Journal of Chromatography, 75 (1973) 137–140

Note

CHROM. 6312

Chromatographic analysis of isomeric isovalerate and isoamyl wax esters

The occurrence of isovalerate wax esters of long-chain fatty alcohols in the head fat of the Atlantic bottle-nosed dolphin (*Tursiops truncatus*) was first reported in 1930 by GILL AND TUCKER[1]. Similar isovalerate esters have since been reported in the head fats of other genera of the Delphinidae family such as the pilot whale *Globicephala melaena*[2,3] and *Sotalia fluviatilis*[4]. Recently, VARANASI AND MALINS[5] identified a small quantity of isoamyl alcohol in addition to large amounts of isovaleric acid in the wax esters of *Tursiops gilli* jaw fat. This isoamyl alcohol is presumably esterified to long-chain fatty acids, indicating the probable existence of isomeric wax esters having the same number of carbon atoms but with the structures of the fatty acid and fatty alcohol chains reversed. For example, two such isomeric wax esters might be hexadecyl isovalerate (*n*-16:0 | *iso*-5:0*) and isoamyl palmitate (*iso*-5:0 | *n*-16:0).

As part of our studies of Odontocete head fats to determine if their unusual chemical structure might be related to their role in echolocation[6], we have developed new chromatographic procedures for the analysis of individual isovalerate and isoamyl wax ester isomers. Our experiments show that isovalerate and isoamyl wax esters can be separated from other lipids and from wax esters of higher carbon number by thin-layer chromatography (TLC) on silicic acid. Isomeric isovalerate and isoamyl forms can then be fully resolved by gas–liquid chromatography (GLC) on polyester capillary columns.

Pure methyl esters of *n*-11:0, *n*-12:0, *n*-13:0, *iso*-14:0, *n*-14:0, *anteiso*-15:0, *n*-15:0, *iso*-16:0, *n*-16:0, 16:1ω7, *anteiso*-17:0, 18:1ω9 and 20:1ω9 fatty acids were purchased from various commercial sources. These were converted to isoamyl esters by KOH-catalyzed alcoholysis[7] with isoamyl alcohol and purified by preparative TLC. A mixture of isoamyl esters of *n*-16:0, *n*-18:0, 18:1ω9, 18:2ω6, and 18:3ω3 was prepared from linseed oil in the same manner. The analogous isovalerate wax esters were prepared from the same starting materials by first reducing the esters to fatty alcohols with $LiAlH_4$. Isovaleroyl anhydride was prepared from isovaleric acid and dicyclohexylcarbodiimide[8]. The isovalerate esters were then synthesized by dissolving the fatty alcohols and excess isovaleroyl anhydride in pyridine, heating in sealed ampules for 3 h at 100°, partitioning the product between equal volumes of petroleum ether and acetonitrile, recovering and evaporating the petroleum ether layer, and purifying the wax ester product by preparative TLC.

The isovalerate and isoamyl wax esters can be separated from common C_{30}–C_{36} wax esters such as *n*-16:0 | *n*-16:0 by TLC on silicic acid (Fig. 1). This resolution of wax esters into C_5- and non-C_5-containing components by TLC is not unexpected,

* The usual shorthand nomenclature for fatty acids and fatty alcohol is *chain length*: *number of double bonds* ω *position of last double bond relative to terminal methyl group*. This terminology has been extended to wax esters using the convention *alcohol*|*acid*.

since we have previously demonstrated the TLC fractionation of beluga whale triglycerides on the basis of their isovalerate content[9]. The isovalerate and isoamyl wax esters have the lower R_F value, no doubt because the smaller bulk of the C_5 chain allows stronger adsorption of the ester group by the silicic acid than a longer hydrocarbon chain permits. As one would predict from their similar structures, the isomeric isovalerate and isoamyl wax esters have identical R_F values in TLC.

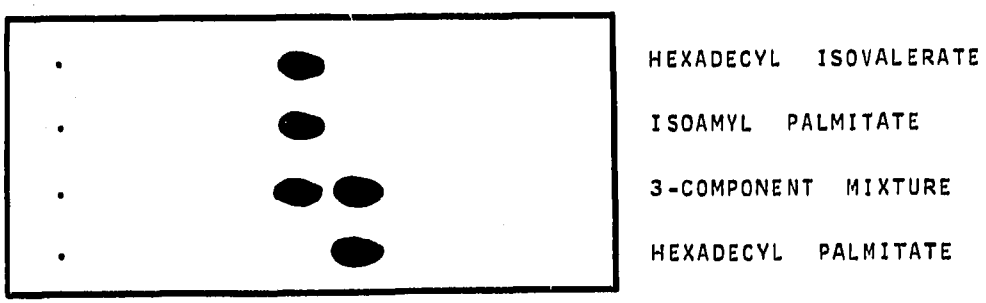

Fig. 1. Resolution of isovalerate and isoamyl wax esters from long-chain wax esters by TLC on silicic acid. Operating conditions: 200 × 200 mm TLC plate coated with 0.25 mm layer of Adsorbosil-1 impregnated with 0.04% Rhodamine 6G; developed once in petroleum ether–diethyl ether (95:5); spots visualized under UV light.

Isomeric pairs of isovalerate and isoamyl wax esters can be fully resolved by GLC on a 46 m × 0.25 mm I.D. capillary column coated with diethyleneglycol succinate polyester (DEGS). Typical results are shown in Fig. 2. The isovalerate isomer elutes before the isoamyl isomer in each case. Similar results were also obtained on capillary columns coated with butanediol succinate polyester or Apiezon L, but only shoulder peaks were observed when isomer pairs were chromatographed on a

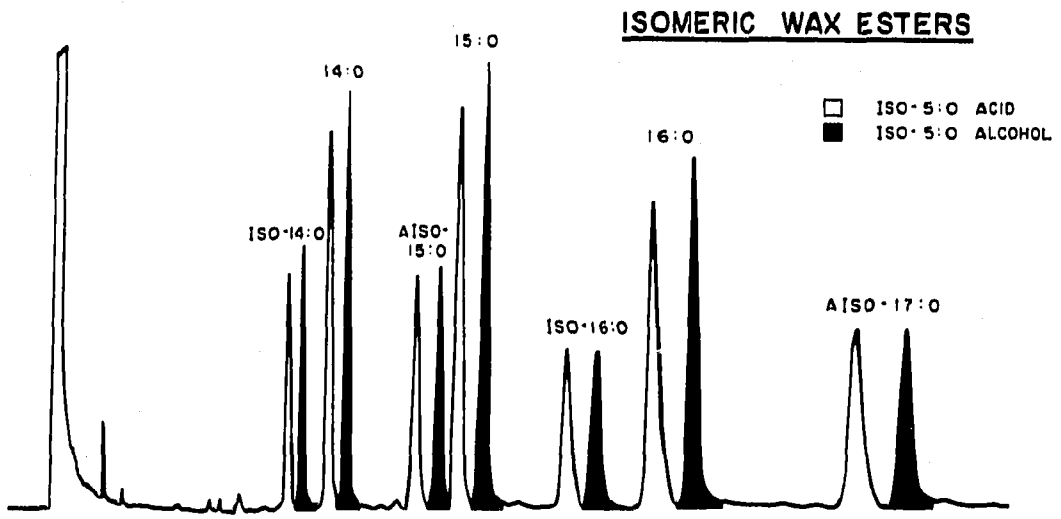

Fig. 2. Resolution of isomeric isovalerate and isoamyl wax esters by GLC on a capillary column coated with DEGS polyester. Operating conditions: 46 m × 0.25 mm I.D. capillary column coated with diethyleneglycol succinate polyester; column temperature, 150°; 60 p.s.i.g. helium carrier carrier gas; Perkin-Elmer Model 226 gas chromatograph.

packed DEGS column (1.83 m × 2.5 mm I.D., 10 % DEGS on 80–100 mesh Chromosorb W, 160°). Obviously the higher resolution of capillary columns is essential for complete separation of these isomers.

Equivalent chain length (ECL) values[10] for all isomers analyzed are reported in Table I. These have been calculated on two different bases: (i) relative to the isovalerate esters of *n*-fatty alcohols, the usual homologous series of wax esters found in dolphin head fats; and (ii) relative to *n*-methyl esters, the more commonly available GLC standards used in lipid analysis. As expected, chain structure homologies can be demonstrated by a semi-log plot of carbon number *vs.* retention time.

TABLE I

EQUIVALENT CHAIN LENGTH VALUES FOR ISOVALERATE AND ISOAMYL WAX ESTERS ON A DEGS POLYESTER CAPILLARY COLUMN

Long-chain moiety	*ECL values relative to isovalerate esters*		*ECL values relative to n-methyl esters*	
	Isovalerate esters	*Isoamyl esters*	*Isovalerate esters*	*Isoamyl esters*
n-11:0	11.00	11.18	13.44	13.62
n-12:0	12.00	12.17	14.43	14.61
n-13:0	13.00	13.16	15.43	15.60
iso-14:0	13.57	13.74	16.03	16.19
n-14:0	14.00	14.16	16.44	16.61
anteiso-15:0	14.70	14.87	17.19	17.34
n-15:0	15.00	15.16	17.45	17.62
iso-16:0	15.60	15.76	18.11	18.28
n-16:0	16.00	16.15	18.51	18.69
16:1ω7	16.30	16.47	18.73	19.00
anteiso-17:0	16.72	16.92	19.29	19.44
n-18:0	18.00	18.19	20.62	20.81
18:1ω9	18.15	18.33	20.76	20.93
18:2ω6	18.69	18.83	21.32	21.48
18:3ω3	19.42	19.57	22.08	22.24
20:1ω9	20.22	20.36	22.82	22.95

ECL values calculated on either basis show an average difference between the isovalerate and isoamyl isomers of 0.17 units. Isovalerate wax esters elute 2.4–2.6 units and isoamyl esters 2.6–2.8 units beyond the corresponding methyl ester having the same long-chain moiety. The actual carbon number difference between a methyl ester and a C_5 ester is 4.0, but the branched structure of the C_5 chain considerably reduces its effect on the ECL value[11]. The difference between the methyl and the C_5 ester ECL values varies with the particular DEGS column employed and often (as here) increases with increasing ester chain length. ACKMAN[11] has previously noted that β-methyl substitution in fatty acid esters has a non-uniform effect on their ECL values.

Fig. 1 and the ECL data in Table I show that it is possible to separate any pair of isovalerate and isoamyl wax ester isomers such as *n*-16:0 | *iso*-5:0 and *iso*-5:0 | *n*-16:0 with baseline separation on a 46-m DEGS capillary column having a resolution of > 25,000 theoretical plates (for methyl palmitate). However, there would probably be some overlapping peaks when complex isovalerate and isoamyl wax ester mixtures

were analyzed. Prior fractionation of wax esters by Ag^+ TLC would be helpful in eliminating overlap between saturated and unsaturated molecules; but Delphinidae head fats mainly contain saturated wax esters[1–5], so it is the overlap among each set of six possible isomeric *iso*-, *anteiso*-, and *n*-chain esters that is most critical. If all six isomers of the same carbon number were chromatographed together, five peaks would be resolved. Both experimental work and ECL calculations from Table I show that the *iso*-5:0 | *iso*-X and *anteiso*-X | *iso*-5:0 isomers elute together and cannot be distinguished. Fortunately, however, very little fatty alcohol with the *anteiso* chain structure has been found in the Delphinidae head fat wax esters examined so far[1–5].

Application of the above TLC and GLC separation techniques to the wax esters found in the jaw fat of the Atlantic bottle-nosed dolphin (*Tursiops truncatus*) has resulted in only a partial identification of the molecular species present. Major GLC peaks representing the isovalerate esters of long-chain fatty alcohols are present, and minor peaks having the same retention times as some isoamyl wax esters are seen. However, numerous other unidentified peaks also appear in the chromatogram; and work is now in progress to identify these.

This investigation was supported in part by grants from the National Science Foundation (GB-30553) and the Rutgers University Research Council.

Department of Biochemistry, CARTER LITCHFIELD
Nelson Biological Laboratories, Rutgers University,
New Brunswick, N. J. 08903 (U.S.A.)

Halifax Laboratory, R. G. ACKMAN
Fisheries Research Board of Canada,
Halifax, Nova Scotia (Canada)

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Received August 21th, 1972