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Molecular Analysis of Intact Preen Waxes of *Calidris canutus* (Aves: Scolopacidae) by Gas Chromatography/Mass Spectrometry

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ABSTRACT: The intact preen wax esters of the red knot *Calidris canutus* were studied with gas chromatography/mass spectrometry (GC/MS) and GC/MS/MS. In this latter technique, transitions from the molecular ion to fragment ions representing the fatty acid moiety of the wax esters were measured, providing additional resolution to the analysis of wax esters. The C_{21}–C_{32} wax esters are composed of complex mixtures of hundreds of individual isomers. The odd carbon-numbered wax esters are predominantly composed of even carbon-numbered *n*-alcohols (C_{14}, C_{16}, and C_{18}) esterified predominantly with odd carbon-numbered 2-methyl fatty acids (C_7, C_9, C_{11}, and C_{13}), resulting in relatively simple distributions. The even carbon-numbered wax esters show a far more complex distribution due to a number of factors: (i) Their *n*-alcohol moieties are not dominated by even carbon-numbered *n*-alcohols esterified with odd carbon-numbered 2-methyl fatty acids, but odd and even carbon-numbered *n*-alcohols participate in approximately equal amounts; (ii) odd carbon-numbered methyl-branched alcohols participate abundantly in these wax ester clusters; and (iii) with increasing molecular weight, various isomers of the 2,6-, 2,8-, and 2,10-dimethyl branched fatty acids also participate in the even carbon-numbered wax esters. The data demonstrate that there is a clear biosynthetic control on the wax ester composition although the reasons for the complex chemistry of the waxes are not yet understood.

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The survival of marine birds depends on an intact and waterproof plumage kept in good shape. A key component in the maintenance systems of the feather coat of birds is the preen (or uropygial) gland, located near the tail, which produces a variety of waxes (1). Yet, we understand very little of the preen gland waxes, but they may fulfill several crucial functions: keeping feathers flexible, protecting against wetting, reducing damage (including ultraviolet protection), playing a role as antiparasitic agents, and acting as pheromones (2; and

references cited therein). Preen gland secretions consist predominantly of monoester waxes (3), which are composed of a fatty acid esterified to an alcohol moiety. These moieties may possess straight-chain, monomethyl alkyl, polymethyl alkyl, or even more complex carbon skeletons. Usually a mixture of fatty acids and alcohols with varying chain lengths and degree and location of branching is used, resulting in a complex mixture composed of hundreds of individual wax esters. The pattern of the lipid constituents has been found to be quite characteristic for a species and differs markedly between various bird taxa (1,4).

The very complex wax composition is usually determined by the analysis of fatty acids and alcohols released after hydrolysis of the monoester preen waxes (2,5). This leads to a loss of information concerning the molecular distribution, which determines the physical properties of the preen wax. In this paper the preen gland wax composition of a long-distance migrating shore bird, the red knot (*Calidris canutus),* was studied in detail using an approach by which the intact waxes were analyzed by capillary gas chromatography/mass spectrometry (GC/MS) and gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS). Recent studies of the preen waxes of male and female *C. canutus* revealed significant changes over the annual cycle in the chemical composition of the preen waxes (6,7) from monoester preen waxes to diester waxes. This warranted a detailed chemical and ecological study of the structure and role of preen waxes in *C. canutus*. Here we focus on the analytical characterization of the complex monoester wax of *C. canutus*.

EXPERIMENTAL PROCEDURES

Birds. Preen wax samples were taken from the red knot, *C. canutus*, subspecies *islandica,* kept in outdoor cages at north-temperate latitudes over several annual cycles. The composition of the preen wax of *C. canutus* has been shown to vary strongly over the annual cycle (6). Here we describe the detailed chemical composition of the preen wax pattern observed in the July–March period. Knot #283 was used for this purpose. To compare the wax patterns of different species, three other species (*Limosa lapponica* 1374068, *Tringa nebularia* 1374069, and *C. alba* H227028) were captured with

mistnets on Augusts 2, 1998, on the intertidal flats near Vlieland, an island in the Dutch Wadden Sea.

Sample processing. Wax samples were taken from the living birds by softly squeezing the gland area (4). The cotton-wool was extracted with 1 mL ethyl acetate and filtered by column chromatography using Na_2SO_4 and ethyl acetate as eluent. The dried extract was analyzed directly by GC, GC/MS, and GC/MS/MS. To study the fatty acid and alcohol composition of the monoesters, wax samples of Knots #294, #382, and #389 were combined for preparative thin-layer chromatography (TLC) analysis (8). TLC bands were scraped off the TLC plate and extracted with ethyl acetate and analyzed by GC and GC/MS. The monoester fraction (representing the largest TLC fraction) was saponified (1 M KOH in 96% methanol), and the fatty acids and alcohols released from the monoesters were derivatized with diazomethane and bis(trimethylsilyl)trifluoroacetamide to their corresponding methyl esters and trimethylsilyl ether derivatives and analyzed by GC/MS.

GC. GC was performed with a Hewlett-Packard 6890 Series II instrument (Palo Alto, CA), using an on-column injector. Detection was accomplished using a flame-ionization detector. Helium was used as the carrier gas. Separation was achieved using a fused-silica capillary column (25 m × 0.32 mm i.d.) coated with CP-Sil 5CB (film thickness 0.12 μm). The samples, dissolved in ethyl acetate, were injected at 70°C, and subsequently the oven was programmed to 130°C at 20°C/min and then 4°C/min to 320°C, where it was held for 30 min. Saponified extracts were injected at 40°C, programmed to 200°C at 4°C/min, 10°C/min to 300°C where it was held for 15 min.

GC/MS. GC/MS was performed on a Hewlett-Packard 5890 Series II gas chromatograph interfaced to a VG Autospec Ultima Q mass spectrometer. GC conditions were identical as described above. Electron impact spectra were obtained at 70 eV using the following conditions: mass range *m/z* 800–50; cycle time 1.6 s; resolution 1000. For GC/MS/MS, dissociation of the parent ions was induced by collision with argon (collision energy 20 eV). The parent ion to daughter ion transitions were analyzed with 20 ms settling and 75 ms sampling periods resulting in a 1.33 s total cycle time. Separation was achieved using the same capillary column and temperature program as described for GC analyses.

RESULTS AND DISCUSSION

Analysis of intact preen waxes. Figure 1 shows the gas chromatograms of the preen gland lipids of four different species, *L. lapponica*, *T. nebularia*, *C. alba,* and *C. canutus islandica* to illustrate the complex distribution of the intact wax lipids. The preen lipids of the four species investigated are dominated by monoester waxes. The *L. lapponica* preen lipids are dominated by C_{22}–C_{38} monoester waxes, those of *T. nebularia* consist mainly of C_{23}–C_{34} monoester waxes, C_{22}–C_{33} monoester waxes for *C. alba,* and C_{21}–C_{32} monoester waxes are dominant in the preen lipids of *C. canutus*. The molecular

weight, fatty acid, and alcohol moiety of the wax esters were established by GC/MS. For example, Figure 2 shows mass spectra of two C_{24} wax esters composed of a C_8 fatty acid esterified with a C_{16} alcohol and a C_{10} fatty acid esterified with a C_{14} alcohol. The base peaks result from loss of the fatty acid substituents with concomitant transfer of two hydrogen atoms (9). Together with the molecular ion, this leads to identification of the wax ester, although no specific information on the carbon skeleton of the fatty acid and alcohol is obtained.

Detailed mass spectrometric analysis. Captive *C. canutus* were used to examine the intact preen gland lipids in detail by GC/MS and GC/MS/MS analysis. Mass chromatography of the molecular ions is a useful tool to analyze individual clusters of wax esters with the same molecular weight in the preen wax. Figure 3 has been constructed on this basis and shows the mass chromatograms of C_{21}–C_{32} wax esters (*m/z* 326 + n × 14). Each individual cluster of wax esters with the same molecular weight has been normalized to the most abundant isomer present. Figure 3 shows that with increasing molecular weight the distribution of wax esters becomes increasingly complex.

To obtain information on the building blocks of the wax esters, a monoester fraction was isolated from the preen wax, hydrolyzed, and the fatty acids and alcohols formed were identified after derivatization. Fatty acids and alcohols were identified based on MS data reported in the literature (4) and the recognition of (pseudo) homologous series through linear Kovats plots (Table 1; Ref. 9). Figure 4 shows the total ion current of the hydrolyzed waxes and summed mass chromatograms of *m/z* 88 + 101 and *m/z* 199 + 213 + 227 + 241 + 255 to show the distributions of the dominant fatty acids and alcohols, respectively. Straight-chain fatty acids are present only in trace quantities and occur in the range C_{16}–C_{28} with even carbon-numbered components predominating. The abundant fatty acids are 2-methyl branched fatty acids with characteristic *m/z* 88 and 101 fragment ions (resulting from McLafferty rearrangements) in their mass spectra. They comprise C_6–C_{15} 2-methyl fatty acids and 2,6-, 2,8-, and 2,10-dimethyl fatty acids (Fig. 4B). The distribution of the C_{14}–C_{18} alcohols is exemplified by a mass chromatogram of M − 15 (Fig. 4C). The even carbon-numbered alcohols show a simple distribution composed of only the straight-chain alcohol isomers. The odd carbon-numbered alcohols have, however, a very different distribution pattern characterized by the straight-chain alcohol and ω-2-, ω-4- and ω-6-methyl and 2-methyl alcohols. These results are in general agreement with those reported by Jacob and Poltz (5), although these authors also reported 4-methyl fatty acids and 4-methyl alcohols, which were not encountered as abundant compounds in this study.

The intact C_{21} wax ester (i.e., the one peak in the mass chromatogram of *m/z* 340, Fig. 3) possesses an *m/z* 131 fragment ion in its mass spectrum, indicating that it contains a C_7 fatty acid moiety. With this information it can be identified as tetradecyl 2-methylhexanoate on the basis of the distribution of the alcohols and fatty acids formed upon hydrolysis
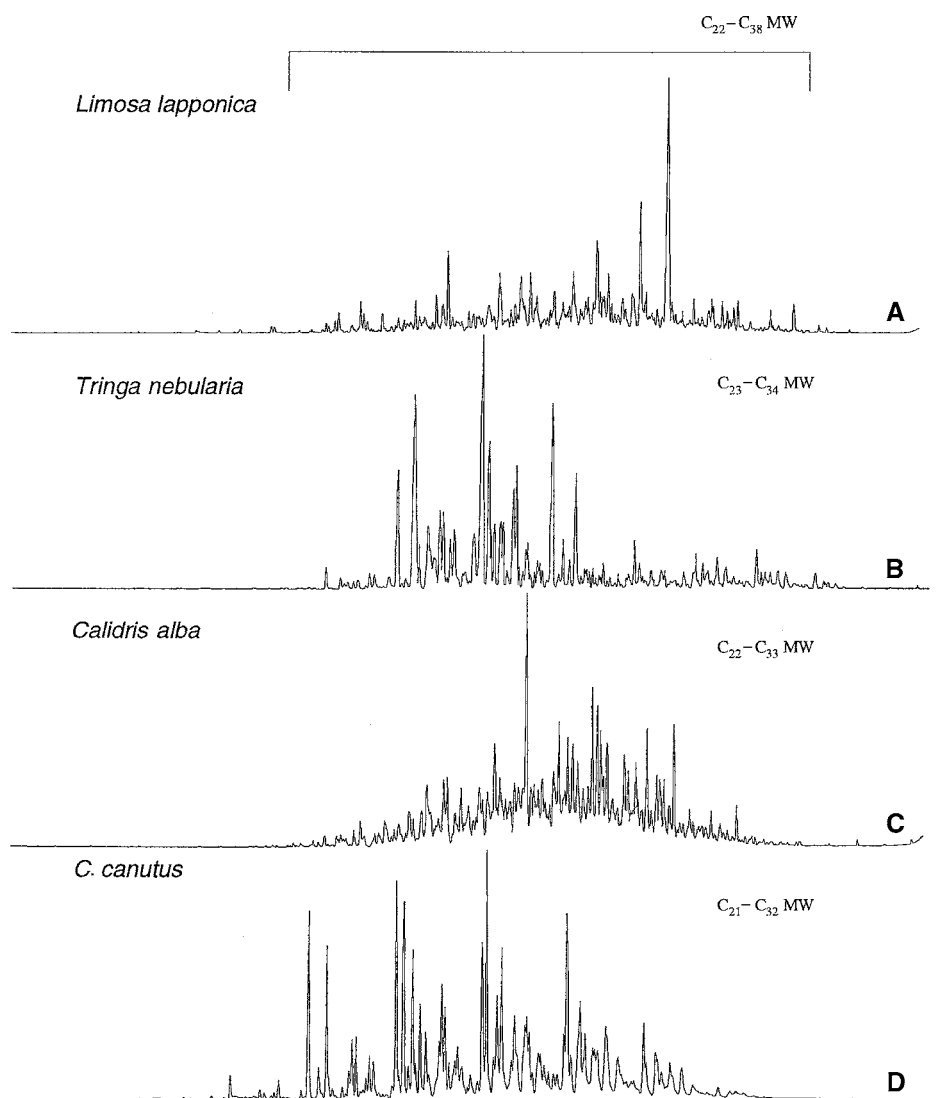
FIG. 1. Partial gas chromatography–flame-ionization detection chromatograms of the intact preen gland wax samples of several species: (A) bar-tailed godwit *Limosa lapponica*; (B) greenshank *Tringa nebularia*; (C) sanderling *Calidris alba*; (D) red knot *C. canutus*.

TABLE 1
Retention Data of Branched Fatty Acids and Alcohols

	Homologous series[a]	RI[b]			Range
		a	b	r^2	
Fatty acid	2-Methylalkanoic acid	100	0	n.a.	$C_6–C_{16}$
	2,6-Dimethylalkanoic acid	96	1	0.9999	$C_8–C_{14}$
	2,8-Dimethylalkanoic acid	93.5	44.5	0.9998	$C_{10}–C_{14}$
	2,10-Dimethylalkanoic acid	90.4	103.7	0.9998	$C_{12}–C_{14}$
Alcohol	*n*-Alkanols	100	0	n.a.	$C_{12}–C_{19}$
	2-Methylalkanols	99.5	−58.3	1	$C_{12}–C_{19}$
	2-Ethylalkanols	100	−96.2	1	$C_{14}–C_{18}$
	ω-2-Methylalkanols	100	−30	1	$C_{15}–C_{17}$
	ω-4-Methylalkanols	100.4	−55.9	0.9997	$C_{14}–C_{19}$
	ω-6-methylalkanols	99.5	−48.3	1	$C_{15}–C_{17}$

[a]Analyzed as their methyl esters or trimethylsilyl ethers.
[b]Retention index (RI) measured on CP-Sil 5CB, 130 to 320°C at 4°C/min, with the homologous series of 2-methylalkanoic acid and *n*-alcohols, respectively, as calibration standards, resulting in Kovats' plots $y = a \cdot x + b$ with correlation coefficient r^2. $RI = 100 \cdot z + 100 \cdot \{t(x) - t(z)\}/\{t(z + 1) - t(z)\}$ where $t(x)$ is retention time of compound for which RI is to be determined, $t(z)$ and $t(z + 1)$ are the retention times of the homologous which bracket the compound, and z is the number of carbon atoms. n.a., not applicable.
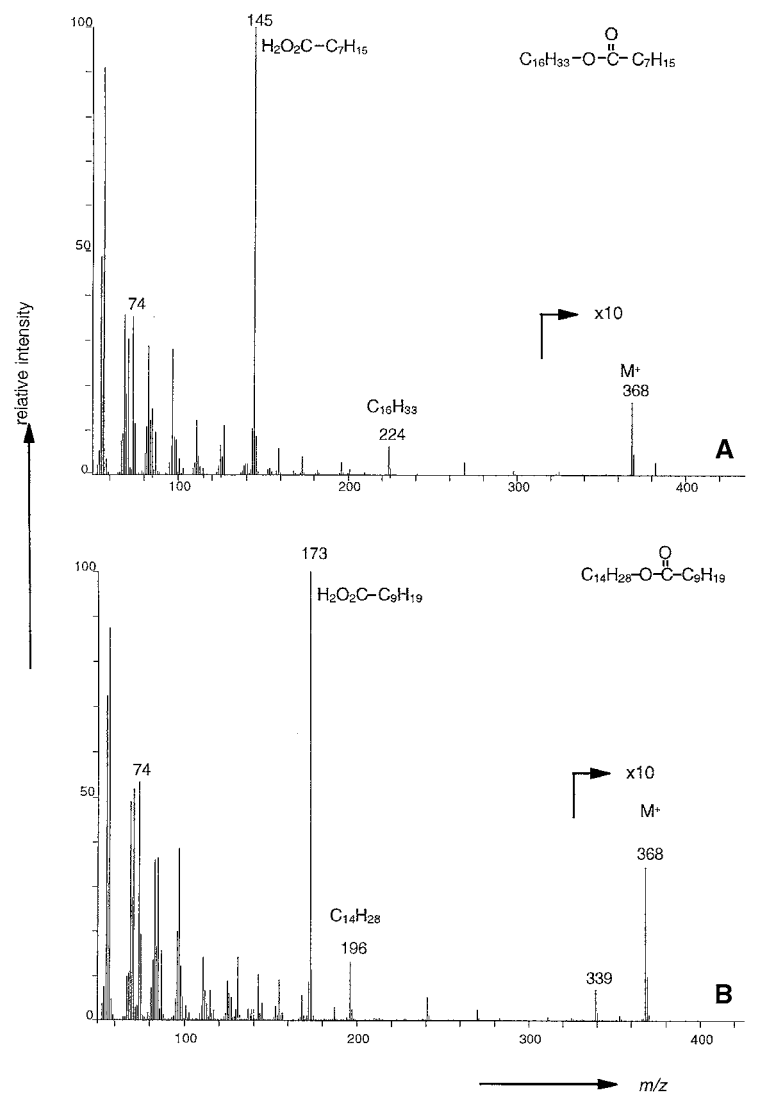
FIG. 2. Mass spectra (corrected for background) of two C_{23} wax esters: (A) hexadecyl 2-methyl-heptanoate (peak number 22 listed in Table 2); (B) tetradecyl 2,6-dimethyloctanoate (peak number 17 listed in Table 2).

(Fig. 4) since this represents the only possible combination of a C_7 fatty acid and C_{14} alcohol moiety to build a C_{21} wax ester. Due to the increasing complexity of the wax ester distribution pattern with increasing molecular weight (Fig. 3), these lines of argument become increasingly difficult to apply. The increasingly complex distribution with increasing molecular weight is due to: (i) the increasing number of possible combinations of fatty acids and alcohols to form a wax ester with a specific total number of carbon atoms and (ii) the increasing number of positional isomers for especially the fatty acids with increasing molecular weight (Fig. 4).

These problems in identifying intact wax esters can be partially overcome by using mass chromatograms of the characteristic fragment ion formed from the fatty acid moiety of the wax ester (i.e., m/z 131 + n·14) in combination with mass chromatograms of the molecular ions. However, since the clusters of wax esters with the same molecular weight par-

tially overlap (Fig. 3), the coupling of molecular weight and specific fatty acid fragment ion becomes unreliable.

GC/MS/MS analysis. One way to overcome this problem in the identification of the intact wax esters is the application of GC/MS/MS. In this technique an ion with a specific mass (parent) is selected by the first mass spectrometer, led into the collision cell of the second mass spectrometer, and selected daughter ions, formed by collision-induced fragmentation, are collected on the second mass detector. Using this GC/MS/MS technique, it is possible to get detailed information on the composition of the preen wax. The major wax esters identified in this way are listed in Table 2.

As an example, the GC/MS/MS results for C_{24} wax esters are shown in Figure 5. These results are based on selection of the molecular ion (m/z 368) in the first mass spectrometer and detection of daughter ions representing the fatty acid part of the wax ester (Fig. 2) in the second mass spectrometer. Fig-
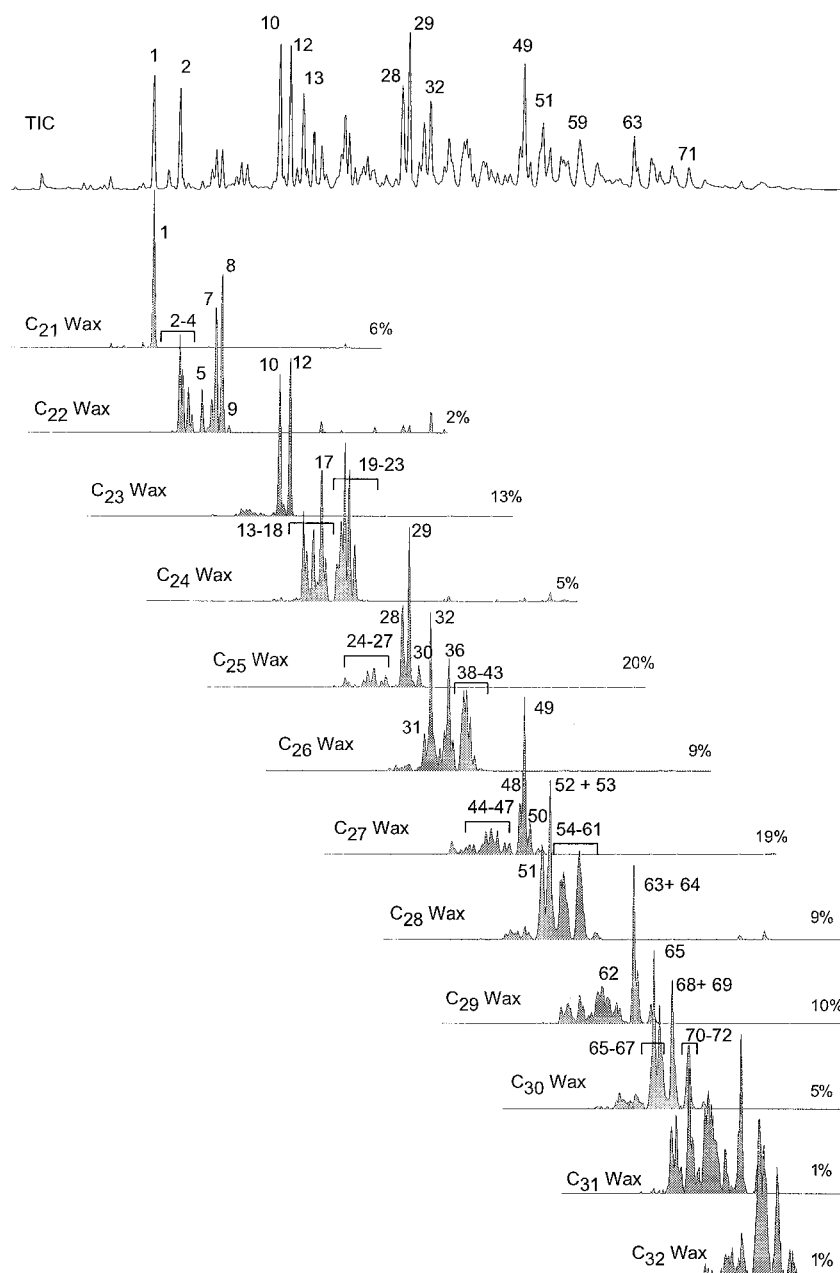
FIG. 3. Partial and mass chromatograms of the molecular ions of the C_{21}–C_{32} wax esters (*m/z* 340, 354, 368, 382, 396, 410, 424, 438, 452, 466, 480, 494) present in the nonsaponified preen wax of *C. canutus* #283. Numbers refer to specific wax ester listed in Table 2. Numbers at the end of the traces (in %) indicate the abundance based on peak height of the most abundance peak of the cluster (total set to 100%). TIC, total ion current; see Figure 1 for other abbreviation.
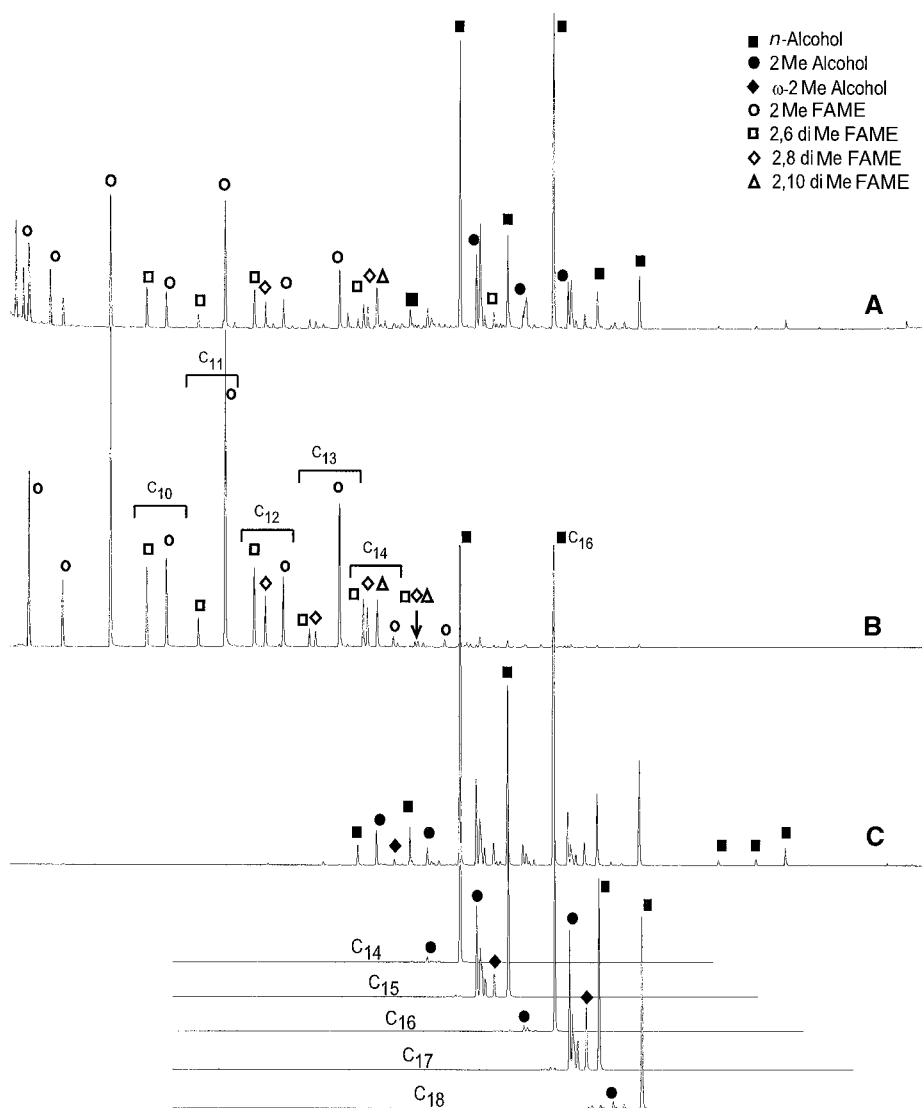
FIG. 4. Partial TIC (A) and (summed) mass chromatograms of *m/z* 88 + 101 (B) and 199 + 213 + 227 + 241 + 255 (C), showing homologous series of 2-methyl and dimethyl fatty acids and linear and methyl alcohols in the saponified monoester fraction of the preen wax of *C. canutus*. Numbers indicate the total number of carbon atoms. FAME, fatty acid methyl esters. See Figures 1 and 3 for other abbreviation.

ure 5D shows the *m/z* 368 → *m/z* 131 transition, representing C_{24} wax esters, containing a C_7 fatty acid moiety and, consequently, a C_{17} alcohol moiety. Hydrolysis has demonstrated that there is only one C_7 fatty acid (i.e., 2-methylhexanoic acid) but at least five structural isomers of the C_{17} alcohol (i.e., heptadecan-1-ol and various methyl-branched C_{17} alcohols) (Fig. 4). In fact, the distribution of peaks in the *m/z* 368 → *m/z* 131 transition is quite similar to the distribution of C_{17} alcohols in the hydrolyzed preen wax (Fig. 3). This is due to the presence of wax esters composed of 2-methylhexanoic acid esterified with all C_{17} alcohol isomers (Table 2). Due to the additivity principle (10), the retention behavior of these wax esters is similar to that of the alcohols. In this way all transitions can be analyzed. The *m/z* 368 → *m/z* 159 transition (Fig. 5F) is rather similar and shows the wax esters of

2-methyloctanoic acid with various C_{15} alcohols. In contrast, the *m/z* 368 → *m/z* 145 transition (Fig. 5E), revealing the C_{24} wax esters comprised of combinations of C_8 fatty acids and C_{16} alcohols, is dominated by one component (hexadecyl 2-methylheptanoate), in agreement with the relatively simple distribution of the C_{16} alcohols (Fig. 4). The *m/z* 368 → *m/z* 173 transition (Fig. 5G), revealing the C_{10}/C_{14} (FA/Alc.) wax esters, is comparatively simple, although in addition to tetradecyl 2-methylnonanoate another earlier-eluting wax ester is present. This is because dimethyl branched fatty acids are present in the hydrolyzed preen wax from C_{10} onward (Fig. 3). The earlier-eluting C_{10}/C_{14} wax ester was therefore identified as tetradecyl 2,6-dimethyloctanoate. The *m/z* 368 → *m/z* 187 and *m/z* 368 → *m/z* 201 transitions (Fig. 5H and 5I) revealed trace amounts of C_{11}/C_{13} and C_{12}/C_{12} wax

TABLE 2
Major Components of the Preen Gland Wax Esters from *Calidris canutus*

| Peak number	MW[a]	Compound	Peak number	MW	Compound
1	326	Tetradecyl 2-methylhexanoate	35	396	Tetradecyl 2,8-dimethyldecanoate
2	340	2-Methyltetradecyl 2-methylhexanoate	36	396	Hexadecyl 2,6-dimethyloctanoate
3	340	8-Methyltetradecyl 2-methylhexanoate	37	396	14-Methylhexadecyl 2-methyloctanoate
4	340	10-Methyltetradecyl 2-methylhexanoate	38	396	Tridecyl 2-methyldodecanoate
5	340	12-Methyltetradecyl 2-methylhexanoate	39	396	Tetradecyl 2-methylundecanoate
6	340	Tridecyl 2-methyloctanoate	40	396	Pentadecyl 2-methyldecanoate
7	340	Tetradecyl 2-methylheptanoate	41	396	Hexadecyl 2-methylnonanoate
8	340	Pentadecyl 2-methylhexanoate	42	396	Heptadecyl 2-methyloctanoate
9	340	Hexadecyl 2-methylpentanoate	43	396	Octadecyl 2-methylheptanoate
10	354	Tetradecyl 2-methyloctanoate	44	410	Tetradecyl 2,6-dimethylundecanoate
11	354	Pentadecyl 2-methylheptanoate	45	410	Tetradecyl 2,8-dimethylundecanoate
12	354	Hexadecyl 2-methylhexanoate	46	410	Hexadecyl 2,6-dimethylnonanoate
13	368	2-Methyltetradecyl 2-methyloctanoate	47	410	Heptadecyl 2,6-dimethyloctanoate
14	368	8-Methyltetradecyl 2-methyloctanoate		410	14-Methylhexanoate 2-methylnonanoate
15	368	10-Methyltetradecyl 2-methyloctanoate	48	410	Tetradecyl 2-methyldodecanoate
16	368	10-Methylhexadecyl 2-methylhexanoate	49	410	Hexadecyl 2-methyldecanoate
17	368	Tetradecyl 2,6-dimethyloctanoate	50	410	Octadecyl 2-methyloctanoate
	368	12-Methylhexadecyl 2-methylhexanoate	51	424	Tetradecyl 2,6-dimethyldodecanoate
18	368	12-Methyltetradecyl 2-methyloctanoate	52	424	Tetradecyl 2,8-dimethyldodecanoate
19	368	Tridecyl 2-methyldecanoate	53	424	Hexadecyl 2,6-dimethyldecanoate
	368	14-Methylhexadecyl 2-methylhexanoate	54	424	Tetradecyl 2,10-dimethyldodecanoate
20	368	Tetradecyl 2-methylnonanoate	55	424	Hexadecyl 2,8-dimethyldecanoate
21	368	Pentadecyl 2-methyloctanoate	56	424	14-Methylhexadecyl 2-methyldecanoate
22	368	Hexadecyl 2-methylheptanoate	57	424	Octadecyl 2,6-dimethyloctanoate
23	368	Heptadecyl 2-methylhexanoate	58	424	Pentadecyl 2-methyldodecanoate
24	382	Tridecyl 2,6-dimethyldecanoate	59	424	Hexadecyl 2-methylundecanoate
	382	8-Methyltetradecyl 2-methylnonanoate	60	424	Heptadecyl 2-methyldecanoate
	382	12-Methyltetradecyl 2,6-dimethyloctanoate	61	424	Octadecyl 2-methylnonanoate
25	382	Tetradecyl 2,6-dimethylnonanoate	62	438	Hexadecyl 2,6-dimethylundecanoate
26	382	Tridecyl 2,8-dimethyldecanoate	63	438	Hexadecyl 2-methyldodecanoate
	382	12-Methylhexadecyl 2-methylheptanoate	64	438	Octadecyl 2-methyldecanoate
27	382	Pentadecyl 2,6-dimethyloctanoate	65	452	Hexadecyl 2,6-dimethyldodecanoate
	382	12-Methyltetradecyl 2-methylnonanoate	66	452	Hexadecyl 2,8-dimethyldodecanoate
28	382	Tetradecyl 2-methyldecanoate	67	452	Octadecyl 2,10-dimethyldecanoate
29	382	Hexadecyl 2-methyloctanoate	68	452	Hexadecyl 2,10-dimethyldodecanoate
30	382	Octadecyl 2-methylhexanoate	69	452	Octadecyl 2,8-dimethyldecanoate
31	396	2-Methyltetradecyl 2-methyldecanoate	70	452	Hexadecyl 2-methyltridecanoate
32	396	Tetradecyl 2,6-dimethyldecanoate	71	452	Heptadecyl 2-methyldodecanoate
33	396	10-Methylhexadecyl 2-methyloctanoate	72	452	Octadecyl 2-methylundecanoate
34	396	12-Methylhexadecyl 2-methyloctanoate			

[a]Molecular weight.

esters. The reconstructed ion current (RIC) from these seven transitions (Fig. 5B) is remarkably similar to the mass chromatogram of *m/z* 368 in the full scan mode (Fig. 5A), indicating that the GC/MS/MS technique can be used not only for qualitative but also for distributional purposes.

For the C_{25} wax esters, a quite different distribution pattern is observed by the GC/MS/MS technique (Fig. 6); components comprised of even carbon-numbered straight-chain alcohols (C_{14}, C_{16}, and C_{18}) esterified with odd 2-methyl fatty acids dominate this cluster. The transitions *m/z* 382 → *m/z* 145 and *m/z* 382 → *m/z* 173 transitions (Fig. 6D and 6F) reveal minor quantities of wax esters containing odd carbon-numbered alcohols, again comprised of various structural isomers. The distribution revealed by the latter transition is especially complex since wax esters composed of straight-chain

and methyl branched C_{15} alcohols with both 2-methylnonanoic acid and 2,6-dimethyloctanoic acid occur. The RIC (Fig. 6B) shows again a good match with the mass chromatogram of *m/z* 382 in the full scan mode (Fig. 6A).

General observations. These examples are indicative of the composition of all clusters of wax esters with the same molecular weight. The odd carbon-numbered wax esters are predominantly composed of even carbon-numbered *n*-alcohols (C_{14}, C_{16}, and C_{18}) esterified predominantly with odd carbon-numbered 2-methyl fatty acids (C_7, C_9 C_{11}, and C_{13}), resulting in relatively simple distributions (Fig. 3). This is in good overall agreement with the results from the hydrolysis (Fig. 4). They show specifically that the distributions of odd carbon-numbered fatty acids and even carbon-numbered alcohols are relatively simple. The even carbon-numbered wax
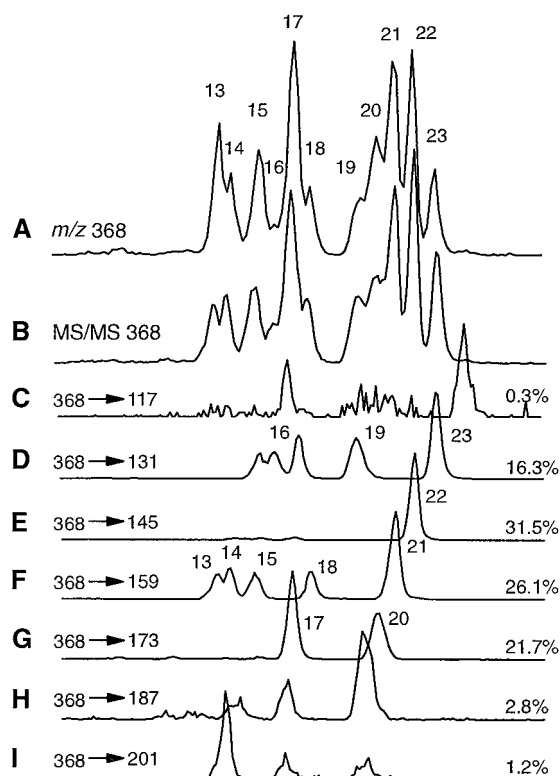
FIG. 5. Gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) transitions (C–I) for C_{24} wax esters in the intact preen wax of *C. canutus* #283. The data were acquired by collision-induced decomposition GC/MSMS. Each trace is identified with the masses of the molecular ion (*m/z* 368) and daughter ions (i.e., C_6 fatty acid *m/z* 117, C_7 fatty acid *m/z* 131, C_8 fatty acid *m/z* 145, C_9 fatty acid *m/z* 159, C_{10} fatty acid *m/z* 173, C_{11} fatty acid *m/z* 187, C_{12} fatty acid *m/z* 201). Trace B shows the reconstructed ion current (RIC) from the measured transitions. The upper trace (A) shows a mass chromatogram of *m/z* 368 (*cf.* Fig. 3) of the GC/MS analysis in full scan mode for comparison. Numbers refer to specific wax esters listed in Table 2. Numbers at the end of the traces (in %) indicate the abundance based on peak height of the most abundance peak of the cluster (total set to 100%). See Figure 1 for other abbreviation.

esters show a far more complex distribution. This is due to a number of factors. First of all, the wax esters containing *n*-alcohol moieties are not dominated by even carbon-numbered *n*-alcohols esterified with odd carbon-numbered 2-methyl fatty acids, but odd and even carbon-numbered *n*-alcohols participate in approximately equal amounts. Second, odd carbon-numbered methyl-branched alcohols participate abundantly in these wax ester clusters (e.g., Fig. 5), leading to many more structural isomers. Third, with increasing molecular weight, the various isomers of the dimethyl branched fatty acids also participate in the even carbon-numbered wax esters. These fatty acid isomers are especially abundant in the clusters of even carbon-numbered fatty acids (Fig. 3), explaining why they are not so abundant in the odd carbon-numbered wax esters, which contain predominantly odd carbon-numbered fatty acid moieties. In the higher molecular weight, even carbon-numbered wax esters containing these dimethyl branched fatty acid moieties predominate as indicated by Fig-
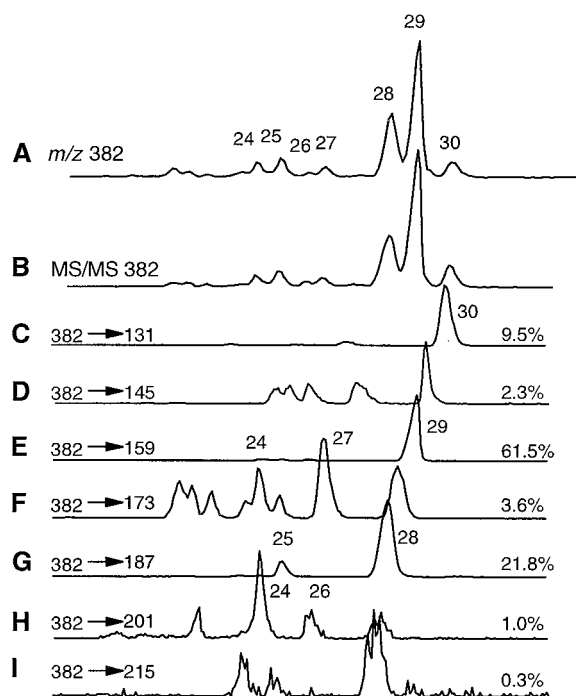
FIG. 6. GC/MS/MS transitions (C–I) for C_{25} wax esters in the intact preen wax of *C. canutus* #283. The data were acquired by collision-induced decomposition GC/MS/MS. Each trace is identified with the masses of the molecular ion (*m/z* 382) and daughter ions (i.e., C_7 fatty acid *m/z* 131, C_8 fatty acid *m/z* 145, C_9 fatty acid *m/z* 159, C_{10} fatty acid *m/z* 173, C_{11} fatty acid *m/z* 187, C_{12} fatty acid *m/z* 201, C_{12} fatty acid *m/z* 215). Trace B shows the RIC from the measured transitions. The upper trace (A) shows a mass chromatogram of *m/z* 382 (*cf.* Fig. 3) of the GC/MS analysis in full scan mode for comparison. Numbers refer to specific wax esters listed in Table 2. Numbers at the end of the traces (in %) indicate the abundance based on peak height of the most abundance peak of the cluster (total set to 100%). See Figures 1 and 5 for abbreviations.

ure 7, showing the important transitions for the C_{28} wax ester cluster. This example also reveals that even with such a powerful technique as GC/MS/MS full resolution of all structural isomers in this cluster is not obtained.

This is the first time that full structural identification of intact wax esters in complex preen waxes is achieved. In contrast to conventional techniques, which identify fatty acids and alcohols after hydrolysis, our method allows the determination of the structure of the components as they occur in the biological system. This is important if we want to understand the physiological role preen waxes play in birds, and their biosynthesis. In view of the rapid changes of the chemical composition of preen waxes in *C. canutus* over the annual cycle (6), this is most relevant, also from an ecological point of view. Our data already demonstrate that the biosynthesis of preen waxes is complex since the distribution of intact wax esters indicates that it is certainly not a random combination of available fatty acids and alcohols. It seems likely that this will influence the physical properties of the preen wax, but presently the reasons for the complex chemistry of the preen waxes are not at all understood.
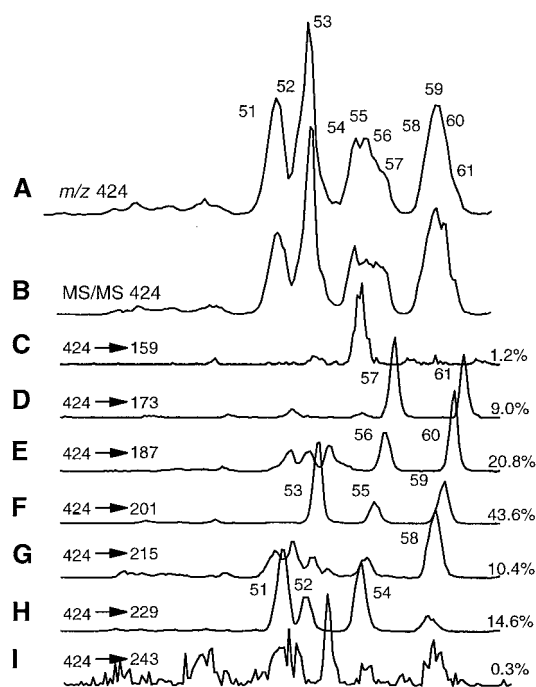
FIG. 7. GC/MS/MS transitions (C–I) for C_{28} wax esters in the intact preen wax of *C. canutus* #283. The data were acquired by collision-induced decomposition GC/MS/MS. Each trace is identified with the masses of the molecular ion (*m/z* 424) and daughter ions (i.e., C_9 fatty acid *m/z* 159, C_{10} fatty acid *m/z* 173, C_{11} fatty acid *m/z* 187, C_{12} fatty acid *m/z* 201, C_{13} fatty acid *m/z* 215, C_{14} fatty acid *m/z* 229, C_{15} fatty acid *m/z* 243). Trace B shows the RIC from the measured transitions. The upper trace (A) shows a mass chromatogram of *m/z* 424 (*cf.* Fig. 3) of the GC/MS analysis in full scan mode for comparison. Numbers refer to specific wax esters listed in Table 2. Numbers at the end of the traces (in %) indicate the abundance based on peak height of the most abundance peak of the cluster (total set to 100%). See Figures 1 and 5 for abbreviations.

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