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COMPOSITION OF LEAF SURFACE WAXES OF *TRITICUM* SPECIES: VARIATION WITH AGE AND TISSUE*

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Key Word Index—*Triticum aestivum*; *T. durum*; Gramineae; wheat; spring, durum; leaf wax; GLC analysis of whole; variation in wax composition with age and part of plant.

Abstract—The composition of cuticular wax from plants of spring wheat (varieties Selkirk and Manitou) and of durum wheat (variety Stewart 63) at various stages of growth, and of wax from different parts of the plants varies considerably. Wax was analysed, without preliminary separation, by GLC using Dexsil 300 as liquid phase. Alcohols are major components of wax from leaf blades and β-diketones are major components of wax from leaf sheaths, especially the flag leaf sheath. Glaucousness of the leaf sheath is due to the high β-diketone content. In the first 50 days after germination, before sheaths and flag leaf are completely developed, the major component is octacosanol (> 50%). At 66 days, when sheath development is complete, β-diketone content is greatest. Hydrocarbon composition differs for wax from leaf blade and leaf sheath and also for different leaf blades and between adaxial and abaxial sides of the flag leaf. From 66 to 100 days ester content of wax increases, especially in Selkirk wheat, apparently due to formation of wax containing high proportions of esters of *trans*-α,β-unsaturated C_{22} and C_{24} acids. The content of these acids in the free fatty acids and of diesters based on these acids also increases during this period.

INTRODUCTION

THE COMPOSITION of wax on the surface of durum and spring wheat plants was determined previously.[1,2] In these investigations plants were cut when the flag leaf was fully developed but before the heads were completely formed and wax was collected from whole plants by washing with hexane. As reported by Troughton and Hall,[3] there are, however, visible differences between blade and sheath of wheat leaves; the sheath generally has a waxy and glaucous look but the blade does not. Electron microscopic examination showed that wax was also present on the blade but usually in the form of platelets, wax on the sheath appeared as rodlets and the appearance varied with the stage of growth. These observations indicated considerable differences in chemical composition of wax from different parts of the wheat plant. Barber and Netting[4] suggested that β-diketones were responsible for glacucousness in wheat and similar conclusions were reached for barley[5] and other plants.[6]

To investigate these possibilities and to find out which components took the place of β-diketones in wax from non-glaucous parts of the plant, wax was collected from wheat plants at weekly intervals from young to ripe plants (24- to 100-days-old) and also from the flag and other leaf blades and from the corresponding leaf sheaths. Durum wheat variety Stewart 63 and spring wheat varieties Selkirk and Manitou were used.

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[*] NRCC No. 13175.

[1] TULLOCH, A. P. and HOFFMAN, L. L. (1971) *Phytochemistry* **10**, 871.

[2] TULLOCH, A. P. and HOFFMAN, L. L. (1973) *Phytochemistry* **12**, 2217.

[3] TROUGHTON, J. H. and HALL, D. M. (1967) *Australian J. Biol. Sci.* **20**, 509.

[4] BARBER, H. N. and NETTING, A. G. (1968) *Phytochemistry* **7**, 2089.

[5] WETTSTEIN-KNOWLES, P. VON (1972) *Planta* **106**, 113.

[6] HALL, D. M., MATUS, A. I., LAMBERTON, J. A. and BARBER, H. N. (1965) *Australian J. Biol. Sci.* **18**, 323

RESULTS

A GLC method of analysing the whole wax at once was necessary to deal with the large number of samples. The carborane–siloxane liquid phase Dexsil 300 gave very sharp peaks and resolved the major components of hydrocarbons, free alcohols (as acetates), free acids (as methyl esters), long chain esters and diesters. Dexsil 300 has also been used in analysis of whole beeswax,[7] but leaf wax analysis is complicated by the presence of free alcohols.

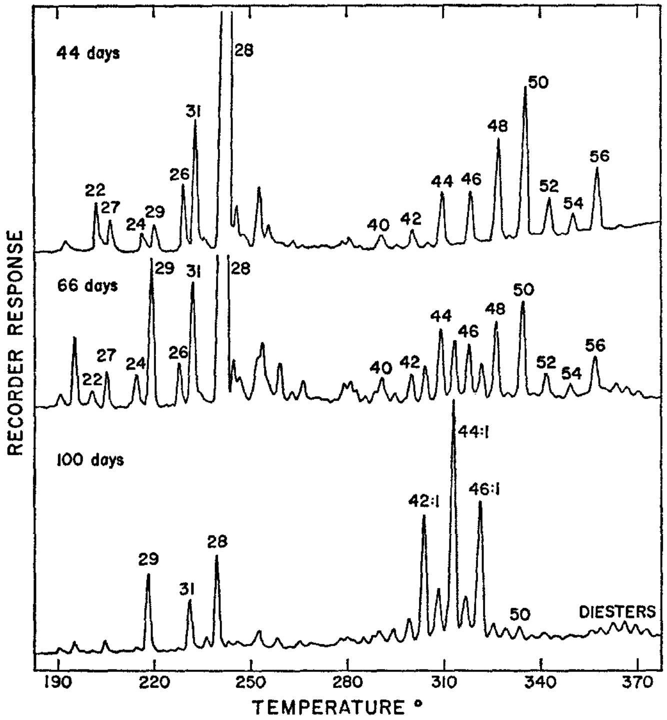

FIG. 1. GLC SEPARATIONS OF LEAF WAX (AFTER ACETYLATION) OF SELKIRK VARIETY OF SPRING WHEAT COLLECTED AT 44, 66 AND 100 DAYS AFTER GERMINATION.

Peaks with even numbers from 22 to 28 are free alcohol acetates, peaks with odd numbers from 27 to 31 are hydrocarbons, peaks with even numbers from 40 to 56 are long chain esters and peaks with numbers 42:1, 44:1 and 46:1 are long chain esters containing *trans* 2,3-unsaturation.

β-Diketones gave peaks in the region of C_{30}–C_{33} methyl esters but the relative response was variable and hydroxy β-diketones were not always separated from the unsubstituted compounds. Free alcohols gave a poor response but could be analysed satisfactorily as acetates. Acetylation of the whole wax caused almost complete disappearance of β-diketone peaks. β-Diketones were therefore estimated by UV spectroscopy[8] before GLC analysis.

Typical GLC separations are shown in Fig. 1. Methyl esters (absent from Fig. 1) appear before acetates of alcohols with the same chain length as the acids. Acetates are followed by hydrocarbons with 5 more carbons. Methyl esters of *trans* 2,3-unsaturated acids coincide with acetates of alcohols of the same chain length. Residual β-diketone peaks prevent

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[7] TULLOCH, A. P. (1972) *J. Am. Oil Chemists' Soc.* **49**, 609.

[8] HORN, D. H. S., KRANZ, Z. H. and LAMBERTON, J. A. (1964) *Australian J. Chem.* **17**, 464.

TABLE 1. VARIATION IN COMPOSITION OF LEAF WAX OF SELKIRK WHEAT WITH AGE AND PART OF PLANT

Days from germination	Feekes-Large scale	Wax %*	Hydro-carbons†	Esters†	Alcohols†	Diesters†	β-Di ketones‡
24	5	0·7	3	11	49	—	2
37	7	0·5	3	11	55	—	6
44	8	0·4	4	10	53	—	4
52	9	0·3	4	12	45	—	13
66	10·1	0·5	6	13	22	—	23
80	11·1	0·4	6	18	17	3	19
93	11·3	0·4	5	21	8	5	15
100	11·4	0·4	4	39	4	9	13
Part of plant							
Leaf 1 (flag)§			5	5	25	—	12
2			7	14	49	—	9
3			4	13	42	—	5
Sheath 1			8	3	1	—	65
2			15	4	2	4	38
3			6	4	3	2	26

* As % of dry wt.

† Wt % by GLC.

‡ From $E^{1\%}_{1\mathrm{cm}}$ at 273 nm assuming pure β-diketone = 260.

§ Leaves numbered with last formed (flag leaf) as 1.

estimation of C_{30} alcohol but this is always a very minor component. The lower curves in Fig. 1 show that long chain esters of *trans* 2:3-unsaturated acids are completely separated from saturated esters by Dexsil 300 (silicone SE-30 gives only partial separation of these components[2]). Unsaturated esters appear in the positions of odd-numbered esters but re-analysis after hydrogenation confirmed that they were unsaturated.

TABLE 2. VARIATION IN COMPOSITION OF LEAF WAX OF MANITOU WHEAT WITH AGE AND PART OF PLANT

Days from germination	Feekes-Large scale	Wax %*	Hydro-carbons†	Esters†	Alcohols†	Diesters†	β-Di-ketones†
24	5	0·7	2	12	40	—	2
37	7	0·5	6	9	56	—	2
44	8	0·5	7	14	53	—	4
52	9	0·4	9	13	39	—	15
66	10·1	0·5	8	13	19	—	29
80	11·1	0·4	8	13	21	—	20
93	11·3	0·5	9	19	11	2	16
100	11·4	0·4	8	24	5	4	19
Part of plant							
Leaf 1 (flag)§			10	10	22	—	23
2			6	9	36	—	21
3			4	12	45	—	9
Sheath 1			4	1	1	—	57
2			16	2	1	2	54
3			25	4	6	5	23

For footnotes see Table 1.

Two internal standards were used to estimate amounts of each class of components; hydrocarbons were compared to *p*-dioctylbenzene, and acetates and esters to octadecyl octadecanoate. Wax collected from ripe Selkirk wheat (after 100 days) was also analysed by silicic acid column chromatography and the results agreed well with those of GLC analysis.

TABLE 3. VARIATION IN COMPOSITION OF LEAF WAX OF STEWART 63 WHEAT WITH AGE AND PART OF PLANT

Days from germination	Feekes-Large scale	Wax %*	Hydro-carbons†	Esters†	Alcohols†	Diesters†	β-Di-ketones‡
24	5	0·6	5	9	58	—	3
37	7	0·5	4	11	60	—	6
44	8	0·5	5	13	71	—	10
52	9	0·4	6	13	65	—	12
66	10·1	0·5	5	7	21	—	54
80	11·1	0·4	4	5	13	—	48
93	11·3	0·5	6	9	18	—	31
100	11·4	0·4	6	12	6	3	40
Part of plant							
Leaf 1 (flags)§			11	12	36	—	27
2			8	13	44	—	19
3			8	13	44	—	12
Sheath 1			3	5	1	—	74
2			4	3	5	—	74
3			8	3	3	—	57

For footnotes see Table 1.

Figure 1 also shows the differences in wax composition of three stages of Selkirk wheat. Variations in composition of waxes collected at different stages of growth (indicated on the Feekes-Large scale[9]) and from different parts of the plant are shown in Tables 1–3 for Selkirk, Manitou and Stewart 63 respectively. In most cases results obtained with the three wheat varieties were similar to each other, though sometimes wax of one variety varied to a greater extent than wax of another. The variation in composition of hydrocarbons, alcohols and esters is illustrated by certain Selkirk samples in Tables 4–6.

TABLE 4. VARIATION IN COMPOSITION OF HYDROCARBONS OF SELKIRK WHEAT LEAF WAX

Chain length	Days from germination			Leaf		Leaf 1		Sheath	
	24	66	100	1	3	Adaxial	Abaxial	1	3
27	18	10	8	9	12	11	10	6	6
29	4	38	50	41	19	31	51	59	59
31	52	41	38	42	51	45	35	31	32
33	26	11	4	8	18	13	4	4	3

Hydrocarbon contents of wax from whole plants of the three varieties were all below 10% but composition changed gradually from major C_{31} before 66 days to major C_{29} after 66 days (Table 4). Hydrocarbon content of wax from leaf blades was similar except in Manitou

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[9] LARGE, E. C. (1954) *Plant Pathology* 3, 128.

where it decreased from flag leaf to third last leaf. In flag leaf wax the major hydrocarbon was C_{29} but in wax of the third last leaf it was C_{31} (except in Stewart 63 in which C_{31} increased but did not exceed C_{29}). Hydrocarbon compositions of wax from the adaxial and abaxial sides of flag leaf were different; C_{29} was the major component of the abaxial and C_{31} of the adaxial. There were differences in hydrocarbon content in sheath wax; in Selkirk it increased from the flag sheath to the second last sheath then fell, in Manitou it rose from last to third last, as high as 25%, and in Stewart 63 there was a slight increase. However, for all varieties and each sheath, C_{29} was the major component.

TABLE 5. VARIATION IN COMPOSITION OF ALCOHOLS OF SELKIRK WHEAT LEAF WAX

Chain	Days from germination			Leaf		Leaf 1		Sheath	
length	24	66	100	1	3	Adaxial	Abaxial	1	3
22	—	1	1	—	—	—	—	—	—
24	—	3	3	1	1	1	2	47	9
26	2	3	4	4	3	3	2	7	10
28	98	93	92	95	96	96	96	46	81

Ester content of each variety was fairly constant for the first 80 days. During the last 20 days ester content of Selkirk wax doubled to 39% and that of Manitou wax increased to 24% but that of Stewart 63 wax increased only slightly. There was also a change in composition of the esters. In Selkirk unsaturated esters increased to over 80% (of total esters, Table 6). In Manitou they increased to 65% and in Stewart 63 to 15%. These esters were also present in wax collected at 66 days.[2] NMR spectroscopy showed that about 35% of the alcohols of esters of Selkirk wax collected at 100 days consisted of *trans* 2-docosen-1-ol.[2]

TABLE 6. VARIATION IN COMPOSITION OF LONG CHAIN ESTERS OF SELKIRK WHEAT LEAF WAX

Chain	Days from germination			Leaf		Leaf 1		Sheath	
length	24	66	100	1	3	Adaxial	Abaxial	1	3
38	—	1	—	—	14	—	—	—	15
38:1	—	—	1	—	—	—	—	—	—
40	2	5	1	4	9	2	6	13	17
40:1	—	—	1	—	—	—	—	—	—
42	2	6	2	4	2	4	7	15	6
42:1	—	6	16	—	—	—	—	—	—
44	7	12	5	19	8	14	17	34	14
44:1	—	9	38	—	—	—	—	—	—
46	31	8	5	17	7	10	9	22	12
46:1	—	4	27	—	—	—	—	—	—
48	15	12	1	21	16	14	12	14	9
48:1	—	—	1	—	—	—	—	—	—
50	20	16	1	22	21	25	13	2	7
52	5	4	1	5	6	7	5	—	—
54	5	3	—	2	4	7	6	—	5
56	13	10	—	5	11	13	20	—	10
58	—	2	—	1	2	2	5	—	5
60	—	2	—	—	—	1	—	—	—

Waxes of the last three leaves, including both sides of the flag leaf, were similar in ester content and composition. Ester content of sheaths was generally below 5% and amounts of

C_{38}–C_{48} esters were greater than in wax from leaf blades. Very little unsaturated esters were found in waxes from blades or sheaths of the three last-formed leaves. Esters of wax collected from the lower parts of plants at 66 days, including shrivelled remains of the fourth and fifth last leaves, contained appreciable amounts of unsaturated esters and these parts were presumably the source of unsaturated esters found in wax of 66 day plants.

Content of diesters, which are diol esters of *trans* 2,3-unsaturated acids,[10] varied in the same way and was only appreciable in wax of 100-day-old Selkirk. Free acid content was determined for some samples and was generally about 5%. Wax from young plants contained major proportions of C_{16} and C_{28} acids but older plants contained major amounts of *trans* 2-docosenoic and tetracosenoic acids and much less C_{28}.

In each variety alcohol content was very high (40–60%) up to 52 days then fell sharply to about 20%, the fall coinciding with a marked increase in β-diketone content. Alcohol content then fell more slowly to about 5% in wax of 100-day-old plants. Wax of the flag leaf contained appreciably less alcohol than that of the second and third last leaves, also wax from the adaxial surface of the flag leaf contained 3 times as much alcohol (45:15%) as did wax from the abaxial surface. Octacosanol was the major component of these samples. Alcohol contents of sheath waxes were extremely low and the percentage of tetracosanol was in most cases much higher than in alcohols of leaf blade waxes.

The percentage of β-diketones (including hydroxy β-diketones) was very low at first but later rose rapidly to a maximum at 66 days and then fell again to an intermediate value. The proportion of hydroxy β-diketones, which had to be determined by silicic acid column chromatography, was only measured for samples isolated from the three varieties at 100 days, and was similar to that previously measured in large samples collected at about 66 days.[1,2] It seems reasonable to assume that the composition of the β-diketones does not vary much.

β-Diketone content of wax from the flag leaf blade ranged from 12% (Selkirk) to 27% (Stewart 63) and was higher than that of the other two leaves.

Wax from flag leaf sheaths contained very high proportions of β-diketones, higher than that of sheath wax from older leaves. Glaucousness of the leaf sheath is no doubt due to high β-diketone content. Wax from immature heads collected at about 66 days contained 38% β-diketones.

DISCUSSION

Clearly change in composition of wax from whole plants with age is related to the stage of development of the plants, particularly to completion of development of flag leaf and of sheaths between 52 and 66 days. Thus the change from a major C_{31} hydrocarbon to a major C_{29} hydrocarbon after 66 days is due to the high percentage of C_{29} in sheath wax and to a lesser extent to the decreased percentage of C_{31} in flag leaf blade wax. Composition of hydrocarbons of leaf wax would not, therefore, be useful in wheat taxonomy. Comparable variations in hydrocarbon compositions of wax from different parts of a number of plants have been reported by Stránský *et al.*[11]

The large variations in the content of alcohols and β-diketones are remarkable. Until all leaves and sheaths are fully developed at 66 days there is an inverse relationship between the amounts of alcohols and β-diketones. At earlier stages (e.g. 44 days), when the sheaths are

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[10] TULLOCH, A. P. (1971) *Lipids* **6**, 641.

[11] STRÁNSKÝ, K., STREIBL, M. and HEROUT, V. (1967) *Coll. Czech. Chem. Commun.* **32**, 3213.

shorter and the flag leaf, which has the largest blade and a lower alcohol content, has not been formed, most of the wax comes from the second and third last leaves. In consequence the alcohol content is very high and β-diketone is very low. At 66 days the last leaf and the sheaths provide a greater proportion of the wax and β-diketone content is higher and alcohol content much lower. Decrease in alcohol content after 66 days to a very low value may be due partly to shrivelling and loss of leaf blades but since β-diketone content also decreases, wax synthesized in the last 30 days may have a different composition from that produced earlier (also see below).

These variations naturally suggest some biosynthetic relationship between alcohols and β-diketones.[4] The biosynthetic route to β-diketones has not yet been elucidated, but Kolattukudy[12] has suggested that tritriacontane-16,18-dione is formed by condensation of two C_{16} acid units with malonate followed by decarboxylation. This route to a C_{31} β-diketone, however, would require participation of both C_{14} and C_{16} units which is unlikely since no C_{29} or C_{33} β-diketones are found.

β-Diketones, like monoketones and paraffins,[13] are more probably formed from long chain precursors closely related to the biosynthesis of long chain acids. Routes to β-diketones and octacosanol could then diverge at a late stage, perhaps octacosanoic acid (a significant component of free acids at 66 days), which could also be reduced to octacosanol.[13]

The principal peculiarity of the esters is the gradual increase in esters of *trans* 2,3-unsaturated C_{22} and C_{24} acids which occurred in all 3 varieties but was particularly marked in Selkirk. These esters must be the result of formation of new wax with a different composition, even in the last 10 days. Ester content of wax from the sheaths was very low at 66 days but may be higher at 100 days since the sheaths must contribute more of the wax due to disintegration of the dry leaf blades. The increase in esters of *trans* 2-docosen-1-ol, free *trans* 2,3-unsaturated C_{22} and C_{24} acids and diesters also indicates production of new wax.

EXPERIMENTAL

Wax collection. Wheat plants were grown outside during May–August; plants were cut close to the ground at weekly intervals (heads were removed from mature plants) and wax was extracted with hexane as before.[14] Flag leaves and the 2nd and 3rd last leaves and their sheaths were collected when the flag leaf was fully developed (about 66 days) and extracted separately. Wax from abaxial and adaxial surfaces of flag leaves was collected by wiping with cotton wool (previously extracted with hexane) dipped in hexane.

Chromatography. GLC analyses were performed using a Hewlett-Packard model 402 gas chromatograph with flame ionization detectors. Columns were 1 m × 3 mm stainless steel packed with 60–80 mesh, acid washed and silanized Chromosorb W coated with 1·5% Dexsil 300 (Analabs, North Haven, Conn.). Temp. was programmed from 125 to 400° at 3°/min and flow rate was 60 ml He/min. Peaks were integrated with an Infotronics Model CRS-104 digital integrator. Wax (15–30 mg) was weighed out and 5 ml of a $CHCl_3$ soln containing about 1 mg each of *p*-dioctylbenzene and octadecyl octadecanoate was added. $CHCl_3$ was removed and the mixture acetylated with Ac_2O and pyridine (0·5 ml each) at 100°. Reagents were removed and the sample analysed by GLC as a $CHCl_3$ soln. In most cases peaks in the β-diketone region (equivalent to C_{30}–C_{33} methyl ester) were quite small but samples with very high β-diketone content, such as sheath wax or acetylated samples which had been kept for some months, sometimes gave tailing peaks which interfered with C_{36} internal standard. Satisfactory results were usually obtained after re-acetylation.

To estimate methyl esters part of the acetylated wax was treated with CH_2N_2 and re-analysed; in calculating methyl *trans* 2-docosenoate and tetracosenoate, allowance was made for the amounts of C_{22} and C_{24} acetates which have the same emergence temps.

The amount of hydrocarbon was calculated from the response relative to *p*-dioctylbenzene and the amounts of alcohols, acids, esters and diesters from the responses relative to octadecyl octadecanoate.

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[12] Kolattukudy, P. E. (1968) *Plant Physiol.* **43,** 1466.

[13] Kolattukudy, P. E. (1970) *Lipids* **5,** 259.

[14] Tulloch, A. P. and Weenink, R. O. (1969) *Can. J. Chem.* **47,** 3119.

Investigation, using internal standards and synthetic triacontane, methyl triacontanoate, triacontyl triacontanoate, and 1,10-decanediol didocosanoate, showed that correction factors were sometimes required. Columns prepared and used under apparently identical conditions required different factors. The column used in the present work did not require correction factors for hydrocarbons or saturated esters but comparison of the results before and after hydrogenation of ripe (100 day) Selkirk wax showed that a factor of 1·33 was required for unsaturated long chain esters and diesters. Since decanediol didocosanoate also required a factor of 1·33 a combined factor of 1·75 was necessary for unsaturated diesters.

Column chromatographic analysis of wax from ripe Selkirk wheat. Wax (5·6 g, collected after 100 days) was freed from β-diketones by treatment with copper acetate.[8] The copper complex (1·32 g), which also contained part of the free acids as copper salts, was recrystallized from hexane to remove co-precipitated material (0·33 g). The remaining uncomplexed wax (4·09 g) was chromatographed on 250 g of Biosil A. Elution with hexane gave hydrocarbons (0·3 g) and elution with hexane–CHCl$_3$ (19:1) gave esters (1·88 g) as 14 fractions. The earliest fractions contained saturated C_{42}–C_{52} esters and the later ones contained unsaturated C_{42}–C_{46} esters. Some of these fractions were used as a source of *trans* 2-docosen-1-ol.[2] Elution with hexane–CHCl$_3$ (4:1) gave unidentified material (0·20 g). Mixtures of diesters with alcohols and with free acids and unidentified components were eluted with hexane–CHCl$_3$ (13:7). The mixture of alcohols and diesters was acetylated and separated on silicic acid, acetates being eluted with hexane–Et$_2$O (99:1) and diesters with hexane–Et$_2$O (97:3). The mixture of free acids and diesters was treated with CH_2N_2 and separated as above (methyl esters eluted with hexane–Et$_2$O (99:1)). Free acids and β-diketones were recovered from the mixture of copper salts and copper complex and separated on silicic acid. β-Diketone (0·37 g) was eluted with hexane–Et$_2$O (49:1), free acids (0·29 g) were eluted with hexane–ether (9:1) and hydroxy-β-diketones (0·17 g) with hexane–Et$_2$O (3:1). Total weights of the components isolated in the above separations were: alcohols (0·22 g), acids (0·54 g) diesters (0·47 g) and unidentified (1·01 g). The percentage composition (GLC results in parentheses) was: hydrocarbons, 6 (4); esters, 37 (39); alcohols, 4 (4); acids, 10 (8); diesters, 9 (9); β-diketone, 7; hydroxy-β-diketones, 4; unidentified, 23. β-Diketones and hydroxy-β-diketones from wax of Manitou and Stewart 63 wheats collected at 100 days were isolated and separated and ratios of β-diketone to hydroxy-β-diketones were close to 2:1 (Manitou) and 4:1 (Stewart 63).

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