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1 **Chemical and Biomolecular Characterization of *Artemisia umbelliformis* Lam., an Important**
2 **Ingredient of the Alpine Liqueur “Genepi”**

3

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13 **Running head: Chemical and Biomolecular Characterization of *Artemisia umbelliformis* Lam.**

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22

23 **ABSTRACT**

24 *A. umbelliformis* Lam., an important Alpine plant used for preparation of flavoured beverages
25 showed a remarkable intraspecific variability, both at genomic and gene products (secondary
26 metabolites) level. The variability of *A. umbelliformis* Lam. currently cultivated in Piedmont (Italy,
27 Au1) and in Switzerland (Au2) was investigated by combining the chemical analysis of essential oil
28 and sesquiterpene lactones and the molecular characterization of the 5S-rRNA-NTS gene by PCR
29 and PCR-RFLP. Marked differences were observed between the two plants. Au1 essential oil
30 contained α - and β -thujone as main components while Au2 1,8-cineole, borneol and β -pinene. Au1
31 sesquiterpene lactone fractions contained *cis*-8-eudesmanolide derivatives and Au2 the *trans*-6-
32 germacranolide costunolide. Specific *A. umbelliformis* Au1 and Au2 primers were designed on the
33 sequence of the 5S-rRNA gene spacer region. Furthermore, a PCR–restriction fragment length
34 polymorphism (PCR–RFLP) method was applied using *RsaI* and *TaqI* restriction enzymes.
35 Chemical and biomolecular data contributed to characterize *A. umbelliformis* chemotypes.

36

37

38 **Keywords:** *Artemisia umbelliformis* Lam.; alpine liqueurs; chemotype discrimination; chemical
39 analysis; biomolecular analysis.

40

41 INTRODUCTION

42 *Artemisia umbelliformis* Lam. is an Alpine species used to prepare “genepi”, a highly praised
43 liqueur characterized by a bitter taste and a peculiar flavour (1). These properties have been traced
44 to the volatile constituents and to the sesquiterpene lactone fraction of the plant, that are
45 characterized by a high contents of α - and β -thujone (2, 3) and by the presence of the *cis*-
46 eudesmanolide sesquiterpene lactones **1-3**, respectively (4-6).

47 Thujone is a natural terpenoid also associated with common wormwood (*Artemisia absinthium* L.)
48 and Roman wormwood (*Artemisia pontica* L.), absinthe’s most widely used ingredients (7 and
49 reference cited there in). There is currently a heated debate on the toxicity of absinthe and thujones
50 (7 and reference cited there in), but the EU legislation have imposed a limit of 35 ppm to the total
51 amount of these compounds in alcoholic beverages (8). To overcome this issue, thujones-free
52 chemotypes of *A. umbelliformis* have been selected by horticultural techniques (9). Remarkably, an
53 investigation on the sesquiterpene lactone fraction of one of these thujones-free chemotypes showed
54 dramatic differences from the wild plant. Thus, the C-8 *cis* sesquiterpene lactones typical of *A.*
55 *umbelliformis* from Western Alps (**1-3**) were replaced by the C-6 *trans* lactones **4-6a,b**, while a
56 structurally unique sesterpene lactone (**7**) was also detected (10) (**Figure 1**). Chemotypes (or
57 chemical phenotypes) are generally considered the phenotypical expression of a genotype, although
58 different chemotypes may derive from the same genotype. This means that, according to
59 environmental conditions, the same genotype may express different chemical patterns, or, on the
60 other side, that different genotype may respond to the same environmental pressure with the same
61 phenotypic expression. In this context, molecular genetic methods have recently been shown to be
62 very effective in genotypic discrimination. Genetic methods focus on genotype rather than
63 phenotype, and DNA based experiments are now widely used for a rapid identification (and
64 therefore authentication) of medicinal plants. Berteau et al. (11) recently showed that molecular
65 approaches are a powerful tool to distinguish the *Acorus calamus* diploid β -asarone-free cytotype
66 from the other cytotypes containing it. The same group also used specific *Salvia divinorum* primers

67 designed on the sequence of the 5S-rRNA gene spacer region (12) to develop a Real-Time PCR-
68 based mathematical model to quantify *S. divinorum* in commercial plant samples or hallucinogenic
69 preparation (13). Given the potential of this approach, it seemed interesting to apply a combination
70 of biomolecular and chemical techniques to characterise the chemotypes of *A. umbelliformis*
71 currently cultivated in Piedmont (Italy, Au1) and in Switzerland (Au2), complementing the analysis
72 of their essential oil and sesquiterpene lactones with a molecular characterization by PCR and PCR-
73 RFLP of the 5S-rRNA-NTS region of their genome.

74

75 MATERIALS AND METHODS

76 **Chemicals** - Thujones standard mixture (mixture of α -thujone and β -thujone purity 99.9%) and all
77 other pure reference compounds were from Sigma-Aldrich (St. Louis MO, USA). Sabinene was
78 from Chromadex TM (Irvine CA, USA); sabinol was kindly supplied by Robertet SA (Grasse,
79 France); sabinyl ester homologous series were synthesized in the authors' laboratory (2). HPLC and
80 analytical grade solvents were from Carlo Erba Reagenti, Rodano, Italy. The sesquiterpene lactones
81 **1-7** were available from previous studies (4-6, 10)

82 **Plant Material** - Forty three samples of *Artemisia umbelliformis* Lam aerial parts from
83 experimental cultivations run in different Alpine valleys [Val Grana (latitude 44°25'N, longitude
84 7°20'E), Valle Stura (44°21'N, 7°26'E), Valle Maira (44°28'N, 7°22'E), Val Chisone (44°57'N,
85 6°52'E)] at an height of least 1300 m a.s.l. Fresh plant material was directly indoor-dried by the
86 farmers under controlled temperature and humidity up to a constant weight, in agreement with the
87 WHO's guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants.
88 Voucher specimen representative of the two chemotypes (native, defined Au1 and selected in
89 Switzerland, defined Au2) are deposited at the Dipartimento di Scienza e Tecnologia del Farmaco
90 (n°231 for Au1 and n°232 for Au2). For each chemotype, batches of 1 kg of aerial dried parts (see
91 above) were supplied by the "Associazione Genepi Occitan" (Cuneo - Italy)

92 **Essential oils and Headspace solid phase-microextraction (HS-SPME) sample preparation -**

93 Essential oils (EOs) were prepared according to the method of the European Pharmacopoeia (14).
94 Ten grams of dried aerial parts was suspended in 250 mL of water in a 500 mL flask for 1 h, and
95 then submitted to hydrodistillation in a Clevenger micro-apparatus for 2 h (2). The resulting EO was
96 left to stabilize for 1 h, then recovered with hexane and then analyzed by GC-MS.

97 The SPME device and the three component CAR/PDMS/DVB fused silica fiber (2 cm long, coating
98 volume: 1.000 μm^3) were purchased from Supelco (Bellafonte, PA, USA), (15). Before use, the
99 fiber was conditioned as recommended by the manufacturer.

100 Each sample (200 mg of *A. umbelliformis* dried aerial parts) hermetically sealed in a 2.0 mL vial
101 was introduced in a thermostatic bath at 80 °C for 15 min; the SPME device was inserted in the
102 sealed vial containing the sample, and the CAR/PDMS/DVB fiber exposed to the matrix headspace
103 (30 min). The vial was vibrated for 10 s every 5 min with an electric engraver (Vibro-Graver V74),
104 (Burgess Vibrocrafter Inc., Brayslake, IL). After sampling, the SPME device was immediately
105 inserted into the GC injector and the fiber thermally desorbed. A desorption time of 5 min at 230 °C
106 was used. Before sampling, each fiber was reconditioned for 20 min in the GC injection port at 230
107 °C.

108 **Sesquiterpene lactone extraction** - One gram of dried aerial parts of both chemotypes were
109 sonicated three times with ethanol 96% (50 mL) for 10 min. The resulting total extract (150 mL)
110 was filtered, and evaporated to dryness under vacuum; the weighed solid residue was dissolved in
111 acetonitrile/water 20/80 at a concentration of 0.1 mg/mL and analyzed by high-performance liquid
112 chromatography-diode array-ultraviolet detection-mass spectrometry (HPLC-DAD-UV-MS).

113 **GC and GC-MS Analyses** - GC analyses were carried out on a Shimadzu QP2010 system provided
114 with a FID and a MS detector, and the results processed by GC Solution software and GC-MS
115 solution software (2.51version) (Shimadzu Italia, Milano Italy). Capillary GC-FID-MS analyses
116 were carried out on two 25 m, 0.25 mm i.d., 0.25 μm columns from MEGA (Milano – Italy), i.e.
117 Mega5 (95% polydimethyl-siloxane, 5% phenyl) and MegaWax (polyethyleneglycol, PEG20M).

118 GC and GC-MS conditions: injection mode: split; split ratio: 1: 20 Temperatures: injector: 230 °C,
119 transfer line: 230 °C; ion source: 200 °C; carrier gas: He flow-rate: 1.0 mL /min in constant flow-
120 mode. MS detector was in electron impact ionization mode (EI) at 70 eV, scan rate was 1111 amu/s
121 and mass range of 35–350 *m/z*. Temperature program: from 50 °C (1 min) to 220 °C (5 min) at 3 °C
122 min⁻¹.

123 EOs and headspace components were identified by comparison of both their linear retention indices,
124 calculated versus a C₈-C₂₅ hydrocarbon mixture, and their mass spectra with those of authentic
125 samples or with data in the literature.

126 Quantitative analysis. Suitable amounts of α + β -thujone commercial standard were diluted with
127 cyclohexane to obtain six concentration levels ranging from 0.5 ng / μ L to 6 ng/ μ L for α -thujone
128 and from 0.04 ng/ μ L to 0.5 ng/ μ L for β -thujone. Calibration curves were obtained by analyzing the
129 resulting standard solutions three times by GC-FID using *n*-nonane as internal standard.

130 **HPLC-DAD-UV analysis** - HPLC-DAD-UV analyses were carried out on a Shimadzu 2010EV
131 system provided with a PDA detector (Shimadzu, Dusseldorf Germany). A 150 x 4.6 mm i.d., 5
132 μ m, Zorbax Stable Bond column (Agilent, Waldbronn Germany) was used. Analysis conditions
133 were: mobile phase: eluent A: 20% acetonitrile/water; eluent B: 100% acetonitrile; mobile phase
134 gradient: from 100% A to 100% B in 25 min. Injection volume: 10 μ L, flow rate: 1 mL/min. UV
135 detection wavelengths: 210nm.

136 Quantitative analysis – Suitable amounts of cusionolide and umbellifolide were diluted with
137 methanol to obtain concentrations ranging from 0.5, 5, 10, 25, 50 and 100 ng/ μ L of each marker,
138 respectively. Umbellifolide (**3**) was adopted as standard for quantitation also for the
139 hydroperoxytelekins **1** and **2**, because of the similarity of structures, their trace abundance and
140 chemical instability; the results are expressed as the sum umbellifolide + hydroperoxytelekins. A
141 calibration curve was made by analyzing the resulting standard solutions three times by HPLC-
142 DAD-UV at 210 nm.

143 **HPLC-MSD analysis** - HPLC-MSD analyses were carried out with a single quadrupole Shimadzu
144 2010EV system (Shimadzu, Dusseldorf, Germany) equipped with an orthogonal atmospheric
145 pressure chemical ionization (APCI) and electrospray ionization (ESI) sources. The same column
146 and mobile phase as for HPLC-DAD-UV analysis was used. Flow rate: 0.8 mL/min. MSD
147 conditions: MS-APCI+; temperature: 400 °C; nebulizer's flow: 2.5mL/min; CDL voltage: 250 °C;
148 Q-Array: MS-ESI+; temp:250 °C; nebulizer's flow: 1,5mL/min; CDL voltage: 250 °C.

149 MSD analysis conditions were optimized by direct flow injection of pure standards of costunolide
150 (4) and umbellifolide (3) and of a fraction containing a mixture of hydroperoxytelekines

151 **Genomic DNA extraction** - Plant material employed for the chemical analyses was also used for
152 genomic DNA extraction. . Fifty mg of dried material was frozen in liquid nitrogen and ground to a
153 fine powder with Tissue Lyser (Qiagen, Hilden, Germany). Genomic DNA was extracted from the
154 ground powder by using the Nucleospin Plant Kit (Macherey Nagel, Düren, Germany) following
155 manufacturer's instruction. The quantity and quality of the DNA were assessed by
156 spectrophotometric analysis by using the Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE,
157 USA) from several samples of the two chemotypes.

158 **PCR amplification, subcloning and sequencing** - Approximately 20 ng of genomic DNA isolated
159 from powdered leaf material of Au1 and Au2 were used as a template for PCR amplification with
160 forward primer 5S-P1 (5' - GTGCTTGGGCGAGAGTAGTA-3') and reverse primer 5S-P2 (5' -
161 TTAGTGCTGGTATGATCGCA-3') flanking the NTS of 5S-rRNA gene (11-13, 16). The
162 amplification was carried out in a 50 µL reaction mixture containing 5 µL 10X PCR reaction buffer
163 (Fermentas), 0.2 mM dNTPs, 20 pmol forward and reverse primers and 0.5 U of *Taq* DNA
164 polymerase (Fermentas, Glen Burnie, MA, USA). The PCR reactions were carried out in a
165 Whatman Biometra T-Gradient Thermalcycler (Whatman Biometra, Goettingen, Germany).
166 Cycling conditions consisted of an initial 4 min at 94 °C, followed by 30 sec denaturing at 94 °C, 1
167 min annealing at 52 °C, and 1 min elongation at 72 °C repeated for 30 cycles and with 5 min final
168 extension at 72 °C.

169 One microliter of the amplification reaction was analyzed by capillary gel electrophoresis (CGE)
170 using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the DNA 1000 LabChip Kit
171 (Agilent Technologies) following manufacturer's instructions. The DNA 1000 LabChip Kit
172 provides sizing and quantitation of dsDNA fragments ranging from 25 to 1000 bp. PCR products
173 were also analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide
174 staining under UV. From this gel a band of about 220 bp for Au1 and about 320 bp for Au2 was
175 purified by using the Nucleospin Extract II Kit (Macherey Nagel) and then subcloned into pGEM-T
176 Easy vector (Promega). The ligated products were transformed into the *Escherichia coli* Subcloning
177 DH5 α Efficiency Competent Cells (Invitrogen). Colonies containing DNA inserts of the correct size
178 were picked and grown overnight in 3 mL of Luria-Bertani (LB) liquid medium. The mini-
179 preparation of plasmid DNAs were performed using QIAprep Spin Miniprep Kit (Qiagen),
180 following manufacturer's instructions. The plasmid DNAs were employed as a template for
181 sequencing. Both strands of DNA were sequenced at least twice and the sequences were aligned by
182 using ClustalX software.

183 **PCR amplification using specific primers for Au1 and Au2** - Sequences derived from powdered
184 leaf material of Au1 and Au2 were aligned in a unique sequence that allowed the design of two
185 forward primers AuF 5' -CTAGGATGGGTGACCTCCTG -3'(which is common to both
186 chemotypes) and Au2F 5'-GCGGTGACAGAGTCGGTAAA-3' and two reverse specific primers:
187 Au2R1 5' -CGTAAAATTCACCGCCTACG -3' and Au2R2 5'-
188 TCCTTTCTCATTGCCTATTTTTC -3', which corresponded respectively to nucleotides 21-40,
189 168-187, 212-231, 253-275 of Au2 non-transcribed spacer (NTS) sequence. The internal primers
190 were used for amplification also in combination with primer 5S-P1 and 5S-P2.

191 The conditions of the PCR reactions were the same as mentioned above. One microliter of the
192 amplification products were separated by CGE with the Agilent 2100 Bioanalyzer (Agilent
193 Technologies) and DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer's
194 instructions.

195 **PCR–RFLP** - The purified PCR products of the 5S-rRNA gene spacer region of both Au1 and Au2
196 chemotypes were either digested with 10 U of RsaI (Amersham Biosciences) at 37 °C for 1h or, in a
197 separate reaction, with 10 U of TaqI (Sigma) at 65 °C for 1 h. One microliter of both digestion
198 reactions was fractionated by CGE using the Agilent 2100 Bioanalyzer (Agilent Technologies) and
199 DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer’s instructions.

200

201 **RESULTS AND DISCUSSION**

202 This study aims to characterize the two chemotypes of *A. umbelliformis* Lam. under investigation
203 by combining results from chemical and genomic analyses of 43 samples from experimental
204 cultivation (Au1, Italian native and Au2, selected in Switzerland).

205 **Chemical analyses** - Chemical analyses investigated the fractions responsible for plant odor and
206 taste i.e. the composition of the volatile fraction including quantitation of α - and β -thujone, and that
207 of the sesquiterpene lactone fraction (i.e. the components responsible for the liqueur bitter taste).
208 The volatile fraction was studied by analyzing both the essential oil (EO) obtained by
209 hydrodistillation and the headspace (HS) sampled by solid phase microextraction (HS-SPME)
210 combined with GC and GC-MS. HS-SPME sampling was applied in view of developing a fully
211 automatic control method, to be run in combination with GC and GC-profile multivariate analysis
212 (Principal Component Analysis (PCA). **Table 1** reports the average percent areas normalized vs. *n*-
213 nonane as internal standard and percent range of the characteristic components of the EOs of the
214 samples investigated together with their Linear Retention Indices (LRI) on both GC columns. **Table**
215 **2** reports calibration curves, mean and range amounts of α - and β -thujone in the samples
216 investigated. This Table considers only ten samples of chemotype Au2 on seventeen because the
217 alpha and beta-thujone peak areas of the remaining seven samples were too low to be correctly used
218 for quantitative determination. From these results it is clear that the two chemotypes are
219 characterized by a different composition: Au1 was found to contain α - and β -thujone and an
220 homologous series of sabinyl esters as main components, whereas in the chemotype Au2, 1,8-

221 cineole, borneol and β -pinene were the major compounds. Moreover, thujones were almost absent
222 from the Au2 chemotype, their total amount accounting from 0.2 to 0.4g/100g of EO, while in the
223 Au1 chemotype thujones ranged from 18 to about 58 g/100g of EO. The results obtained by HS-
224 SPME-GC analysis, although not directly comparable, were in full agreement with those of the
225 EOs, as shown by the PCA scatterplot of **Figure 2**. Each chemotype is clearly discriminated and the
226 samples belonging to the same chemotype analysed by HS-SPME-GC and through their EO are
227 coherently positioned in the PCA scatterplot (**Figure 2**).

228 Significant differences can also be found in the composition of the non-volatile bitter fraction.
229 **Figure 3** reports the HPLC-DAD-UV profiles of two samples belonging to Au1 and Au2
230 chemotypes, respectively. The bitter taste of the native Au1 chemotype is mainly due to
231 sesquiterpene lactones of the *cis*-8-eudesmanolide type [5-deoxy-5-hydroperoxy-5-epitelekin (**1**), 5-
232 deoxy-5-hydroperoxytelekin (**2**), umbellifolide (**3**) (4-6)]. On the other hand, the Au2 chemotype is
233 characterized by high amounts of costunolide (**4**), a germacranolide typical of *A. genipi* Weber (4),
234 and by the presence of an unusual sesterpene lactone, named genepolide (**7**) (10). An in depth
235 investigation of the Au2 ethanolic extract composition after fractionation by column
236 chromatography in combination with NMR, and analysis by HPLC-UV and HPLC-single-
237 quadrupole-MS revealed that costunolide (**4**) was accompanied by a series of related oxygenated
238 sesquiterpene lactones (artemorine (**5b**), santamarine (**6a**), and reynosine(**6b**)) (4). On the other
239 hand, in the Au1 chemotype the presence of both telekine hydroperoxides and umbellifolide was
240 confirmed.

241 Costunolide and the sum of hydroperoxytelekins and umbellifolide were adopted as markers of the
242 two chemotypes to evaluate quantitatively the bitter fraction of the 43 samples under investigation.
243 These analyses showed an average amount of umbellifolide + hydroperoxytelekins expressed as
244 umbellifolide ($y = 29.9392x - 1.6795$; $R^2: 0.99999$) of 0.11 g/100g of dried plant material in a range
245 varying between 0.03 and 0.37 g/100g for the Au1 chemotype, and an average amount of
246 costunolide ($y = 101.1709x - 31.0592$; $R^2: 0.99975$) of 0.56 g/100g of dried plant material in a range

247 between 0.20 and 0.93 g/100g for the Au2 chemotype. Costunolide was also detected in very low
248 amounts in some samples of the Au1 chemotype, its percentage never exceeding 0.05 %

249 **Molecular characterization of the two *A. umbelliformis* chemotypes** - In higher eukaryotes, the
250 5S-rRNA gene occurs in tandemly repeated units consisting of an 120 bp coding region separated
251 by a non-transcribed spacer varies from species to species (16). Thus the diversity of the spacer
252 region can be used as an identification basis (17).

253 Here, two primers flanking the spacer region of 5S-rRNA, already successfully employed for
254 differing *Acorus calamus* chemotypes (16), *A. calamus* cytotypes (11) and *Salvia divinorum* both as
255 pure plants (12) and in plant mixtures (13) were used in PCR analysis of genomic DNA isolated
256 from the two chemotypes Au1 and Au2.

257 A single fragment of approximately 220 bp was produced by Au1 (**Figure 4**, lane 1) and a single
258 fragment of about 320 bp was produced by Au2 (**Figure 4**, lane 2). Fragments derived from both
259 chemotypes were ligated into pGEM-T Easy vector and the nucleotide sequence was determined.
260 The sequenced region spans 224 bp for Au1 (NCBI GenBank Accession No. EU816950) and 327
261 bp for Au2 (NCBI GenBank Accession No. EU816951).

262 Sequence alignment of the 5S-rRNA spacer region flanked by the 3'-and 5'-ends of the coding
263 region is shown in **Figure 5**. Surprisingly, Au1 presented a difference of 103 nucleotides with
264 respect to Au2. This difference is quite consistent but not uncommon between chemotypes or
265 cytotypes, as it has been previously demonstrated with other plant species (11, 16).

266 In order to characterize better the two chemotypes and to simplify the identification method,
267 nucleotide sequences of the 5S-rRNA gene spacer region were used to design four specific primers
268 (**Figure 6**).

269 PCR products derived from all possible combinations of Au1 and Au2 specific primers also used
270 with the primers designed on the coding regions of the plant 5S-rRNA gene, were analyzed. In the
271 chemotype Au1 a single fragment of 204 bp in length was amplified using the primer AuF in
272 combination with the primer 5S-P2 (**Figure 4**, lane 3). The same strategy used with the chemotype

273 Au2 produced a single fragment of 307 bp in length (**Figure 4**, lane 4). The three additional specific
274 primers designed for Au2 gave a combination of single fragments as follows: 231 bp with 5S-P1
275 and Au2R1 (**Figure 4**, lane 5), 275 bp with 5S-P1 and Au2R2 (**Figure 4**, lane 6), 64 bp with Au2F1
276 and Au2R1 (**Figure 4**, lane 7), 108 bp with Au2F1 and Au2R1 (**Figure 4**, lane 8), 213 bp with AuF
277 and Au2R1 (**Figure 4**, lane 9) and 255 bp with AuF and Au2R2 (**Figure 4**, lane 10). All
278 amplifications occurred only in Au2 when the Au2 specific primers were used and no PCR products
279 were detected when Au1 DNA was employed as a template.

280 In addition, a PCR–RFLP method was applied. From the identified sequences, two RsaI sites could
281 be found in both chemotype 5S-rRNA spacer regions at 18 bp and 141 bp position in Au1 and at 18
282 and 301 bp in Au2 (**Figure 5**). Purified PCR products obtained by using 5S-P1 and 5S-P2 primers
283 were digested with Rsa I. As expected, PCR products from the chemotype Au1 could be digested by
284 RsaI giving two major fragments of 123 bp and 82 bp respectively, and a minor fragment of 19 bp
285 not visible in the gel because out of the resolution capacity of the instrument (**Figure 4**, lane 11).
286 When purified PCR products from the chemotype Au2 were digested using RsaI, a completely
287 different RFLP profile was observed. RsaI cleaved Au2 5S-rRNA spacer region giving a major
288 fragment of 283 bp and two minor fragments of 25 bp (barely visible in the gel) and 19 bp (not
289 visible in the gel) (**Figure 4**, lane 12). A TaqI site was also identified in the sequence of the
290 chemotype Au2 (see **Figure 5**). As expected, PCR products from the chemotype Au1 could not be
291 digested by Taq I (**Figure 4**, lane 13), whereas purified PCR products from the chemotype Au2.
292 Thus, our biomolecular characterization provides a useful tool for the unequivocal characterization
293 of the two chemotypes. If some intermediate chemotypes due to meiotic rearrangements were
294 already present in the population, they would be detected by using universal primers (different
295 fragment size or different nucleotide composition), but this was not the case, since all samples gave
296 the same results. Besides, this marker has been successfully used for *Acorus calamus* chemotype
297 determination (16) and it represents a powerful tool to deduce genetic relationship of medicinal
298 plants, especially at the intra-specific level.

299 In conclusion, these results clearly support the view that *A. umbelliformis*, a valuable plant for
300 Alpine agriculture, shows a remarkable intraspecific variability, both at the genomic and gene
301 products (secondary metabolites) levels. This multidisciplinary study, by showing remarkable
302 chemical variation in the terpenoid profile, and consistent genomic difference in the 5S-rRNA
303 spacer regions, has identified two chemotypes of *A. umbelliformis*. A multidisciplinary approach
304 based on the combination of metabolome- and genome-derived product analysis enabled the
305 unequivocal chemical and biomolecular fingerprinting of these two chemotypes. Combined “omics”
306 approaches are becoming a useful tool not only for basic science but also for industrial plant
307 characterization. Owing to the commercial relevance of *A. umbelliformis* and to the regulatory
308 issues related to the presence of thujones, the identification of a *RsaI* and *Taq* sites can be used for a
309 rapid and precise chemotype identification of the plant chemotypes, complementing the chemical
310 analysis of the essential oil and the sesquiterpene lactones.

311

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365

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371

372 **FIGURES CAPTIONS**

373

374 **Figure 1** - Structures of the sesquiterpene lactones identified in both chemotypes of *A.umbelliformis*

375 Lam. **1:** 5-desoxy-5hydroperoxy-5-epitelekin; **2:** 5-desoxy-5-hydroperoxytelekin; **3:**
376 umbellifolide; **4:** costunolide; **5a:** verlоторin; **5b:** artemorine; **6a:** santamarine; **6b:** reynosine; **7:**
377 genepolide

378

379 **Figure 2** - PCA scatterplot of the cumulative elaboration of both EOs and headspaces sampled by
380 HS-SPME of both *Artemisia umbelliformis* chemotypes. Capital letters: EO GC analysis, lower-
381 case letters: HS-SPME GC analysis.

382 **Figure 3** - HPLC-DAD-UV profiles at 210 nm of *Artemisia umbelliformis* chemotypes Au1 and
383 Au2.

384

385 **Figure 4** - PCR products derived from all possible combinations of Au1 and Au2 specific primers
386 designed on the coding and non-transcribing regions of the plant 5S-rRNA gene. Lane 1, a
387 single fragment of approximately 224 bp is produced by Au1. Lane 2, a single fragment of
388 about 327 bp is produced by Au2. Lane 3, a single fragment of 204 bp in length amplified using
389 the primer AuF in combination with the primer 5S-P2 in the chemotype Au1. Lane 4, a single
390 fragment of 307 bp in length using the primer AuF in combination with the primer 5S-P2 in the
391 chemotype Au2. Lane 5, a single fragments of 231 bp with primers 5S-P1 and Au2R1 in the
392 chemotype Au2. Lane 6, a single fragments of 275 bp with primers 5S-P1 and Au2R2 in the
393 chemotype Au2. Lane 7, a single fragments of 64 bp with primers Au2F1 and Au2R1 in the
394 chemotype Au2. Lane 8, a single fragments of 108 bp with primers Au2F1 and Au2R1 in the
395 chemotype Au2. Lane 9, a single fragments of 213 bp with primers AuF and Au2R1 in the
396 chemotype Au2. Lane 10, a single fragments of and 255 bp with primers AuF and Au2R2 in the
397 chemotype Au2. Lane 11, purified PCR products obtained by using 5S-P1 and 5S-P2 primers

398 digested with Rsa I give two major fragments of 123 bp and 82 bp respectively, and a minor
399 fragment of 19 bp not visible in the gel because out of the resolution capacity of the instrument
400 in the chemotype Au1. Lane 12, RsaI cleaved Au2 5S-rRNA spacer region gives a major
401 fragment of 283 bp and two minor fragments of 25 bp (barely visible in the gel) and 19 bp (not
402 visible in the gel). Lane 13, PCR products from the chemotype Au1 not digested by Taq I. Lane
403 14, purified PCR products from the chemotype Au2 digested using Taq I producing two
404 fragments of 197 and 130 bp.

405

406 **Figure 5** - Alignments of the nucleotide sequences of 5S-rRNA gene spacer region of *Artemisia*
407 *umbelliformis* chemotypes Au1 and Au2. Universal primer sequences are indicated in squared
408 solid boxes. *Artemisia umbelliformis* forward primers are indicated in bold. Identical sequences
409 are indicated by (*). Gaps (-) are introduced for the best alignment. RsaI site is evidenced in the
410 squared large dot box, whereas the TaqI site is evidenced in the squared small dots box.
411 Forward and reverse specific primers of the Au2 chemotype are indicated.

412

413 **Figure 6** - Position of the universal primers (5S-P1 and 5S-P2) flanking the spacer region of 5S-
414 rRNA gene and specific *Artemisia umbelliformis* forward (AuF) and *A. umbelliformis*
415 chemotype Au2 forward (Au2F1) and reverse (AuR1 and AuR2) specific primers used for PCR
416 amplification of the 5S-rRNA spacer region.

Table 1 - Components Characterising the Essential Oils of *Artemisia umbelliformis* Chemotypes Au1 and Au2.

Compounds	MEGA5 ^a	MEGA5 ^b	CW	Au1		Au2	
				Mean	Range	Mean	Range
α -Thujene	930	929	1038	0.05	tr-0.10	0.13	tr-0.57
α -Pinene	936	936	1031	0.26	tr-0.40	0.60	0.33-1.20
Camphene	954	951	953	0.06	tr-0.30	0.43	tr-1.10
Sabinene	975	975	1132	0.35	0.09-1.40	0.15	tr-0.36
β -Pinene	979	978	1117	1.74	0.10-2.30	5.12	1.73-11.50
β -Myrcene	991	993	1174	0.18	tr-0.30	0.68	0.10-2.90
α -Terpinene	1017	1017	1189	0.18	tr-0.30	0.34	0.10-0.55
<i>p</i> -Cymene	1025	1026	1280	0.27	0.10-0.40	0.21	0.10-0.70
1,8-Cineole	1031	1033	1213	4.86	0.30-9.50	7.58	4.05-14.00
γ -Terpinene	1060	1061	1257	0.35	0.10-0.50	0.75	0.43-1.00
<i>cis</i> -Sabinene hydrate	1070	1069	1268	1.17	0.30-1.50	7.16	2.70-20.08
α -Terpinolene	1089	1089	1292	0.10	tr-0.10	0.21	0.10-0.30
<i>trans</i> -Sabinene hydrate	1098	1097	1488	0.65	0.30-1.00	2.19	1.30-3.35
α -Thujone	1102	1108	1420	36.99	29.70-51.90	0.37	tr-2.00
β -Thujone	1114	1118	1446	9.11	4.60-19.53	0.27	tr-0.65
Sabinol	1142	1141	1708	0.90	0.28-4.10	0.20	0.10-0.50
Camphor	1143	1145	1516	0.15	tr-1.54	0.75	tr-1.70
Borneol	1169	1168	1712	1.14	tr-3.90	11.58	0.09-19.43
Terpinen-4-ol	1177	1177	1619	1.21	0.40-1.48	4.75	3.30-8.15
α -Terpineol	1189	1190	1714	0.29	tr-0.50	1.08	0.40-2.20
Bornyl acetate	1289	1286	1378	0.05	tr-0.10	0.51	tr-1.97
α -Terpinyl acetate	1349	1351	1494	0.58	tr-1.10	2.30	0.70-4.19
Sabinyll isobutirrate	1416 ^c	1416	1494	1.35	1.00-3.83	0.01	tr-0.09
β -Caryophyllene	1419	1418	1594	0.40	tr-0.56	2.24	1.00-4.60
Sabinyll isovalerianate	1503 ^c	1503	1577	3.94	3.10-7.13	0.16	tr-0.50
Sabinyll valerianate	1516 ^c	1516	1605	2.69	1.30-7.28	0.14	tr-0.30
Caryophyllene oxide	1583	1581	1965	1.89	0.57-3.30	3.61	2.15-5.40
Neryl isovalerianate	1584	1585	1679	4.11	1.08-6.90	4.72	1.40-10.00

a: LRI from Adams library (18);

b: experimental LRI

c: LRI from standards synthesized in the authors' laboratory (2)

Mean and range values are expressed as percent areas normalized vs. nonane as internal standard.

Table 2 – Mean and Range Amount of α - + β - thujone Expressed as g/100g of Essential Oil in the Investigated Samples of *Artemisia umbelliformis* Chemotypes Au1 and Au2.

	n	Range	Mean	SD
Chemotype AU1	26	18.0-57.3	36.8	14.7
Chemotype AU2	10	0.2-0.4	0.3	0.1

α -thujone ($Y = 0.3105x + 3.311, R^2 = 0.9995$)

β -thujone ($Y = 0.2896x + 0.1380 ; R^2 = 0.9994$)

Figure 1

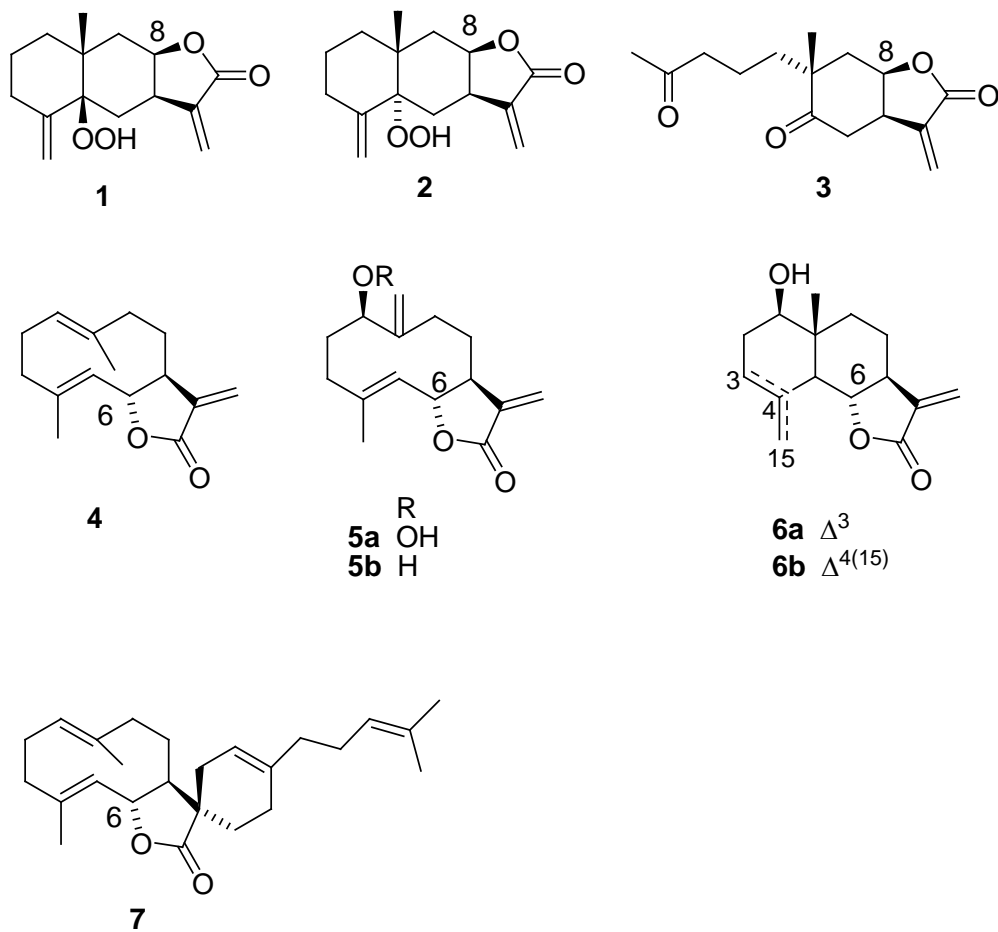


Figure 2

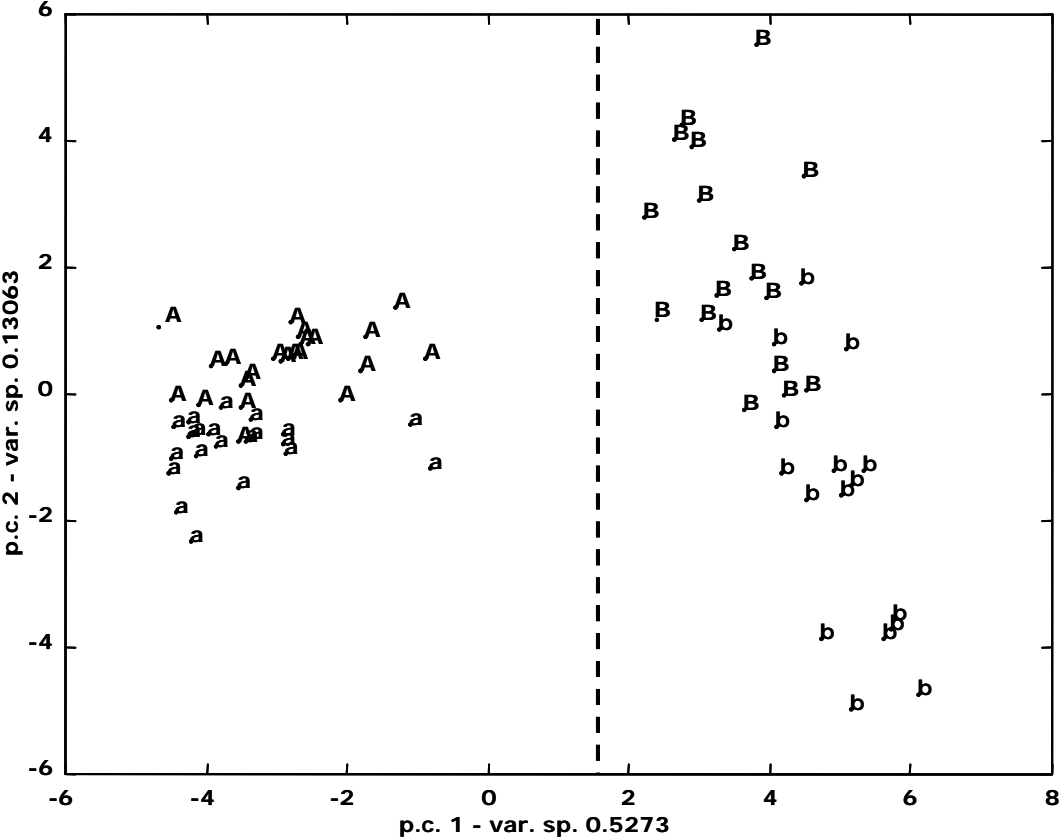


Figure 3

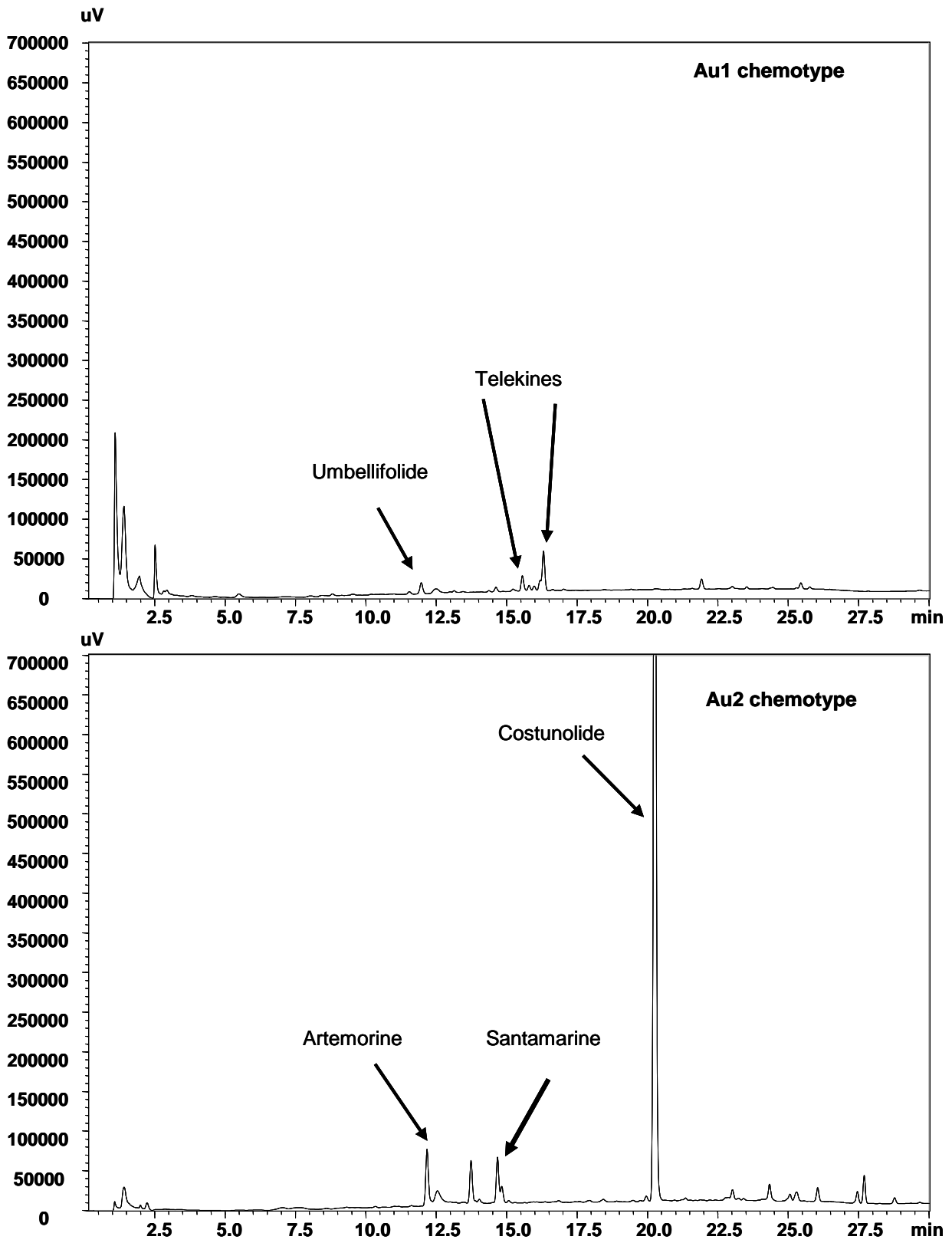


Figure 5

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Au1      GTGCTTGGGCGAGAGTAGTAC TAGGATGGGTGACCTCCTGGGAAGTCCTCGTGTGCACC 60
Au2      GTGCTTGGGCGAGAGTAGTAC TAGGATGGGTGACCTCCTGGGAAGGCCTCGTGTGCATC 60
          *****
          5S-P1      AuF

Au1      CC-----TAAATTTTTGTTTTTTTAAATTTTAAATTTTTTT-----TCTTTA 101
Au2      CCTTTTTTTCGTTTTGTTTTATTTTTTACATGATCTTAAATTTTTTTTTTTTCATATTTACA 120
          **                * * **** * * **** * * **** * * **** * * *

Au1      TTATA-ATTTCCGGAATGATTATTGTAATCGGGAAATTTGTGTGGCT-TGAGGGAG- 158
Au2      TTAAATATCTCGAAGAGACGTGTTTGA AAAACAAGAGACGGTGGGTGGGCGGGTGACAGAGT 180
          *** * * * * * * * * * * * * * * * * * * * * * * * * * * *
          Au2F1

Au1      -----GAGAGGGGTTTCGCTATAAAATGACTATTCGACACAATACATTT----- 200
Au2      CGGTAAAGAGTAAAACGGTTAAAAGAAAACGCGTAGGCGGTGAATTTTACGTTTTTTTGG 240
          **** * * * * * * * * * * * * * * * * * * * * *
          Au2R1

Au1      ----- 200
Au2      TAACTTAAGTGA GAAAAATAGGCAATGAGAAAGGAA TAAAGATAATTTGGCAATAGAAAT 300
          Au2R2

Au1      ---CGGCTGCGATCATAACCAGCACTAA 224
Au2      GTACGGATGCGATCATAACCAGCACTAA 327
          *** *****
          5S-P2

```

GTAC = Rsa I site

TCGA = Taq I site

Figure 6

