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Studies on the Composition of Horse Oil

1. COMPOSITION OF HORSE OIL IN RELATION TO THE DEPOT FATS OF OTHER PASTURE-FED ANIMALS

BY E. G. BROOKER AND F. B. SHORLAND
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

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Many investigators (cf. Schuette, Garvin & Schwoegler, 1934) have determined the characteristics of fat from various parts of the horse, indicating in particular a relatively high iodine value (74·8–110·7) (cf. Lewkowitsch, 1922). There is, however, little exact information concerning the fatty-acid composition. Heiduschka & Steinruck (1921) deduced the presence of oleic, linoleic and linolenic acids by the preparation of hydroxy and bromo derivatives, and by fractional crystallization showed the saturated acids to be palmitic and stearic. They also gave the relative proportions of the fatty acids present, but it is doubtful whether the methods used were quantitative. More recently, Schuette *et al.* (1934) confirmed the results of these investigators and determined the relative proportions of the acids by the Kaufmann (1926) thiocyanometric procedure, together with the ester fractionation analysis of the 'solid' acids from the lead salt-ethanol separation. Of particular interest is the fact that these workers recorded the presence of 4·48% linolenic acid; a result which is consistent with the yield and melting point of the hexabromides of horse oil recently recorded by Crowell (1944). According to Bloor (1943), however, linolenic acid is not normally found in animal fats.

It has been stated in the literature (Armstrong & Allan, 1924; Hilditch, 1947) that horses fed continuously on grass lay down fat very similar to that of mutton or beef tallow. This, no doubt, has led to the general impression that horse oil contains little or no linolenic acid, as has been shown to be the case with beef and mutton tallow.

Recently, Beadle, Wilder & Kraybill (1948) observed that rats fed on flax seed, equivalent to 6·6% linolenic acid in the diet, deposit up to 27·57% of linolenic acid in their depot fats. In addition, they noted that yellow fat from hogs may contain up to 11·4% linolenic acid. Vickery (1928), quoted by Banks, Hilditch & Jones (1933), found 9·0% of linolenic acid, based on the weight of hexabromide isolated, in the perinephric fat of the rabbit. However, taking into consideration the fact that not more than one-quarter to one-third of the linolenic acid is precipitated as ether-insoluble polybromide (cf. White & Brown, 1949), it may be estimated that the true linolenic acid content was 27–36%. From these results it is apparent that certain species, far from rapidly metabolizing the dietary linolenic acid, readily store considerable proportions of this acid in their depots.

Further to a preliminary note published elsewhere by one of us (Shorland, 1949*a*), it will now be shown that oils from pasture-fed horses contain considerably greater proportions of linolenic acid than has hitherto been generally suspected, and that the fatty-acid composition of such oils differ markedly from that of beef and mutton tallow.

EXPERIMENTAL

Material

The material used in this work was obtained from horses killed at Auckland Farmers' Freezing Company, Southdown, New Zealand, during the 1946–7 season. The horses were of mixed origin, typically semi-draughts from 3 to 5 years old, taken from various parts of the Auckland Province, and invariably pasture fed. Since the horses were being used for the production of canned and pickled meat for export, some attention was paid to the meat qualities, and old animals and foals were rejected. On the basis of a total of 786 horses killed between 28 March and 3 April 1947 the average carcass weight was 618 lb. (280 kg.). After dissection of the useful meat, the practice has been to subdivide the residual carcasses into: (1) heads, trotters and offal; and (2) bones with indefinite amounts of adhering flesh and meat trimmings, giving respectively 'offal oil' and 'bone oil'. (The 'bone oil' was thus not solely from bone but admixed on the average with 1·44 parts of oil from the meat trimmings.) On the average there has been obtained 4·00 gal. (18 l.) of bone oil and 1·33 gal. (6 l.) of offal oil per carcass, but the yield has been obviously affected by the condition of the horse. The bone and offal oils used in this work were representative of the season's production, comprising approximately 2000 horses. In addition, a sample of hoof oil was separately steam-rendered for us from a line of sixty-five horses killed on 7 November 1947. The characteristics of the oils used in this investigation are given in Table 1.

Table 1. *Characteristics of horse oils used in this investigation*

Oil	Saponification equiv.	Iodine val.	Free fatty acid (%)	Unsaponifiable matter (%)
Bone	283·5	99·0	1·1	0·72
Offal	283·5	97·9	1·5	0·97
Hoof	285·9	109·3	2·3	1·11

Methods and Results

The fatty-acid composition was determined essentially as described by Shorland & De la Mare (1945), but using the fatty acids in place of the methyl esters for the cold acetone separation at −30°. The esters were fractionated under a vacuum of approx 0·1 mm. in a column of the type described by Longenecker (1937). Duplicate analyses of the bone oil were made on 750 g. samples, using a 100 cm. column. For the hoof oil and the offal oil respectively 250 g. and 100 g. samples were used and the fractionation was carried out on a 60 cm. column. To illustrate the course of the distillation data are given for the bone oil in Table 2. Percentages of the component fatty acids are given in Table 3.

The composition of C_{18} unsaturated acids was determined by spectrophotometric analysis before and after alkali isomerization using the procedure of Hilditch, Morton & Riley (1945). For the conjugated diene C_{18} unisomerized acids the $E^{1\%}_{1\,cm.}$ 234 mμ. value of 1200 (cf. Hilditch & Jasperson, 1945) was used. The results are shown in Tables 4 and 5.

Bromination of 11·04 g. acids from fraction L9 in ether at 0° yielded 1·57 g. ether-insoluble bromides, m.p. 180·5° (not depressed on admixture with an authentic specimen of hexabromostearic acid, m.p. 181°). (Found: C, 28·6, 28·5; H, 4·2, 4·2. Calc. for $C_{18}H_{28}O_2Br_6$: C, 28·5; H, 4·0%.) From the ether-soluble fraction there was obtained 0·48 g. light petroleum insoluble bromides which charred at 167° and melted indefinitely at 172°.

In order to determine the proportions of fully saturated glycerides the bone oil (100 g.) was recrystallized three times from acetone (15 ml./g., based on the original weight) at −30° and then three times at 0° yielding 1·4 g. insoluble glycerides, iodine val. 24·0. If it is assumed that the un-

Table 2. *Fractionation of the methyl esters of horse 'bone' oil*

Methyl esters of 'liquid' acids (Weight fractionated 505·0 g.)				Methyl esters of 'solid' acids (Weight fractionated 209·2 g.)			
Fraction no.	Wt. (g.)	Sap. equiv.	Iodine val. (Wijs 1 hr.)	Fraction no.	Wt. (g.)	Sap. equiv.	Iodine val. (Wijs 1 hr.)
L1	6·05	232·1	14·3	S1	9·96	262·1	1·1
L2	19·11	244·9	19·4	S2	32·14	267·6	1·1
L3	22·77	267·1	74·9	S3	23·18	269·3	1·1
L4	28·24	277·7	104·0	S4	24·87	269·3	1·1
L5	17·14	277·8	101·9	S5	17·84	269·8	1·2
L6	54·11	289·4	135·6	S6	15·17	269·7	1·3
L7	53·69	289·4	140·0	S7	35·03	272·4	3·0
L8	37·20	292·2	141·2	S8	31·15	283·3	9·7
L9	60·31	293·9	148·5	S9	9·92	295·8	14·6
L10	33·87	293·9	148·3	SR†	10·36	322·6	—
L11	61·16	294·0	149·9	Total	209·62		
L12	39·28	294·3	150·4				
L13	35·31	294·4	152·6				
LR*	37·44	324·2	151·2				
Total	505·68						

* Liquid residues (non-volatile). For esters in this fraction, excluding non-saponifiable matter; saponification equiv., 298; iodine val., 138·8.

† Solid residues (non-volatile). For esters in this fraction, excluding non-saponifiable matter; saponification equiv., 300·1; iodine val., 17·54.

Table 3. *Component fatty acids of horse oils*

(Values in parentheses are mean unsaturations, i.e. number of H atoms short of saturation.)

Oil	Saturated C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	Unsaturated C_{12}	C_{14}	C_{16}	C_{18}	C_{20}
						(*a*) Weight percentages				
Bone	0·2	3·1	25·3	4·1	0·1	Trace	0·6 (−2·0H)	8·3 (−2·0H)	57·6 (−3·5H)	0·7 (−4·0H)
	0·2	2·6	25·0	5·1	—	Trace	0·8 (−2·0H)	7·8 (−2·0H)	57·2 (−3·5H)	1·3 (−4·0H)
Offal	—	1·1	27·4	1·7	—	Trace	0·8 (−2·0H)	10·5 (−2·0H)	56·8 (−3·4H)	1·7 (−4·0H)
Hoof	—	0·8	17·9	2·5	0·7	Trace	0·6 (−2·0H)	18·8 (−2·0H)	56·3 (−3·5H)	2·4 (−4·0H)
						(*b*) Molecular percentages				
Bone	0·3	3·8	27·3	4·0	0·1	Trace	0·7 (−2·0H)	9·0 (−2·0H)	54·2 (−3·5H)	0·6 (−4·0H)
	0·2	3·1	27·1	5·0	—	Trace	1·0 (−2·0H)	8·5 (−2·0H)	53·9 (−3·5H)	1·2 (−4·0H)
Offal	—	1·4	29·6	1·6	—		0·9 (−2·0H)	11·5 (−2·0H)	53·4 (3·4H) –	1·6 (−4·0H)
Hoof	—	1·0	19·5	2·4	0·6		0·7 (−2·0H)	20·5 (−2·0H)	53·1 (−3·5H)	2·2 (−4·0H)

saturation is due to oleopalmitostearin (iodine val. 29·6), it will be seen that the fully saturated glyceride content is less than 0·3%. Oxidation of 50 g. by the method of Hilditch & Lea (1927) yielded 0·57 g. of unoxidized product, iodine val. 5·4, corresponding to a fully saturated glyceride content of less than 1·8%.

Table 4. *Spectrophotometric analyses of* C_{18} *unsaturated acids of horse 'bone' oil*

Fraction no.	Unisomerized $E^{1\%}_{1\,cm.}$ 234 mμ.	268 mμ.	Isomerized (170°, 15 min.) $E^{1\%}_{1\,cm.}$ 268 mμ.	Isomerized (180°, 60 min.) $E^{1\%}_{1\,cm.}$ 234 mμ.
L9	8·7	0·6	168·5	263·8
L11	29·3	2·4	160·2	289·5
L12	14·5	0·6	166·2	237·9
L13	26·2	1·3	185·1	278·5

DISCUSSION

The oils used in this work were from pasture-fed horses, and it is therefore interesting in assessing the influence of dietary fat on the depot fats to consider the fatty-acid composition of other pasture-fed animals. Comparative figures are given in Table 6.

It will be seen that while the fatty-acid compositions of the ox and sheep depot fats are similar and agree with the findings of other workers (cf. Hilditch, 1947), they bear little resemblance to that of the pasture glycerides. The horse and rabbit fats have a fatty-acid composition like that of the pasture fat, but still retaining greater proportions of palmitic acid to conform approximately with the 30 ± 3 mol. % characteristic of animal body fats (Hilditch, 1949). The absence of more than traces of fully saturated glycerides in horse oil agrees well with Hilditch's (1949) view that the glycerides of animal body fats are a special case of 'even' distribution in which an animal fat containing little or no stearic acid has negligible proportions of fully saturated glycerides. Considering the unsaturated acids, it is not possible to comment on the proportions of the lower unsaturated acids in rabbit fat since the analysis was not made by the more modern techniques. The pentadecenecarboxylic acid content of

Table 5. *Component acids of fractions of horse 'bone' oil*

(All percentages are w/w.)

Fraction no.	Heptadecadienecarboxylic Non-conjugated (%)	Conjugated (%)	Heptadecatrienecarboxylic Non-conjugated (%)	Conjugated (%)	Oleic (by difference) (%)	Iodine value (methyl esters) Calc.	Found
L9	8·3	0·7	31·7	Trace	59·3	149·0	148·5
L11	9·8	2·2	29·7	0·5	57·8	149·1	149·9
L12	5·6	1·6	31·1	Trace	62·2	145·9	150·4
L13	6·0	2·2	34·5	0·2	57·1	153·5	152·6

Table 6. *Comparison between dietary fats of pasture-fed animals*

(Proportions of acids are given in % (w/w). Values in parentheses are mean unsaturations.)

	Fatty acids										
	Saturated				Unsaturated						
	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	Remarks
Ox	—	2·7	27·8	21·6	—	—	0·3 (−2·0H)	2·5 (−2·05H)	43·3 (−2·05H)	1·8 (−6·0H)	Commercial sample of beef tallow supplied by Abels Ltd., Newmarket, Auckland (Shorland, 1949*a*)
Sheep	1·3	3·5	25·0	22·2	0·7	—	0·5 (−2·0H)	1·7 (−2·0H)	44·2 (−2·0H)	0·9 (−2·0H)	Representative of sample of fat from fatty tissues of New Zealand lamb and mutton (Shorland, 1948)
Horse	0·2	3·1	25·3	4·1	0·1	—	0·6 (−2·0H)	8·3 (−2·0H)	57·6 (−3·5H)	0·7 (−4·0H)	'Bone' oil (this investigation)
Rabbit	—	4·5	23·0	4·0	—	—	—	—	65·8 (−4·0H)	—	Perinephric fat, Vickery (1928), cf. Banks, Hilditch & Jones (1933)
Cock's-foot	—	1·4	11·2	2·6	1·5	—	0·4 (−2·0H)	6·4 (−2·0H)	76·5 (−5·1H)	—	Mean value of 10 samples taken at intervals through the year (Shorland, 1945)
Mixed pasture	2·9	3·3	9·4	1·5	0·7	0·3 (−2·0H)	0·4 (−2·0H)	3·0 (−2·0H)	78·5 (−4·6H)	—	Jasperson & Burke quoted by Hilditch (1947)

horse oil (8·3 %) is, however, rather greater than found in pasture fats or in beef and mutton tallow. Information concerning the exact composition of the C_{18} unsaturated acids of pasture fat is still lacking. It is almost certain, however, that oleic acid is a minor constituent. Smith & Chibnall (1932) investigated the fatty acid of cock's-foot and ryegrass glycerides, and showed by bromination and by oxidation with alkaline permanganate (Lapworth & Mottram, 1925) that the unsaturated constituents comprised approximately two-thirds heptadecadienecarboxylic acid (isomeric with linoleic) and one-third linolenic acid. The absence of dihydroxystearic acid after oxidation suggested that not more than traces of oleic acid were present in the original acids. Shorland (1945), using low-temperature crystallization followed by alkaline permanganate oxidation, obtained traces of dihydroxystearic acid equivalent to 0·6 % of oleic acid in the total fatty acids. On the other hand, although Hilditch & Jasperson (1945) did not isolate oleic acid from pasture-grass fat, they estimated the following composition for the total fatty acids, using the iodine value and spectrophotometric measurements before and after alkali isomerization: saturated 0·7, oleic 32·4, heptadecadienecarboxylic 14·5 and heptadecatrienecarboxylic 52·4 %. The value for oleic acid, however, is probably too high, since no allowance is made for the presence of C_{14} and C_{16} monoethenoid acids. It may also be calculated from the mean unsaturation of the C_{18} acids (Shorland, 1945, 1949*b*) of glycerides from cock's-foot (−5·1 H), ryegrass (−5·4 H) and white clover (−5·4 H), that the maximum oleic acid content would not exceed 22·5, 15 and 15 %, respectively assuming linolenic and oleic acids only to be present. On the other hand, it is almost certain that some heptadecadienecarboxylic acid would also occur, so that the proportion of oleic acid in New Zealand pasture, which, on the more highly productive farms, comprises mostly ryegrass and white clover, would be very small indeed.

In beef and mutton tallow nearly all the C_{18} unsaturated acids are monoethenoid (mainly oleic acid), and it is somewhat doubtful whether linolenic occurs in these fats, though from the work of Knight, Jordan & Swern (1946) it is possible that the traces of ether-insoluble bromides (m.p. 170–174°), which they found in beef tallow may represent impure hexabromostearic acid.

In the present work, the bromination data together with the spectrophotometric analysis of the alkali-isomerized acid show that the C_{18} unsaturated acids of horse oil contain considerable proportions, about 30 %, of triethenoid acids (mainly linolenic), and about 9 % of a diethenoid acid, which, from the melting point of light petroleum-insoluble bromides is not linoleic acid. The remaining acids are monoethenoid, presumably mainly oleic acid, according to

the results of the previous workers (cf. Heiduschka & Steinruck, 1921).

The hoof oil from the horse compared with the depot fat shows much the same variations as in the case of the ox, where the hoof oil (neat's-foot oil) is distinguished from the depot fat by its lower content of palmitic acid which is compensated for by increased proportions of pentadecenecarboxylic acid. In both the horse and the ox the composition of the C_{18} unsaturated acids is approximately the same in the hoof oil as in the depot fat (cf. Hilditch & Shrivastava, 1948). In the ox the depot fats are rich in stearic acid, while neat's-foot oil contains only minor proportions of this constituent. In the horse the depot fats contain little stearic acid, as does the hoof oil.

Hilditch & Jasperson (1944) recently examined the fatty-acid composition of milk fat from a mare on a pasture diet, and noted the unusual composition (for a milk fat) of the C_{18} unsaturated acids which comprised 38% heptadecatrienecarboxylic (linolenic acid), 44% heptadecadienecarboxylic (not linoleic) and 18% heptadecenecarboxylic acid. They suggested that: 'Perhaps similar factors which lead to the presence of a small proportion (2%) of linolenic acid in the body fat of the horse (Heiduschka & Steinruck, 1921) also influence the appearance of this acid in the milk fat.' However, from the results of the present work it is clear that horse depot fat does in fact contain appreciable proportions of linolenic acid, about 20%, and the presence of this acid in the milk fat on this basis is, therefore, to be expected.

In considering reasons for the difference in fatty-acid composition between the depot fats of pasture-fed animals it may be speculated that in ruminants, such as the ox and sheep, linolenic acid is largely destroyed in the rumen during prolonged digestion. Some linolenic acid, however, must enter the blood-stream, since Kelsey & Longenecker (1941) have found 9·2% of the fatty acids combined as cholesterol esters in cow's blood comprise linolenic acid, and in addition, Hilditch & Shorland (1937) deduced the presence of linolenic acid in the glycerides and phosphatides of sheep liver by isolation of hexabromostearic acid (m.p. 175–176°, not depressed by the addition of authentic hexabromostearic acid). On the basis of the weight of hexabromostearic acid isolated, and allowing for the fact that only one-third to one-quarter of the linolenic acid is precipitated as ether-insoluble polybromide, it may be estimated that linolenic acid comprised 3·9–5·2% of the total fatty acids. It is interesting that no linolenic acid was found in ox liver, and this may indicate differences in metabolism between ox and sheep with respect to this acid. It is thus possible that, in ruminants, linolenic acid is either metabolized or hydrogenated in the body after absorption into the blood stream. In the case of the pig, Beadle *et al.* (1948) have shown that large amounts of linolenic acid may accumulate in the depot fats, but it has not yet been shown that this phenomenon is not limited to pigs which have an unusual fat metabolism. This possibility is suggested from the work of Ellis & Isbell (1926), who found that while linoleic acid from soya bean diet was readily absorbed into the pig's fat depots, only very small amounts, and in some cases none, of the dietary linolenic acid could be found in the depots. On the other hand, it is now perfectly clear that linolenic acid in the case of the rat, rabbit and horse is readily assimilated from the diet and deposited in the depot fat. The final linolenic acid content of the depot fats will presumably be related to the linolenic acid content of the diet, but other factors such as the rate of growth will influence the result. If the rate of growth is rapid (cf. Callow, 1935) the amount of fat derived by synthesis from non-fatty sources will be relatively large in relation to the amount derived directly from the dietary fat. Since fat derived by synthesis from carbohydrate or protein does not contain linolenic acid (cf. Hilditch, Lea & Pedelty, 1939; Shorland & De la Mare, 1945) the linolenic acid content of the depot fats may be expected to be less in fast-growing than in slow-growing animals. Horse depot fat may thus be visualized as being made up to the extent of about one-third (to judge from the respective linolenic acid contents of the pasture and of the horse oil) directly from the fatty acids of the grass, and the remainder by synthesis from carbohydrate or by alteration of the dietary fatty acids. It is hoped shortly, by comparison of the glycerides from horse oil and from the pasture, to obtain evidence as to whether the pasture glycerides are directly incorporated with the fatty depots of the horse, or whether the fatty acids of the pasture glycerides are rearranged to give new types of glycerides.

SUMMARY

1. Representative commercial samples of horse oil from pasture-fed animals have been submitted to ester-fractionation analysis. On a weight percentage basis, the saturated acids were found to comprise 25% palmitic together with minor proportions (< 5%) of stearic and myristic. The unsaturated acids contained minor proportions of tridecenecarboxylic acid, with 8–10% pentadecenecarboxylic and minor amounts of C_{20} acids. The C_{18} unsaturated acids, comprising 57% of the total, were made up of oleic acid 60%, heptadecadienecarboxylic 10% and heptadecatrienecarboxylic (linolenic) acid 30%.

2. Horse-hoof oil, as compared with the depot fat, contained less palmitic acid, but increased proportions of pentadecenecarboxylic acid. This relationship resembles that between the fatty acid composition of neat's-foot oil and of beef tallow.

3. The virtual absence of linolenic acid in the ox and sheep, as compared with its presence in quantity in the horse, the rabbit, and the pasture on which these animals feed, would indicate a difference in the nature of fat metabolism of these animals.

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Bile Pigment Precursors in Normal Human Erythrocytes

BY C. GARDIKAS, J. E. KENCH AND J. F. WILKINSON
Department of Haematology, Manchester Royal Infirmary

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In studies on the *in vitro* breakdown of haemoglobin Lemberg, Lockwood & Legge (1941) have obtained evidence of the occurrence of a chromoprotein intermediate, to which they have given the name choleglobin. The presence of this substance in the reaction mixture of ascorbic acid and oxyhaemoglobin is revealed by the appearance of a new absorption spectrum, and treatment with acetic acid will then liberate the bile pigments, biliverdin and bilipurpurin.

Reduced choleglobin has a well defined absorption band in the red at 628–630 mμ., reacts with carbon monoxide, and can be oxidized to the ferric compound, the maximum absorption of which occurs at 670 mμ. The pigment will therefore be green whether oxidized or reduced. Because many of its physical characteristics are similar to those of haemoglobin, Lemberg and his co-workers have been unable to effect separation of choleglobin from the great excess of its precursor. There is a paucity of direct knowledge of the pigment, and it is not known if it can undergo reversible oxygenation. Chemical evidence indicates that choleglobin arises by oxidative removal of the methene group in the prosthetic group of haemoglobin. The haem molecule is thereby opened, but otherwise unchanged, and the iron and globin linkages remain undisturbed. This chemical change is sufficient to endow the new protein