Pergamon

PII: S0031-9422(97)00272-0

Phytochemistry, Vol. 46, No. 1, pp. 83–96, 1997

0031-9422/97 $17.00+0.00

EFFECTS OF ENVIRONMENT ON THE COMPOSITION OF EPICUTICULAR WAX ESTERS FROM KALE AND SWEDE

T. SHEPHERD,* G. W. ROBERTSON, D. W. GRIFFITHS and A. N. E. BIRCH†

Department of Chemistry; †Department of Zoology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, U.K.

(*Received in revised form* 18 *February* 1997)

Key Word Index—*Brassica oleracea* var. *acephola*; kale; *B. napus* var. *rapifera*; swede; Cruciferae; environment; leaf epicuticular waxes; wax ester composition.

Abstract—The composition of intact leaf epicuticular wax esters of two individual genotypes each of kale and swede grown indoors (I) and outdoors (O) at SCRI, Scotland, and outdoors at Wädenswil in Switzerland (S), were determined by GC-mass spectrometry. For all genotypes (I, O, S) esters were found to consist of unbranched (*n*-) and branched *anteiso*- (*a*-) and *iso*- (*i*-) components in the *a*:*a*, *a*:*i*, *i*:*a*, *a*:*n*, *n*:*a*, *n*:*n* and *i*/*n*:*n*/*i* acid–alcohol combinations. Esterification was non-random, *n*:*n* and doubly branched *br*-/*br*- combinations were favoured over mixed *n*-/*br*- combinations. Combinations with extremes of acid and alcohol chain-length were generally uncommon, although longer-chain alcohols were more predominant in some swede esters. There were considerable compositional differences between indoor-grown plants (I) and those grown outdoors (O and S). In general, *i*:*n*/*n*:*i*, *i*:*a* and *a*:*i* esters were relatively more abundant in (O and S) and *n*:*n* and *n*:*a* esters were more abundant in (I), whereas *a*:*n* and *a*:*a* esters were of similar abundance in all (I, O and S). Generally, (I)-grown plants were found to have proportionally more esters of longer chain-length and (O, S)-grown plants proportionally more esters of shorter chain-length. For kale *a*:*a*, *n*:*n*, *a*:*n* and *n*:*a* esters, this was particularly related to variation in alcohol chain-length. There were also major compositional differences between kale and swede esters, long-acid–short-alcohol combinations were more prominent in the former, while short-acid–long-alcohol combinations dominated in the latter.

INTRODUCTION

Epicuticular waxes protect plants from disease and pests, and are of particular significance in plant–insect interactions, such as predation of the *Brassicae* by root fly [1, 2]. The swede (*Brassica napus* var. *rapifera*) genotypes Doon Major and GRL, and kale (*Brassica oleracea* var. *acephola*) genotypes Fribor and DGC show a range of resistance and susceptibility to the turnip root fly (*Delia floralis*) [1]; we recently described the effects of environmental conditions on the composition of their epicuticular waxes [2]. Plants were grown indoors (I) and outdoors (O) at SCRI, and outdoors (S) at Wädenswil in Switzerland. Waxes from (O and S)-grown plants had more alkanes, octacosanoic acid, primary alcohols and long-chain esters, but less aldehydes, ketones, ketols and secondary alcohols than waxes from (I)-grown plants. Outdoor-grown plants also had proportionally more compounds of shorter chain-length and (I)-grown plants more compounds of longer chain-length [2]. Our report included a detailed compositional analysis of all epicuticular wax components identified, other than the intact wax esters and free primary alcohols, and, herein, we present the data for the latter.

In most species, epicuticular wax esters usually consist of straight-chain (*n*-) fatty acids esterified to *n*-fatty alcohols [3–6]. However, in brassicas, most wax esters are branched-chain (*br*-) compounds, with branches in the acid and alcohol moieties, which were identified originally as *anteiso*- (*a*-) and *iso*- (*i*-) compounds following hydrolysis of the esters [7–10]. These *br*- components, together with *n*-acids and alcohols, give highly complex mixtures of unbranched, singly- and doubly-branched esters, of which there are nine possible different structural combinations. In earlier studies, the structures of intact esters were not determined because of the difficulty of introducing them into a mass spectrometer over the temperatures required for their separation by GC [7–9]. Advances in technology have simplified the analysis and we recently described methodology for analysis of intact brassica wax esters [10], which were found to consist of complex mixtures in the range C_{42}–C_{49}. On analysis by GC or GC-mass spectrometry, most chro-

* Author to whom correspondence should be addressed.

Table 1. Chemical composition of epicuticular waxes from leaves of kale genotypes Fribor and DGC, and swede genotypes Doon Major and GRL*

	Fribor†			DGC†			GRL†			Doon Major†		
	I	O	S	I	O	S	I	O	S	I	O	S
Main group‡ (*n*-acyl derived)	87.70 ±1.12	79.73 ±0.15	85.94	85.74 ±0.12	81.79 ±1.50	87.64	85.75 ±1.72	80.73 ±2.20	85.18	83.11 ±0.68	74.08 ±1.62	85.49
Primary‡ alcohols	0.92 ±0.05	1.36 ±0.05	1.07	1.15 0.27	1.56 ±0.42	2.24	0.52 ±0.02	1.04 ±0.01	1.18	0.60 ±0.00	1.49 ±0.08	0.89
Esters‡	8.79 ±0.29	16.07 ±0.59	9.09	11.32 ±0.50	14.62 ±0.89	8.56	11.81 ±1.97	16.24 ±1.86	11.10	13.94 ±0.75	22.73 ±1.26	11.28

* I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

† Results of two replicates (I and O), percentage composition values given are based on capillary GC peak areas; compounds were identified by GC-MS.

‡ Values taken from ref. [2].

matographic peaks contained several compounds, and individual esters were identified from the ions $[RCO_2R']^+$ and those $[RCO_2H_2]^+$ derived from the acid moieties by McLafferty rearrangement [10]. Additional evidence came from consideration of all acid–alcohol combinations and their chromatographic relative retention times in terms of equivalent chain-lengths [10]. All possible ester structures are clearly identified, except for *i*:*n* and/or *n*:*i* acid–alcohol combinations, which co-elute on GC, but cannot be distinguished from mass spectral data, and the *i*:*i* combinations, which are not detected [10].

RESULTS AND DISCUSSION

Chromatographic separation of kale and swede epicuticular waxes, following TMSi derivatization, gave two groups of peaks. The first (R_t 23–34 min) included alkanes, ketones, aldehydes, primary and secondary alcohols, α- and β-hydroxy ketones and an acid, while the second group (R_t > 40 min) were the wax esters [2, 10]. The overall composition of the brassica epicuticular waxes, divided into the main group of wax components (total non-ester *n*-acyl derived compounds) [2], total free primary alcohols, and total esters, are shown in Table 1.

The results of the initial analysis of the brassica wax esters by GC are shown in Table 2; individual GC peaks, each containing several esters, are identified by a letter (a, b, c, d . . . p), corresponding to their order of elution as used in our previous study [10]. Capillary GC peaks a, f/e, k/j and o contained *i*:*n* and/or *n*:*i* esters and *a*:*a* esters [Table 2(A)]; peaks b, g and l contained *n*:*n* esters [Table 2(B)]; peaks c, h and m contained *i*:*a* and *a*:*i* esters [Table 2(C)]; and peaks d, i, n and p contained *n*:*a* and *a*:*n* esters [Table 2(D)].

Individual esters present in each GC peak were identified and quantified by GC-mass spectrometry using the method described previously [10]. Details for the 37 major esters are shown in Table 3, arranged according to structural type. The *i*:*n*/*n*:*i* esters are shown in Table 3(A1), *a*:*a* esters in Table 3(A2), *n*:*n* esters in Table 3(B), *i*:*a* esters in Table 3(C1), *a*:*i* esters in Table 3(C2), *n*:*a* esters in Table 3(D1) and *a*:*n* esters in Table 3(D2). Individual esters are arranged in order of increasing acid moiety carbon number, then esters with the same acid are arranged in order of increasing alcohol moiety carbon number. Details of a further 17 esters identified and quantified as minor components are omitted from the tables, although their identities are listed in the table footnotes. Percentage distributions of the individual acid and alcohol moieties comprising these esters (derived from Table 3), and individual free alcohols of each structural type, are shown in Table 4. Percentages of total esters, acid and alcohol moieties and free alcohols are shown in Tables 5(a–c), respectively.

Epicuticular wax ester composition

The most abundant individual esters found in the brassica waxes and the major component acid and alcohol moieties, irrespective of species or environmental growth conditions, are shown in Fig. 1. Overall, *a*:*a* esters were most abundant (35.9–54%) with *n*:*a* (18.4–33.9%), *n*:*n* (3.7–12.1%), *a*:*i* (2.7–9.5%), *a*:*n* (4.1–8.8%), *i*:*n*/*n*:*i* (1.4–6.6%) and *i*:*a* (0.3–3.8%) compounds making up the remainder. *Anteiso*-acids (49.4–69.3%) and alcohols (64–77.2%) and *n*-acids (23.3–44.4%) and alcohols (8.4–19.5%) were the major components with *i*-acids (0.3–3.8%) and alcohols (2.7–9.5%), and *i*/*n*-acids and *n*/*i*-alcohols (1.4–6.6% each) as minor components. The overall distribution of individual esters and their component acid and alcohol moieties were similar to those reported previously for various brassica species [7–10].

Effects of environment on wax ester composition

Statistical analysis of the capillary GC data (Table 2) indicated that there were significant differences in wax ester composition between (I)- and (O, S)-grown plants, irrespective of genotype and species. Com-

Table 2. GC analysis of long-chain esters in epicuticular waxes from leaves of kale genotypes Fribor and DGC, and swede genotypes Doon Major and GRL*

Ester§ C_n CL	Fribor† I	Fribor† O	Fribor† S	DGC† I	DGC† O	DGC† S	GRL† I	GRL† O	GRL† S	Doon Major† I	Doon Major† O	Doon Major† S
(A) *i*:*n*/*n*:*i*; *a*:*a* esters (GC peaks a; e, f; j, k; o)‡												
42 (a)	$1.28^{a,a}$	$3.60^{b,a}$	$2.77^{b,a}$	$1.35^{a,a}$	$2.86^{b,ab}$	$2.22^{ab,ab}$	$1.32^{a,a}$	$1.60^{a,b}$	$1.16^{a,b}$	$1.11^{a,a}$	$1.58^{a,b}$	$1.18^{a,b}$
41; 40	±0.04	±0.07		±0.07	±0.21		±0.01	±0.04		±0.03	±0.02	
44 (e, f)	$23.57^{a,a}$	$24.13^{a,a}$	$24.13^{a,a}$	$22.37^{a,a}$	$20.69^{a,a}$	$27.58^{a,a}$	$18.74^{a,a}$	$28.43^{b,a}$	$29.97^{b,a}$	$18.33^{a,a}$	$27.05^{b,a}$	$29.79^{b,a}$
43; 42	±0.41	±1.29		±0.31	±0.81		±1.39	±0.81		±0.01	±1.04	
46 (j, k)	$21.40^{a,ab}$	$18.45^{a,a}$	$20.59^{a,a}$	$23.76^{a,a}$	$22.19^{a,a}$	$24.24^{a,a}$	$16.48^{a,b}$	$20.15^{a,a}$	$22.17^{a,a}$	$18.60^{a,b}$	$21.44^{a,a}$	$22.47^{a,a}$
45; 44	±0.02	±0.15		±0.39	±0.66		±1.05	±0.17		±0.43	±0.72	
48 (o)	$4.20^{a,ab}$	$2.73^{b,a}$	$3.17^{ab,a}$	$4.59^{a,a}$	$3.54^{a,a}$	$3.73^{a,a}$	$3.25^{a,b}$	$3.45^{a,a}$	$3.94^{a,a}$	$3.92^{a,ab}$	$3.81^{a,a}$	$3.99^{a,a}$
47; 46	±0.07	±0.02		±0.08	±0.11		±0.14	±0.03		±0.16	±0.04	
(B) *n*:*n* esters (GC peaks b; g; l)‡												
42 (b)	$3.97^{a,a}$	$3.97^{a,a}$	$2.67^{a,a}$	$3.03^{a,a}$	$3.05^{a,ab}$	$1.91^{a,a}$	$3.55^{a,a}$	$1.37^{b,b}$	$1.27^{b,a}$	$4.33^{a,a}$	$1.97^{b,b}$	$1.25^{b,a}$
42	±0.09	±0.00		±0.14	±0.35		±0.32	±0.10		±0.16	±0.23	
44 (g)	$4.33^{a,ab}$	$2.84^{b,a}$	$2.22^{b,a}$	$3.57^{a,a}$	$2.92^{ab,a}$	$1.75^{b,a}$	$4.36^{a,ab}$	$1.92^{b,a}$	$1.73^{b,a}$	$5.17^{a,b}$	$2.21^{b,a}$	$1.62^{b,a}$
44	±0.19	±0.01		±0.40	±0.21		±0.51	±0.17		±0.10	±0.35	
48 (l)	$1.90^{a,a}$	$0.87^{b,a}$	$0.77^{b,a}$	$1.57^{a,a}$	$0.89^{b,a}$	$0.60^{b,a}$	$2.62^{a,b}$	$1.03^{b,a}$	$0.98^{b,a}$	$2.61^{a,b}$	$0.85^{b,a}$	$0.80^{b,a}$
48	±0.16	±0.07		±0.46	±0.06		±0.46	±0.17		±0.04	±0.16	
(C) *i*:*a*/*a*:*i* esters (GC peaks c; h; m)‡												
43 (c)	$1.63^{a,a}$	$4.04^{b,a}$	$3.98^{b,a}$	$1.55^{a,a}$	$3.01^{b,b}$	$3.06^{b,b}$	$1.66^{a,a}$	$4.17^{b,a}$	$3.41^{b,a}$	$1.36^{a,a}$	$3.35^{a,ab}$	$3.73^{a,b}$
41	±0.04	±0.04		±0.08	±0.12		±0.15	±0.19		±0.01	±0.21	
45 (h)	$2.96^{a,ab}$	$6.49^{b,a}$	$6.59^{b,a}$	$3.22^{a,a}$	$6.00^{b,ab}$	$5.75^{b,ab}$	$2.22^{a,b}$	$5.95^{b,ab}$	$5.19^{b,b}$	$2.16^{a,b}$	$5.51^{b,b}$	$5.53^{b,b}$
43	±0.13	±0.14		±0.01	±0.18		±0.17	±0.14		±0.01	±0.29	
47 (m)	$0.85^{a,a}$	$1.35^{b,a}$	$1.39^{b,ab}$	$0.88^{a,a}$	$1.34^{b,a}$	$1.28^{b,a}$	$0.97^{a,a}$	$1.89^{b,b}$	$1.70^{b,b}$	$1.00^{a,a}$	$1.84^{b,b}$	$1.72^{b,b}$
45	±0.04	±0.03		±0.01	±0.02		±0.04	±0.02		±0.02	±0.01	

(*Continued overleaf.*)

Table 2. (*Continued*)

Ester§ C_n CL	Fribor† I	Fribor† O	Fribor† S	DGC† I	DGC† O	DGC† S	GRL† I	GRL† O	GRL† S	Doon Major† I	Doon Major† O	Doon Major† S
(D) *n*:*a*/*a*:*n* esters (GC peaks d; i; n; p)‡												
43 (d)	$11.87^{a,ab}$	$12.71^{a,a}$	$12.25^{a,a}$	$11.08^{a,a}$	$10.91^{a,a}$	$11.07^{a,a}$	$16.89^{a,b}$	$11.07^{b,a}$	$10.43^{b,a}$	$15.64^{a,b}$	$12.05^{ab,a}$	$10.86^{b,a}$
42	±0.03	±0.03		±0.48	±0.32		±0.12	±0.08		±0.11	±0.16	
45 (i)	$12.56^{a,a}$	$9.09^{b,a}$	$9.38^{b,a}$	$12.88^{a,a}$	$10.69^{ab,a}$	$9.94^{b,a}$	$17.23^{a,b}$	$11.63^{a,b}$	$10.96^{b,a}$	$16.92^{a,b}$	$11.46^{b,a}$	$11.35^{b,a}$
44	±0.29	±0.09		±0.50	±0.02		±0.26	±0.64		±0.26	±0.78	
47 (n)	$5.63^{a,a}$	$2.97^{b,a}$	$3.45^{b,a}$	$5.44^{a,a}$	$3.69^{b,ab}$	$3.68^{b,a}$	$7.79^{a,b}$	$4.64^{b,b}$	$4.56^{b,b}$	$6.96^{a,b}$	$3.98^{b,ab}$	$4.56^{b,b}$
46	±0.15	±0.10		±0.22	±0.08		±0.40	±0.45		±0.19	±0.50	
49 (p)	$0.00^{a,a}$	$0.00^{a,a}$	$0.00^{a,a}$	$0.92^{a,b}$	$0.16^{b,a}$	$0.00^{b,a}$	$0.77^{a,b}$	$0.29^{a,a}$	$1.02^{a,b}$	$0.58^{a,b}$	$0.58^{a,b}$	$0.72^{a,b}$
48				±0.11	±0.16		±0.77	±0.29		±0.58	±0.08	

* I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

† Results of two replicates (I and O), percentage composition values given are based on capillary GC peak areas. Values followed by different letters are significantly different to at least $p = 0.05$ by analysis of variance. First superscript = comparison of the same genotype under different environmental conditions; second superscript = comparison of different genotypes under the same environmental conditions.

‡ General ester structures, showing acid:alcohol combinations. *i*, *iso*-branched-chain; *a*, *anteiso*-branched chain; *n*, *n*-straight-chain. Letters (a–p) correspond to individual peaks detected during capillary GC analysis of derivatised epicuticular wax; peaks e, f and j, k could not be resolved on capillary GC alone, but were resolved during GC-MS analysis (see Table 3). For details see ref. [10].

§ The esters and component ester acid:alcohol moieties have carbon numbers C_n and maximum continuous chain lengths CL. For *a*- and *i*-moieties, $CL = C_n - 1$, for *n*-moieties $CL = C_n$.

Table 3. Composition of individual long-chain esters in epicuticular waxes from leaves of kale genotypes Fribor and DGC, and swede genotypes Doon Major and GRL*

Ester, acid:alcohol§ C_n CL	C_n:C_n CL:CL	Fribor† I	Fribor† O	Fribor† S	DGC† I	DGC† O	DGC† S	GRL† I	GRL† O	GRL† S	Doon Major† I	Doon Major† O	Doon Major† S
(A1) *i*:*n*/*n*:*i* esters‡††													
42 (a)	16:26††	1.28[f]	3.60[f]	2.77[f]	1.35[f]	2.86[f]	2.22[f]	1.32[f]	1.60[f]	1.16[f]	1.11[f]	1.58[f]	1.18[f]
41	15:26/16:25	*1.28*	*3.73*	*2.85*	*1.33*	*2.89*	*−1.79*	*1.29*	*−1.38*	*1.03*	*1.19*	*1.62*	*−0.90*
44 (f)	16:28††	0.09[f]	0.56[f]	0.33[f]	0.25[f]	0.45[f]	0.27[d]	0.34[d]	0.05[d]	0.36[d]	0.46[d]	0.81[d]	0.30[d]
43	15:26/17:27	*0.09*	*−0.43*	*−0.25*	*0.27*	*0.42*	*+0.37*	+0.42	*+0.28*	*+0.51*	*0.46*	0.84	*+0.37*
46 (k)	16:30††	0.00[d]	0.00[d]	0.02[f]	0.00[d]	0.00[d]	0.02[d]	0.18[f]	0.04[f]	0.04[f]	0.19[f]	0.13[f]	0.00[d]
45	15:30/16:29	*0.00*	*0.00*	*0.02*	*0.00*	*0.00*	+0.35	*−0.13*	*−0.03*	*−0.02*	*−0.16*	*−0.06*	*+0.21*
44 (f)	18:26††	0.00[d]	1.50[f]	0.74[f]	0.90[f]	2.31[f]	1.52[d]	0.34[d]	0.01[d]	0.74[d]	0.17[f]	1.50[f]	0.75[f]
43	17:26/18:25	*+0.10*	*1.38*	*0.68*	*0.97*	*2.35*	*+2.03*	*+0.42*	*+0.27*	*+0.86*	*0.18*	*1.51*	*+1.08*
46 (k)	18:28††	0.00[d]	0.44[f]	0.00[d]	0.26[f]	0.38[f]	0.56[f]	0.26[f]	0.32[f]	0.56[f]	0.09[f]	0.85[f]	0.56[f]
45	17:28/18:27	*0.00*	*−0.16*	*+0.06*	*−0.19*	*−0.34*	*−0.42*	*−0.14*	*−0.05*	*−0.42*	*−0.07*	*0.78*	*−0.45*
46 (k)	20:26††	0.04[f]	0.12[f]	0.25[f]	0.29[f]	0.56[f]	0.28[f]	0.17[f]	0.24[f]	0.24[f]	0.15[f]	0.30[f]	0.13[f]
45	19:26/20:25	*0.04*	*0.11*	0.23	*−0.24*	*−0.49*	*−0.20*	*−0.12*	*−0.20*	*0.25*	*−0.10*	*−0.24*	*−0.08*
(A2) *a*:*a* esters‡													
44 (e)	15:29	0.00[d]	0.00[d]	0.00[d]	0.04[d]	0.04[d]	0.06[f]	0.13[f]	0.00[d]	0.11[f]	0.06[f]	0.07[d]	0.00[d]
42	14:28	*+0.04*	*+0.02*	*+0.04*	*+0.05*	*+0.05*	*0.06*	*−0.07*	*+0.03*	*−0.06*	*−0.05*	*+0.08*	*+0.04*
46 (j)	15:31	0.25[f]	0.15[f]	0.23[f]	0.31[f]	0.31[f]	0.34[f]	0.15[f]	0.15[f]	0.23[f]	0.14[f]	0.25[f]	0.19[f]
44	14:30	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*	*−0.00*
44 (e)	17:27	23.17[f]	21.70[f]	22.53[f]	20.81[f]	17.53[f]	25.12[f]	17.79[d]	28.36[f]	28.61[f]	17.55[d]	24.51[f]	28.61[f]
42	16:26	*−21.97*	*22.33*	*24.14*	*21.34*	*18.81*	*25.66*	*+19.70*	*30.69*	*30.41*	*+20.97*	*26.89*	*31.26*
46 (j)	17:29	6.67[f]	4.36[f]	4.95[f]	5.78[f]	3.89[f]	4.79[f]	7.72[f]	8.42[f]	9.55[f]	9.57[f]	9.46[f]	10.12[f]
44	16:28	*−5.83*	*−3.90*	*−3.83*	*−4.59*	*−2.94*	*4.67*	*−6.67*	*8.17*	*9.05*	*−6.38*	*8.99*	*10.00*
44 (e)	19:25	0.31[f]	0.37[f]	0.52[f]	0.36[f]	0.36[f]	0.61[f]	0.14[f]	0.01[f]	0.16[f]	0.09[f]	0.16[f]	0.10[f]
42	18:24	*−0.08*	*−0.10*	*−0.15*	*−0.15*	*−0.17*	*−0.18*	*−0.04*	*−0.00*	*−0.11*	*−0.02*	*−0.04*	*−0.02*
46 (j)	19:27	14.44[f]	13.38[d]	15.14[d]	17.09[f]	17.05[f]	18.25[f]	7.93[f]	10.99[d]	11.15[f]	8.47[f]	10.39[f]	11.46[f]
44	18:26	*15.20*	*+15.21*	*+17.10*	*17.20*	*17.85*	*18.95*	*7.75*	*+12.21*	*11.05*	*−7.31*	*10.67*	*11.55*
48 (o)	19:29	4.20[f]	2.73[f]	2.57[f]	3.90[f]	2.65[f]	2.97[d]	2.63[f]	3.49[f]	2.17[d]	0.01[d]	3.24[d]	3.51[f]
46	18:28	*4.04*	*2.65*	*2.71*	*3.70*	*2.79*	*+3.45*	*2.62*	*3.25*	*+3.29*	*−0.00*	*+3.57*	*3.70*

(*Continued overleaf.*)

Table 3. (*Continued*)

Ester, acid:alcohol§ C_n CL	C_n:C_n CL:CL	Fribor† I	Fribor† O	Fribor† S	DGC† I	DGC† O	DGC† S	GRL† I	GRL† O	GRL† S	Doon Major† I	Doon Major† O	Doon Major† S
(B) *n*:*n* esters‡													
42 (b)	16:26	3.97[f]	3.97[f]	2.67[f]	3.03[f]	2.94[f]	1.91[f]	3.55[f]	1.37[f]	1.27[f]	4.33[f]	1.97[f]	1.25[f]
42	16:26	*−2.04*	*−1.98*	*−1.50*	*−1.65*	*−1.69*	*−0.96*	*−2.87*	*−0.83*	*−0.80*	*4.16*	*−1.33*	*−0.82*
44 (g)	*16:28*	*2.95*[f]	1.52[f]	1.14[f]	0.86[f]	0.62[f]	0.27[f]	2.44[f]	1.85[f]	0.40[d]	2.54[f]	0.72[f]	0.32[d]
44	16:28	*−1.25*	*−0.55*	*−0.41*	*−0.53*	*−0.31*	*−0.20*	*−1.90*	*−0.77*	*+0.46*	*−1.77*	*−0.55*	*+0.42*
44 (g)	18:26	1.38[f]	1.32[f]	1.08[f]	2.57[f]	2.19[f]	1.48[f]	1.92[f]	0.07[d]	1.33[f]	2.63[f]	1.49[f]	1.30[f]
44	18:26	*−1.16*	*−0.81*	*−0.63*	*−1.43*	*−1.21*	*−0.78*	*−1.62*	*+0.48*	*−0.72*	*−1.85*	*−0.93*	*−0.77*
46 (l)	18:28	0.80[f]	0.00[d]	0.00[d]	0.84[f]	0.25[f]	0.43[f]	1.21[f]	0.51[f]	0.98[f]	0.48[d]	0.56[f]	0.80[f]
46	18:28	*−0.71*	*+0.22*	*+0.17*	*−0.46*	*−0.22*	*−0.17*	*−1.07*	*−0.45*	*−0.41*	*+0.79*	*−0.38*	*−0.40*
46 (l)	20:26	1.10[f]	0.00[d]	0.00[d]	0.73[f]	0.64[f]	0.17[f]	0.60[f]	0.24[f]	0.00[d]	1.08[f]	0.29[f]	0.00[d]
46	20:26	*−0.47*	*+0.20*	*+0.27*	*−0.47*	*−0.39*	*−0.15*	*−0.43*	*−0.17*	*+0.22*	*−0.19*	*−0.24*	*+0.11*
(C1) *i*:*a* esters‡													
43 (c)	16:27	0.00[d]	0.70[f]	0.39[f]	0.22[f]	0.35[f]	0.23[f]	0.60[f]	0.83[f]	0.56[f]	1.36[f]	0.69[f]	0.51[f]
41	15:26	*0.00*	*−0.47*	*−0.26*	*−0.15*	*−0.24*	*−0.19*	*−0.35*	*−0.64*	*−0.36*	*−0.84*	*−0.52*	*−0.40*
45 (h)	*18:27*	*0.00*[d]	2.72[f]	2.45[f]	1.21[f]	2.37[f]	2.58[f]	0.56[f]	1.53[f]	1.90[f]	0.48[f]	1.73[f]	1.77[f]
43	17:26	*+0.18*	*−1.81*	*−1.65*	*−0.81*	*−1.60*	*2.38*	*−0.49*	*−1.12*	*−1.54*	*−0.30*	*−1.25*	*−1.44*
47 (m)	18:29	0.29[f]	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.75[f]	0.30[f]	0.22[d]	0.52[f]	0.00[d]	0.38[d]	0.55[f]
45	17:28	*−0.05*	*+0.32*	*+0.26*	*+0.17*	*+0.25*	*−0.43*	*−0.17*	*+0.30*	*−0.46*	*0.00*	*+0.42*	*−0.46*
47 (m)	*20:27*	*0.00*[d]	0.42[f]	0.00[d]	0.08[f]	0.16[f]	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.08[f]	0.00[d]
45	19:26	*0.00*	*-0.28*	*0.00*	*−0.05*	*−0.11*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*−0.00*	*0.00*
(C2) *a*:*i* esters‡													
43 (c)	17:26	1.63[f]	3.34[f]	3.59[f]	1.33[f]	2.66[f]	2.83[f]	1.06[f]	3.34[f]	2.85[f]	0.00[d]	2.66[f]	3.22[f]
41	16:25	*1.61*	*−2.52*	*−2.72*	*−0.93*	*−1.86*	*−2.12*	*−0.73*	*−2.31*	*−2.07*	*+0.34*	*−2.00*	*−2.44*
45 (h)	17:28	0.39[f]	0.88[f]	1.08[f]	0.70[f]	0.76[f]	0.71[f]	0.98[f]	3.12[f]	2.16[f]	0.89[f]	2.29[f]	2.34[f]
43	16:27	*0.38*	*−0.73*	*1.03*	*−0.39*	*−0.66*	*−0.50*	*−0.52*	*−2.61*	*−1.73*	*0.83*	*−1.63*	*−1.77*
43 (c)	19:24	0.27[f]	0.31[f]	0.35[f]	0.08[f]	0.21[f]	0.18[f]	0.00[d]	0.00[d]	0.00[d]	0.05[f]	0.11[f]	0.00[d]
41	18:23	*−0.07*	*−0.09*	*−0.10*	*−0.02*	*−0.07*	*−0.05*	*0.00*	*0.00*	*0.00*	*−0.01*	*−0.02*	*0.00*
45 (h)	*19:26*	*2.57*[f]	2.90[f]	2.94[f]	1.32[f]	2.81[f]	2.42[f]	0.67[f]	1.12[f]	1.13[f]	0.78[f]	1.31[f]	1.28[f]
43	18:25	*−1.11*	*2.65*	*−1.93*	*−0.75*	*−1.76*	*−1.56*	*−0.29*	*−0.92*	*−0.75*	*−0.12*	*−0.79*	*−0.90*
47 (m)	19:28	0.61[f]	0.93[f]	1.39[f]	0.42[f]	1.18[f]	0.53[f]	0.42[f]	1.67[f]	1.18[f]	1.00[f]	0.95[f]	0.92[f]
45	18:27	*−0.26*	*−0.50*	*−0.73*	*−0.32*	*−0.62*	*−0.37*	*−0.20*	*−1.04*	*−0.63*	*−0.29*	*−0.65*	*−0.65*
(D1) *n*:*a* esters‡													
43 (d)	16:27	9.19[d]	9.94[d]	9.11[f]	9.09[d]	8.32[f]	9.02[f]	13.86[f]	8.36[f]	7.61[f]	12.11[d]	8.13[f]	7.53[f]
42	16:26	*+11.75*	*+11.36*	*9.85*	*+10.05*	*9.01*	*8.91*	*14.62*	*9.00*	*7.23*	*+15.45*	*7.88*	*6.90*

Table 3. (*Continued*)

Ester, acid:alcohol§ C_n	C_n:C_n CL:CL	Fribor† I	Fribor† O	Fribor† S	DGC† I	DGC† O	DGC† S	GRL† I	GRL† O	GRL† S	Doon Major† I	Doon Major† O	Doon Major† S
45 (i)	16:29	2.86^{f}	1.64^{d}	1.66^{f}	1.97^{f}	1.48^{f}	1.27^{d}	4.75^{f}	2.19^{f}	2.09^{f}	4.95^{f}	2.45^{f}	2.04^{f}
44	16:28	*3.12*	*+1.98*	*1.56*	*2.16*	*−0.41*	*+1.62*	*4.95*	*2.40*	*2.15*	*4.70*	*2.63*	*2.21*
45 (i)	18:27	7.40^{f}	4.76^{f}	5.02^{f}	8.20^{f}	6.46^{f}	6.57^{f}	8.74^{f}	6.34^{f}	5.98^{f}	8.02^{f}	5.75^{f}	6.29^{f}
44	18:26	*6.69*	*4.66*	*−4.12*	8.75	*6.46*	*7.27*	*8.26*	*−5.24*	*6.52*	*−6.88*	*5.53*	*6.51*
47 (n)	18:29	1.23^{d}	0.92^{f}	0.00^{d}	1.38^{d}	0.61^{d}	1.70^{f}	2.49^{d}	1.26^{f}	1.96^{f}	0.00^{d}	1.52^{d}	2.11^{f}
46	18:28	*+1.78*	*−0.81*	*+0.65*	*+1.88*	*+1.01*	*−1.32*	*+2.80*	*1.40*	*1.94*	*0.00*	*+1.85*	*2.08*
47 (n)	20:27	3.26^{f}	1.71^{f}	2.60^{f}	3.36^{f}	2.33^{f}	1.83^{f}	3.16^{f}	2.71^{f}	2.10^{f}	0.00^{d}	1.60^{f}	1.54^{f}
46	20:26	*−2.70*	*−1.14*	*−1.76*	*−2.84*	*−2.06*	*−1.43*	*−2.21*	*−1.89*	*1.98*	*0.00*	*1.45*	*−0.95*
49 (p)	20:29	0.00^{d}	0.00^{d}	0.00^{d}	0.92^{f}	0.16^{d}	0.00^{d}	0.77^{f}	0.29^{d}	1.02^{f}	1.18^{f}	0.58^{f}	0.72^{f}
48	20:28	*+0.72*	*+0.20*	*+0.28*	*−0.61*	*+0.32*	*+0.26*	*0.75*	*+0.50*	*−0.59*	*−0.21*	*−0.48*	*−0.31*
(D2) *a*:*n* esters‡													
43 (d)	17:26	2.41^{d}	2.47^{d}	2.80^{d}	1.91^{d}	2.31^{d}	1.86^{d}	3.00^{d}	2.64^{f}	2.82^{d}	3.46^{d}	3.81^{d}	3.33^{d}
42	16:26	*+3.81*	*+3.92*	*+3.68*	*+3.50*	*+3.53*	*+2.76*	*+3.86*	*2.81*	*+3.38*	*+5.65*	*+4.53*	*+3.72*
45 (i)	17:28	1.20^{d}	0.80^{d}	0.79^{d}	0.84^{d}	0.74^{f}	0.61^{f}	2.57^{f}	2.00^{d}	1.81^{f}	2.45^{f}	1.90^{f}	2.03^{f}
44	16:28	*+2.33*	*+1.07*	*+1.01*	*+1.13*	*0.65*	*0.59*	*2.56*	*+2.61*	*1.92*	*2.40*	*1.86*	*1.91*
45 (i)	19:26	1.11^{d}	1.89^{d}	1.91^{d}	1.73^{d}	1.89^{d}	1.41^{d}	1.03^{d}	1.10^{f}	1.08^{d}	1.43^{d}	1.31^{d}	0.98^{d}
44	18:26	*+2.64*	*+2.65*	*+2.61*	*+2.82*	*+3.35*	*+2.03*	*+1.52*	*1.12*	*+1.23*	*+1.97*	*+1.80*	*+1.37*
47 (n)	19:28	1.14^{d}	0.34^{d}	0.50^{d}	0.69^{d}	0.31^{d}	0.15^{d}	0.66^{d}	0.67^{d}	0.50^{d}	0.00^{d}	0.51^{d}	0.38^{d}
46	18:28	*+1.61*	*+0.73*	*+0.72*	*+0.91*	*+0.62*	*+0.43*	*+1.01*	*+1.04*	*+0.70*	*0.00*	*+0.74*	*+0.71*

* I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

† The values are based on capillary GC peak areas (Table 2), with the proportions of component esters in each chromatographic peak derived from integration of the respective reconstituted single ion chromatogram (SIC) peaks for $[RCO_2H_2]^+$ obtained by GC-MS. For details see ref. [10]. Values shown in italics are the expected distribution of esters following random combination of acid and alcohol moieties (from Table 4); a negative sign means less expected than found, a positive sign means more expected than found (difference > ±10%). Ester formation is categorised as 'favoured', superscript f = amount found ⩾ random, or as 'disfavoured', superscript d = amount found < random.

‡ General ester structures, showing acid:alcohol combinations. *i*, *iso*-branched-chain; *a*, *anteiso*-branched chain; *n*, *n*-straight-chain. Letters (a–p) correspond to individual peaks detected during capillary GC analysis of derivatised epicuticular wax (see Table 2); peaks e, f and j, k could not be resolved on capillary GC alone, but were resolved during GC-MS analysis. For details see ref. [10].

§ The esters and component ester acid:alcohol moieties have carbon numbers C_n and C_n:C_n and maximum continuous chain lengths CL and CL:CL respectively. For *a*- and *i*-moieties, CL = C_n−1, for *n*-moieties CL = C_n. Underlined values for C_n and CL are the upper and lower limits of alcohol and acid length apparently favoured for ester formation.

¶ These *iso*-acid:*n*-alcohol/*n*-acid:*iso*-alcohol combinations cannot be distinguished. In addition to the above, the following esters (C_n(CL)GC peak; [C_n:C_n(CL:CL)]) were quantified but were minor components. *i*:*n*/*n*:*i* 48 (47) (o), [20:28 (19:28/20:27); 18:30 (17:30/18:29)]; *a*:*a*: 46 (44) (j), [21:25(20:24)]; 48 (46) (o), [15:33 (14:32); 21:27 (20:26)]; *n*:*n*: 46 (46) (l), [16:30 (16:30)]; *i*:*a*: 45 (43) (h) [*16:29* (*15:28*)]; *a*:*i*: 45 (43) (h), [15:30 (14:29)]; 47 (45) (m), [15:32 (14:31); 21:26 (20:25); 17:30 (16:29)]; *n*:*a*: 43 (42) (d), [18:25 (18:24)]; 45 (44) (i), [*20:25* (*20:24*)]; *a*:*n*: 43 (42) (d), [15:28 (14:28)]; 45 (44) (i), [*15:30* (*14:30*)]; 47 (46) (n), [17:30 (16:30); 21:26 (20:26)]. All were categorised overall as 'disfavoured' except those in italics, which were borderline between 'favoured/disfavoured' for swede. The following esters were detected in trace amounts: C_{41}: *a*17:*n*24, C_{43}: *a*19:*n*24, *n*20:*a*23; C_{44}: *i*20:*n*24/*n*20:*i*24; C_{46}: *i*22:*n*24/*n*22:*i*24, *a*23:*a*23; C_{47}: *a*15:*n*32, *a*23:*i*24, *a*23:*n*24; C_{48}: *a*23:*a*25. The following general combinations were detected, but specific combinations could not be identified. C_{48}: *n*:*n*; C_{49}: *a*:*i*, *i*:*a*, *a*:*n*, *n*:*a*; C_{50}: *a*:*a*.

Table 4. Percentage composition of individual acid and alcohol moieties in epicuticular wax esters, and free primary alcohols from leaves of kale genotypes Fribor and DGC, and swede genotypes Doon Major and GRL*

	Fribor†			DGC†			GRL†			Doon Major†		
C_n	I	O	S	I	O	S	I	O	S	I	O	S
Acid moiety‡												
*a*15	0.26	0.16	0.35	0.36	0.43	0.48	0.35	0.16	0.34	0.24	0.41	0.19
*a*17	35.47	33.55	35.73	31.75	27.89	35.92	34.25	47.88	47.80	33.92	45.30	50.44
*a*19	24.65	22.85	25.32	25.59	26.46	26.52	13.48	19.05	17.37	11.83	17.98	18.63
*a*21			0.95	0.71	1.33		1.28		1.05		0.63	
*i*16		0.70	0.39	0.22	0.35	0.26	0.60	1.00	0.56	1.36	0.87	0.65
*i*18	0.29	2.72	2.45	1.21	2.37	3.33	0.86	1.75	2.42	0.48	2.11	2.32
*i*20		0.42		0.08	0.16						0.08	
*n*16	18.97	17.07	14.58	14.95	13.36	12.47	25.41	14.05	11.37	24.98	13.27	11.14
*n*18	10.81	7.00	6.10	12.99	9.58	10.18	14.36	8.18	10.25	11.13	9.32	10.50
*n*20	4.36	1.71	2.60	4.23	3.06	2.00	3.85	2.95	3.12	1.14	2.44	1.54
*i*16/*n*16§	1.37	4.16	3.12	1.60	3.31	2.51	1.84	1.69	1.56	1.76	2.52	1.48
*i*18/*n*18§	0.00	1.54	0.74	1.16	2.69	2.84	0.60	0.33	1.30	0.26	2.61	1.79
*i*20/*n*20§	0.04	0.12	0.25	0.29	0.56	0.28	0.17	0.24	0.37	0.15	0.38	0.13
Alcohol moiety‡												
*a*25	0.31	0.37	0.52	0.52	0.52	0.61	0.30	0.01	0.57	0.15	0.22	0.10
	—	—	—	—	—	—	—	—	—	—	—	—
*a*27	57.46	55.33	57.84	60.75	55.46	63.60	53.26	59.12	58.55	47.99	53.35	57.71
	39.13	*28.68*	*19.63*	*16.96*	*28.85*	*33.48*	*34.61*	*27.88*	*29.66*	*38.33*	*25.50*	*25.84*
*a*29	15.25	9.65	9.18	13.07	8.67	11.57	18.02	15.75	17.42	14.59	17.84	18.47
	2.17	*6.62*	*7.48*	*10.43*	*7.05*	*8.04*	*11.54*	*12.50*	*14.41*	*18.33*	*14.09*	*13.48*
*a*31	0.25	0.15	0.23	0.31	0.31	0.34	0.15	0.15	0.23	0.14	0.25	0.19
	—	—	—	—	—	—	—	—	—	—	—	—
*i*24	0.27	0.31	0.35	0.08	0.21	0.18	—	—	—	0.05	0.11	—
	—	—	—	—	—	—	—	—	—	—	—	—
*i*26	4.20	6.24	6.53	2.65	5.47	5.25	1.98	4.46	3.98	0.78	3.97	4.50
	7.61	*13.20*	*7.48*	*8.69*	*13.48*	*12.94*	*3.85*	*3.85*	*3.39*	*1.67*	*7.38*	*6.74*
*i*28¶	1.00	1.81	2.47	1.12	1.94	1.24	1.40	4.79	3.34	1.89	3.24	3.26
	6.52	*10.29*	*16.82*	*7.83*	*8.97*	*7.59*	*9.61*	*15.38*	*15.25*	*6.67*	*18.12*	*20.22*
*i*30	—	—	0.12	0.38	0.06	—	—	—	—	—	0.43	0.25
	tr	*tr*	*tr*	*tr*	*tr*	*tr*	*tr*	*tr*	*tr*	*tr*	*tr*	*tr*
*n*26	9.97	9.65	8.81	9.97	10.41	6.83	10.44	5.42	6.50	12.93	8.98	6.68
	26.09	*27.94*	*27.10*	*25.22*	*28.85*	*25.89*	*15.38*	*18.27*	*19.95*	*18.33*	*20.80*	*15.73*
*n*28	6.09	2.66	2.43	3.23	1.92	1.46	6.91	5.03	3.69	5.49	3.70	3.53
	15.22	*12.50*	*17.76*	*17.39*	*12.18*	*11.16*	*19.23*	*20.19*	*17.80*	*6.67*	*14.09*	*16.85*
*n*30¶	—	—	—	—	0.02	0.08	1.98	0.28	—	1.07	0.28	0.54
	3.26	*2.20*	*2.80*	*2.61*	*0.64*	*0.89*	*3.85*	*1.92*	*2.54*	*1.67*	*0.67*	*1.12*
*n*26/*i*26§	1.32	5.22	3.76	2.54	5.73	4.02	1.83	1.85	2.14	1.43	3.38	2.06
	—	—	—	—	—	—	—	—	—	—	—	—
*n*28/*i*28§	0.09	0.60	0.33	0.51	0.83	0.83	0.60	0.37	1.05	0.55	1.74	0.86
	—	—	—	—	—	—	—	—	—	—	—	—
*n*30/*i*30§	—	—	0.02	—	—	0.78	0.18	0.04	0.04	0.19	0.13	0.48
	—	—	—	—	—	—	—	—	—	—	—	—

* I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

† The values shown are based on the distribution of individual esters as shown in Table 3 including those minor components (data not shown) listed in the table footnotes. Also found in minor quantities were *i*-C_{32} (0.01%) and *a*-C_{33} (0.03%) alcohol moieties from Doon Major (O). Values shown in italics are for free primary alcohols.

‡ *i*, *iso*-branched-chain; *a*, *anteiso*-branched chain; *n*, *n*-straight-chain.

§ Components of *iso*-acid: *n*-alcohol/*n*-acid: *iso*-alcohol combinations which cannot be distinguished.

¶ Free primary alcohols did not fully resolve on analysis by GC, values were derived from reconstituted single ion chromatogram (SIC) peak areas for $[M\text{-}15]^+$ on GC-MS analysis.

parison of the distribution of the esters, and their component acids and alcohols (total and individual) under the different environmental conditions, was made in terms of the ratios O/I and S/I of their respective percentage compositions. If the ratio was > 1, the component was more abundant (O) than (I), and vice versa. Effects of environment on chain-length were deduced from the way the ratio O/I changed with C_n. For example, if O/I diminished with increasing C_n, then longer chain-lengths were relatively more abun-

Table 5. Total esters, acid and alcohol moieties of each structural class in epicuticular waxes from leaves of kale genotypes Fribor and DGC, and swede genotypes Doon Major and GRL*

	Fribor†			DGC†			GRL†			Doon Major†		
	I	O	S	I	O	S	I	O	S	I	O	S
(a) Esters‡												
i:n§	1.41	6.22	4.11	3.05	6.56	5.63	2.61	2.26	3.23	2.17	5.17‡	3.40
n:i	*1.41*	*6.22*	*4.11*	*3.05*	*6.56*	*5.63*	*2.61*	*2.26*	*3.23*	*2.17*	*5.17*	*3.40*
n:n	10.20	6.81	4.89	8.03	6.64	4.26	10.53	4.32	3.98	12.11	5.03	3.67
	−5.78	*−3.68*	*−2.96*	*−4.70*	*−3.79*	*−2.26*	*−9.01*	*−2.87*	*−2.67*	*−8.68*	*−3.41*	*−2.72*
i:a	0.29	3.84	2.84	1.51	2.88	3.59	1.46	2.75	2.98	1.84	3.06	2.97
	−0.22	*−2.92*	*−2.18*	*−1.23*	*−2.20*	*−3.00*	*−1.11*	*−2.17*	*−2.43*	*−1.37*	*−2.37*	*−2.38*
a:i	5.47	8.36	9.47	4.23	7.68	6.67	3.38	9.25	7.32	2.72	7.76	8.01
	−3.48	*−5.49*	*−6.67*	*−2.66*	*−5.06*	*−4.60*	*−1.75*	*−6.51*	*−5.17*	*−1.45*	*−5.30*	*−5.77*
n:a	23.94	18.97	18.39	25.06	19.45	20.39	33.86	21.15	20.76	26.31	19.62‡	20.23
	+26.39	*19.60*	*17.83*	*26.89*	*19.95*	*20.58*	*33.80*	*20.13*	*20.15*	*28.53*	*19.06*	*19.16*
a:n	5.86	5.50	6.35	5.17	5.71	4.11	8.80	6.41	6.21	7.38	7.06‡	7.26
	+10.23	*+8.08*	*+7.92*	*+8.29*	*+8.14*	*+5.78*	*+10.02*	*+7.55*	*+7.19*	*+10.39*	*+8.26*	*7.87*
a:a	49.04	42.69	46.54	49.00	42.72	52.14	37.18	51.42	53.03	35.88	44.93‡	53.99
	46.66	*42.99*	*47.76*	*47.45*	*42.90*	*52.54*	*37.59*	*53.02*	*54.20*	*34.14*	*46.19*	*55.61*
(b) Acids‡												
a-	60.37	56.55	62.36	58.40	56.11	62.92	49.36	67.08	66.56	45.98	59.75	69.26
i-	0.29	3.84	2.84	1.51	2.88	3.59	1.46	2.75	2.98	1.84	3.06	2.97
n-	34.14	25.78	23.28	33.09	26.09	24.65	44.39	25.47	24.74	38.42	24.65	23.90
i/n-§	1.41	6.22	4.11	3.05	6.56	5.63	2.61	2.26	3.23	2.17	5.17	3.40
(c) Alcohols‡												
a-	73.23	65.50	67.77	75.57	65.05	76.12	72.50	75.32	76.77	64.03	67.61	77.19
	41.30	*35.30*	*27.11*	*37.39*	*35.60*	*41.52*	*46.15*	*40.38*	*44.07*	*56.66*	*39.59*	*39.32*
i-	5.47	8.36	9.47	4.23	7.68	6.67	3.38	9.25	7.32	2.72	7.76	8.01
	14.13	*23.49*	*24.30*	*16.52*	*11.43*	*20.53*	*13.46*	*19.23*	*18.64*	*8.34*	*25.50*	*26.96*
n-	16.06	12.31	11.24	13.20	12.35	8.37	19.33	10.73	10.19	19.49	12.09	10.93
	44.57	*42.64*	*47.66*	*45.22*	*41.67*	*37.94*	*38.46*	*40.38*	*37.29*	*36.67*	*35.56*	*33.70*
i/n-§	1.41	6.22	4.11	3.05	6.56	5.63	2.61	2.26	3.23	2.17	5.17	3.40

* I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

† The values shown are based on the distribution of individual esters (Table 3) and ester acid and alcohol moieties (Table 4). Values shown in italic for esters are the expected distribution of total esters following random combination of acid and alcohol moeities, based on values for individual components in Table 3. A negative sign means less expected than found, a positive sign means more expected than found (difference $> \pm 10\%$). Values shown in italic for alcohols are for free primary alcohols.

‡ Acid–alcohol combinations; *i*, *iso*-branched-chain; *a*, *anteiso*-branched chain; *n*, *n*-straight-chain.

§ Components of *iso*-acid: *n*-alcohol/*n*-acid: *iso*-alcohol combinations which cannot be distinguished.

dant (I) than (O), and vice versa. Such values were calculated for all components but are not shown.

Intact wax esters

Total esters. As total esters, the structural classes were differentiated under the different conditions [Table 5(a)]. Most *i:n*/*n:i*, *i:a* and *a:i* esters were more abundant in (O and S) than in (I), *n:n* and *n:a* esters were more abundant in (I) and *a:n* esters were of similar abundance under all conditions. However, some swede (GRL) *i:n*/*n:i* and *a:n* esters were more abundant in (I). Amounts of *a:a* esters were greater in (I) for kale, but were greater in (O, S) for swede.

Individual esters. The GC data for the kale *a:a* plus *i:n*/*n:i*, *n:n*, *i:a*/*a:i* and *n:a*/*a:n* ester groupings [Tables 2(A–D)] indicates that, in general, longer esters were relatively more abundant in (I) than in (O). Similar effects were also seen for some swede wax ester groupings. Variation in ester length arise from changes in the length of the alcohol and/or acid moieties, and the extent to which these were effected by environment can be deduced from the data for individual esters. On this basis, individual kale esters were clearly differentiated into two groups.

a:a, n:n, a:n *and* n:a *esters.* In general, individual kale *a:a*, *n:n*, *n:a* and *a:n* esters were more abundant in (I) than in (O) [Tables 3(A2), (B), (D1), (D2)], as for total esters [Table 5(a)], and for esters with the same acid their abundance in (I) relative to (O) increased with length of alcohol. For esters with the same alcohol, changes in acid length distribution under the different conditions were more varied. Patterns for (S) and (O)-grown plants were generally similar.

Variations in the alcohol chain-length distribution between (O) and (I) for some swede *n:a* and *a:n* esters, particularly those from DM, were similar to those of kale; otherwise there were no distinct patterns in relation to acid and alcohol chain-length.

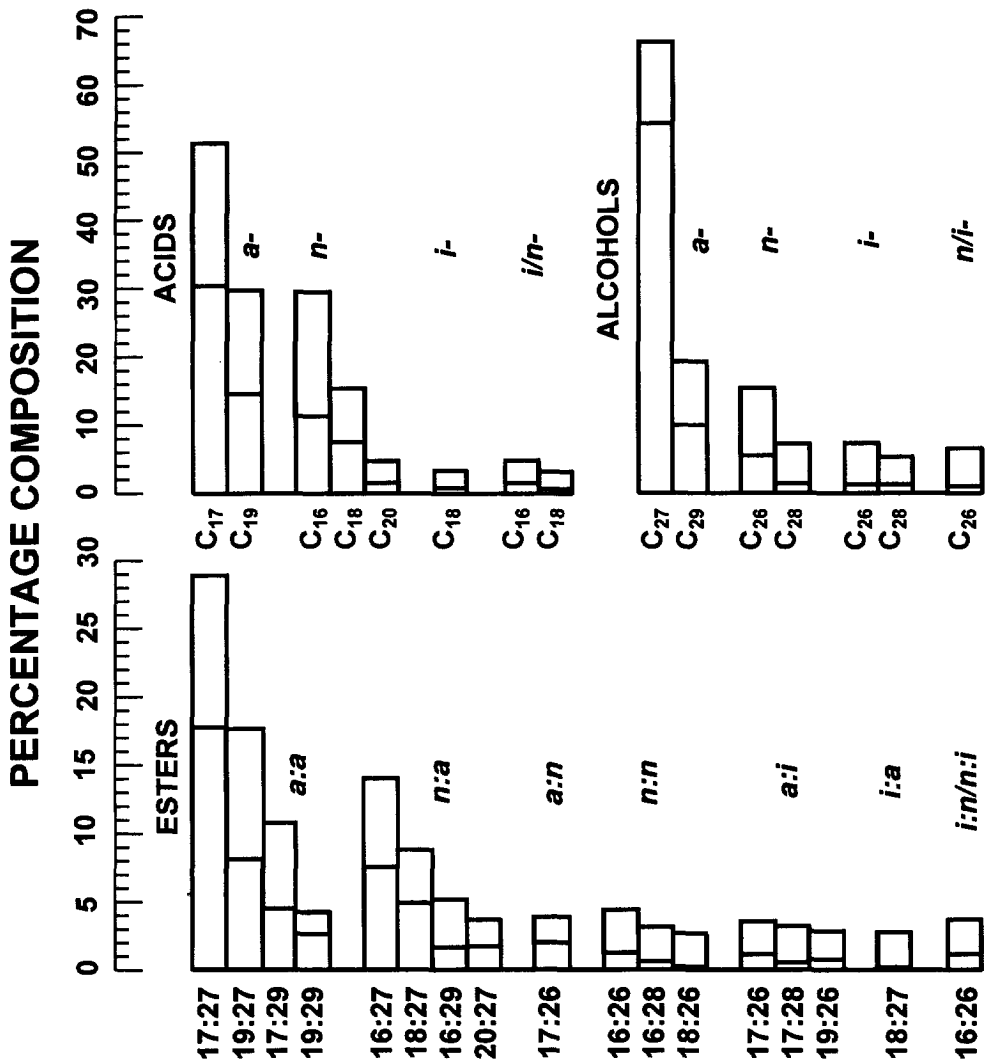

Fig. 1. Major components of long-chain esters, and acid and alcohol moieties of esters, in epicuticular wax from leaves of kale genotypes Fribor and DGC, and swede genotypes GRL and Doon Major. Esters have acid–alcohol combinations as shown, with *a*-, *anteiso*-branched chain, *i*-, *iso*-branched chain, and *n*-straight chain acid and alcohol moieties. The upper and lower values show the range of values found across all species and genotypes, under all environmental conditions.

i:n/n:i, i:a *and* a:i *esters*. Individually, *i:n/n:i*, *i:a* and *a:i* esters were relatively more abundant in (O and S), than in (I) [Table 3(A1), (C1), (C2)], as for total esters [Fig. 5(a)], with the exceptions of some swede *i:n/n:i*, *a:i* and *a:i* esters and some kale *i:a* esters. Effects of environment on the distribution of acid and alcohol chain-lengths in (O and S) relative to (I), were more varied for these ester classes than for the *a:a*, *n:n*, *n:a* or *a:n* esters; no clear patterns were evident.

The overall effects of environmental variation on the distribution of the wax esters is dependent on the combined effects of environment on the two principal stages of ester synthesis, namely formation of the precursor acid and alcohol moieties, and the esterification process itself. These possibilities were examined as follows.

Formation of wax ester precursors. The *n*-components of wax esters are formed by addition of C_2 units to a C_2 precursor (acetate) in the synthesis *de novo* of acyl chains up to C_{16}–C_{18}, followed by chain extension up to *ca* C_{18}–C_{22} (*n*-acids) and *ca* C_{26}–C_{32} (*n*-alcohols) [2, 11–13]. Formation of these components, along with the free primary *n*-alcohols found in the wax, is believed to involve an elongation–decarboxylation–oxidation–reduction (EDOR) system separate from that which produces the non-ester *n*-wax components [2, 14–16]. Evidently, the *i*- and *a*-compounds which are the major wax ester components in the *brassicae*, are similarly synthesised from C_4 (from valine) and C_5 (from isoleucine) precursors, respectively; this may involve a different EDOR system to that producing the *n*-ester components [2, 7, 9, 10, 17, 18]. Variation in environmental conditions may have several effects, including changes in the relative proportions of the *n*-, *i*- and *a*-ester components, and

changes in their chain-length distribution, as found previously for the non-ester *n*-components of these waxes [10].

The distribution of acid and alcohol moieties of each structural type changed under the different environmental conditions. In terms of total acid and alcohol moieties, *a*-acids and alcohols were of similar abundance in esters under all environmental conditions (O, S and I), except for swede, where *a*-acids were more abundant in (O and S) than in (I) [Tables 5(b and c)]. In general, *i*- and *i*/*n*-acids and alcohols were more abundant in (O and S) than in (I), while the *n*-acids and alcohols were more abundant in (I). Overall, there were more *br*-acid and alcohol moieties in (O, S) and more *n*-moieties in (I). These variations probably reflect differences in the availability and/or utilisation of the C_2, C_4 and C_5 precursors of the *n*- and *br*- acyl CoA chains from which the acid and alcohol moieties were derived [2, 7, 9, 10, 17, 18].

As suspected, the chain-length distributions of individual acid and alcohol moieties changed under the different environmental conditions (Table 4). The general increase in abundance of longer chains in (I) relative to (O), seen for kale and swede *n*-alcohols and kale *n*-acids, mirror those seen for the *n*-non-ester wax components [10]. This points to a similarity between the EDOR systems producing *n*-ester and non ester components, and may suggest a common origin for both. Similarly, increased abundance of longer chains in (I), relative to (O), was seen for some *br*- moieties, noticeably kale *a*-alcohols, some *i*-alcohols (kale, DGC) and *n*/*i*-alcohols (kale, DGC and swede, GRL). Conversely, longer chains were relatively more abundant in (O) than in (I) for most *i*-acids, *n*-acids (swede), and some *i*-alcohols (kale, Fribor and swede, Doon Major) and *n*/*i*-alcohols (kale, Fribor). Other patterns were less distinct, although mid-length chains were relatively more abundant than the shorter and longer chains in (I) than (O) for some *a*-acids (kale, DGC and swede, Doon Major), *i*-acids (kale, Fribor), *n*-alcohols (kale) and *i*/*n*-acids (swede, GRL). Conversely, they were relatively more abundant in (O) than (I) for *a*-acids (swede, GRL), *i*-alcohols (kale, Fribor and swede, GRL), *n*-alcohols (swede, GRL), *i*/*n*-acids (kales and the swede, Doon Major) and *n*/*i*-alcohols (swede, Doon Major). Patterns of variation for (S) and (O)-grown plants, relative to (I)-grown plants, were generally similar, although there were differences, particularly for *br*-moieties.

The *a*-C_{17} and C_{19} acids and C_{27} and C_{29} alcohols, and the *i*-, *n*- and *i*/*n*-C_{16} and C_{18} acids and C_{26} and C_{28} alcohols contributed most to the overall distribution of total acids and alcohols [Tables 5(b and c)].

These observations suggest that the EDOR chain-length specificity is more variable for *br*- than *n*-precursors under the different environmental conditions, and may be evidence for different systems with specificities for *n*- and *i*- and *a*-substrates, respectively. Comparison between the distribution of individual acid and alcohol moieties of the same structural classes (Table 5) showed similarities for some (*n*-compounds, kale; *a*- and *i*-compounds, swede), but differences for others (*n*-compounds, swede; *a*- and *i*-compounds, kale). This suggests differences in the chain-length specificity or activity of the elongation systems giving rise to C_{15}–C_{21} acids and C_{25}–C_{31} alcohols respectively; such differences appear to be structure and species related.

Esterification. Currently, little is known about the esterification process. Three mechanisms proposed for *B. oleracea* are direct esterification of fatty acids with alcohols, transfer of acyl groups from phospholipids to alcohols, and acyl transfer from acyl-CoA to alcohols [11]. Reaction of alcohols with aldehyde equivalents, derived from acyl chains by reduction, is also a possibility [11]. Esterification may be sensitive to acid and alcohol chain-length, and the distribution of esters may differ from that for random combination of acids and alcohols [11, 14, 19]. In wild-type maize, the combination of mid-range acids and alcohols was preferred at the seedling stage; but for young and mature plants the preference was for shorter acids with longer alcohols [14]. The latter acid/alcohol combination was also reported for pea wax esters [11, 19]. Formation of Brussels sprout wax esters was reported to favour involvement for *br*- over *n*-alcohols, leading to an enrichment of the latter in the free primary alcohol pool [7]. However, the composition of the intact esters could not be determined with certainty, therefore this possibility requires further investigation. In our previous study of swede wax esters, the combination of acids and alcohols was generally non-random, although the distribution of the major components, the *a*:*a* combinations, was close to that predicted for random combination [10].

Selectivity of acid–alcohol combination within kale and swede wax esters. Esterification selectivity was evaluated by comparison of the measured distributions of individual and total wax esters, with those expected for random combination of acids and alcohols. The latter were calculated from the data for acid and alcohol moieties (Table 4) and are shown (in italics) in Tables 3 and 5, respectively, alongside the experimental values. Predicted abundances were categorised as less than (negative sign), or more than (positive sign), or close to (no sign) those measured experimentally.

For total esters of each class, a consistent picture emerged for all genotypes under all environmental conditions. Measured concentrations of *n*:*n*, *i*:*a* and *a*:*i* esters were greater than those expected for random combination, amounts of *a*:*n* esters were less than expected, and those for *n*:*a* and *a*:*a* esters were close to random. *Anteiso*-alcohols were depleted in the free primary alcohol pool, while the *n*- and *i*-alcohols were enriched [Table 4(c)], a pattern similar to that reported previously for Brussels sprouts [7]. These observations indicate a clear non-random preference for certain combinations. The relative preference for *n*:*n* and

doubly *br-* esters over mixed *br-*/*n*-esters is of particular note, and may suggest the operation of different esterification systems with primary specificities for *br-* and *n*-substrates, respectively, but which display also a degree of cross-over.

Comparison between data for individual esters (Table 3) revealed the apparent chain-length requirements for individual acids and alcohols during esterification. For comparison of esters of different structural types, the longest continuous carbon chains (CL) in each acid and alcohol moiety were used. These are one and two carbons less than the carbon number (C_n) for singly and doubly *br-* esters, respectively. Esters were categorised as favoured (Table 3, superscript f) if the observed abundance was greater than or close to that expected for random combination, or as disfavoured (Table 3, superscript d) if the observed abundance was less than expected or if the ester was not detected. In some instances, a particular ester was mostly favoured or disfavoured across the different genotypes and growth conditions. In other cases, the overall categorisation was less clear, and is considered indicative of a chain-length limit, where esterification becomes less favourable. Comparison between esters of similar type with the same acid, but with alcohols of different lengths, or vice versa, also suggested apparent upper and lower limiting values for C_n and CL, for both acids and alcohols. These are represented in Table 3 by underlined acid or alcohol moiety carbon number and chain-length.

For doubly *br-* esters, the optimum continuous chain-lengths were 42, 44 and 46 (C_{44}, C_{46} and C_{48} *a*:*a* esters), and 41 and 43 (C_{43} and C_{45} *i*:*a* and *a*:*i* esters). Most acid–alcohol combinations were favoured. However, those with extreme lengths of acid or alcohol and some with the shortest acids (*a*:*a* and *a*:*i* esters) were disfavoured, especially for kale, while for swede longer chain-lengths were more favoured. For both species, alcohols of longer chain length were more favoured within *a*:*a* esters than *i*:*a* esters.

Optimum chain-lengths for singly *br-* esters were 41 and 45 (C_{42} and C_{46} *i*:*n*/*n*:*i* esters) and 42, 44, 46 (C_{43}, C_{45} and C_{47} *n*:*a* esters). Additionally 43 (C_{44} *i*:*n*/*n*:*i* esters) was favoured for kale but not swede. As with doubly *br-* esters, extremes of acid- or alcohol-length were generally disfavoured for kale, but were more favoured for swede, and *n*:*a* esters with the shortest alcohol were also disfavoured for both species. All *a*:*n* esters were generally disfavoured, although combinations with acids and alcohols of mid chain length were relatively more favoured, especially for swede.

The optimum CL for unbranched *n*:*n* esters were 42, 44 and 46. As with the other ester classes, extremes of acid- and alcohol-length were disfavoured for kale but were more favoured for swede.

From comparison between the data for the different structural classes, the optimum ranges of CL are 41–46 (kale) and 41–48 (swede) for the esters, 16–20 (kale and swede) for the acid moieties, and 24–28 (kale) and 24–30 (swede) for the alcohol moieties. Ester formation appears to have a particular sensitivity to the structure and length of the alcohol moieties.

The apparent preference for certain acid–alcohol combinations, in relation to both structural type and chain-length, was essentially independent of genotype, species and environmental conditions. Variation in ester composition between the different growth conditions, between species, and between genotypes within a species, must therefore depend primarily on differences in the abundance of the acid and alcohol moiety precursors supplied to the esterification process.

These results, particularly for kale *a*:*a*, *n*:*n*, *a*:*n* and *n*:*a* esters, indicate that the distribution of individual esters under the different environmental conditions is greatly influenced by alcohol chain-length. This evidently arises from the greater sensitivity of the esterification process to length of alcohol than to length of acid in combination with the regular variation in the chain-lengths of the acid and alcohol precursors, particularly the latter, under the different environmental conditions.

Statistical analysis of the GC data indicated that there were also significant intra- and inter-species variations in wax ester composition between plants grown under the same environmental conditions. Intra-species variations were evaluated in terms of the ratios F/DGC (kale) and GRL/DM (swede) of their percentage compositions, and inter-species variations (kale/swede) in terms of F/GRL, F/DM, DGC/GRL and DGC/DM.

Intra-species differences

There was little variation in the distribution of total esters or acid and alcohol moieties between the genotypes of each species, with the exception of some *i*:*n*/*n*:*i*, *i*:*a*, and *a*:*n* esters, *i*- and *i*/*n*-acids and *n*/*i*-alcohols [Tables 5(a–c)]. Although there was some variation in the chain-length distribution of individual acids, alcohols and intact esters, no clear patterns were evident.

Inter-species differences

Total esters. Kale and swede were clearly differentiated in terms of total esters [Table 4(a)], particularly for (I)-grown plants. Most noticeably, the *a*:*a* and *a*:*i* esters were more abundant in kale than swede in (I), the *n*:*a* and *a*:*n* esters were generally more abundant in swede than kale in (I, O and S). Whereas, total *i*:*n*/*n*:*i*, *i*:*a* and *n*:*n* esters were generally more abundant in kale wax than swede in (O and S), this was reversed in (I).

Individual esters. The most striking feature to the variation in wax ester distribution between kale and swede was that there were proportionally more esters with longer alcohols in swede wax relative to kale, although the reverse was seen for some ester groupings, principally in (I and S). Esters with longer acids

were relatively more abundant in kale wax than swede for all kale *a*:*a*, *a*:*n*, *i*:*a*, most *a*:*i* and some *n*:*n*, *n*:*a* and *i*:*n*/*n*:*i* esters, while for some *n*:*n*, *n*:*a* and *i*:*n*/*n*:*i* esters groupings the reverse was seen.

Total acid and alcohol moieties. The major inter-species differences were found for *a*-acids (I), *i*-acids (O), *n*-alcohols (O) and *i*/*a* and/or alcohols (O, S) which were more abundant in kale esters than swede, whereas in (I), *i*-acids, and *n*-acids and alcohols were more abundant in swede esters than kale [Tables 5(b and c)].

Individual acid and alcohol moieties. In general, data for the individual *i*-acids, most *a*-acids, some *n*- and *i*/*n*-acids and most individual *i*, *a*- and *n*-alcohols shows that the longer wax ester acid precursors were more prevalent in kale than swede, whereas the longer alcohol precursors were more prevalent in swede than kale.

Overall in (I), there were more *br* -ester components in kale, and more *n*-components in swede, while in (O and S) levels of *br*- and *n*-components were closer in both species (O, S). Kale wax esters had higher proportions of the longer acid moieties and long acid–short alcohol combinations, and swede esters higher proportions of the longer alcohol moieties and short acid–long alcohol combinations. This is further evidence for variation in both the availability and use of the precursors to the *n*- and *br*- acyl CoA chains, and in the activities/specificities of the respective elongation systems from which they were derived, and also of the apparent sensitivity of esterification to alcohol length, particularly for swede, where longer alcohols are more favoured [2, 7, 9, 10, 17, 18].

A number of conclusions can be drawn about wax ester synthesis in kale and swede. Separate EDOR systems are likely for synthesis of *n*- and *br*- ester precursors; the former is related to that producing *n*-non ester components. During elongation of *n*- and *br*- acyl CoA chains from, e.g. C_{16}, each sequential addition of C_2 is probably mediated by a separate enzyme system, particularly for the steps C_{16}–C_{18}, C_{18}–C_{20}, C_{20}–C_{22}, C_{26}–C_{28} and C_{28}–C_{30} (or equivalent for *br*-chains). This is in accord with previously studies with leek epidermal cells, *Brassica*, *Arabidopsis* and barley [8, 9, 13, 20]. Changes in the activities of these elongation components would explain the environmental and inter-species variations in chain-length distribution found for kale and swede. At least two esterification systems are present, with primary specificities for *n*- and *br*- precursors, and which show a general preference for mid-range acids and alcohols.

EXPERIMENTAL

Plant growth and epicuticular wax collection. Details of plant growth and sample collection are given elsewhere [2].

Sample preparation and analysis by GC and GC-MS. Details of sample preparation, chemical derivitization, analytical instrumentations, chromatographic conditions and analytical methodology are given in ref. [10].

Statistical analysis of capillary GC data. These were analysed by ANOVA using the Genstat 5 software package.

Acknowledgements—The authors thank Drs R. Baur and E. Städler of the Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture, Wädenswil, Zurich, Switzerland, for providing plant material; Mr J. W. McNicoll of Biomathematics and Statistics Scotland for conducting the statistical analyses; Mr P. Smith for provision of data transcription software; and The Scottish Office Agriculture, Environment and Fisheries Department for financial support.

REFERENCES

1. Baur, R., Birch, A. N. E., Hopkins, R.J., Griffiths, D. W., Simmonds, M. S. J. and Städler, E., *Entemology and Experimental Applications*, 1996, **78**, 61.
2. Shepherd, T., Robertson, G. W., Griffiths, D. W., Birch, A. N. E. and Duncan, G., *Phytochemistry*, 1995, **40**, 407.
3. Franich, R. A., Goodin, S. A. and Volkman, J. K., *Phytochemistry*, 1985, **24**, 2949.
4. Gülz, P.-G., Markstadter, C. and Riederer, M., *Phytochemistry*, 1994, **35**, 79.
5. Miwa, T. K., *Journal of the American Oil Chemists Society*, 1984, **61**, 407.
6. Spencer, G. F. and Plattner, R. D., *Journal of the American Oil Chemists Society*, 1984, **61**, 90.
7. Baker, E. A. and Holloway, P. J., *Phytochemistry*, 1975, **14**, 2463.
8. Holloway, P. J., Brown, G. A., Baker, E. A. and Macey, M. J. K., *Chemistry and Physics of Lipids*, 1977, **19**, 114.
9. Netting, A. G., Macey, M. J. K. and Barber, H. N., *Phytochemistry*, 1972, **11**, 579.
10. Shepherd, T., Robertson, G. W. and Griffiths, D. W., *Phytochemical Analysis*, 1994, **6**, 65.
11. Kolattukudy, P. E., Croteau, R. and Buckner, J. S., in *Chemistry and Biochemistry of Natural Waxes*, ed. P. E. Kolattukudy. Elsevier, Amsterdam, 1976, p. 289.
12. Bianchi, G., in *Waxes: Chemistry, Molecular Biology and Functions*, ed. R. J. Hamilton. The Oily Press, Dundee, 1995, p. 175.
13. Wettstein-Knowles, P. von, in *Waxes: Chemistry, Molecular Biology and Functions*, ed. R. J. Hamilton. The Oily Press, Dundee, 1995, p. 91.
14. Avato, P., Bianchi, G. and Pogna, N., *Phytochemistry*, 1990, **29**, 1571.
15. Avato, P., Bianchi, G., Gentinetta, E. and

Salamini, F., *Journal of Experimental Botany*, 1984, **35**, 245.
16. Mikkelsen, J. D., *Carlseberg Research Communications*, 1978, **43**, 15.
17. Kolattukudy, P. E., *Plant Physiology*, 1968, **43**, 1423.
18. Macey, M. J. K. and Barker, N. H., *Phytochemistry*, 1970, **9**, 12.
19. Kolattukudy, P. E., *Lipids*, 1969, **5**, 398.
20. McNevin, J. P., Woodward, W., Hannoufa, A., Feldmann, K. A. and Lemieux, B., *Genome*, 1993, **36**, 610.