Composition of the hydrocarbon fraction of goats' milk[1]

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Abstract The hydrocarbon fraction of the neutral lipids of goats' milk was chromatographically purified and analyzed by gas–liquid chromatography and mass spectrometry. The goats' milk samples, which were collected during the spring of the year, represent a cross-sectional analysis; the purified hydrocarbon fraction displays a broad spectrum of compounds. The major components of the hydrocarbon fraction identified for the first time in goats' milk were 3,7,11,15-tetramethylhexadec-2-ene (phytene-2) (1.5%), squalene (~2.5%), and n-$C_{29}H_{60}$ (4.2%); in addition, a series of odd and even carbon number n-alkanes (C_{15} to C_{33}), a series of alkenes (C_{16} to C_{23}), and a series of branched chain hydrocarbons were found. The goats' milk hydrocarbon fraction, in comparison to the known distribution from cows' milk, contains a good deal less squalene and phytene, and is more complex. One human milk hydrocarbon fraction isolated from a longitudinal composite sample from one lactation displays a distribution that appears to be more closely related to that of human skin lipids (1983. *J. Lipid Res.* **24:** 120–130) than to those of goats' and cows' milk. — **Cerbulis, J., V. P. Flanagan, and H. M. Farrell, Jr.** Composition of the hydrocarbon fraction of goats' milk. *J. Lipid Res.* 1985. **26:** 1438–1443.

Supplementary key words lipids • milk fat • gas–liquid chromatography • mass spectrometry

Studies on the lipid composition of cows' milk (1, 2) have shown the occurrence of a hydrocarbon fraction which may account for up to 70 ppm of the total lipid (3). In cows' milk this complex fraction has been studied in detail (4, 5); it contains squalene and phytene derivatives as major components. The hydrocarbon fraction, or a portion of it, may be associated primarily with the milk fat globule membranes (6). The hydrocarbon fraction of goats' milk, however, has apparently not been investigated, and in the case of human milk, only squalene has been identified as a component (7).

As a part of our study of the lipid composition of goats' milk (8–10) the hydrocarbon fraction was investigated. This report deals with the identification of the major components of the hydrocarbon fraction of goats' milk and comparison with one similar fraction from human milk.

MATERIALS AND METHODS

Materials

Raw goats' milk samples were obtained with the cooperation of a large commercial goat dairy company, and were maintained at 5°C in transit to the laboratory. Upon receipt, the samples were lyophilized and stored at −20°C. Before lipid extraction, equal weights of five samples obtained from several geographical areas in Pennsylvania and New Jersey during the months of April through June were mixed together to minimize nutritional, environmental, and breed differences.

Human milk samples were obtained from a single donor experiencing a normal delivery and in good health throughout the lactation. Samples were from fractions of full milkings taken between 3 and 6 months after parturition and represent a total of 1000 ml of human milk. Samples were frozen upon receipt, then lyophilized and pooled.

Reagents

All solvents were of Nanograde quality. Unisil silicic acid (100–200 mesh), silica gel G Uniplates, and precoated SIL G-25 TLC plates were obtained from Clarkson Chemical Company (Williamsport, PA), Analtech, Incorporated (Newark, DE), and Brinkmann Instruments, Incorporated (Westbury, NY), respectively. The plates were prewashed with chloroform–methanol 2:1 prior to activation. Silica gel was from Davison Chemical Corporation (Baltimore, MD).

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; RIC, reconstructed ion chromatograph.

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Lipid extraction

The neutral lipids were obtained as previously described in detail by Cerbulis, Parks, and Farrell (8). Briefly, the freeze-dried samples were extracted four times with Nanograde petroleum ether (bp 30–65°C); polar lipids were obtained by three subsequent extractions with chloroform–methanol 2:1 (v/v). The neutral lipid (petroleum ether-extractable) fraction contains glyceride, cholesterol, cholesteryl ester, and hydrocarbon fractions and accounts for 96.8% of the total milk lipid. The polar lipid (chloroform–methanol-extractable) represents 3.2% of total lipid and contains nearly all of the phospho- and glyco-lipids as well as some residual neutral lipid (8). Lipid extracts and column chromatographic fractions were analyzed by thin-layer chromatography; the developing solvents were petroleum ether–diethylether–acetic acid 90:10:1, or benzene–hexane 20:80. Sulfuric acid–acetic acid–$FeCl_3$ (25 ml of concentrated H_2SO_4:25 ml of glacial acetic acid:3 g of $FeCl_3 \cdot 6\ H_2O$) at 120°C was used to visualize the lipid components.

Silicic acid column separation for purification of the hydrocarbon fraction

Silicic acid (100–200 mesh), activated by treatment at 200°C for 12 hr, was used for all column separations (11). Free or neutral lipids (100 g) were dissolved in 500 ml of hexane and applied to a column (4 × 50 cm) of silica equilibrated with hexane. Stepwise elution was carried out with 1 liter each of hexane, benzene, 10% ether in benzene, and ether at a flow of 2 ml per min; the eluant from each step was collected in five 200-ml fractions. Excess solvent was evaporated under a stream of N_2 at 50°C.

As expected, the hydrocarbon fraction was found to occur in the hexane eluant and these samples were pooled and rechromatographed on the same column until free of the sterol ester bands. The purity of the hydrocarbon fraction was followed by TLC using the benzene–hexane solvent system. The sterol ester-free fractions, after removal of solvent under a stream of N_2, were dissolved in a minimum volume of redistilled Nanograde hexane and placed on a column containing 3 g of Al_2O_3, hydrated to 6%, and equilibrated with the hexane. The first 5 ml eluted was collected into a tube previously washed with acetone and hexane. Solvent was removed under N_2; the residue was transferred to a Teflon-lined, washed screw-cap vial and stored under N_2 at −25°C.

Both the goats' and human milk hydrocarbon fractions were prepared from large scale operations. The human milk was pooled from mid-lactation samples. Since the silica gel, glass wool, and solvents could be sources of hydrocarbons, control experiments with no lipid present were run and the products were analyzed.

Gas–liquid chromatography and mass spectrometry

The gas chromatograph was a Hewlett Packard HP-5750 interfaced to a Hitachi RMU-6E mass spectrometer via a single-stage glass separator. The glass column was 1.8 m × 2.0 mm I.D. packed with 1% OV-101 on Gas Chrom Q (100/120 mesh) with a helium flow of 20 ml/min. The thermal program was 100°C to 240°C at 2°/min. The GLC effluent was continuously scanned. The 70 eV electron impact spectra were recorded with a Finnigan Incos 2300 data system.

RESULTS

The total extracted neutral lipids (free lipids) of goats', cows', ewes', and human milk were compared by TLC (**Fig. 1**). The pattern for each species shows the predominant triglyceride fraction as well as cholesterol, a trace of sterol esters, and a small hydrocarbon fraction. The hydrocarbon fraction was isolated from both the goats' and human milk samples by silicic acid column chromatography as described above.

Fig 2 shows the reconstructed ion chromatograph (RIC) for the goats' milk hydrocarbon fraction. Twenty major peaks were detected on the chromatogram, as well as a number of minor peaks falling between the numbered ones. The peaks were subjected to continuous scanning mass spectral (MS) analysis to identify the structure of the components. Control experiments showed little or no

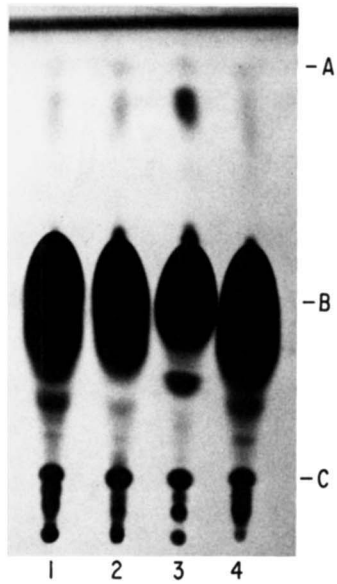

Fig. 1. Thin-layer chromatograms of the neutral lipid fractions of: (1) cows', (2) goats', (3) human, and (4) ewes' milk. The plates were silica gel G and were washed before using. The solvent was freshly prepared petroleum ether–diethyl ether–acetic acid 90:10:1. Fractions of interest are from the top (A) hydrocarbon, (B) triglycerides, and (C) cholesterol.

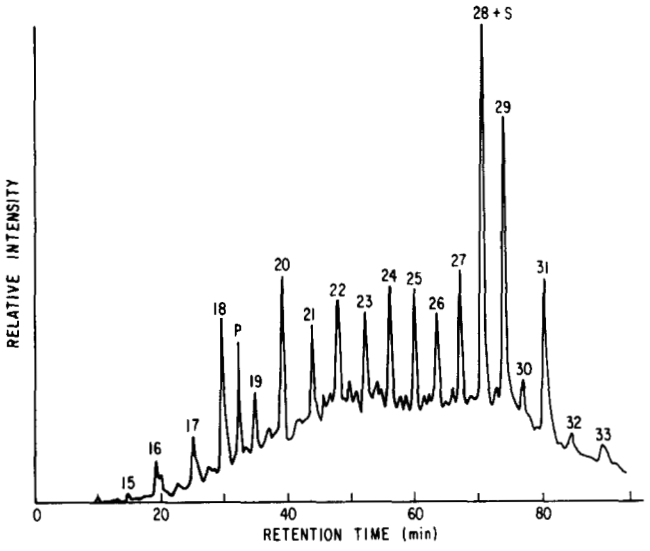

Fig. 2. Reconstructed ion chromatograms (RIC) for goats' milk hydrocarbon fraction obtained as described in Materials and Methods. Components are identified in Table 1.

recovery of hydrocarbon from the solvent alone, however, hexane-soluble material was recovered from the mock-column elution; gravimetrically this was <1% of a recovered hydrocarbon fraction. The RIC of this control showed about ten components, only two of which were in common with minor components of Fig. 2. To obtain the theoretical outer limit of contamination, the relative areas for these common peaks were compared to the total sample area. These analyses indicated that no more than 5% of the total area represented in Fig. 2 could be from contaminants, even if all of the components of the mock run were carried over to the sample; more likely contamination is <1%. The mass spectra of the peaks of Fig. 2 exhibited the typical ion fragmentation pattern associated with long chain hydrocarbons with the exception of phytene and squalene. Positive identification by MS was made when the molecular ion could be determined. The retention times and fragmentation patterns were in agreement with authentic compounds or literature data. On this basis the minor peaks were tentatively identified as being composed of mixtures of branched chain hydrocarbons. The major peaks consisted of a series of normal and monoene hydrocarbons and squalene. Typical of this is peak 18 (Fig. 2). Close examination of the reconstructed ion chromatogram (RIC) region for peak 18 showed it to be a doublet indicating at least two compounds (**Fig. 3**). Examination of the total ion spectrum of the RIC peak centered around 30 min (scan 222) showed a molecular ion (M^+) at M/E 252 corresponding to the monoene hydrocarbon $C_{18}H_{36}$ (**Fig. 4A**). Also, observed in this scan are the fragment ions of the $C_{18}H_{36}$ monoene. The location and configuration of the double bonds in this and in the other peaks are unknown since they are known to migrate up and down the chain upon electron impact (12). The later part of this RIC doublet (scan 226) had an M^+ of M/E 254, as well as associated fragments indicative of n-$C_{18}H_{38}$ alkane (Fig. 4B). These compounds were not completely resolved. On the other hand, in the mass fragmentogram, the respective M^+'s (Fig. 3A and B) were clearly resolved. Also, in scan 226 (Fig. 4B) it can be seen that several ions are present that are characteristic of phytene-2, namely, M/E 280, 210, 196, 140, and 70. These ions are probably due to the minor phytene isomer previously reported by Urbach and Stark (13). In a similar fashion components of the peaks labeled 15 to 33 from the RIC were identified. **Table 1** summarizes the major compounds found in the goats' milk hydrocarbon fraction. Inspection of the table shows that one of the prominent components of the fraction is squalene; n-$C_{29}H_{60}$ and 3,7,11,15-tetramethylhexadec-2-ene (phytene-2) are also prominent. The remainder of the numbered components (Fig. 2, Table 1) is a series of odd and even normal chain hydrocarbons and monoenes, ranging from C_{16} to C_{29} with the n-alkanes predominating. The peaks observed between the major com-

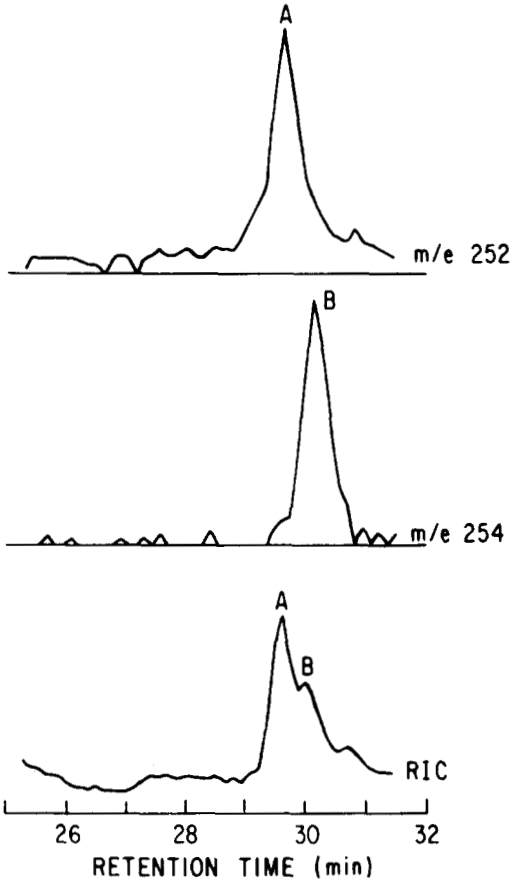

Fig. 3. RIC and mass chromatograms for the peak labeled 18 in Fig. 2. Retention time relates to Fig. 2 as well.

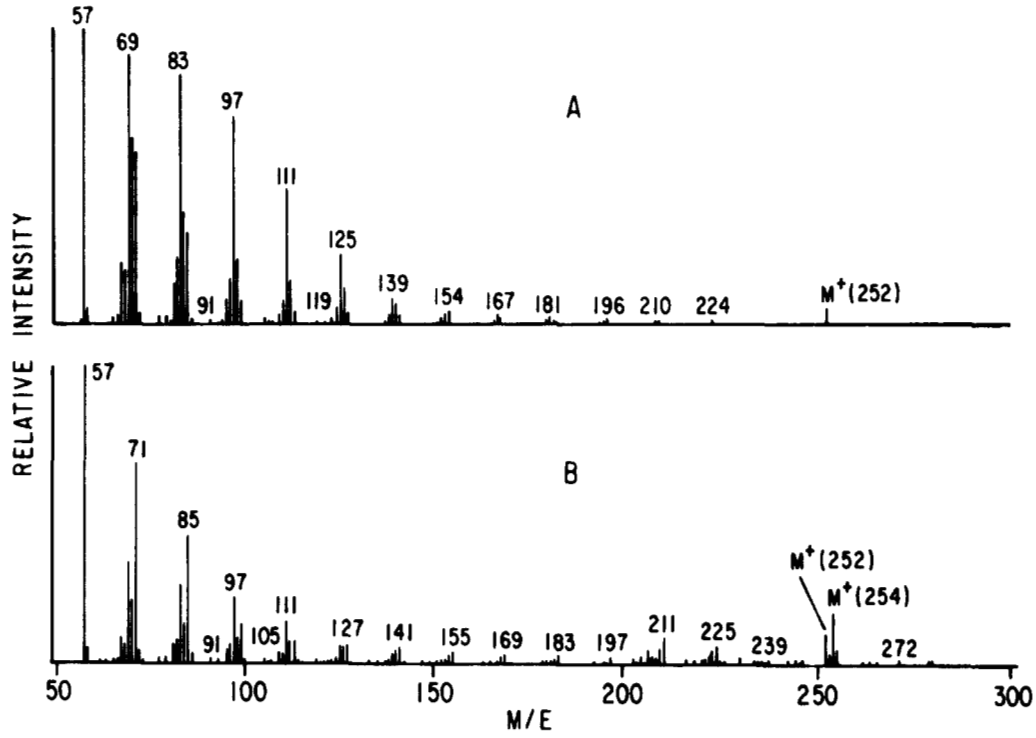

Fig. 4. Mass spectral analysis of the areas 18A and B from Figs. 2 and 3 (scans 222 and 226, respectively). Analysis identifies A as $C_{18}H_{36}$ and B as a mixture of the former and n-$C_{18}H_{38}$.

ponents in the goats' milk samples probably represent complex mixtures of branched hydrocarbons. However, analyses of these mixtures were not carried further for the goats' milk samples.

The RIC for the human milk hydrocarbon fraction is shown in **Fig. 5.** Compared to goats' milk, human milk contains fewer shorter chain hydrocarbons, and exhibits a simpler spectrum of compounds. In human milk, the branched chain compounds that occur between the major peaks are more clearly resolved and the total carbon length could be determined for most peaks. In each case the branched chain hydrocarbon has a slightly shorter retention time than its n-alkane homologue. However, analyses of these compounds indicated that they do not seem to fit a specific series (e.g., a 2-methyl or a 3-methyl series), but may consist of mixtures of monomethylated or multi-branched hydrocarbons. In contrast to the goats' milk samples, the human milk, with the exception of squalene, appeared to contain only saturated alkanes. In addition, phytene appears to be essentially absent when compared to the goats' milk sample.

DISCUSSION

Studies on the composition of the hydrocarbon fraction of cows' milk have shown that the components are subject to considerable seasonal, lactational, and dietary variation (3). However, squalene and various phytenes are the dominant components (3, 14); these two are usually accompanied by several minor series of n-alkanes, n-alkenes, and branched hydrocarbons (4, 5, 12). In the case

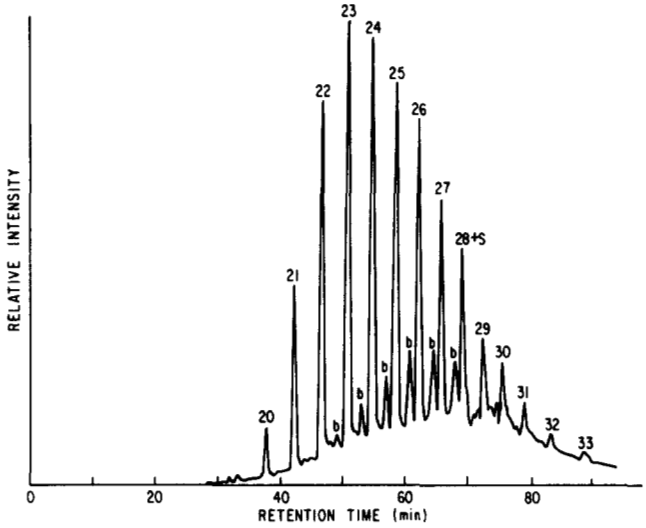

Fig. 5. RIC for the hydrocarbon fraction of human milk. Peaks labeled 20–27 and 29–33 represent a series of n-alkanes ranging from C_{20} to C_{33}; peaks labeled B represent the branch chain homologue of the following n-alkane; peak 28-S is a mixture of n-C_{28} and squalene.

TABLE 1. Qualitative and quantitative identification of the major components of goats' milk hydrocarbon fraction

Peak No.[a]	Hydrocarbon Components[b]	Relative Area %[c]
15	$n\text{-}C_{15:0}$	Tr
16	$C_{16:1}$ $n\text{-}C_{16:0}$	0.93 ± 0.52
17	$C_{17:1}$ $n\text{-}C_{17:0}$	0.91 ± 0.32
18	$C_{18:1}$ $n\text{-}C_{18:0}$	3.1 ± 0.8
P	Phytene-2	1.5 ± 0.6
20	$C_{20:1}$ $n\text{-}C_{20:0}$	3.5 ± 1.1
21	$C_{21:1}$ $n\text{-}C_{21:0}$	1.9 ± 0.6
22	$C_{22:1}$ $n\text{-}C_{22:0}$	3.1 ± 1.1
23	$n\text{-}C_{23:0}$	3.2 ± 1.1
24	$C_{24:1}$ $n\text{-}C_{24:0}$	3.2 ± 0.6
25	$n\text{-}C_{25:0}$	2.4 ± 0.6
26	$C_{26:1}$ $n\text{-}C_{26:0}$	2.7 ± 0.7
27	$n\text{-}C_{27:0}$	2.5 ± 0.5
28 + S	$n\text{-}C_{28:0}$ Squalene	5.6 ± 0.3
29	$n\text{-}C_{29:0}$	4.2 ± 0.8
30	$n\text{-}C_{30:0}$	1.7 ± 0.4
31	$n\text{-}C_{31:0}$	3.0 ± 0.4
32	$n\text{-}C_{32:0}$	1.2 ± 0.4
33	$n\text{-}C_{33:0}$	1.5 ± 0.2

[a] Peak number as in Fig. 1.
[b] Positive identification by scanning mass spectrometer.
[c] Average of three determinations ± σ; calculated as % area from three RIC's, Fig. 1 being typical.

of goats' milk, although squalene is a prominent component, there appears to be a broader spectrum of components which account for the bulk of the hydrocarbon fraction. Goats are highly seasonal in their estrus cycles, and the samples in this study were all collected in the spring of the year; thus lactational and seasonal variations are not responsible for the wide spectrum of compounds observed in this composite sample. The variation may be more related to the variety of dietary conditions prevailing in the goats' milk industry. The overall goal of this study (8, 9) was to achieve a large cross-sectional sample of mid-lactation milk samples for analysis of the lipid composition of goats' milk, and thus to reflect an average composition available to the consumer.

The human milk hydrocarbon fraction, in comparison with the goats' milk sample and published values for cows' milk, exhibits a very different composition. Phytene and its related compounds appear to be absent, and squalene is a minor component. The majority of the compounds in the human milk sample constitute a series of odd and even n-alkanes. The pattern of compounds observed here is similar though not identical to the hydrocarbon fraction recently isolated from human skin lipid (15). In the latter case, a bell-shaped profile of odd and even n-alkanes centering around C_{25} was found in lipid samples from four different body sites. These compounds were carefully isolated, and the authors concluded that the hydrocarbons were a true component of the human skin lipid and were not an environmental contaminant. In comparison with the skin lipid hydrocarbons, the milk hydrocarbon distribution appears to center more around C_{23}, and shows the occurrence of a series of branched, saturated hydrocarbons as well. This report thus represents a more in-depth study of the hydrocarbon fraction of human milk than previously reported (7, 16). However, to achieve the large volume needed for this isolation, the milk was obtained from only one individual and thus may not be representative of a true cross-section of the general population. This fraction does represent multiple samplings across the mid-range of human lactation, and is thus a composite-longitudinal sample. It has been noted (16) that other lipid components of human milk vary considerably with diet.

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