JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Volatile Organic Nitrogen-Containing Constituents in Ambrette Seed *Abelmoschus moschatus* Medik (Malvaceae)

ZHIZHI DU,[†] ROBIN A. CLERY,*[,‡] AND CHRISTOPHER J. HAMMOND[‡]

Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China; and Givaudan UK Ltd., Kent, TN24 0LT, United Kingdom

A detailed investigation of the basic fraction of a CO_2 extract of ambrette seeds (*Abelmoschus moschatus*) revealed a total of 58 nitrogen-containing compounds. The identification of these compounds was carried out by GC-MS and NMR. All the identified nitrogen-containing compounds are reported here for the first time in ambrette seeds. Among these are 27 pyrazine derivatives and 12 pyridines, including the tentative identification of four new natural compounds, 1-(6-ethyl-3-hydroxypyridin-2-yl)ethanone (**1**), 1-(3-hydroxy-5,6-dimethylpyridin-2-yl)ethanone (**2**), 1-(3-hydroxy-6-methylpyridin-2-yl)ethanone (**3**), and 1-(3-hydroxy-5-methylpyridin-2-yl)ethanone (**4**). The odor of the basic fraction was assumed to be due to these pyrazines and pyridines and also the presence of seven thiazoles. The odors described suggest that these N-compounds contribute to what is described in perfumery terms as the "natural and rounded" character of the ambrette extract.

KEYWORDS: ***Abelmoschus moschatus*; ambrette seed; basic extract; nitrogenous compounds; pyridines; pyrazines**

INTRODUCTION

Natural plant materials are an important source of flavor and fragrance materials. Flavor and fragrance researchers are constantly looking for new molecules to satisfy consumer demands and to create new flavors and provide new ingredients to extend their portfolia. During the last two decades, attention has been paid to the characterization and sensory properties of nitrogen compounds from natural isolates due to their low perception thresholds (*1, 2*). Volatile nitrogen compounds occur in many flower scents and essential oils. Although the concentration of these compounds in natural isolates is often lower than 100 ppm, they can contribute significantly to the sensory properties of the natural material (*2*). A great number of such compounds have been discovered, but further studies of natural products benefit from the constant improvement in extraction methods and analytical techniques, and new compounds continue to be identified (*3–7*).

Abelmoschus moschatus. Medik. (syn. *Hibiscus abelmoschus* L.) of the Malvaceae, commonly known as ambrette, is a plant native to India, southern China, tropical Asia, and some Pacific islands. The essential oil obtained by steam-distillation from ambrette seeds is a valuable material, noted for a rich, sweet, floral-musky, distinctly winelike or brandylike odor, which finds application both in flavor and fragrance formulations (*8*). Ambrette seed oil has a much smoother odor than synthetic musk compounds, and the major compounds responsible for the characteristic musky odor include ambrettolide ((*Z*)-7-hexadecen-16-olide) and (*Z*)-5-tetradecen-14-olide (*9*). Despite a long history of use of ambrette seed oil in perfumery, there are few published studies about the constituents in the oil (*10–13*). During a preliminary study of the chemical composition of ambrette oil, several nitrogen containing compounds were detected. This prompted an in-depth study of the volatile nitrogen-containing compounds in ambrette seeds.

MATERIALS AND METHODS

Materials. Ambrette CO_2 extract (500 g) was purchased from Danisco SA, Seillans, France. Commercially available chemicals and solvents were purchased from Sigma-Aldrich and used without further purification. Reference materials were purchased from Sigma-Aldrich, Oxford Chemicals Ltd., Fluka AG, or taken from the in-house laboratory reference collection.

Extraction. Ambrette CO_2 extract (450 g) was washed with 10% (v/v) aqueous sulfuric acid solution (3 × 200 mL portions) followed by water (2 × 100 mL portions). The acidic washings were combined and back washed with pentane (2 × 200 mL portions) to remove any remaining residues. The acidic aqueous layer was treated with sodium carbonate until it reached pH 8. The neutralized extract was washed with ethyl acetate (4 × 150 mL portions) to remove basic compounds. The ethyl acetate extracts were combined and washed with distilled water (4 × 100 mL portions) to remove any residual acid. The ethyl acetate extract was dried over anhydrous magnesium sulfate, filtered, and concentrated on a rotary evaporator at 40 °C

* To whom correspondence should be addressed. E-mail: robin.clery@givaudan.com.
[†] Chinese Academy of Sciences.
[‡] Givaudan UK Ltd.

10.1021/jf800958d CCC: $40.75
Published on Web 07/26/2008

under reduced pressure to yield a dark brown residue (basic extract, 0.6 g). GC-FID/NPD analysis of the basic extract revealed the presence of a number of nitrogen-containing compounds.

Fractionation by Flash Chromatography (FC). Flash column chromatography was used to obtain concentrated samples of nitrogenous compounds for subsequent analyses. The basic extract (0.5 g) of ambrette CO_2 oil was loaded onto a silica 60 (40−63 μm, 4 cm × 7 cm, Biotage) FC column and eluted with a stepwise solvent gradient of acetone in hexane (0% 250 mL; 3% 600 mL; 5% 300 mL; 10% 320 mL; 20% 450 mL; 40% 200 mL; 100% 300 mL). Fractions were collected and concentrated on a rotary evaporator for GC analysis. These were combined to afford 18 fractions (fr 1−18) based on composition by GC analysis.

Isolation. Compounds not readily identified by GC-MS were isolated for spectroscopic analysis. Six compounds were isolated by further column chromatography and preparative fraction collection (PFC). The major fraction from flash chromatography (fr 1, 200 mg) was dissolved in hexane and loaded onto a Flash Si II column (10 g, Isolute SPE column, International Sorbent Technology) and eluted with diethyl ether in hexane (3% 100 mL, 5% 100 mL, 10% 100 mL). The total of 21 fractions were collected and combined into 10 subfractions (fr 1a−1j) based on GC analysis. Compound **1** (0.1 mg) was obtained from fr 1a using PFC. Two further compounds were isolated from fr 1b by PFC: **2** (0.1 mg) and **3** (1.0 mg). Compounds **4** (0.7 mg) and **5** (1.2 mg) were isolated from fr 1e by PFC, and fr 1f was purified to afford **6** (0.6 mg).

Gas Chromatography−Flame Ionization Detector (GC-FID). Analysis was carried out using a Hewlett-Packard 6890 gas chromatograph, fitted with a HP-5 (5% diphenyl polysiloxane) capillary column (50 m × 0.2 mm i.d., 0.33 μm film thickness, J & W Scientific), split injection (30:1) with helium carrier gas (initial head pressure 50 psi, flow 2.0 mL/min) controlled via an Atas OPTIC 3 injection system. Oven program: 50 °C (0 min), 10 °C/min ramp to 280 °C (hold for 6 min). The injector and detector temperatures were held constant at 200 and 300 °C, respectively. Data was acquired and processed using HP ChemStation software (Rev. A.10.02 [1757]).

Analysis of Nitrogen-Containing Compounds by GC-FID/NPD. Analysis was carried out using a Hewlett-Packard 6890 gas chromatograph, fitted with a HP-5 (5% diphenyl polysiloxane) capillary column (25 m × 0.2 mm i.d., 0.33 μm film thickness, J&W Scientific), split injection (50:1) with hydrogen carrier gas (initial head pressure 13.1 psi, flow 1.2 mL/min). A glass Y-shaped column splitter leads to separate FID and nitrogen phosphorus detector (NPD). Oven program: 50 °C (0 min), 10 °C/min ramp to 280 °C (hold for 8.5 min). The injector and detector temperatures were held constant at 250 and 300 °C, respectively. Data was acquired and processed using HP ChemStation software (Rev. A.10.02 [1757]).

Gas Chromatography−Mass Spectrometry (GC-MS) and GC-TOFMS. Identification was carried out using an Agilent 6890N gas chromatograph, fitted with a Ultra 2 (5% diphenyl polysiloxane) or Ultra 1 capillary column (50 m × 0.2 mm i.d., 0.33 μm film thickness, J & W Scientific), with helium carrier gas, and an initial head pressure of 46.3 psi (1.7 mL/min). The column effluent was split equally between an Agilent 5975 inert MSD spectrometer (ionization potential 70 eV) and a FID detector via a Capillary Flow Technology splitter plate (pressure held at 3.8 psi). The FID detector temperature was held at 300 °C and the MSD transfer line at 280 °C, with the injector set at 225 °C. Oven program: 50 °C (0 min), 2 °C/min ramp to 280 °C (hold for 20 min). All injections were performed in split mode (50:1). Data was acquired and processed using MSD ChemStation software (Rev. D.02.00.275). Identification was also carried out using an Agilent 6890A gas chromatograph, fitted with a Stabilwax column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Restek). Oven program: 30 °C (0 min), 2.5 °C/min ramp to 220 °C (hold for 45 min). All injections were performed in split mode (40:1). Accurate mass measurements for isolated compounds were carried out by GC-TOFMS using Agilent 6890N fitted with a Ultra 2 (5% diphenyl polysiloxane) capillary column (50 m × 0.2 mm i.d., 0.33 μm film thickness, J & W Scientific), with helium carrier gas, an initial head pressure of 40 psi (1.26 mL/min), and connected to a Micromass GCT mass spectrometer. Oven program: 50 °C (0 min), 2 °C/min ramp to 280 °C (hold for 20 min). The system was calibrated with chloropentafluorobenzene and heptacosa, with data processed using Mass Lynx software (version 4.1).

Quantitation. The approximate concentration of each nitrogen-containing compound in the basic extract was calculated using FID response factors for pyrazine or pyridine determined by external calibration with authentic reference materials and peak area values. The pyridine response factor was applied to all pyridine derivatives and other compounds containing only one nitrogen atom (e.g., pyrroles). The remaining compounds (e.g., pyrazine derivatives and compounds containing two or more nitrogen atoms) were quantified using the pyrazine response factor. On the basis of the yield of basic extract from original ambrette CO_2 extract, the concentration of each compound in the original CO_2 extract was calculated (**Table 1**).

Gas Chromatography−Mass Spectrometry/Olfactometry (GC-MS/O). Odor analysis was carried out using an Agilent 6890N gas chromatograph, fitted with a HP-5 (5% diphenyl polysiloxane) capillary column (50 m × 0.32 mm i.d., 0.52 μm film thickness, J & W Scientific), with helium carrier gas, and an initial head pressure of 22.0 psi (3.0 mL/min). A pressure ramp of −0.02 psi/min was applied to achieve actual retention time consistent with in-house library values, a form of retention time locking. The column effluent was split between an Agilent 5975 inert MSD spectrometer (ionization potential 70 eV) and a specially modified Gerstel odor detection port (ODP2) via a Capillary Flow Technology splitter plate. The injector and MSD transfer line were held constant at 250 °C. Oven program: 60 °C (0 min), 5 °C/min ramp to 280 °C (hold for 16 min). Data was acquired and processed using MSD ChemStation (Rev. D.02.00.275) and Gerstel ODP Recorder software (version 2.7.5.2).

Preparative Fraction Collection (PFC). Isolation of compounds for NMR analysis was performed using a Hewlett-Packard 5890 series II gas chromatograph coupled to a Gerstel PFC unit and fitted with a BPX-5 (5% diphenyl polysiloxane) capillary column (30 m × 0.53 mm i.d., 1.0 μm film thickness, SGE). The initial head pressure of helium carrier gas was 10.0 psi. The detector temperature was held at 300 °C, and the transfer line and splitter were held at 250 °C. Injection was made using a Gerstel PTV injector programmed from 100 to 220 °C, ramp at 12 °C/sec. Oven program: 60 °C (0 min) to 250 °C, ramp at 10 °C/min. Data was acquired and processed using HP ChemStation software (Rev.A.10.02 [1757]).

Nuclear Magnetic Resonance Spectroscopy. NMR spectra were recorded on a Bruker Avance 500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR), with a TCI cryoprobe probehead (Bruker), using C_6D_6. Chemical shift values (δ) were calibrated against a residual benzene signal set at 7.15 ppm (^{1}H NMR) and 128.0 ppm (^{13}C NMR). Assignments by heteronuclear single-quantum correlation (HSQC), heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) experiments were performed with TopSpin software from Bruker. The mixing time in the NOESY experiment was 1.7 s.

Compound Identification. Identifications were made by comparison of mass spectra and retention indices with those of reference compounds of both in-house and commercial libraries including NIST, Adams, and Wiley (*14–16*). Linear retention indices were calculated against *n*-alkanes and calibrated from C_8 to C_{30} according to Van den Dool and Kratz (*17*). The compounds, which could not be identified by mass spectra and retention indices, were isolated, and their structures were elucidated by spectroscopic analysis including 1D and 2D NMR.

*1-(6-Ethyl-3-hydroxypyridin-2-yl)ethanone (**1**).* Compound shown in **Figure 1**. GC-TOFMS: *m/z* 165.1891 (calcd 165.079), $C_9H_{11}NO_2$. GC-MS (EI) *m/z* (%): 165 (100, M^+), 164 (40), 137 (25), 122 (62), 108 (20), 94 (13), 80 (18), 67 (8), 43 (20). GC retention index: $RI_{HP\text{-}5}$ 1250. ^{1}H NMR: δ 12.18 (s, 1, OH), 6.91 (d, 1, J = 8 Hz, CHCHC), 6.54 (d, 1, J = 8 Hz, CHCHC), 2.52 (q, 2, J = 7 Hz, CH_2CH_3), 2.47 (s, 3, $OCCH_3$), 1.13 (t, 3, J = 7 Hz, CH_2CH_3). ^{13}C NMR: δ 207.5 (q), 157.3 (s), 153.6 (s), 135.3 (s), 128.9 (d), 126.4 (d), 30.4 (t), 25.3 (q), 13.6 (q).

*1-(3-Hydroxy-5,6-dimethylpyridin-2-yl)ethanone (**2**).* Compound shown in **Figure 1**. GC-TOFMS: *m/z* 165.0799 (calcd 165.079), $C_9H_{11}NO_2$. GC-MS (EI) *m/z* (%): 165 (100, M^+), 137 (25), 122 (57), 108 (10), 94 (35), 80 (7), 67 (16), 43 (22). GC retention index: $RI_{HP\text{-}5}$ 1310. ^{1}H

Table 1. Nitrogen-Containing Compounds Identified in Ambrette CO_2 Extract

no.	compd	retention index RI_{HP-5}	retention index RI_{wax}	retention index RI_{U1}	conc (mg/kg)[a,b]	odor (GC-olfactometry)	identification[c]	reference source
1	2-methylpyrazine	824	1254	795	1	nutty, roasted	MS, RI, RC	Oxford Chemicals
2	2,5-dimethylpyrazine	909	1308	883	10	typical pyrazine slightly coffee	MS, RI, RC	Oxford Chemicals
3	2,6-dimethylpyrazine	911	1315	882	7	typical pyrazine slightly coffee	MS, RI, RC	Givaudan
4	2-ethylpyrazine	914	1322	887	10	earthy pyrazine potato	MS, RI	
5	2,3-dimethylpyrazine	918	1332	892	2	strong dry pyrazine	MS, RI, RC	Oxford Chemicals
6	4,5-dimethylthiazole	929	1360	1060	1	earthy potato skin	MS, RI, RC	Oxford Chemicals
7	2-ethyl-6-methylpyrazine	998	1372	970	23	metallic pyrazine	MS, RI, RC	Givaudan
8	2,4,5-trimethylthiazole	ND[d]	1362	972	tr		MS, RI	
9	2-ethyl-5-methylpyrazine	1001	1378	974	75	pyrazine earth strong slightly coffee	MS, RI	
10	2,3,5-trimethylpyrazine	1002	1389	975	11	roast slightly coffee	MS, RI, RC	Oxford Chemicals
11	2-ethyl-3-methylpyrazine	1005	1391	977	2	smoky roast	MS, RI, RC	Oxford Chemicals
12	2-formyl pyrrole	1008	2010	971	5		MS, RI	
13	2-propylpyrazine	1009	1404	980	2	green vegetable	MS, RI	
14	2-ethenyl-6-methylpyrazine[e]	1017	1476	986	1	soup vegetable	MS	
15	2-acetylthiazole	1020	1634	979	tr		MS, RI, RC	Penta
16	2-acetylpyridine	1034	1587	998	10	strong smoky mousey slightly amine	MS, RI, RC	Sigma-Aldrich
17	2-acetyl pyrrole	1060	1960	1022	1		MS, RI, RC	Takasago
18	4-ethyl-2,5-dimethylthiazole	1065	1426	1043	tr	green slightly pyridine like	MS, RI, RC	Oxford Chemicals
19	2-isopropenylpyrazine[e]	1073	1580	1041	5	burnt	MS	
20	2,6-diethylpyrazine	1080	1421	1059	14	strong earthy pyrazine	MS, RI	
21	2-ethyl-4,5-dimethylthiazole	1082	ND[d]	1060	tr	weak cocoa	MS, RI, RC	Oxford Chemicals
22	2,5-dimethyl-3-ethylpyrazine	1085	1431	1054	4	strong earthy musty	MS, RI	
23	2,3-dimethyl-5-ethylpyrazine	1087	1447	1057	18	earthy	MS, RI	
24	1-(3-hydroxypyridin-2-yl)ethanone (**5**)	1089	1622	1055	12	nutty, earthy, oily	MS, NMR	
25	2,5-diethylpyrazine	1090	1445	1064	50	green pyrazine metallic	MS, RI	
26	2-methyl-3-propylpyrazine	1094	1461	1065	2	moldy beanlike	MS, RI	
27	2-methylthiopyridine	1099	1635	ND[d]	2	dirty oily metallic	MS, RI, RC	Fluka
28	2-acetyl-6-methylpyridine (**6**)	1107	1614	1073	13	green, vegetable	MS, NMR	
29	6,7-dihydrocyclopentapyrazine	1111	1619	1070	1		MS, RI, RC	Givaudan
30	4,5-dimethyl-2-isopropylthiazole	1129	1436	ND[d]	1		MS, RI	
31	1-(2-pyridinyl)-1-propanone	1137	1676	1102	9	like dry pandan leaves	MS, RI	
32	5-methyl-6,7-dihydrocyclopentapyrazine	1143	1598	1107	2	earthy, baked	MS, RI, RC	Givaudan
33	C_8H_9NO isomer (**6a**)	1147	1627	1109	2		MS	
34	2,3-diethyl-5-methylpyrazine	1157	1502	1220	3	earthy, nutty	MS, RI, RC	Oxford Chemicals
35	3,5-diethyl-2-methylpyrazine	1159	1481	1133	7		MS, RI	
36	C_8H_9NO isomer (**6b**)	1161	1710	1123	2		MS	
37	1-(3-hydroxy-6-methylpyridin-2-yl)ethanone (**3**)[f]	1167	1662	1135	7	caramel, toffee, sugar, vegetable	MS, NMR	
38	2-acetyl-3-ethylpyrazine[e]	1168	1502	1142	3		MS	
39	2,6-dimethyl-3-propylpyrazine	1182	1497	1140	2		MS, RI	
40	$C_8H_9NO_2$ isomer **3a**	1186	ND[e]	1155	1		MS	
41	$C_8H_9NO_2$ isomer **3b**	1193	1682	1158	2		MS	
42	2-methyl-5(1-propenyl)pyrazine	1196	1687	1158	7		MS, RI	
43	5,6,7,8-tetrahydroquinoxaline	1212	ND[d]	1173	1	strong and earthy potato	MS, RI	
44	1-(3-hydroxy-5-methylpyridin-2-yl)ethanone (**4**)[f]	1214	1745	1179	10	vegetable, medicated	MS, NMR	
45	2-isobutyl-4,5-dimethylthiazole	ND[d]	1514	1186	tr		MS, RI	
46	2,5-dimethyl-6,7-dihydro-5*H*-cyclopentapyrazine	1225	1652	1190	1		MS, RI, RC	Oxford Chemicals
47	quinoline	1242	1903	1200	1		MS, RI, RC	Givaudan
48	1-(6-ethyl-3-hydroxypyridin-2-yl)ethanone (**1**)[f]	1250	1715	1219	4	faint, oily, slightly earthy	MS NMR	
49	$C_9H_{11}NO_2$ isomer (**1a**)	1260	1720	1230	1		MS	
50	$C_9H_{11}NO_2$ isomer (**1b**)	1264	1966	1233	tr		MS	
51	2-(2-furyl)pyrazine	1265	1967	1225	tr		MS, RI	
52	$C_8H_9NO_2$ isomer (**3c**)	1266	ND[d]	ND[d]	tr		MS	
53	2-methylquinoxaline	1304	1936	1262	tr		MS, RI, RC	Givaudan
54	1-(3-hydroxy-5,6-dimethylpyridin-2-yl)ethanone (**2**)[f]	1310	1826	1276	2	slightly caramel, very weak	MS, NMR	
55	$C_9H_{11}NO_2$ isomer (**1c**)	1312	1822	1307	2		MS	
56	2,5-dimethyl-3-(3-methylbutyl)pyrazine	1318	1645	1291	2		MS, RI	
57	8-methylquinoline	1323	1929	1283	1		MS, RI	
58	5-methylquinoxaline	1332	1992	1271	tr		MS, RI	
59	$C_9H_{11}NO_2$ isomer (**1d**)	1353	1996	1303	tr		MS	
60	6-methylquinoline	1358	ND[d]	1318	tr		MS, RI, RC	Givaudan
61	8-hydroxyquinoline	1362	2153	1339	4		MS, RI	
62	4-methylquinoline	1383	ND[d]	1347	tr		MS, RI, RC	Sigma-Aldrich
63	3-phenylpyridine	1469	2207	1423	14	weak dry slightly metallic	MS, RI, RC	Sigma-Aldrich
64	2-benzylpyridine	1482	2168	1432	1		MS, RI	
65	caffeine	1859	3108	1772	4		MS, RI, RC	Givaudan
66	1-acetylcarboline	2027	3206	1958	9		MS, RI	

[a] Approximate concentrations in mg/kg of ambrette CO_2 extract. [b] Concentrations below 1 mg/kg are noted as trace (tr). [c] MS, matched with commercial or in-house library mass spectrum; RI, matched with retention index of reference compound recorded previously in-house; RC, matched with mass spectrum and retention index of reference compound recorded in the author's laboratory; NMR, NMR of compound isolated as part of this study. [d] Not determined. [e] Compound tentatively identified. [f] New natural compound, not previously reported.

NMR: δ 12.27 (s, 1, OH), 6.75 (s, 1, CC<u>H</u>C), 2.54 (s, 3, OCCH_3), 2.13 (s, 3, CH_3C), 1.59 (s, 3, CH_3C). ^{13}C NMR: δ 207.1 (s), 158.0 (s), 148.0 (s), 140.0 (s), 133.8 (s), 126.5 (d), 25.4 (q), 21.7 (q), 19.1 (q).

*1-(3-Hydroxy-6-methylpyridin-2-yl)ethanone (**3**).* Compound shown in **Figure 1**. GC-TOFMS: *m/z* 151.0635 (calcd 151.0633), $C_8H_9NO_2$. GC-MS (EI) *m/z* (%): 151 (100, M^+), 136 (6), 123 (32), 108 (58), 94

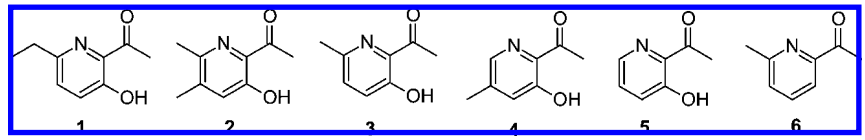

Figure 1. Structures of compounds **1**−**6**.

(10), 80 (41), 53 (33), 43 (31). GC retention index: $RI_{HP\text{-}5}$ 1167. ^1H NMR: δ 12.20 (s, 1, H), 6.88 (d, 1, J = 8 Hz, CHCHC), 6.48 (d, 1, J = 8 Hz, CCHCH), 2.48 (s, 3, $OCCH_3$), 2.18 (s, 3, CCH_3). ^{13}C NMR: δ 207.4 (s), 157.3 (s), 148.7 (s), 135.4 (s), 129.9 (d), 126.5 (d), 25.4 (q), 23.4 (q).

*1-(3-Hydroxy-5-methylpyridin-2-yl)ethanone (***4***).* Compound shown in **Figure 1**. GC-TOFMS: m/z 151.0642 (calcd 151.0633), $C_8H_9NO_2$. GC-MS (EI) m/z (%): 151 (100, M^+), 136 (12), 123 (20), 108 (64), 94 (8), 80 (37), 53 (33), 43 (25).GC retention index: $RI_{HP\text{-}5}$ 1214. ^1H NMR: δ 12.33 (s, 1, OH), 7.80 (s, 1, NCHC), 6.73 (s, 1, CCHC), 2.51 (s, 3, $OCCH_3$), 1.62 (s, 3, CCH_3). ^{13}C NMR: δ 207.1 (s), 159.1 (s), 141.7 (d), 141.2 (s), 134.7 (s), 125.7 (d), 25.4 (q), 18.1 (q).

*1-(3-Hydroxypyridin-2-yl)ethanone (***5***).* Compound shown in **Figure 1**. GC-TOFMS: m/z 137.0478 (calcd 137.0477), $C_7H_7NO_2$. GC-MS (EI) m/z (%): 137 (100, M^+), 122 (9), 109 (33), 94 (58), 80 (8), 67 (36), 43 (40), 39 (37). GC retention index: $RI_{HP\text{-}5}$ 1089. ^1H NMR: δ 12.21 (s, 1, OH), 7.84 (dd, 1, J = 4, 1 Hz, NCHCH), 6.88 (dd, 1, J = 8, 1 Hz, CCHCH), 6.54 (dd, 1, J = 8, 4 Hz, CHCHCH), 2.45 (s, 3, $OCCH_3$). ^{13}C NMR: δ 207.6 (s), 159.0 (s), 140.4 (d), 136.7 (s), 129.7 (d), 125.9 (d), 25.4 (q).

*2-Acetyl-6-methylpyridine (***6***).* Compound shown in **Figure 1**. C_8H_9NO. GC-MS (EI) m/z (%): 135 (73, M^+), 107 (29), 93 (100), 65 (34), 43 (25), 39 (20). GC retention index: $RI_{HP\text{-}5}$ 1107. ^1H NMR: δ 7.87 (d, 1, J = 7 Hz, CCHCH), 6.98 (t, 1, J = 7 Hz, CHCHCH), 6.58 (d, 1, J = 7 Hz, CCHCH), 2.63 (s, 3, $OCCH_3$), 2.28 (s, 3, $CCH3$). ^{13}C NMR: δ 199.2 (s), 157.8 (s), 153.8 (s), 136.7 (d), 126.2 (d), 118.6 (d), 25.2 (q), 24.2 (q).

RESULTS AND DISCUSSION

A total of 58 nitrogen-containing compounds have been identified in the basic fraction of a CO_2 extract of ambrette seeds (**Table 1**). All the identified nitrogen-containing compounds are reported here for the first time in ambrette seeds. Among these are 27 pyrazine derivatives and 12 pyridines, including four pyridine derivatives (**1**−**4**), which are new natural compounds. The odor of the basic fraction was assumed to be due to these pyrazines, pyridines, and the presence of seven thiazoles.

Flash chromatography was used to further fractionate the basic extract. Fractions were selected on the basis of their odor, and sequential separations were made to isolate individual materials for identification by NMR. Several fractions contained unknown peaks, which had a characteristic odor when assessed by GC-MS/O, and these were isolated by GC-trapping. Six compounds were isolated with yields between 30 μg and 1 mg. The majority of compounds in the fractions of interest were identified by mass spectra and retention indices, and in these cases, GC-MS/O was used to obtain a broad odor description without further isolation. The predominant odor types were "earthy", "green", "vegetable", "roasted", and "nutty" and are already well-known for this type of compound (*2, 18, 19*). The odor of the isolated compounds was determined by both GC-MS/O and direct odor assessment from smelling strip.

The isolated compounds comprise a series of substituted 2-acetyl-3-hydroxy pyridines, and in addition to these, there were several similar unknown materials present at levels too low to permit isolation and identification.

Identification of Unknown Pyridine Derivatives. The molecular formula, $C_9H_{11}NO_2$, was determined by GC-TOFMS (m/z 165.1891). The ^1H NMR spectrum of **1** shows two methyl proton signals at δ 1.13 and 2.47, two methylene protons at δ 2.52, two aromatic protons at δ 6.54 and 6.91, which were correlated by the HSQC experiment to the corresponding carbon signals at δ 13.6, 25.3, 30.4, 128.9, and 126.4, as well as one aldehydic proton at δ 12.2. The DEPT NMR data indicates that **1** contains four quaternary carbons, including one ketone signal at δ 207.5 and three unsaturated quaternary carbon signals at δ 135.3, 153.6, and 157.3. The existence of a trisubstituted pyridine skeleton is suggested by the five unsaturations and NMR data. The structure of (6-ethyl-3-hydroxypyridin-2-yl)ethanone was elucidated by the ^1H, ^{13}C long-range correlations and ^1H, ^1H correlations data from the HMBC and NOESY experiments. The key ^1H and ^{13}C corrections found by HMBC are shown in **Figure 2**.

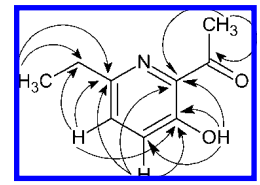

Figure 2. Structure of **1** and the long-range ^1H, ^{13}C correlations found by HMBC.

The structures of **2**−**6** were identified by 1D and 2D NMR data including HMQC, HMBC, and NOESY data and by comparison with those of **1**.

Isomers of **1** *and* **2**. Four compounds ($C_9H_{11}NO_2$ isomers **1a**, **1b**, **1c**, and **1d**) were found to have mass spectra similar to compounds **1** and **2**, which suggests that they may be positional isomers of **1** or **2** with respect to the ethyl or dimethyl substituents but the precise structure could not be determined. Isomer **1a**, GC retention index: $RI_{HP\text{-}5}$ 1260. GC-MS (EI) m/z (%): 165 (100, M^+), 137 (25), 123 (42), 122 (40), 95 (18), 94 (40), 67 (15), 43 (23), 41 (14), 39 (16). Isomer **1b**, GC retention index: $RI_{HP\text{-}5}$ 1264. GC-MS (EI) m/z (%): 165 (74, M^+), 150 (35), 137 (81), 136 (77), 109 (99), 108 (100), 81 (30), 80 (48), 53 (59), 52 (20). Isomer **1c**, GC retention index: $RI_{HP\text{-}5}$ 1312. GC-MS (EI) m/z (%): 165 (75, M^+), 150 (26), 137 (34), 123 (30), 122 (100), 80 (61), 65 (19), 53 (16), 43 (47), 41 (16). Isomer **1d**, GC retention index: $RI_{HP\text{-}5}$ 1353. GC-MS (EI) m/z (%): 165 (100, M^+), 164 (55), 150 (15), 146 (21), 137 (17), 136 (25), 123 (14), 122 (29), 94 (15), 43 (28).

Isomers of **3** *and* **4**. Three compounds ($C_8H_9NO_2$ isomers **3a**, **3b**, and **3c**) were found to have mass spectra similar to compounds **3** and **4**, which suggests that they may be positional isomers but the precise structure could not be determined. Isomer **3a**, GC retention index: $RI_{HP\text{-}5}$ 1186. GC-MS (EI) m/z (%): 151 (100, M^+), 123 (15), 109 (34), 108 (40), 81 (9), 53 (23), 51 (75), 43 (18). Isomer **3b**, GC retention index: $RI_{HP\text{-}5}$ 1193. GC-MS (EI) m/z (%): 151 (64, M^+), 136 (33), 123 (85), 122 (70), 96 (15), 95 (76), 94 (100), 67 (31), 66 (22), 39 (43). Isomer **3c**, GC retention index: $RI_{HP\text{-}5}$ 1266. GC-MS (EI) m/z (%): 151 (100, M^+), 136 (68), 108 (41), 82 (10), 81 (9), 80 (23), 53 (13), 43 (30), 39 (11).

Isomers of **6**. Two compounds (C_8H_9NO isomers **6a** and **6b**) were found to have mass spectra similar to compound **6**, which suggests that they may be positional isomers of **6** with respect to the methyl substituents but the precise structure could not be determined. Isomer **6a**, GC retention index: $RI_{HP\text{-}5}$ 1147. GC-

MS (EI) *m/z* (%): 135 (80, M^+), 107 (25), 93 (100), 92 (75), 66 (24), 65 (32), 43 (20), 39 (18). Isomer **6b**, GC retention index: $RI_{HP\text{-}5}$ 1161. GC-MS (EI), *m/z* (%): 135 (93, M^+), 107 (32), 93 (94), 92 (100), 66 (20), 65 (38), 43 (22), 39 (18).

A direct comparison of the original CO_2 extract with a steam-distilled essential oil of ambrette injected at the same concentration on the GC-FID/NPD system showed that the nitrogen-containing compounds found in the CO_2 extract were not detected in the steam-distilled essential oil. Unfortunately, it is not possible to determine whether this is due to differences in the extraction process, the drying and preparation of the seeds, or the origin of the plant material. It is likely that these N-compounds do contribute to the odor as they are present at levels above their reported odor thresholds. The odors described above suggest that these N-compounds contribute to what is described in perfumery terms as the "natural and rounded" character of the ambrette extract, as compared to synthetic ambrettolide.

ACKNOWLEDGMENT

We thank Gerhard Brunner for NMR analysis at the research facility of Givaudan Schweiz AG in Dübendorf, and the analytical group at the Ashford research facility of Givaudan UK Ltd. for GC-MS and NMR analyses as well as helpful discussions.

LITERATURE CITED

(1) Breme, K.; Fernandez, X.; Meierhenrich, U. J.; Brevard, H.; Joulain, D. Identification of new, odor-active thiocarbamates in cress extracts and structure-activity studies on synthesizedhomologues. *J. Agric. Food Chem.* **2007**, *55*, 1932–1938.

(2) Boelens, M. H.; Gemert, L. J. Characterization and sensory properties of volatile nitrogen compounds from natural isolates. *Perfum. Flavor.* **1994**, *19*, 51–65.

(3) Clery, R.; Hammond, C.; Wright, A. Nitrogen-containing compounds in black pepper oil (*Piper nigrum* L.). *J. Essent. Oil Res.* **2006**, *18*, 1–3.

(4) Clery, R.; Hammond, C.; Wright, A. Nitrogen compounds from Haitian vetiver oil. *J. Essent. Oil Res.* **2005**, *17*, 591–592.

(5) Naef, R.; Velluz, A.; Mayenzet, F.; Starkenmann, C.; Sun, H. D. Volatile constituents of *Semnostachya menglaensis* Tsui. *J. Agric. Food Chem.* 200*553*, 9161–9164.

(6) Joulain, D.; Casazza, A.; Laurent, R.; Portier, D.; Guillamon, N.; Pandya, R.; Le, M.; Viljoen, A. Volatile flavor constituents of fruits from southern Africa: Mobola Plum (*Parinari curatellifolia*). *J. Agric. Food Chem.* **2004**, *52*, 2322–2325.

(7) Thomas, A.; Bassols, F. Occurrence of pyridines and other bases in orange oil. *J. Agric. Food Chem.* **1992**, *40*, 2236–2243.

(8) Arctander, S. *Perfume and Flavor Materials of Natural Origin*; Arctander: Elizabeth, NJ, 1960; pp 58−60.

(9) Grieder, A.; Maurer, B. (*Z*)-5-Tetradecen-14-olide, a new macrocyclic lactone, and two unsaturated straight chain acetates from ambrette seed absolute. *Helv. Chim. Acta* **1977**, *60*, 1155–1160.

(10) Rout, P. K.; Rao, Y. R.; Jena, K. S.; Sahoo, D.; Mishra, B. C. Extraction and composition of essential oil of ambrette (*Abelmoschus moschatus*) seeds. *J. Essent. Oil Res.* **2004**, *16*, 35–37.

(11) Cravp, L.; Perineau, F.; Gaset, A. Study of the chemical composition of the essential oil, oleoresin and its volatile product obtained from ambrette (*Abelmoschus moschatus* Moench) seeds. *Flavour Fragrance J.* **1992**, *7*, 65–67.

(12) Tang, Y. J.; Zhou, T. S.; Ding, J. K.; Sun, H. D. The chemical constituents of the essential oil from ambrette seeds. *Acta Bot. Yunnanica* **1990**, *12*, 113–114.

(13) Buil, J. P.; Laurent, R.; Fourniol, J. Ph.; Joulain, D.; Hardy, P.-Y. Composition chimique de l'huile essentielle de graine d'ambrette. *Parfum., Cosmet., Aromes* **1989**, *10*, 95–96.

(14) National Institute of Standards and Technology. Mass Spectral Search Program, version 2.0d; NIST: Gaithersburg, MD, 2005.

(15) Adams R. P. *Identification of Essential Oil Components by Gas Chromatography Quadrupole Mass Spectroscopy*; Allured Publishing Corporation: Carol Stream, IL, 2001.

(16) Wiley Register of MS Data.

(17) Van den Dool, H.; Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* **1963**, *II*, 463–471.

(18) Pittet, A. O.; Hruza, D. E. Comparative study of flavor properties of thiazole derivatives. *J. Agric. Food Chem.* **1974**, *22*, 264–269.

(19) Flament, I. *Coffee Flavor Chemistry*; John Wiley & Sons, Ltd.: West Sussex, England, 2001; pp 284−328.

Received for review March 27, 2008. Revised manuscript received June 17, 2008. Accepted June 21, 2008. This study was carried out with funding for Zhizhi Du from the European Community's Sixth Framework Programme under the Marie-Curie Action Incoming International Fellowship (MC-IFF-039253).

JF800958D