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ARTICLE

Chemical and Biomolecular Characterization of *Artemisia umbelliformis* Lam., an Important Ingredient of the Alpine Liqueur "Genepì"

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Artemisia umbelliformis Lam., an important alpine plant used for the preparation of flavored beverages, showed a remarkable intraspecific variability, at both genomic and gene product (secondary metabolites) levels. The variability of *A. umbelliformis* Lam. currently cultivated in Piedmont (Italy, Au1) and in Switzerland (Au2) was investigated by combining the chemical analysis of essential oil and sesquiterpene lactones and the molecular characterization of the 5S-rRNA-NTS gene by PCR and PCR-RFLP. Marked differences were observed between the two plants. Au1 essential oil contained α- and β-thujones as the main components, whereas Au2 contained 1,8-cineole, borneol, and β-pinene. Au1 sesquiterpene lactone fractions contained *cis*-8-eudesmanolide derivatives and Au2 the *trans*-6-germacranolide costunolide. Specific *A. umbelliformis* Au1 and Au2 primers were designed on the sequence of the 5S-rRNA gene spacer region. Furthermore, a PCR−restriction fragment length polymorphism (PCR-RFLP) method was applied using *Rsa*I and *Taq*I restriction enzymes. Chemical and biomolecular data contributed to the characterization of *A. umbeliformis* chemotypes.

KEYWORDS: *Artemisia umbelliformis* Lam.; alpine liqueurs; chemotype discrimination; chemical analysis; biomolecular analysis

INTRODUCTION

Artemisia umbelliformis Lam. is an Alpine species used to prepare "genepì", a highly prized liqueur characterized by a bitter taste and a peculiar flavor (*1*). These properties have been traced to the volatile constituents and to the sesquiterpene lactone fraction of the plant, which are characterized by high contents of α- and β-thujones (*2*, *3*) and by the presence of the *cis*-eudesmanolide sesquiterpene lactones **1**−**3**, respectively (*4*−*6*).

Thujone is a natural terpenoid also associated with common wormwood (*Artemisia absinthium* L.) and Roman wormwood (*Artemisia pontica* L.), absinthe's most widely used ingredients (*7* and references cited therein). There is currently a heated debate on the toxicity of absinthe and thujones (*7* and reference cited therein), but European Union legislation has imposed a limit of 35 ppm on the total amount of these compounds in alcoholic beverages (*8*). To overcome this issue, thujone-free chemotypes of *A. umbelliformis* have been selected by horticultural techniques (*9*). Remarkably, an investigation of the sesquiterpene lactone fraction of one of these thujone-free chemotypes showed dramatic differences from the wild plant. Thus, the C-8 *cis*-sesquiterpene lactones typical of *A. umbelliformis* from the western Alps (**1**−**3**) were replaced by the C-6 *trans*-lactones **4**−**6a**,**b**, whereas a structurally unique sesterpene lactone (**7**) was also detected (*10*) (**Figure 1**). Chemotypes (or chemical phenotypes) are generally considered to be the phenotypical expression of a genotype, although different chemotypes may derive from the same genotype. This means that, according to environmental conditions, the same genotype may express different chemical patterns, or, conversely, that different genotypes may respond to the same environmental pressure with the same phenotypic expression. In this context, molecular genetic methods have recently been shown to be very effective in genotypic discrimination. Genetic methods focus on genotype rather than phenotype, and DNA-based experiments are now widely used for the rapid identification (and therefore autentication) of medicinal plants. Bertea et al. (*11*) recently showed that molecular approaches are a powerful tool to distinguish the *Acorus calamus* diploid β-asarone-free cytotype from the other cytotypes containing it. The same group also used specific *Salvia divinorum* primers designed on the sequence of the 5S-rRNA gene spacer region (*12*) to develop a real-time PCR-based mathematical model to quantify *S. divinorum* in commercial plant samples or hallucinogenic preparation (*13*). Given the potential of this approach, it seemed interesting to apply a combination of biomolecular and chemical techniques to characterize the chemotypes of *A. umbelliformis* currently cultivated in Piedmont (Italy, Au1) and in Switzerland (Au2),

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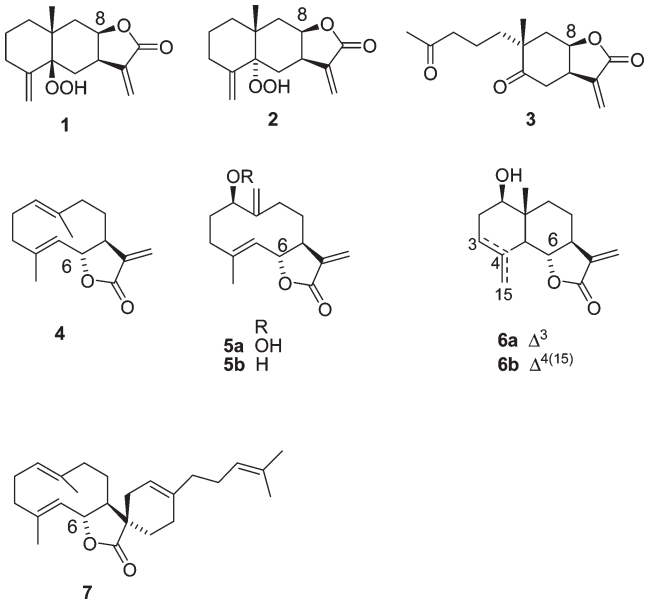

Figure 1. Structures of the sesquiterpene lactones identified in both chemotypes of *A.umbelliformis* Lam.: **1**, 5-desoxy-5-hydroperoxy-5-epitelekin; **2**, 5-desoxy-5-hydroperoxytelekin; **3**, umbellifolide; **4**, costunolide; **5a**, verlotorin; **5b**, artemorine; **6a**, santamarine; **6b**, reynosine; **7**, genepolide.

complementing the analysis of their essential oil and sesquiterpene lactones with a molecular characterization by PCR and PCR-RFLP of the 5S-rRNA-NTS region of their genome.

MATERIALS AND METHODS

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

Plant Material. Forty-three samples of *A. umbelliformis* Lam. aerial parts were from experimental cultivations run in different alpine valleys [Val Grana (latitude 44° 25′ N, longitude 7° 20′ E), Valle Stura (44° 21′ N, 7° 26′ E), Valle Maira (44° 28′ N, 7° 22′ E), and Val Chisone (44° 57′ N, 6° 52′ E)] at a height of least 1300 m above sea level. Fresh plant material was directly indoor-dried by the farmers under controlled temperature and humidity to a constant weight, in agreement with the WHO guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants. Voucher specimens representative of the two chemotypes (native, Au1; and selected in Switzerland, Au2) are deposited at the Dipartimento di Scienza e Tecnologia del Farmaco (no. 231 for Au1 and no. 232 for Au2). For each chemotype, batches of 1 kg of aerial dried parts (see above) were supplied by the Associazione Genepì Occitan (Cuneo, Italy).

Essential Oils (EOs) and Headspace Solid Phase Microextraction (HS-SPME) Sample Preparation. EOs were prepared according to the method of the European Pharmacopoeia (*14*). Ten grams of dried aerial parts was suspended in 250 mL of water in a 500 mL flask for 1 h and then submitted to hydrodistillation in a Clevenger microapparatus for 2 h (*2*). The resulting EO was left to stabilize for 1 h, then recovered with hexane, and analyzed by GC-MS.

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

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

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

GC and GC-MS Analyses. GC analyses were carried out on a Shimadzu QP2010 system provided with a FID and a MS detector, and the results were processed by GC Solution software and GC-MS solution software (2.51 version) (Shimadzu Italia, Milano Italy). Capillary GC-FID-MS analyses were carried out on two 25 m, 0.25 mm i.d., 0.25 μm columns from MEGA (Milano, Italy), that is, Mega5 (95% polydimethylsiloxane, 5% phenyl) and Mega-Wax (polyethyleneglycol, PEG20M).

GC and GC-MS conditions were as follows: injection mode, split; split ratio, 1:20; injector temperature, 230 °C; transfer line temperature, 230 °C; ion source temperature, 200 °C; carrier gas, He; flow rate, 1.0 mL/min in constant flow mode. The MS detector was in electron impact ionization mode (EI) at 70 eV, the scan rate was 1111 amu/s, and the mass range was m/z 35−350. The temperature program was from 50 °C (1 min) to 220 °C (5 min) at 3 °C min^{-1}.

EOs and headspace components were identified by comparison of both their linear retention indices, calculated versus a C_8−C_{25} hydrocarbon mixture, and their mass spectra with those of authentic samples or with data in the literature.

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

HPLC-DAD-UV Analysis. HPLC-DAD-UV analyses were carried out on a Shimadzu 2010EV system provided with a PDA detector (Shimadzu, Dusseldorf Germany). A 150 × 4.6 mm i.d., 5 μm, Zorbax Stable Bond column (Agilent, Waldbronn, Germany) was used. Analysis conditions were as follows: mobile phase, eluent A, 20% acetonitrile/water; eluent B, 100% acetonitrile; mobile phase gradient, from 100% A to 100% B in 25 min; injection volume, 10 μL; flow rate, 1 mL/min; UV detection wavelength, 210 nm.

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

HPLC-MSD Analysis. HPLC-MSD analyses were carried out with a single-quadrupole Shimadzu 2010EV system equipped with an orthogonal atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources. The same column and mobile phase as for HPLC-DAD-UV analysis were used. The flow rate was 0.8 mL/min. MSD conditions were as follows: MS-APCI+; temperature, 400 °C; nebulizer flow, 2.5 mL/min; CDL voltage, 250 °C; MS-ESI+; temperature, 250 °C; nebulizer flow, 1.5 mL/min; CDL voltage, 250 °C.

MSD analysis conditions were optimized by direct flow injection of pure standards of costunolide (**4**) and umbellifolide (**3**) and of a fraction containing a mixture of hydroperoxytelekins.

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

PCR Amplification, Subcloning, and Sequencing. Approximately 20 ng of genomic DNA isolated from powdered leaf material of Au1 and Au2 was used as a template for PCR amplification with forward primer 5S-P1 (5′-GTGCTTGGGCGAGAGTAGTA-3′) and reverse primer 5S-P2 (5′-TTAGTGCTGGTATGATCGC A-3′) flanking the NTS of the 5S-rRNA gene (*11*−*13*, *16*). The amplification was carried out in a 50 μL reaction mixture containing 5 μL of 10× PCR reaction buffer (Fermentas), 0.2 mM dNTPs, 20 pmol of forward and reverse primers, and 0.5 U of *Taq* DNA polymerase (Fermentas, Glen Burnie, MA). The PCR reactions were carried out in a Whatman Biometra T-Gradient Thermalcycler (Whatman Biometra, Goettingen, Germany). Cycling conditions consisted of an initial 4 min at 94 °C, followed by 30 s of denaturing at 94 °C, 1 min of annealing at 52 °C, and 1 min of elongation at 72 °C, repeated for 30 cycles and with 5 min of final extension at 72 °C.

One microliter of the amplification reaction was analyzed by capillary gel electrophoresis (CGE) using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the DNA 1000 LabChip Kit (Agilent Technologies) following the manufacturer's instructions. The DNA 1000 LabChip Kit provides sizing and quantitation of dsDNA fragments ranging from 25 to 1000 bp. PCR products were also analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. From this gel bands of about 220 bp for Au1 and about 320 bp for Au2 were purified by using the Nucleospin Extract II Kit (Macherey Nagel) and then subcloned into pGEM-T Easy vector (Promega). The ligated products were transformed into *Escherichia coli* Subcloning DH5α Efficiency Competent Cells (Invitrogen). Colonies containing DNA inserts of the correct size were picked and grown overnight in 3 mL of Luria−Bertani (LB) liquid medium. The mini-preparation of plasmid DNAs was performed by using QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer's instructions. The plasmid DNAs were employed as a template for sequencing. Both strands of DNA were sequenced at least twice, and the sequences were aligned by using ClustalX software.

PCR Amplification Using Specific Primers for Au1 and Au2. Sequences derived from powdered leaf material of Au1 and Au2 were aligned in a unique sequence that allowed the design of two forward primers, AuF 5′-CTAGGATGGGTGACCT CCTG-3′(which is common to both chemotypes) and Au2F 5′-GCGGTGACAGAGTCGGTAAA-3′, and two reverse specific primers, Au2R1 5′-CGTAAAATTCACCGCCTACG-3′ and Au2R2 5′-TCCTTTCTCATTGCCTATTTTTC-3′, which correspond, respectively, to nucleotides 21−40, 168−187, 212−231, and 253−275 of the Au2 nontranscribed spacer (NTS) sequence. The internal primers were used for amplification also in combination with primers 5S-P1 and 5S-P2.

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

PCR-RFLP. The purified PCR products of the 5S-rRNA gene spacer region of both Au1 and Au2 chemotypes were digested

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

				Au1		Au2	
compound	MEGA5[a]	MEGA5[b]	CW	mean[d]	range[d]	mean[d]	range[d]
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
p-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
cis-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
trans-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
sabinyl isobutyrate	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
sabinyl isovalerianate	1503[c]	1503	1577	3.94	3.10−7.13	0.16	tr−0.50
sabinyl 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*). [d] Mean and range values are expressed as percent areas normalized vs nonane as internal standard. tr, trace.

either with 10 U of *Rsa*I (Amersham Biosciences) at 37 °C for 1 h or, in a separate reaction, with 10 U of *Taq*I (Sigma) at 65 °C for 1 h. One microliter of both digestion reactions was fractionated by CGE using the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip Kit (Agilent Technologies) following the manufacturer's instructions.

RESULTS AND DISCUSSION

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

Chemical Analyses. Chemical analyses investigated the fractions responsible for plant odor and taste, that is, the composition of the volatile fraction including quantitation of α- and β-thujones and that of the sesquiterpene lactone fraction (i.e., the components responsible for the liqueur bitter taste). The volatile fraction was studied by analyzing both the EO obtained by hydrodistillation and the HS sampled by HS-SPME combined with GC and GC-MS. HS-SPME sampling was applied with a view to develop a fully automatic control method, to be run in combination with GC and GC-profile multivariate analysis [principal component analysis (PCA)]. **Table 1** reports the average percent areas normalized versus *n*-nonane as internal standard and percent range of the characteristic components of the EOs of the samples investigated together with their linear retention indices (LRI) on both GC columns. **Table 2** reports calibration curves and mean and range amounts of α- and β-thujones in the samples investigated. This table considers only 10 samples of chemotype Au2 of 17 because the α- and β-thujone peak areas of the remaining 7 samples were too low to be correctly used for quantitative determination. From these results it is clear that the two chemotypes are characterized by different compositions: Au1 was found to contain α- and β-thujones and a homologous series of sabinyl esters as the main components, whereas in chemotype Au2, 1,8-cineole, borneol, and β-pinene were the major compounds. Moreover, thujones were almost absent from the Au2 chemotype, their total amount accounting for from 0.2 to 0.4 g/100 g of EO, whereas in the Au1 chemotype thujones ranged from 18 to about 58 g/100 g of EO. The results obtained by HS-SPME-GC analysis, although not directly comparable, were in full agreement with those of the EOs, as shown by the PCA scatterplot of **Figure 2**. Each chemotype is clearly discriminated, and the samples belonging to the same chemotype analyzed by HS-SPME-GC and through their EO are coherently positioned in the PCA scatterplot (**Figure 2**).

Significant differences can also be found in the composition of the nonvolatile bitter fraction. **Figure 3** reports the HPLC-DAD-UV profiles of two samples belonging to Au1 and Au2 chemotypes, respectively. The bitter taste of the native Au1 chemotype is mainly due to sesquiterpene lactones of the *cis*-8-eudesmanolide type [5-deoxy-5-hydroperoxy-5-epitelekin (**1**), 5-deoxy-5-hydroperoxytelekin (**2**), umbellifolide (**3**) (*4*−*6*)]. On the other hand, the Au2 chemotype is characterized by high amounts of costunolide (**4**), a germacranolide typical of *A. genipi* Weber (*4*), and by the presence of an unusual sesterpene lactone, named genepolide (**7**) (*10*). An in-depth investigation of the Au2 ethanolic extract composition after fractionation by column chromatography in combination with NMR and analysis

Table 2. Mean and Range Amount of α- + β-Thujones[a] Expressed as Grams per 100 g of Essential Oil in the Investigated Samples of *Artemisia umbelliformis* Chemotypes Au1 and Au2

chemotype	*n*	range	mean	SD
AU1	26	18.0−57.3	36.8	14.7
AU2	10	0.2−0.4	0.3	0.1

[a] α-Thujone: $Y = 0.3105x + 3.311$, $R^2 = 0.9995$. β-Thujone: $Y = 0.2896x + 0.1380$; $R^2 = 0.9994$.

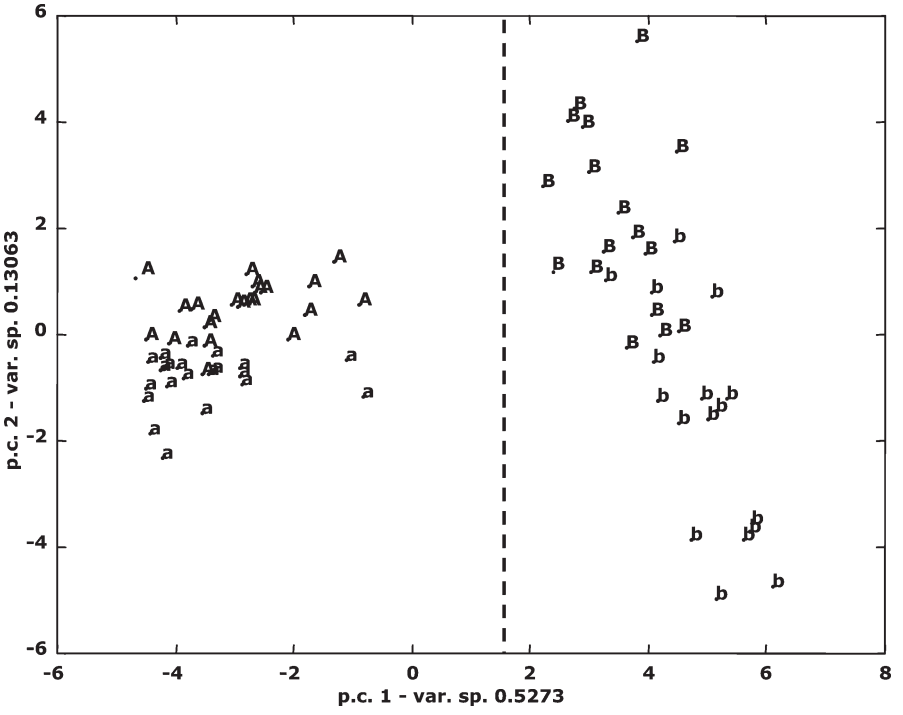

Figure 2. PCA scatterplot of the cumulative elaboration of both EOs and headspaces sampled by HS-SPME of both *Artemisia umbelliformis* chemotypes. Capital letters, EO GC analysis; lower case letters, HS-SPME GC analysis.

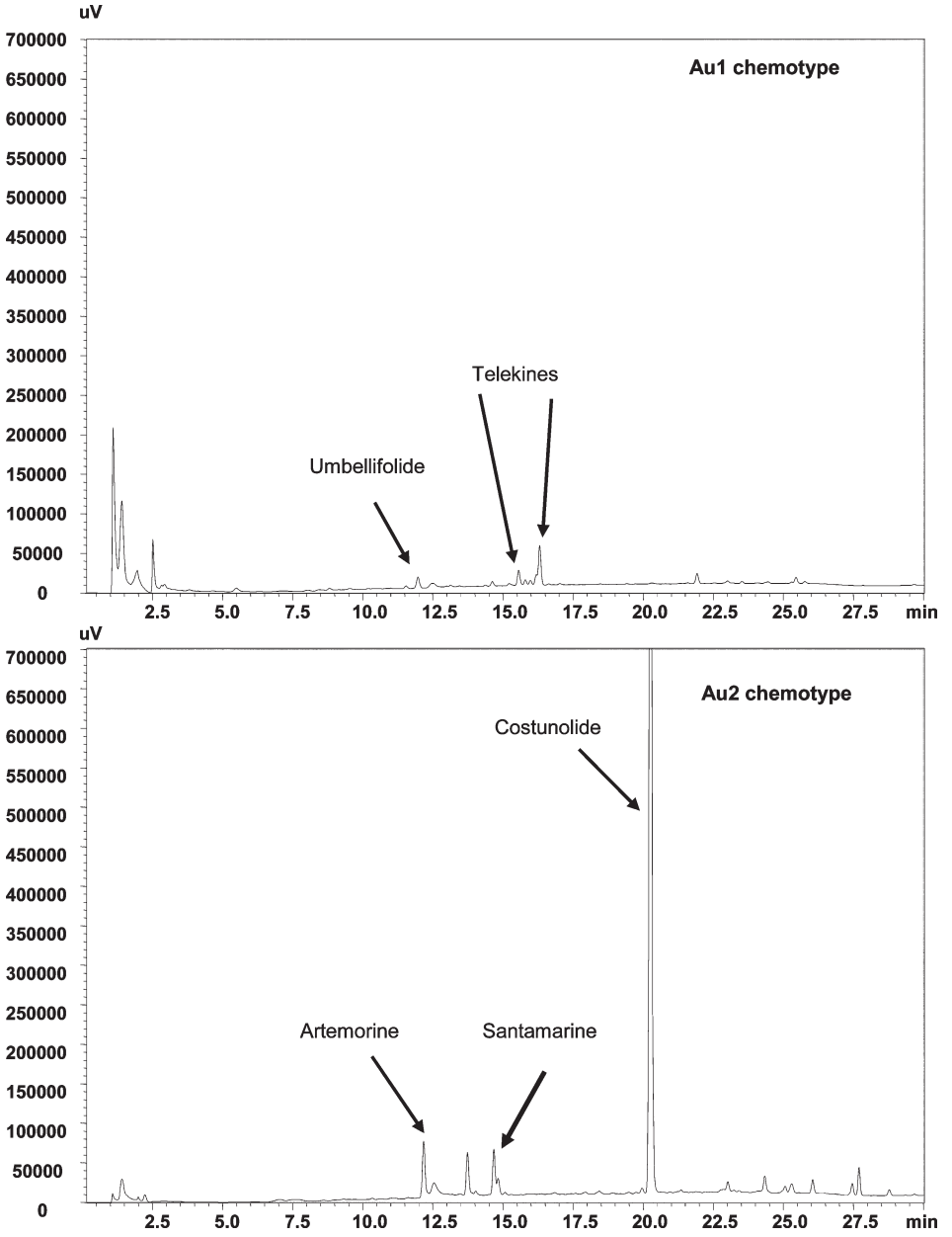

Figure 3. HPLC-DAD-UV profiles at 210 nm of *Artemisia umbelliformis* chemotypes Au1 and Au2.

by HPLC-UV and HPLC single-quadrupole MS revealed that costunolide (**4**) was accompanied by a series of related oxygenated sesquiterpene lactones [artemorine (**5b**), santamarine (**6a**), and reynosine(**6b**)] (*4*). On the other hand, in the Au1 chemotype the presence of both telekin hydroperoxides and umbellifolide was confirmed.

Costunolide and the sum of hydroperoxytelekins and umbellifolide were adopted as markers of the two chemotypes to evaluate quantitatively the bitter fraction of the 43 samples under investigation. These analyses showed an average amount of umbellifolide + hydroperoxytelekins expressed as umbellifolide ($Y = 29.9392x - 1.6795$; $R^2 = 0.99999$) of 0.11 g/100 g of dried plant material in a range varying between 0.03 and 0.37 g/100 g for the Au1 chemotype, and an average amount of costunolide ($Y = 101.1709x - 31.0592$; $R^2 = 0.99975$) of 0.56 g/100 g of dried plant material in a range between 0.20 and 0.93 g/100 g for the Au2 chemotype. Costunolide was also detected in very low amounts in some samples of the Au1 chemotype, its percentage never exceeding 0.05%.

Molecular Characterization of the Two *A. umbelliformis* Chemotypes. In higher eukaryotes, the 5S-rRNA gene occurs in tandemly repeated units consisting of a 120 bp coding region separated by a nontranscribed spacer that varies from species to species (*16*). Thus, the diversity of the spacer region can be used as an identification basis (*17*).

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

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

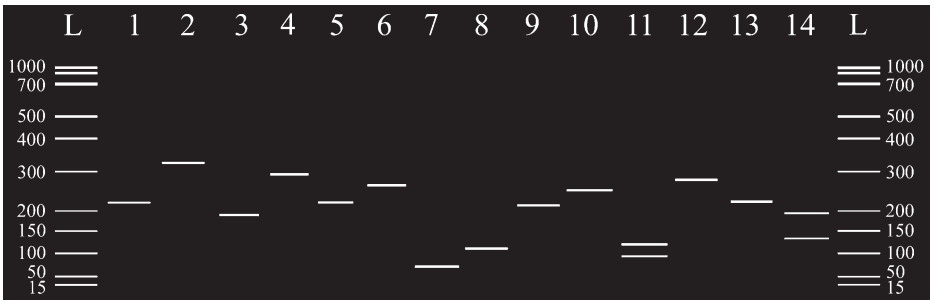

Figure 4. PCR products derived from all possible combinations of Au1 and Au2 specific primers designed on the coding and nontranscribing regions of the plant 5S-rRNA gene. Lanes: 1, a single fragment of approximately 224 bp is produced by Au1; 2, a single fragment of about 327 bp is produced by Au2; 3, a single fragment of 204 bp in length amplified using the primer AuF in combination with the primer 5S-P2 in chemotype Au1; 4, a single fragment of 307 bp using the primer AuF in combination with the primer 5S-P2 in chemotype Au2; 5, a single fragment of 231 bp with primers 5S-P1 and Au2R1 in chemotype Au2; 6, single fragments of 275 bp with primers 5S-P1 and Au2R2 in chemotype Au2; 7, single fragments of 64 bp with primers Au2F1 and Au2R1 in chemotype Au2; 8, single fragments of 108 bp with primers Au2F1 and Au2R1 in chemotype Au2; 9, single fragments of 213 bp with primers AuF and Au2R1 in chemotype Au2; 10, single fragments of 255 bp with primers AuF and Au2R2 in chemotype Au2; 11, purified PCR products obtained by using 5S-P1 and 5S-P2 primers digested with *Rsa*I to give two major fragments of 123 and 82 bp, respectively, and a minor fragment of 19 bp not visible in the gel because of the resolution capacity of the instrument in chemotype Au1; 12, *Rsa*I cleaved Au2 5S-rRNA spacer region gives a major fragment of 283 bp and two minor fragments of 25 bp (barely visible in the gel) and 19 bp (not visible in the gel); 13, PCR products from chemotype Au1 not digested by *Taq*I; 14, purified PCR products from chemotype Au2 digested using *Taq*I producing two fragments of 197 and 130 bp.

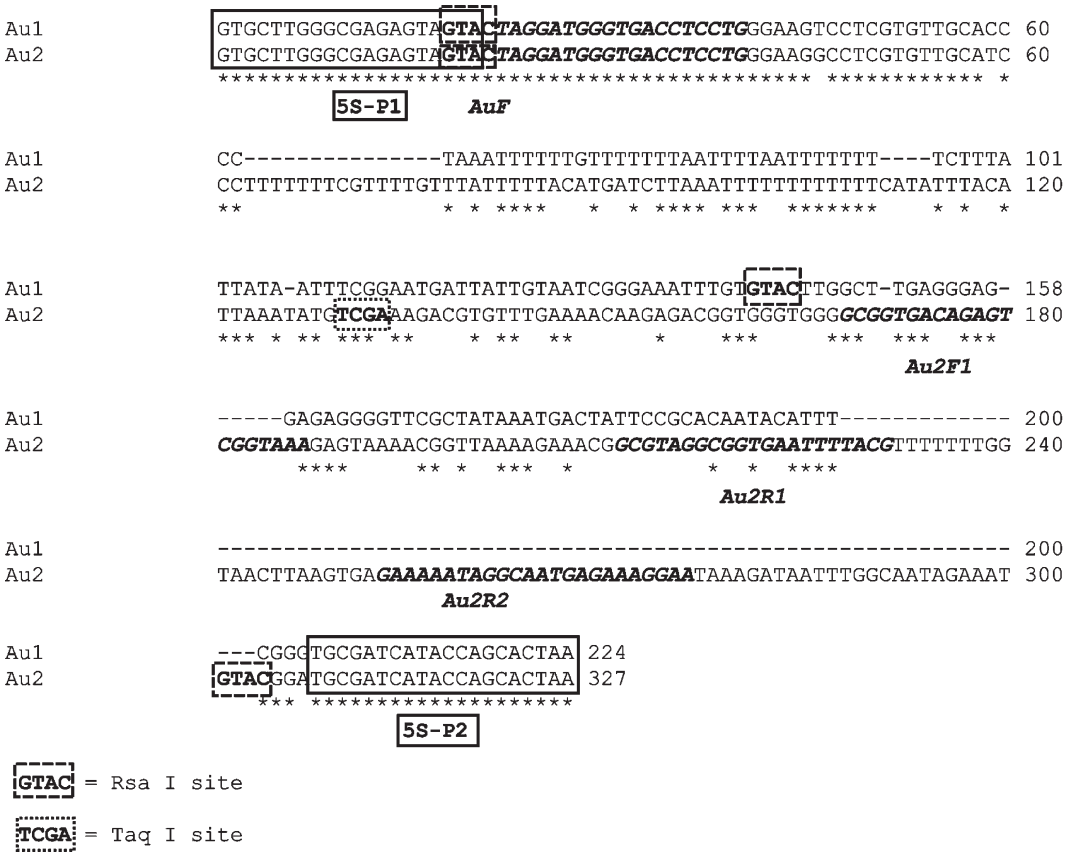

Figure 5. Aligments of the nucleotide sequences of 5S-rRNA gene spacer region of *Artemisia umbelliformis* chemotypes Au1 and Au2. Universal primer sequences are indicated in squared solid boxes. *A. umbelliformis* forward primers are indicated in bold. Identical sequences are indicated by (∗). Gaps (−) are introduced for the best alignment. *Rsa*I site is evidenced in the dashed box, whereas the *Taq*I site is evidenced in the dotted box. Forward and reverse specific primers of the Au2 chemotype are indicated.

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

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

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

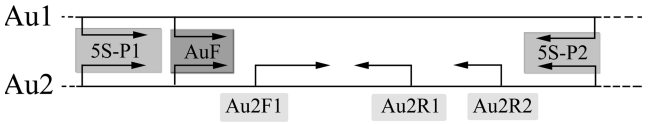

Figure 6. Position of the universal primers (5S-P1 and 5S-P2) flanking the spacer region of 5S-rRNA gene and specific *Artemisia umbelliformis* forward (AuF) and *A. umbelliformis* chemotype Au2 forward (Au2F1) and reverse (AuR1 and AuR2) specific primers used for PCR amplification of the 5S-rRNA spacer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

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

In addition, a PCR-RFLP method was applied. From the identified sequences, two *Rsa*I sites could be found in both chemotype 5S-rRNA spacer regions at 18 and 141 bp positions in Au1 and at 18 and 301 bp in Au2 (**Figure 5**). Purified PCR products obtained by using 5S-P1 and 5S-P2 primers were digested with *Rsa*I. As expected, PCR products from chemotype Au1 could be digested by *Rsa*I, giving two major fragments of 123 and 82 bp, respectively, and a minor fragment of 19 bp not visible in the gel because it was out of the resolution capacity of the instrument (**Figure 4**, lane 11). When purified PCR products from chemotype Au2 were digested using *Rsa*I, a completely different RFLP profile was observed. *Rsa*I cleaved the Au2 5S-rRNA spacer region, giving a major fragment of 283 bp and two minor fragments of 25 bp (barely visible in the gel) and 19 bp (not visible in the gel) (**Figure 4**, lane 12). A *Taq*I site was also identified in the sequence of chemotype Au2 (see **Figure 5**). As expected, PCR products from chemotype Au1 could not be digested by *Taq*I (**Figure 4**, lane 13), whereas purified PCR products from chemotype Au2 could be.

Thus, our biomolecular characterization provides a useful tool for the unequivocal characterization of the two chemotypes. If some intermediate chemotypes due to meiotic rearrangements were already present in the population, they would be detected by using universal primers (different fragment size or different nucleotide composition), but this was not the case, because all samples gave the same results. Besides, this marker has been successfully used for *A. calamus* chemotype determination (*16*), and it represents a powerful tool to deduce genetic relationships of medicinal plants, especially at the intraspecific level.

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

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