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Qualitative and Quantitative Analyses of the Essential Oils of Red and Ladino White Clovers

Takayasu Kami

The essential oils of red and Ladino white clovers were respectively isolated by steam distillation of the fresh grasses with yields of 0.0005 and 0.0032%. The essential oils were analyzed by combined gas chromatography–mass spectrometry and gas chromatographic comparison with authentic specimens, directly or after the fractionation to each functional group, to identify, respectively, about 80 compounds consisting of acids, phenol, aldehydes, ketones, alcohols, esters, hydrocarbons, and miscellaneous. Both essential oils showed some differences in the components of aldehydes, alcohols, and esters. Quantitative analyses were further carried out on the essential oils: red clover was rich in hydrocarbons, while Ladino white clover was rich in alcohols and esters.

Under the assumption that the aromas of forages might have some connection with the palatability or herbage intake of domestic animals, investigations of the aromatic constituents of forage crops were undertaken. In the present work, the essential oils of red and Ladino white (ladino) clovers, which are leguminous pasture plants, were analyzed by means of combined gas chromatography–mass spectrometry and gas chromatographic comparison with authentic specimens, in connection with the previous papers (Kami, 1975, 1977) on the analyses of the essential oils of Hybridsorgo and Sudangrass, which are gramineous forage plants.

Since the different clovers (*Trifolium*) are very important for the nutrition of domestic animals, many agricultural species of clovers are widely cultivated in the world. In the southwestern warm district of Japan, red clover (*Tr. pratense* L.) and ladino clover (*Tr. repens* L., giant or mammoth white clover) are especially cultivated as the major roughage of dairy cattle. This is due to the adaptation to the climate and soil of this district, to the suitability for the mixed sowing with forage grasses, and to the superiority in their palatability for dairy cattle.

EXPERIMENTAL SECTION

Materials. Red clover (Kenland-early variety) and ladino clover were cultivated on a farm of the Faculty of Fisheries and Animal Husbandry, Hiroshima University. The former was harvested in May 1971 and the latter in July 1974 by mower. The harvest times corresponded to the first flowering stage of each clover.

Isolation of the Essential Oils. The fresh crops (red, 260 kg; ladino, 170 kg) were, respectively, steam distilled in 26–30-kg lots under 0.8 kg/cm^2 pressure for 1 h using a boiler and sterilization kettle in a cannery of the Faculty of Fisheries and Animal Husbandry, Hiroshima University. The distillates were collected into a series of three traps cooled with water, ice–water, and dry ice–methanol; in total, red clover and ladino clover gave, respectively, about 130 and 100 L of cloudy aqueous liquids in the first trap, 26.2 and 19.2 g of colorless aqueous liquids in the second trap, and 15.4 and 0.5 g of colorless aqueous liquids in the third trap. Each cloudy aqueous liquid collected in the water-cooled trap was saturated with sodium chloride and extracted twice with distilled diethyl ether to yield a dark-brown essential oil with an unpleasant bitter odor (red, 1.25 g, pH 2.0; ladino, 5.4 g, pH 2.0). These essential oils were stored in sealed tubes at 3 °C, as were the aqueous condensates from the second and third traps of both clovers.

Fractionation of the Essential Oils. A portion (red, 670 mg; ladino, 2990 mg) of each essential oil was sequentially shaken with 10% sodium carbonate, 3% sodium hydroxide, and 3% hydrochloric acid aqueous solutions to separate it into acid (brown viscous; red, 30 mg; ladino, 384 mg), phenolic (orange viscous; red, 51 mg; ladino, 65 mg), and basic (drab viscous; red, 24 mg; ladino, 92 mg) fractions (Kami, 1975). Among them, the acid fraction was further converted to the methyl esters with diazomethane (Vorbeck et al., 1961). The remaining neutral oil layer (red, 345 mg; ladino, 1821 mg) was extracted in *n*-pentane and then diethyl ether with silicic acid to separate it into nonpolar (white crystal; red, 19 mg; ladino, 186 mg) and polar (yellow brown liquid; red, 105 mg; ladino, 907 mg) fractions (kami, 1977).

Analyses of the Essential Oils. In the beginning, the unfractionated essential oils of both clovers were injected to a combined apparatus of a Hitachi K53 gas chromatograph with a Carbowax 20M column and a Hitachi RMU-6E mass spectrometer, and the mass spectra of all components were taken (GC–MS). In addition, the fractions of red clover oil, except for the basic fraction, were

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Table I. Analyses of the Essential Oils of Red and Ladino Clovers[a]

compound	fraction detected	red clover, %	ladino clover, %
ethyl formate (T)	H	0.1	0.3
methyl acetate	W_2, H	1.2	4.5
ethyl acetate	W_1, W_2, A, O, H	Tr	Tr
isovaleraldehyde	W_2, H	0.7	4.2
ethanol	W_1, W_2, A, O, H	1.3	1.2
methyl *n*-butyrate	W_2		Tr
methyl 2-methylbutyrate	W_2		Tr
methyl isovalerate	W_2		
ethyl *n*-butyrate	W_2		1.4
ethyl 2-methylbutyrate	W_1, W_2	Tr	0.9
ethyl isovalerate	W_1, W_2	Tr	1.2
n-hexanal	W_1, W_2	0.1	0.1
isoamyl acetate	W_1, W_2	Tr	0.2
ethyl *n*-valerate	W_1, W_2	0.1	0.2
n-amyl acetate	W_1, W_2	Tr	Tr
methyl caproate	W_2		Tr
n-heptanal	W_1	Tr	
3-methyl-1-butanol	W_1, W_2	0.1	0.3
ethyl caproate	W_1, W_2	Tr	0.9
3-octanone	W_2, O	Tr	0.7
p-cymene	W_2		Tr
n-hexyl acetate	W_1, W_2	Tr	Tr
n-octanal	W_1	Tr	
cis-3-hexen-1-yl acetate	W_1, P, O	Tr	
ethyl oenanthate	W_1, W_2	Tr	Tr
n-tridecane	N	Tr	Tr
n-hexanol	W_1	0.2	
cis-3-hexen-1-ol	W_1	0.2	
1-octen-3-yl acetate	W_1, W_2	Tr	1.9
n-nonanal	W_1	0.6	
acetic acid	W_2, P, O	0.1	1.6
1-octen-3-ol	W_1, W_2, O	0.8	7.3
furfural	W_2		
n-tetradecane	N		
ethyl caprylate	W_1, W_2	Tr	0.6
n-octyl acetate	W_2		0.4
methyl pelargonate	W_2		
benzaldehyde	W_1, W_2, P, O	0.3	3.5
ethyl pelargonate	W_1, W_2, O	0.8	0.4
n-pentadecane	N		
diethyl malonate	W_2		Tr
phenylacetaldehyde	W_1, W_2	0.3	1.1
ethyl benzoate	W_1, W_2	0.3	0.7
diethyl succinate	W_2		
n-hexadecane	N		
ethyl caprate	W_1, W_2, O	0.4	0.4
cis-3-hexen-1-yl caproate	W_1, O	0.3	
benzyl acetate	W_1, W_2, P, O	0.4	0.4
methyl phenylacetate	W_2	Tr	0.3
n-heptadecane	N		
ethyl phenylacetate	W_1, W_2	Tr	0.9
phenethyl acetate	W_1, W_2, O	1.4	0.1
benzyl alcohol	W_1, P	1.4	0.7
n-octadecane	N		
ethyl laurate	W_1, W_2	2.3	2.7
n-nonadecane	N		
phenol	P	0.9	1.4
methyl eugenol	W_1, W_2, O	0.1	0.9
n-eicosane	N	0.1	0.2
ethyl myristate	W_1, W_2, O	1.1	0.4
6,10,14-trimethylpentadecan-2-one	W_1, W_2, O	2.1	3.2
n-heneicosane	N		
ethyl pentadecanoate	W_1, W_2	0.1	0.4
ethyl palmitate	W_1, W_2, O	2.3	3.5
n-docosane (T)	N		
n-tricosane	W_1, N	0.6	0.2
indole	W_1, O	0.3	
n-tetracosane (T)	N	1.0	0.1
ethyl stearate	W_1, W_2, O	1.7	0.2
ethyl oleate	W_1, O	1.1	0.4
ethyl linoleate	O	0.7	0.6
phytol	O		
n-pentacosane	W_1, N	2.9	0.2
n-hexacosane (T)	N	0.3	0.1
n-heptacosane	W_1, N	3.3	0.8
n-octacosane	W_1, N	1.1	0.1
n-nonacosane	W_1, N	22.7	1.4

Table I (*Continued*)

compound	fraction detected	red clover, %	ladino clover, %
2-methylbutyric acid	A	0.2	Tr
caproic acid	A	1.1	0.4
oenanthic acid	A	0.3	Tr
caprylic acid	A	0.1	0.3
pelargonic acid	A	1.3	0.3
benzoic acid	A	1.9	6.6
capric acid	A		
undecanoic acid	A	0.1	0.1
p-toluic acid	A	Tr	Tr
phenylacetic acid	A	0.5	1.1
salicylic acid	A	0.1	Tr
lauric acid	A	0.1	0.2
tridecanoic acid (T)	A	Tr	Tr
myristic acid	A	0.3	0.2
anisic acid	A	0.3	0.4
pentadecanoic acid	A	0.1	0.2
palmitic acid	A	0.6	1.2
heptadecanoic acid (T)	A	0.1	0.1
veratric acid	A	0.5	1.6
stearic acid	A	0.7	0.9
oleic acid	A	0.5	0.2
triethylamine (T)	B	9.4	5.7
isobutylamine (T)	B	0.5	0.3

[a] W_1, unfractionated essential oil of red clover; W_2, unfractionated essential oil of ladino clover; A, P, N, O, and B, methylated acid, phenolic, nonpolar, polar, and basic fractions of red clover; H, headspace vapors of ice-water cooled traps of red and ladino clovers; (T), tentative. Percentage compositions of low-boiling compounds, acids, amines, and remaining compounds were calculated from GC of Carbowax 1500, Carbowax 20M, Triethanolamine, and Carbowax 20M columns, respectively.

also analyzed by GC–MS, and further identity of the assigned compounds was performed by gas chromatographic comparison with authentic samples on an FID-type Yanagimoto GCG-550T gas chromatograph with a Carbowax 20M column (programmed temperature GC). However, the component identification in the ladino clover fractions was carried out only by gas chromatographic comparison with the red clover data, because they had a strong resemblance to each other. In these GC–MS and programmed temperature GC, diethyl ether was used as injection solvent, and the operating conditions were the same as those of the previous papers (Kami, 1975, 1977). The basic fractions of both clovers were heated with 2 N solution of sodium hydroxide, and the regenerated gases of amines were analyzed by isothermal GC with a Triethanolamine column at 65 °C (Kami, 1975). For the analysis of low-boiling compounds, the headspace vapors in ice–water and dry ice cooled traps of both clovers were directly chromatographed on the same procedure as previously reported (Kami et al., 1972).

Percentage Compositions of the Essential Oils. Percentage compositions of the essential oils were calculated according to the procedure previously described (Kami, 1977). The relative peak areas in GC of the unfractionated essential oils, acidic fractions (after methylation), and basic fractions (after regeneration of amines) of both clovers were calculated from the paper cutout weights of the peaks in each chromatogram, because acids and amines could not be analyzed by programmed temperature GC of the unfractionated essential oils. The relative peak areas in each chromatogram were then multiplied by the yield percent of the corresponding fractions, which was 13, 10, and 77%, respectively, for the acidic, basic, and the remaining fractions of red clover oil, while 23, 6, and 71%, respectively, for those of ladino clover oil. Among the low-boiling compounds, only ethyl formate could not be detected by programmed temperature GC of the unfractionated essential oils, and so its percentage composition was calculated on the basis of the relative areas of the ethanol peaks in analyses of the headspace vapors of ice–water cooled traps. These were 1.3 and 1.2%, respectively, in the essential oils of red and ladino clovers.

RESULTS AND DISCUSSION

Identification of the Components in the Essential Oils. The first crops of red and ladino clovers were, respectively, distilled with steam to collect the essential oils. When the essential oils were subjected to GC–MS without any fractionation, red clover oil exhibited 73 peaks and ladino clover oil 74 peaks on the GC. Among them, 45 compounds [six aldehydes, one ketone, six alcohols, 16 ethyl esters, eight acetates, five hydrocarbons (C_{23}–C_{29}), two miscellaneous (methyl eugenol and indole), and *cis*-3-hexen-1-yl caproate] or 46 compounds [five aldehydes, two ketones, three alcohols, 16 ethyl esters, two diethyl esters, nine acetates, six methyl esters, one hydrocarbon (*p*-cymene), one miscellaneous (methyl eugenol), and acetic acid], which are indicated by W_1 or W_2 in Table I, were, respectively, assigned from red clover oil or ladino clover oil through comparison of mass spectra with those of authentic specimens and/or with authentic spectra (Stenhagen et al., 1969). The other peaks, however, could not be assigned because their mass spectra were those of mixed state.

For the purpose of more precise analysis, the essential oils were fractionated into acidic, phenolic, basic, nonpolar, and polar fractions by combining usual chemical procedure and adsorption method with silicic acid. Regarding the red clover oil, the other fractions except the basic fraction were analyzed directly or via methylation by GC–MS and gas chromatographic comparison with authentic samples, and 3-octanone, nine *n*-alkanes from C_{13} to C_{21}, phenol, ethyl linoleate, phytol, 14 aliphatic acids from C_2 to C_{18}, and six aromatic acids were additionally identified apart from the compounds identified in the unfractionated essential oil (A, P, N, and O in Table I). However, three missing *n*-alkanes (C_{22}, C_{24}, and C_{26}) and two missing aliphatic acids (C_{13} and C_{17}) in the GC–MS of the fractions of red clover oil could be detected, respectively, as small peaks in the programmed temperature GC of the nonpolar

Table II. Relative Amounts of Functional Groups in the Essential Oils of Clovers and Sorghums

forage	hydrocarbons	oxygen-containing compounds							amines	miscellaneous
		aldehydes	ketones	alcohols	esters	acids	phenols	total		
red clover	32	3	2	4	14	9	1	33	10	0.4
ladino clover	4	8	4	11	24	14	1	62	6	1
Hybridsorgo	5	10	6	5	12	19	24	76	10	1
Sudangrass	5	21	11	6	11	12	5	66	8	2

and acid fractions. The basic fraction of red clover oil was heated with alkali, and the regenerated gas was examined by isothermal GC to tentatively identify triethylamine and isobutylamine (B in Table I). In addition, the headspace vapors of the ice–water and dry ice cooled traps of both clovers were analyzed by isothermal GC with a Carbowax 1500 column in order to characterize the top note of the essential oils, and only ethyl formate was tentatively identified (H in Table I). The fractions of ladino clover oil resembled closely the corresponding fractions of red clover oil in the GC pattern, and so they were analyzed by GC alone using the fractions of the red clover oil as a standard.

Thus, it was confirmed that the essential oils bore some differences in a small amount of components: red clover oil lacked in six methyl esters, ethyl *n*-butyrate, *p*-cymene, furfural, *n*-octyl acetate, two diethyl esters, while ladino clover oil lacked in *n*-alkanals (C_7–C_9), *n*-hexanol, *cis*-3-hexen-1-ol (leaf alcohol), its acetate, its caproate, and indole. Since Takei et al., who are the pioneers in the chemical investigation of leaf alcohol, have stated that the concentration of the alcohol in the essential oil of white clover decreased in summer (Takei et al., 1938), the lack of leaf alcohol and its esters in ladino clover oil may be attributable to the harvest time of July. Nevertheless, this is the first time that *cis*-3-hexen-1-yl caproate [mass: *m/e* 82 (100), 43 (86), 67 (64), 41 (51), 55 (48), 29 (31), 71 (29), 198 (trace, M^+)] was identified in the plant. On the other hand, in comparison of the components of clovers and sorghums, many esters, alkanals, and alkenols were detected in clovers, while many phenols, methyl ketones, and amines were detected in sorghums.

Percentage Compositions of the Essential Oils. Percentage compositions of the essential oils of both clovers were subsequently calculated to clarify the aroma character, and the results are listed in Table I, in which the compounds are arranged in the order appearing in programmed temperature GC.

The compounds containing more than 3% of essential oil were *n*-heptacosane, *n*-nonacosane, and triethylamine in red clover oil, while in ladino clover oil, methyl acetate, isovaleraldehyde, 1-octen-3-ol, benzaldehyde, 6,10,14-trimethylpentadecan-2-one, ethyl palmitate, benzoic acid, and triethylamine contained more than 3%. It can be said that red clover oil is relatively rich in hydrocarbons, while ladino clover oil is relatively rich in oxygen-containing compounds. When the percentage compositions of the compounds were readjusted according to the functional groups, the finding became more evident as shown in Table II. Furthermore, the essential oil of ladino clover was characterized by high contents of alcohols and esters. Accordingly, the difference in the essential oils was especially obvious in their content of hydrocarbons, alcohols, and esters. Since the stems of red clover are terrestrial and those of ladino clover are stoloniferous, it was assumed that much stems admixed in the material of red clover when they were harvested by mower. This may be the reason that red clover oil was rich in hydrocarbons. On the other hand, Hybridsorgo oil was relatively rich in phenols, while Sudangrass oil was relatively rich in carbonyl compounds (Table II).

In Japan it is generally believed that clovers are superior to sorghums in their palatability for dairy cattle and that Sudangrass is superior to Hybridsorgo. Such qualitative and quantitative differences of the aromatic constituents seem to have some connection with their palatability for dairy cattle. However, further investigation of the relation between the odor of forages and its palatability for domestic animals is necessary.

CONCLUSION

From the essential oil of red clover, 78 compounds including 14 hydrocarbons, six aldehydes, two ketones, seven alcohols, 26 esters, 20 acids, phenol, and two miscellaneous were identified by GC–MS and GC, while from ladino clover, 80 compounds including 15 hydrocarbons, five aldehydes, two ketones, three alcohols, 33 esters, 20 acids, phenol, and one miscellaneous were identified by GC–MS and GC. In addition, ethyl formate, three *n*-alkanes, two aliphatic acids, and two amines were tentatively identified by GC alone. Two essential oils showed some differences between the compounds of aldehydes, alcohols, and esters. However, there was a remarkable difference between the percentage compositions: red clover was rich in hydrocarbons, while ladino clover was rich in alcohols and esters.

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