ACTA CHEMICA SCANDINAVICA 22 (1968) 1717–1721

Volatile Carbonyl Compounds in the Milk Fat of Normally- and Synthetically-fed Cows

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Carbonyl compounds in the volatiles from 1.8 kg of butteroil obtained from the milk of synthetically-fed cows (zero milk) have been compared with those obtained from normally-fed cow milk butteroil. Thirty-seven carbonyls (as 2,4-dinitrophenylhydrazones) were found in the latter whereas 28 were detected in the zero milk fat. One carbonyl compound was detected in zero milk fat volatiles which was not present in normal milk fat volatiles. Both fats contained a class of volatile carbonyls which have not been previously reported in butterfat.

The feasibility of feeding dairy cows a diet containing urea and ammonium salts as the sole source of nitrogen, has been reviewed by Virtanen.[1] In a continuing effort to compare the composition of the milk from normally-fed and synthetically-fed cows (so-called zero milk), we have examined the volatiles obtained from the fat of each type of milk qualitatively for carbonyl compounds.

EXPERIMENTAL

Preparation of butteroil. Pooled morning milk from three Ayrshire cows well-adapted to the synthetic feed,[1] and pooled milk from normally-fed Ayrshire cows was separated, the cream cooled to 4°C, and churned by agitation in an electric drink mixer. The butter was isolated, liquefied by immersion in hot tap water and then permitted to resolidify undisturbed at 4°C. When solid, a hole was punched through the fat layer and the majority of the buttermilk was poured off. The fat was reliquefied and centrifuged at 40°C for 20 min at 6000 rpm. The oil layer was decanted off and passed over a plug of fine glass wool contained in a chromatography tube. This treatment removed any turbidity and also some moisture and yielded an optically clear oil. The entire procedure for obtaining the butteroil was executed in several hours. The oil was stored overnight at 4°C and was distilled the following morning.

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Distillation. Volatiles from six 300 g batches of butteroil were obtained by distillation using the carbon dioxide-sweeping procedure of Honkanen and Karvonen [2] but substituting carbonyl-free hexane for diethyl ether where the latter was indicated. The hexane was rendered carbonyl-free by the method of Hornstein and Crowe [3] but using a greatly reduced flow rate (approximately one liter/24 h) than was recommended by these authors. The purity of the hexane was checked by the method of Schwartz and Parks.[4]

Distillation was conducted at 60°C at a residual pressure of 1—7 mm Hg and for a period of 4—5 h. At the end of the process, the traps were rinsed with a total of 40 ml of purified hexane and the rinsings were passed immediately over a column of Analytical Grade Celite impregnated with a phosphoric acid solution of 2,4-dinitrophenylhydrazine to convert the carbonyls into 2,4-dinitrophenylhydrazones.[4] The hydrazones from the six distillations were then pooled and fractionated as described below.

Fractionation of 2,4-dinitrophenylhydrazones into classes. The pooled hydrazones were dissolved in hexane and passed over a column (10 g) of 6 % hydrated alumina (Merck, Darmstadt) and the classical monocarbonyl fraction was eluted from the adsorbent with 100 ml of benzene:hexane (1:1).[4] The solvents were removed on the steam bath under a stream of N_2, the residue taken up in $CHCl_3$ and separated into classes on a Seasorb 43:Celite 545 column (15 g:15 g) as described by Schwartz *et al.*[5] using a gradient of methanol in $CHCl_3$. Overlapping bands were collected and rechromatographed in the same system until the overlapping band was separated cleanly. Each class thus obtained was spotted on a MgO:Celite plate along with authentic methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals in order to establish the proper classification of the unknown band. The thin-layer procedure was identical to that described by Schwartz *et al.*[6]

Separation of the members of each class by thin-layer partition chromatography (TLPC). Resolution of the individual members present in each band obtained in the class separation was accomplished by TLPC as described by Schwartz *et al.*[6] The band from the class separation was streaked across the origin of the plate and the plate developed twice. At the end of the second development, the bands were scraped from the plate and the hydrazones (plus the stationary phase, polyethylene glycol 400) were eluted with $CHCl_3$. The solvent was removed by evaporation under an N_2 stream, the residue dissolved in about 1 ml of benzene and passed over about 500 mg of the 6 % hydrated alumina contained in a medicine dropper. The column was washed with 2 column volumes of benzene to elute the hydrazones. The polyethylene glycol 400 remained adsorbed on the alumina.

The purified hydrazones were then rechecked for their proper classification on the MgO:Celite class plates and when the proper classification was firmly established by this technique (and also by the absorption maximum when possible) they were then rerun by TLPC along with authentic members of the class in order to establish their chain-length.

RESULTS AND DISCUSSION

The results of the analyses are summarized in Table 1. A total of 28 carbonyl compounds were detected in the volatiles from the fat of synthetically-fed cows, whereas 37 carbonyl compounds were found in normal milk fat volatiles. The zero milk fat contained one compound that was not identified which was absent from normal milk fat. Normal milk fat contained a series of ten 2-enals in very low amounts which were undetected in zero milk fat. No 2,4-dienals were detected in either milk fat.

A series of 8 unidentified carbonyl compounds which constitute a previously undescribed class in butterfat was isolated from both zero and normal milk fat, but none of the members were identified. Some properties of this class are described below (unidentified class). Further work toward identification of this class will be attempted.

Table 1. Carbonyl compounds detected in the volatiles from normally- and synthetically-fed cow's milk fat.

Class	*Present (+) or absent (−) in* *Zero milk fat*	*Normal milk fat*
Methyl ketones		
2-Propanone	+	+
2-Butanone	+	+
2-Pentanone	+	+
2-Hexanone	+	+
2-Heptanone	+	+
2-Nonanone	+	+
2-Undecanone	+	+
2-Tridecanone	+	+
Saturated aldehydes		
Methanal	+	+
Ethanal	+	+
Propanal	+	+
Butanal	+	+
Hexanal	+	+
Heptanal	+	+
Octanal	+	+
Nonanal	+	+
Decanal	+	+
Undecanal	+	+
Dodecanal	+	+
2-Enals		
Acrolein	−	+
Crotonal	−	+
Pent-2-enal	−	+
Hex-2-enal	−	+
Hept-2-enal	−	+
Oct-2-enal	−	+
Non-2-enal	−	+
Dec-2-enal	−	+
Undec-2-enal	−	+
Dodec-2-enal	−	+
Unidentified class containing 8 members	+	+
Unidentified compound	+	−

Experience in the senior author's Laboratory indicates that direct analysis of fresh butterfat yields very little methyl ketones other than acetone. In the present study on both fats, the methyl ketones made up the bulk of the carbonyls in the volatiles. On this basis, one would expect the C_5, C_7, C_9, C_{11}, C_{13} (and C_{15}) ketones to be thermally generated during distillation. Lawrence has also stated that this is the case.[7]

Most of the saturated aldehydes and 2-enals found in this study were reported in fresh, unoxidized butteroil by Parks *et al.*[8] who used the direct

reaction method of Schwartz *et al.*[9] for the analysis. The short chain aldehydes and 2-enals, however, were not reported in their study.

A discussion of the new unidentified class, which occurred (incidentally, in approximately equal concentration) in both fats follows.

The fraction did not adsorb from $CHCl_3$ onto the MgO-Celite column. It was detected by the yellow color in the effluent. The latter was evaporated on the steam bath under N_2 and the residue taken up in hexane and applied to a 3 g×3 g column of Seasorb 43:Celite 545 prepared in hexane. The fraction was adsorbed now as a greyish band. The column was washed with 100 ml of hexane followed by 50 ml of hexane:benzene (1:1). The band was then readily eluted with 10 % $CHCl_3$ in benzene. Evaporation of this effluent gave a residue containing the unknown fraction but heavily contaminated with a wax-like substance which gave a positive hydroxamic acid test. To purify the unknown hydrazone, the residue was taken up in methanol and passed over a short column of Dowex-1×8 in the hydroxyl form according to the procedure of Schwartz, Johnson and Parks.[10] The unknown fraction was weakly held as a reddish-violet zone but on recycling through a longer ion-exchange column, the class was retarded sufficiently to be freed of the waxy impurities although some loss of the fraction was also incurred.

The unknown fraction was then subjected to thin-layer chromatography on silica Gel G in the solvent system $CHCl_3$:benzene (1:1) and moved as a single spot. However, when the unknown fraction was chromatographed in the thin-layer partition system described earlier, it separated into 8 components. Exposure of the completed chromatogram to diethylamine vapor did not cause the spots to darken. This was unusual in that all hydrazones, which we have had experience with, will darken when subjected to this treatment. In order to account for the unusually weakly acidic properties of the hydrazones in this fraction, the assumption was made that the parent molecule contained a basic nitrogen center. Moreover, it was also assumed that if the parent molecule did contain a basic nitrogen atom, it would have to be weakly basic owing to the fact that the hydrazones in this fraction passed through the original 2,4-dinitrophenylhydrazine-H_3PO_4 acid column which has a pH of about 1.5. With this in mind, the partition plate containing the 8 separated members was exposed for a few minutes to HCl vapors. The position of the original spots was marked on the plate and the plate was redeveloped twice in the solvent. No movement of any of the spots was discerned and it was assumed that a salt had been formed. However, when the plate was now exposed to diethylamine vapor the spots turned violet. Redevelopment of the plate in the solvent after the violet spots had faded back to their original yellow color (during evaporation of the diethylamine) also resulted in no movement. It was therefore concluded that exposure of the hydrazones to strongly acidic conditions caused a rearrangement resulting in a more polar molecule. This was verified also by exposing a plate containing the separated members to formic acid vapor and also to acetic acid vapor. Exposure to formic acid vapor for 1 h resulted in the same characteristics as described for exposure to HCl vapor. However, exposure to the weaker acetic acid vapor resulted in partial immobilizing of the spots, *i.e.*, part of the original spot moved and part

was immobilized. The latter colored violet, the mobile spots did not, when the plate was exposed to diethylamine vapor.

On passage of the unknown fraction dissolved in MeOH over Dowex 50 (H), most of the fraction was irreversibly held to the resin. Thus, it appears that the molecule is altered on exposure to strongly acidic conditions and this may account for the fact that this class has not been described in the literature when volatiles from butterfat are analyzed as 2,4-dinitrophenylhydrazones, since 2,4-dinitrophenylhydrazine in 1 or 2 N HCl is usually used to form the dinitrophenylhydrazones. That these are true 2,4-dinitrophenylhydrazones cannot be unequivocally proved at this time, but the absorption maximum (375 mμ) is in the range normally found for aliphatic 2,4-dinitrophenylhydrazones and the color of the derivatives appears to be normal. Moreover, the molecule does color under strongly alkaline conditions, although other dinitro compounds also color.[11]

Acknowledgement. This research has been financed in part by a grant made by the *United States Department of Agriculture, Agricultural Research Service.*

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Received January 9, 1968.