reference compound.
[f] Tentative identification.
[g] Identification based on coincidence of the GC-O retention time with those of a reference compound.
[h] m/z (%) = 203 (5), 202 (31), 187 (6), 159 (18), 147 (7), 146 (41), 145 (16), 132 (13), 131 (100), 129 (11), 128 (6), 117 (5), 116 (5), 115 (10), 107 (5), 105 (8), 91 (17).
[i] m/z (%) = 202 (49), 188 (15), 187 (100), 159 (16), 146 (13), 145 (73), 143 (18), 133 (13), 132 (15), 131 (43), 129 (15), 128 (21), 121 (15), 119 (28), 115 (16), 109 (12), 105 (20), 93 (48), 91 (22), 79 (12).
[j] m/z (%) = 220 (50), 205 (17), 200 (16), 185 (24), 177 (17), 163 (15), 149 (15), 138 (100), 137 (77), 135 (24), 123 (37), 121 (32), 120 (46), 119 (15), 110 (71), 109 (48), 108 (24), 107 (56), 105 (37), 96 (20), 95 (28), 93 (43), 91 (52), 77 (31), 69 (22), 55 (24), 41(30).

Table 2
GC-O Analysis of the Odorant Constituents of *Cedrus atlantica* wood essential oil.

LRI[a]	Constituent[b]	Olfactory Description[c]	Individual log4(FD)[d]												AM[e]
			1	2	3	4	5	6	7	8	9	10	11	12	
1084	*p*-cresol **18**	Burnt plastic[1, 9]; wood[4]; woody, powdery, rancid[6]; phenolic[7, 8]; urine[12]	2		0	1	0	1	2	1	1		0	1	
1112	–	Olive[6, 7]; phenolic[8, 10, 12]; smoky, guaiacol-like [10]						1	1	0	1	3		1	
1152	4-acetyl-1-methylcyclohexene **9**	Typically cedarwood[1–5, 7–12]; orange peel, buttery[6]; orange[9]; powdery, sweet, dill[10]; cacao, floral, fruity[12]	4	3	2	3	2	4	3	4	3	3	3	4	**3.1**
1213	4-methylacetophenone **15**	Almond[1, 3, 5, 8]; wet wood[2]; floral, jasmine[6]; fresh, woody[7]; cedarwood[9]; cypress, soapy[11]; fatty, buttery, rancid[10]; floral, fresh leaves[12]	1	1	1	0	0	1	1	1	0	1	1	2	**0.7**
1305	undecan-2-one **16**	Fruity[1]; dry leaf, cedarwood[2]; almond[5]; capric, fatty[6]; stink bug[8]; mint, citrus[9]; soap[9–11]; floral, fruity, fresh leaves[12]	0	1	1	0	0	2	0	1	2		3	2	
1310	–	Honey, fatty[6]; anise, fennel[7]; fruity[9]; petrol, moldy[11]; rotten, fruity, cyclopentanone-like, balsamic, valeric[10]				0		3	1		1	2	1		
1392	–	Almond[1, 5, 8]; incense[6]; hot, dust, milky[7]; coconut[8]; cinnamon[9]; sweet, powdery cedarwood-like[10]; lemon[11]; fruity[12]	0				0	0	0	1	0	0	1	1	
1412	β-damascenone **17**	Lemon, fresh fruit[4]; soapy, floral, violet, blackberry[6]; fruity, damascenone-like[8, 10]; orange, fruity[9]; fresh, cedarwood[11]	0			1		1		2	0	2	1	1	
1473	vestitenone **7**	Floral[1, 10]; cedarwood[2–5, 7, 12]; lemon[4–5, 8–9, 11]; lavender, pine, Marseille soap[6]; liquorice[7]; powdery[7, 10]; eucalyptus[9]; slightly minty, fruity[10]; pepper[11]	4	5	1	4	4	4	0	3	4	2	4	5	**3.1**
1500	–	Cedarwood[2–4, 7–8, 10–12]; citrus peel[6]; ginger, fruity[9]; dill[10]; apricot[12]	1	2	2	1	0	5	2	3	4	0	1	5	**2.0**
1560	–	Herb[1]; unpleasant[3]; solvent, washing powder[6]; phenolic[7]; plastic[8, 12]; woody, dry, mushroom, mossy[10]; rust, mint[12]	0		3			1	2	1		1		0	
1573	–	Lemon[1, 5]; clove[6]; spicy, cinnamon[7]; eugenol[8]	0				0	0	1	0					
1623	–	Cedarwood, rose[2]; camphoraceous[8]; flowery, powdery, perfume[10]; minty[12]		1		0				2	1	1		4	
1657	–	Lemon[1, 3, 8]; cedarwood[4]; smoked saffron[6]; woody, fruity, vetiver, vertofix-like[10]	1		1	0		2		1		0			
1703	–	Vegetal[1]; cedarwood[2–3, 7, 8, 10]; almond[4]; lemon[5]; rotten clementine[6]; honey[7]; lactonic, coconut[8]; fir tree[9]; vetiver, buttery, fatty, fruity, powdery[10]; curds, wood[11]; apricot, sweet[12]	1	1	1	1	0	3	1	2	2	1	2	3	**1.3**
1736	–	Soap, fruity[4, 10]; rosy, powdery[7]; floral, vetiver[8]; sweet[9, 7]; fruity, damascenone[10]				1			1	2	2	0			
1748	–	Cedarwood[2]; dust, smoke, mushroom[6]; metallic, mushroom[8]; fruity, woody, mushroom[10]; dry wood, pine, sauna[11]; fermented leaves[12]		2	1			0		1		0	0	4	
1809	–	Soap, almond[4]; cedarwood, damascenone[9]; fruity, plum-like[10]; lemon, cypress[11]; floral[12]			1	1				1	0	0	1	5	
1817	–	Fruity[1]; fatty, soap[6]; lactonic[8]; spicy, cypress, tamarix[11]	1	1				1		0	0		0		
1828	–	Cedarwood[2]; citrus[2, 9]; fruity[4, 8, 10]; rose[5, 2]; almond[4–5]; violet, candy[6]; cypress[11]; damascenone[8, 10]; fig[7]; grape[12]	0	2	0	2	0	3	2	3	2	4	4	3	**1.8**

[a] Linear retention index in GC-O on HP5 column.
[b] Odorants identified unambiguously by coinjection of reference compounds. -: Non identified. Additional constituents identified unambiguously by coinjection of reference compounds, and detected by less than half of the panelists: Guaiacol ($LRI_{GC\text{-}O} = 1105$) and 4-ethylphenol ($LRI_{GC\text{-}O} = 1172$).
[c] Individual olfactory descriptions (corresponding panelist letter in superscript).
[d] Log_4(FD) of each individual perceived odor zone.
[e] Arithmetic mean of the log_4(FD), for each odor zone unanimously perceived.

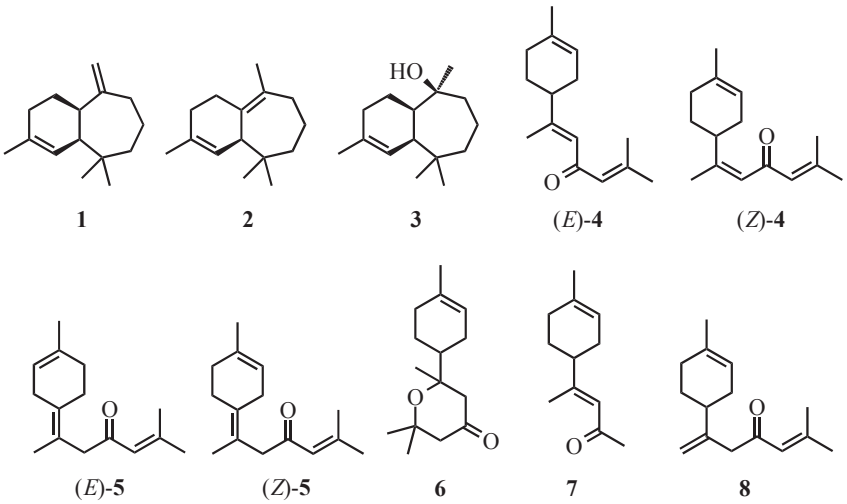

Fig. 1. Typical constituents of *Cedrus atlantica* wood essential oil.

reference compounds, obtained preferably from another origin than the raw material itself. Therefore, we synthesized pure samples of **7** and **9** (Fig. 2), which we used in subsequent coinjection experiments in GC-MS and GC-O. Several expeditious procedures are known for the preparation of **9**, either based on the Diels-Alder addition of isoprene and methylvinylketone (Alder and Vogt, 1949) or the oxidation of limonene (Baucherel et al., 2001). In our study, we followed an original synthetic approach, which, albeit longer, permitted to prepare three additional reference constituents (**10**–**12**) to confirm their identification by coinjection, since preliminary GC-MS investigations suggested that they could be actual constituents of the essential oil. As our chiral GC investigations showed that, not surprisingly, **9** was present in the essential oil as a racemate, we decided to synthesize **9** in racemic form to ensure that our synthetic reference compound would display the same olfactory properties than the natural constituent. Hence, hydroboration of (±)-limonene by 9-BBN afforded the primary alcohol **10**, which was oxidized with PCC to aldehyde **11**. The application of an original oxidative decarbonylation procedure developed in our laboratory (Baldovini et al., 2013) permitted to convert **11** in alcohol **12** containing a small amount of **9**. In these reactions, compounds **10**–**12** were obtained as ca. 1/1 diastereomeric pairs, and we noticed that these three constituents were also contained in this form in the essential oil. Eventually, the crude mixture of **9** and **12** led to **9** by PCC oxidation. Vestitenone **7** was synthesized from **9** by a three-step sequence (Wittig-Horner-Wadsworth-Emmons reaction, saponification, and treatment by 2 equivalents of methyllithium). Both synthetic samples were used in coinjection experiments which confirmed unambiguously that **7** and **9** were the actual contributors of the two main odor zones. Four other odorants (at $LRI_{GC-O} = 1213$, 1500, 1703, and 1828) were detected by all of the panelists, and also reported to possess an Atlas cedarwood character by some of them, though less unanimously than for **7** and **9**. The characterization of the first one as 4-methylacetophenone **15** was easily confirmed by coinjection of a commercial reference sample. However, despite our extensive analysis of the essential oil sample, the identification of the three other odorants could not be achieved. These constituents possess probably an extremely low detection threshold, since they produced relatively strong olfactory stimuli for many panelists in spite of their very low amount in the sample. It demonstrates that the GC-O analyses of complex natural mixtures can be useful for the discovery of new families of potent odorants, with many potential applications in the fragrance industry. Three additional odorants detected by almost all of the panelists could be identified unambiguously by coinjection of authentic reference samples: undecan-2-one **16** (soap, fatty, stink bug) and β-damascenone **17** (fruity) were constituents of the neutral part of the essential oil, and *para*-cresol **18** (animalic, leather) could be identified in the acidic extract of the oil. In the context of an analysis devoted to the identification of odor-active compounds, such a preliminary acido-basic fractionation is extremely useful since many naturally occurring carboxylic acids and phenols have a low detection threshold and can contribute significantly to the odor of the whole mixture (Boelens, 1996; Cerutti-Delasalle et al., 2016). Indeed, in addition to **18**, 4-ethylphenol and guaiacol were also identified in this fraction and detected by some panelists as odorant contributors bringing the specific phenolic, animalic, sheepfold-like tonality of Cedarwood oil.

3. Conclusions

The determination of the most important olfactory contributors of a natural mixture can be an extremely long and complex task, which requires the combination of very efficient analytical techniques with sensorial analyses involving a large number of panelists. Consequently, the lack of accurate knowledge about the main odoriferous constituents is rather common for many essential oils and extracts obtained from fragrant plants. Even in modern studies, the identification of the most important odor-active constituents is often neglected, and this situation is paradoxical when it concerns materials used for their odorant properties in the flavor and fragrance industry.

In this study, we reported that 4-acetyl-1-methylcyclohexene **9** and vestitenone **7** are the two most important contributors to the typical odor of Atlas cedarwood, together with some compounds of minor importance like *para*-cresol **18**, undecan-2-one **16** and 4-methylacetophenone **15** (Fig. 3). Many other olfactorily important unidentified constituents contribute to the overall odor of this material, but in contrast with most of the previously published data, we conclude that the atlantones play a negligible role in the olfactory character of Atlas cedarwood. In such kind of studies on

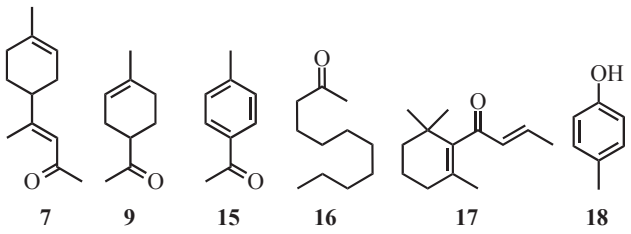

Fig. 3. Most important identified *Cedrus atlantica* wood essential oil odorants.

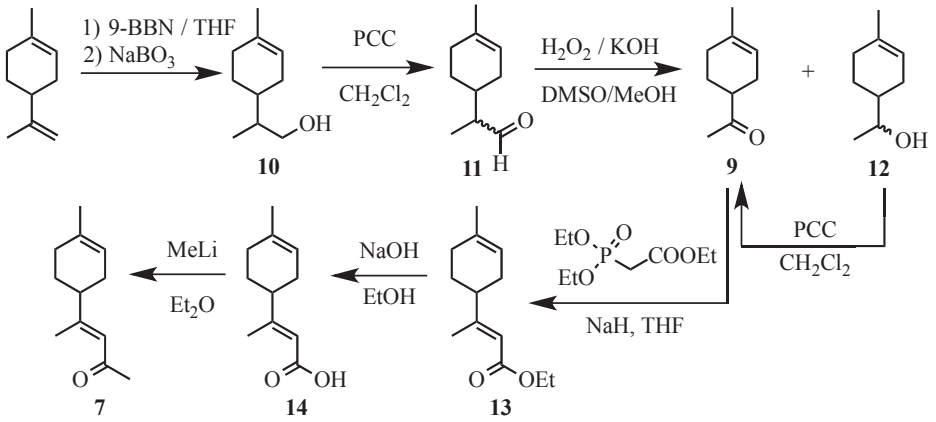

Fig. 2. Synthesis of *Cedrus atlantica* wood essential oil constituents.

the key odorants of natural raw materials, the main analytical issue is due to the fact that the key contributors are often strongly potent odorant constituents contained in low (or even trace) amounts. Consequently, their identification requires an extensive analysis of the whole mixture, involving several fractionation steps of a significant amount of material. Moreover, the use of authentic standard compounds is often necessary to ensure the validity of the identification of the odor active constituents. For instance, in this study, the third most potent cedarwood-like odorant displayed a LRI_{GC-O} of 1500, identical to the LRI_{GC-O} of α-himachalene **1**, but the GC-O analysis of several fractions demonstrated that **1** was odorless, hence the olfactory perception was actually due to a coeluting potent odorant. Finally, the large differences between all the panelists illustrate how strongly can vary the olfactory representations of complex mixtures from an individual to another. This last observation justifies the use of large panels and the report of the individual responses of each panelist, in order to give the best possible representation of the actual identity of the key odorants of a given raw material. In conclusion, this study is another demonstration of the difficulty to characterize the main odorant contributors of a mixture, because of the selectivity, the sensitivity and the interindividual variability of the human olfactory system.

4. Experimental

4.1. General experimental procedures

All reagents and solvents were from Sigma Aldrich (L'Isle d'Abeau, France). THF was dried by distillation over sodium/benzophenone before use. Reference compounds **7**, **9**–**12** were synthesized according to the procedures described hereafter. NMR Analyses were performed on a Bruker NanoBay Avance III HD (400 MHz) spectrometer (Bruker, FR-Wissembourg) at 25 °C in $CDCl_3$. All reference compounds, reagents and solvents were from Sigma Aldrich (L'Isle d'Abeau, France) except sodium perborate (Prolabo, France), undecan-2-one (Verley, France), and borneol (Janssen, Belgium). THF and Diethylether were dried by distillation over sodium/benzophenone before use.

4.2. Essential oil sample

The essential oil of *Cedrus atlantica* (Endlicher) Carrière (Pinaceae) was purchased from Aromaris Company (Taounate, Morocco). It was obtained by distillation of Atlas Cedarwood sawdust generated by the sawing of the wood in an industrial sawmill processing exclusively this species. The wood was collected in the Ain Leuh forest (area of Ifrane, Morocco, GPS coordinates: 33.209974, −5.424671). The distillation unit was a 1800 L inox apparatus, in which a 500 kg charge of sawdust was hydrodistilled during 7 h, to furnish the essential oil with a 8% yield. The chemical composition and olfactory characteristics of the sample were evaluated by trained experts who validated its conformity with conventional Atlas cedarwood essential oil samples.

4.3. Essential oil fractionation

A sample of *Cedrus atlantica* essential oil (659 g) was dissolved in diethylether (1L) and extracted 3 times with 250 ml of a 1M aqueous sodium hydroxide solution. The basic aqueous phases were then gathered, washed 3 times with 250 ml of diethyl ether, and poured in an Erlenmeyer flask with 250 ml of dichloromethane. The content of the flask was cooled in an ice bath, and slowly acidified by the dropwise addition of 37% hydrochloric acid (65 ml) under vigorous stirring. The mixture was then decanted, and the aqueous phase reextracted twice with dichloromethane (250 ml). The organic phases were then gathered, washed twice with brine, dried with magnesium sulfate and evaporated to furnish 350 mg of a dark brown thick oil. The ethereal phases containing the neutral compounds were evaporated, and the resulting oil was distilled under vacuum using a vacuum jacketed 50 cm column filled with metallic packing. A total of eleven fractions (F1-F11) were collected, and 13.5 g of residue (F12) was recovered in the boiler. On the basis of the results of the GC-MS and GC-O experiments (see Tables 1 and 2), the characterization of several non-identified odorant constituents required continuing the fractionations, and F1, F2, F9 and F12 were further fractionated by successive column chromatography on silica gel using increasing amounts of diethyl ether in petroleum ether for elution.

4.4. GC-MS analyses

Gas chromatography - Mass spectrometry (GC-MS) analyses were carried out using an Agilent 6890N gas chromatograph coupled to an Agilent 5973N mass selective detector working in electron impact (EI) mode at 70 eV (scanning over 35–350 amu range in SCAN mode). The gas chromatograph was equipped a fused silica capillary column HP-5MS (60 m × 0.2 mm i.d., film thickness: 0.3 μm). The analytical parameters were the following: The carrier gas was helium at a flow rate of 0.8 ml/min. The oven temperature was programmed from 50 to 210 °C at 2 °C/min and held isothermal for 10 min. The injector (split mode, ratio 1/100) temperature was 240 °C. The temperatures of the ion source and transfer line were 230 and 250 °C, respectively. LRI were determined from the retention times of a series of *n*-alkanes with linear interpolation. The constituents were identified by comparison of their mass spectra and LRI with those of pure compounds registered in commercial libraries and literature data, and with a laboratory-made database built from authentic compounds.

4.5. GC-FID analyses

Gas chromatography – Flame Ionization Detector (FID) analyses were performed in the same chromatographic conditions than those described for the GC-MS, with a FID temperature set at 250 °C. The quantification of the constituents was performed using predicted response factors as described by Tissot et al. (2012) using methyl octanoate as an internal standard.

4.6. GC-O analyses

GC-O analyses were performed on a Shimadzu GC-2010 Gas Chromatograph equipped with a HP-5 capillary column (50 m × 0.32 mm i.d.; 0.53 μm film thickness), a FID detector and an ATAS olfactory port OP275 mounted with a glass nasal cone. Samples were analyzed under the following conditions: injection volume: 1.0 μL, in splitless mode. Injector temperature: 250 °C. Oven temperature program: 100 °C–250 °C at 5 °C/min, then isothermal for 5 min. Carrier gas (nitrogen) flow: 1.50 mL/min 60% of the flow was directed to the FID while 40% was directed into the heated sniffing port. FID temperature: 250 °C.

The AEDA methodology (Ullrich and Grosch, 1987) was applied to characterize the main odor active components: at first, a 2% solution of the essential oil was analyzed (3–5 times) by each panelist who was asked to describe freely her/his olfactory perceptions, and to specify if the odor was reminiscent of the typical smell of Atlas cedarwood. These experiments were then followed by the injection of serial successive (1/4) dilutions until no more olfactory perception was detected. Twelve evaluators participated in this study. All of the panelists not familiar with the typical smell of Atlas Cedarwood essential oil were asked to memorize the odor

of the sample studied in this work prior GC-O experiments.

4.7. Identification of the odorant constituents

To identify the odorant constituents corresponding to the odor zones detected in the GC-O experiments, the linear correlation $LRI_{GC\text{-}MS} = f(LRI_{GC\text{-}O})$ was determined by the measurement of the retention indices of a series of typical EO constituents in GC-MS and GC-O. This correlation helped to predict the $LRI_{GC\text{-}MS}$ of the presumed odorant corresponding to a given odor zone. GC-O experiments on the various fractions and subfractions helped the search for candidate odorants, by investigating chromatograms of lower complexity. The identification of all odorants was confirmed by coinjection of samples of reference compounds, either commercially purchased or synthesized. In these coinjection experiments, a panelist performed a GC-O experiment of the essential oil spiked with the compound, and checked that a single odor perception was detectable at the expected retention time, with a qualitative description similar to those perceived in the non-spiked essential oil analysis.

4.8. Synthesis

4.8.1. β,4-Dimethyl-3-cyclohexene-1-ethanol **10**

A 0.5 M THF solution of 9-BBN (162 ml, 81 mmol, 1.1 eq) was added dropwise to (±)-limonene (10.0 g, 73 mmol, 1 eq) at 0 °C under inert atmosphere. The cooling bath was removed 10 min after the end of the addition, and the solution was stirred for 3 h at room temperature. The reaction mixture was then cooled to 0 °C and a suspension of sodium perborate (56 g, 364 mmol, 5 eq) in 150 ml water was slowly added under stirring. After 2 h stirring at 0 °C, 200 ml of saturated NH_4Cl solution and 200 ml of diethyl ether were added and the mixture was decanted. The aqueous layer was extracted twice with 200 ml diethyl ether, the organic layers were combined, washed with brine, dried over $MgSO_4$ and concentrated under vacuum to afford a yellow oil which was purified by column chromatography on silica gel with petroleum ether/diethylether (8/2) to furnish 9.06 g (80%) of *β,4-dimethyl-3-cyclohexene-1-ethanol* **10** (1/1 mixture of diastereomers) as a slightly yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.38 (s, 1H), 3.66–3.64 (m, 1H), 3.51–3.47 (m, 1H), 1.95 (s, 3H), 1.65 (s, 3H), 1.88–1.50 (m, 5H), 0.95–0.91 (dd, J = 3.6, 17.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 134.01, 133.95, 120.71, 120.64, 66.35, 66.23, 40.13, 39.95, 35.27, 35.12, 30.73, 30.59, 29.80, 27.65, 27.22, 25.44, 23.44, 13.65, 13.23. MS (EI, 70 eV, m/z %): Diastereomer 1: 40 (56), 67 (28), 77 (39), 79 (85), 91 (61), 92 (29), 93 (100), 94 (73), 107 (41), 121 (56), 136 (53), 154 (M^+, 6). Diastereomer 2: 40 (48), 67 (30), 77 (50), 79 (91), 91 (49), 93 (100), 94 (89), 95 (32), 107 (35), 121 (38), 136 (50), 154 (M^+, 7).

4.8.2. α,4-Dimethyl-3-cyclohexene-1-acetaldehyde **11**

To a suspension of PCC (22.5 g, 104.6 mmol, 2 eq) and celite (22.8 g) in 345 mL dichloromethane at 0 °C, a solution of **10** (7.87 g, 51 mmol, 1 eq) in 106 ml dichloromethane was added dropwise, and the reaction mixture was stirred at room temperature during 2 h after the addition. The crude mixture was then filtered through a short pad of celite and silica gel, rinsed with dichloromethane and distilled on a Kugelrohr apparatus to give 5.70 g (74%) of **9** (1/1 mixture of diastereomers) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.61–9.57 (m, 1H), 5.29 (s, 1H), 2.27–2.18 (m, 1H), 2.03–1.77 (m, 6H), 1.71–1.59 (m, 1H), 1.57 (s, 3H), 1.00 (t, J = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.48, 205.40, 134.04, 134.01, 119.95, 119.88, 50.96, 50.65, 34.32, 34.25, 30.13, 29.97, 29.65, 28.04, 27.29, 25.43, 23.39, 10.31, 10.22. MS (EI, 70 eV, m/z %): Diastereomer 1: 67 (16), 68 (10), 77 (12), 79 (59), 91 (16), 93 (12), 94 (100), 95 (17), 119 (7), 134 (5), 152 (M^+, 5). Diastereomer 2: 67 (13), 77 (11), 79 (53), 91 (20), 93 (13), 94 (100), 95 (14), 105 (5), 119 (7), 134 (6), 152 (M^+, 5).

4.8.3. α,4-Dimethyl-3-cyclohexene-1-methanol **12**

To a solution of **11** (1.98 g, 13.0 mmol, 1 eq) diluted in a mixture of 7.6 ml dimethylsulfoxide and 18 ml isopropanol, a 10.2 M aqueous solution of hydrogen peroxide (13 ml, 148 mmol, 11 eq) was added at room temperature and stirred for 5 min. A 10.6 M aqueous solution of potassium hydroxide (8.32 ml, 88 mmol, 6.8 eq) was then added dropwise for 90 min, and an exothermic reaction occurred before the end of the addition. The mixture was then heated at 80 °C and stirred for 2 h. After cooling, 100 ml of water and 50 ml diethyl ether were added, and the mixture was decanted. The aqueous layer was extracted 3 times with 100 ml diethyl ether, the organic phases were combined, washed with brine, dried over $MgSO_4$, and evaporated to give 1.67 g of a slightly yellow oil which was purified by column chromatography on silica gel with petroleum ether/diethyl ether (8/2) to furnish 840 mg (46%) of **12** as a c.a. 6/4 mixture of diastereomers, together with 209 mg (12%) of 4-acetyl-1-methylcyclohexene **9**. **12**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.31 (s, 1H), 3.53 (dq, J = 38.0, 6.1 Hz, 1H), 2.10–1.60 (m, 7H), 1.57 (s, 3H), 1.42 (s, large, 1H), 1.13–1.09 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.16, 132.87, 119.25, 119.09, 70.64, 70.50, 40.11, 39.93, 29.09, 26.87, 26.10, 24.32, 23.95, 22.43, 19.75, 19.56. MS (EI, 70 eV, m/z %): Diastereomer 1: 40 (17), 67 (23), 77 (22), 79 (36), 91 (38), 93 (100), 94 (19), 105 (17), 107 (39), 122 (49), 140 (M^+, 8). Diastereomer 2: 67 (20), 77 (19), 79 (35), 81 (13), 91 (34), 93 (100), 94 (21), 105 (14), 107 (40), 122 (48), 140 (M^+, 8).

4.8.4. 4-Acetyl-1-methylcyclohexene **9**

A solution of the crude mixture of **12** and **9** (1.48 g) in dichloromethane (8 ml) was added dropwise to a suspension of PCC (2.69 g, 7.77 mmol) and celite (2.67 g) in dichloromethane (40 mL) at 0 °C. The mixture was stirred at room temperature during 2 h after the addition, and then filtered through a short pad of celite and silica gel, using dichloromethane for rinse. After evaporation of the solvent, the crude mixture was purified by filtration on silica gel using petroleum ether/diethyl ether (8/2) for elution, to give eventually 410 mg of **9** as a slightly yellow oil. When purified **12** (543 mg, 3.9 mmol) was used as a starting material, the same protocol gave 500 mg (93%) of **9**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.31 (s, 1H), 2.50–2.39 (m, 1H), 2.16–1.84 (m, 6H), 2.10 (s, 3H), 1.58 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 211.99, 133.77, 119.23, 47.20, 29.46, 27.92, 27.02, 24.87, 23.36. MS (EI, 70 eV, m/z %): 43 (32), 67 (36), 77 (17), 79 (26), 93 (18), 95 (78), 105 (17), 123 (50), 138 (M^+, 100).

4.8.5. 3-(4-Methylcyclohex-3-en-1-yl)-2-butenoic acid **14**

A 60% dispersion of sodium hydride in mineral oil (167 mg, 4.2 mmol) was rinsed 3 times with dry petroleum ether under inert atmosphere. Dry THF (8 ml) was then added, and the suspension was cooled to 0 °C. Ethyl 2-(diethylphosphoryl)acetate (988 mg, 4.4 mmol) was then added dropwise and stirred until the end of the emission of hydrogen. 403 mg of crude **9** were then added dropwise, and the mixture thus obtained was then stirred for 30 min at 0 °C, and then for 6 h at room temperature. 40 ml of saturated aqueous ammonium chloride solution was then added, and after decantation the aqueous layer was extracted with 3 × 40 ml of diethyl ether. The combined organic layers were dried on $MgSO_4$ and evaporated to give 400 mg of a colorless oil which was purified by column chromatography on silica gel with petroleum ether/diethyl ether (95/5) for elution to furnish 177 mg of (*E*)-ethyl-3-(4-methylcyclohex-3-en-1-yl)-2-butenoate **13** as a colorless oil: ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.65–5.58 (m, 1H), 5.39–5.27 (m, 1H), 4.08 (q, J = 7.14 Hz, 2H), 2.23–2.06 (m, 1H), 2.09 (d, J = 1.28 Hz, 3H),

2.04–1.34 (m, 5H), 1.58 (s, 3H), 1.26–1.15 (m, 1H), 1.21 (t, $J = 7.13$ Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.18, 163.98, 133.86, 120.01, 114.36, 59.49, 44.25, 30.29, 30.27, 27.32, 23.40, 17.14, 14.33. MS (EI, 70 eV, m/z %): 39 (17), 41 (66), 43 (13), 53 (12), 55 (47), 56 (25), 57 (10), 67 (65), 68 (28), 69 (100), 70 (17), 71 (28), 81 (70), 82 (64), 83 (15), 95 (66), 96 (13), 109 (28), 123 (44), 138 (22), 156 (10), 208 (M^+, 0.1). To a solution of crude **13** (362 mg) in ethanol (2 ml), a solution of sodium hydroxide (1.2 g, 30 mmol) in 9 ml water was slowly added. The reaction mixture was heated at reflux during 6 h, and eventually acidified by the addition of 6M hydrochloric acid (5 ml). 5 ml of ethyl acetate were then added, and after decantation, the aqueous layer was extracted with 3 × 20 ml of ethyl acetate, and the combined organic phases were washed with brine, dried over $MgSO_4$, and evaporated to give a light orange solid which was purified by column chromatography on silica gel using dichloromethane/methanol (95/5) to give 144 mg of **14**. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.65 (s, 1H), 5.33 (s, 1H), 2.28–2.04 (m, 1H), 2.10 (d, $J = 1.14$ Hz, 3H), 2.04–1.37 (m, 5H), 1.59 (s, 3H), 1.32–1.11 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.35, 167.31, 133.91, 119.87, 113.82, 44.55, 30.23, 30.19, 27.25, 23.40, 17.50. EI–MS m/z (%): 39 (14), 41 (15), 53 (14), 67 (52), 68 (87), 69 (12), 77 (18), 79 (29), 81 (10), 87 (14), 91 (24), 93 (31), 94 (100), 95 (18), 97 (10), 105 (15), 107 (14), 111 (25), 119 (12), 120 (10), 121 (12), 135 (15), 147 (11), 180 (M^+, 8).

4.8.6. *Vestitenone* **7**

To a stirred solution of **14** (102 mg, 0.56 mmol, 1 eq) in 3 ml dry diethylether, a 1.6 M solution of methyllithium in diethylether (0.72 ml, 1.2 mmol, 2 eq) was added dropwise at 0 °C and stirred for 90 min at this temperature, and then 3 h at room temperature. The reaction mixture was then treated by the addition of 4 ml of a 0.5 M solution of hydrochloric acid, followed by the addition of 4 ml diethylether. After decantation, the aqueous layer was extracted with 3 × 10 ml of diethylether. The combined organic phases were washed with brine, dried over $MgSO_4$, and evaporated to give a colorless oil which was purified by column chromatography on silica gel with petroleum ether/diethylether to give 55 mg (55%) of **7** as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.01 (s, 1H), 5.32 (s, 1H), 2.16–1.82 (m, 6H), 2.10 (s, 3H), 2.04 (s, 3H), 1.75–1.66 (m, 1H), 1.58 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.03, 162.42, 133.74, 122.13, 119.93, 44.37, 31.80, 30.25, 30.20, 27.30, 23.34, 17.48. EI-MS m/z (%): 43 (36), 67 (43), 68 (30), 77 (21), 79 (30), 91 (28), 93 (35), 94 (30), 95 (100), 105 (38), 107 (37), 109 (61), 120 (41), 121 (22), 135 (65), 145 (39), 163 (22), 178 (M^+, 35).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2017.09.017.

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