Mold Induced Changes in Cacao Lipids[1]

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Abstract

The free fatty acid content of sound cacao beans of nine geographic origins varied from 2.7 to 4.8 meq./100 g fat. Fungi grown on cacao beans under humid environmental conditions have remarkable lipolytic activity. Values as high as 200 meq. free acid/100 g fat were obtained for badly deteriorated samples. Titration of the free fatty acids of cacao fat is suggested as a simple control procedure for detecting cocoa butter obtained from moldy beans. Percentage composition of the free fatty acid fraction changes as a result of mold growth. Stearic and palmitic acid increased while oleic and linoleic acids decreased. Tracer experiments show no observable conversion of oleic acid to stearic. The changes suggest oxidative reactions to form carbonyl compounds.

Introduction

Careful grading of cacao bean shipments has greatly minimized the flavor problem associated with moldy beans. However, the presence of a relatively small number of mold damaged beans may be sufficient to affect flavor; hence, a sporadic problem exists. Starkle's observation in 1924 (8) of the formation of methyl ketones by molds grown on cacao fat was the first information concerning the chemical changes occurring in moldy cacao beans. In 1965 Boyd et al. (1) suggested that the variation they found in the methyl ketone content of cacao beans might have been related to moldiness. Hansen and Keeney (2,3) made direct comparisons of the carbonyls in moldy and non-moldy beans and found that moldy samples always contained greater concentrations of total monocarbonyls, methyl ketones, 2-enals and 2,4-dienals.

In a continuation of the study of Hansen and Keeney (2,3), we observed that the 2,4-dinitrophenylhydrazones (2,4-DNPH) bands of moldy bean extracts moved further down Celite/Seasorb columns than the corresponding bands of non-moldy extracts. Subsequent investigations demonstrated that extracts of fat from moldy cacao beans were high in acid content compared to fat from non-moldy beans. The resulting increase in polarity was responsible for the greater mobility of the 2,4-DNPH bands.

As a consequence of this finding, a more extensive study of the effect of mold on the free fatty acid content of cacao fat was initiated. The results of this investigation are the subject of this report.

Experimental Procedures

Samples

Cacao beans from major producing areas were obtained from several chocolate manufacturers, as were the original samples of moldy beans. The latter were used in the preparation of additional quantities of moldy beans by transferring mold spores to previously uncontaminated whole bean samples using sterile water. Storage for several weeks at 24 C generally produced satisfactory moldy samples when the inoculated beans were placed in quart polyethylene containers and covered with moist filter paper to increase the humidity.

Lipid samples were obtained either by solvent extraction or by hydraulic pressing. For the former, beans (25 g) and Celite 545 (Fisher Scientific) (25 g), were pulverized in a Waring Blendor to obtain a fine powder and then packed into a 3 × 55 cm glass column. Fat was eluted from the column using 150 ml of the appropriate solvent (either hexane, or diethyl ether, or 1:4 v/v methanol-chloroform). Pressed fat samples were obtained using a Carver laboratory hydraulic press equipped with heating plates (120 C). Ground bean samples were subjected to 15,000 psi pressure for 10–15 min.

The free acid content of the fat was determined by titration of petroleum ether solutions with 0.01 M alcoholic KOH, using phenolphthalein as indicator. Sample sizes were 1 g and 0.1 g for fat from non-moldy and moldy samples respectively. Peroxide numbers were determined using the iodometric method of Wheeler (9).

Lipid Analysis

Cacao fat samples were separated into neutral lipid and free fatty acid fractions using KOH treated silicic acid columns [McCarthy and Duthie (5)]. Methyl esters were prepared from these fractions by refluxing the fat (or fatty acids) with excess methanol in the presence of sulfuric acid or by the use of BF_3-methanol reagent (Applied Science Laboratories, State College, Pa.). These were then chromatographed on a Hewlett Packard F & M Model 5750 gas chromatograph equipped with hydrogen flame detectors, using a 255 × 0.32 cm stainless steel column packed with 2% phosphoric acid treated diethylene glycol adipate (10%) on 80–100 mesh Gas Chrom A (Applied Science Labs.). All samples were chromatographed isothermally at 190° with a nitrogen flow rate of 30 ml/min. Analyses of known mixtures using GLC peak areas to determine percentage composition showed less than 1% difference from the known weight per cent composition for each of the components.

Radiochemistry

^{14}C-Carboxyl labeled triolein (10 μC) (Applied Science Labs.) was added to a solution of cacao fat (8.0 g) in ether (20 ml). After thorough mixing, equal portions of the mixture were transferred to eight petri dishes and the solvent was allowed to evaporate. Mold spores were then transferred to the residues (six dishes) using a platinum loop. To provide additional nutrients, 2 ml tryptic soy broth (Difco) was added and the dishes were stored at 24 C under humid conditions to assure satisfactory mold growth.

Analyses of the fat for free acid content and isotopic labeling were performed on the contents of the dishes after five and eight weeks. Distribution of the isotope label was determined on a Barber-Colman Model 5000 gas chromatograph equipped with a Model 5190 radioactivity monitoring system for the detection of organic ^{14}C as $^{14}CO_2$. Samples were chromatographed isothermally (190 C) on a U-shaped glass column (180 × 0.6 cm) packed with 15% diethylene glycol succinate

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TABLE I

Free Fatty Acid Content of Pressed Cacao Fat From Bean Varieties Representing Major Producing Areas (meq./100 g fat)

Variety	Acid content	Variety	Acid content
Samoa	4.3	Ivory Coast	6.3
Sanchez	3.9	Costa Rica	3.6
Trinidad	2.7	Accra	3.2
Caracas	4.8	Arriba	2.9
Ghana	4.5	Bahia	3.2
Sanchez (moldy)	220	Arriba (moldy)	82
Trinidad (moldy)	138	Bahia (moldy)	53

(Hi-EFF) on 80/100 mesh Gas-Chrom P (Applied Science Labs.).

Results and Discussion

As is readily evident in Table I, the free fatty acid content of non-moldy beans was found to be reasonably uniform, generally 3–4 meq./100 g fat. These values are generally comparable with those obtained in industrial quality control laboratories. The one sample with a higher acid content, Ivory Coast, displayed traces of shell moldiness. Furthermore, the values observed were essentially the same whether the fat was recovered by pressing or by solvent extraction. A slight increase in free fatty acids was detected when the beans were roasted.

Moldy beans displayed markedly higher free acid contents than non-moldy samples (Table I). Generally the acid content of the fat was related to the degree of moldiness; the greater the observed mold growth the higher the titer. It is, of course, inconceivable that beans deteriorated to the extent shown in Table I would go undetected by the chocolate manufacturer, except when present in trace amounts. However, the demonstrated relationship between moldiness and free fatty acid content suggests the titration of cacao fat may provide a reasonable basis for the detection of fungal deterioration (excepting perhaps where the fat has been deodorized or subjected to alkali refining).

Having demonstrated that extensive increases in acid occur when mold proliferates on cacao beans (presumably due to lipolysis), a study was made of the changes in relative amounts of individual fatty acids in several varieties of beans (both moldy and non-moldy), Table II. It is evident that, with the exception of Arriba beans, mold growth significantly altered the relative amounts of saturated and unsaturated acids. Apparent increases in the palmitic and stearic acid contents of the fat occurred, while the amounts of oleic and linoleic acids decreased proportionately. Two explanations of these results seemed reasonable, namely (a) the partial reduction by the mold of unsaturated acids to the corresponding saturated compounds (considered less likely) or (b) the preferential degradation of the unsaturated acids of the fat. (The reason for the lack of observable change in the Arriba beans is not clear, although in contrast to the other samples the beans were firm with comparatively little penetration of the mold into the interior of the bean. While this may be a characteristic of Arriba beans, it seems more likely that the differences observed between bean species are due to differences in the nature of the mold as well as the environmental conditions.

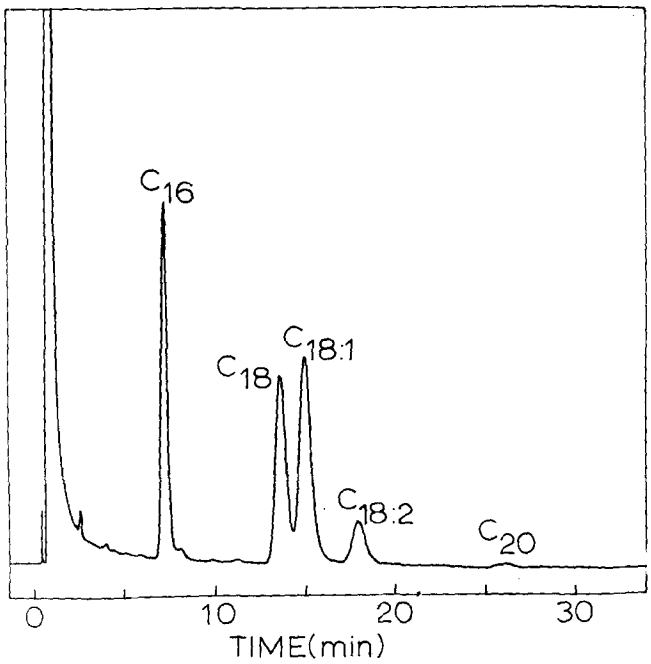

FIG. 1. Gas chromatographic analyses of the free fatty acids (methyl esters) present in non-moldy cacao beans.

The possibility that the unsaturated acids were being hydrogenerated by the mold was investigated using radioactive labeling experiments. In view of the difficulties involved in uniformly distributing the label in bean samples, mold was grown on cacao fat containing ^{14}C carboxyl labeled triolein and the rancid fat was subsequently examined for distribution of the label. The results of this study indicate that conversion of unsaturated to saturated acids, if it occurs at all, is not important. Certainly, no (<1%) conversion of 1-^{14}C-oleic acid to 1-^{14}C-stearic acid was achieved by the mold. Each moldy sample did however display the characteristic lipolysis (approaching 80% in some cases) and the characteristic fatty acid

TABLE II

Relative Fatty Acid Content of Several Varieties of Moldy and Non-Moldy Beans[a,b]

Acid	Bahia		Sanchez		Arriba	
	Moldy	Non-Moldy	Moldy	Non-Moldy	Moldy	Non-Moldy
Whole fat						
Palmitic	25.3	22.8	28.0	25.9	21.9	22.0
Stearic	37.1	33.3	40.1	34.0	36.9	37.3
Oleic	33.5	38.9	28.9	35.8	37.2	36.2
Linoleic	3.2	4.0	2.1	3.3	3.1	3.3
Arachidic	0.9	0.8	1.0	0.9	0.8	1.3
Neutral lipids						
Palmitic	22.4	23.4	23.6	23.1	23.8	23.7
Stearic	34.3	32.6	36.4	36.0	35.1	37.3
Oleic	39.0	39.3	36.0	36.8	37.0	35.2
Linoleic	3.6	3.8	3.1	2.9	3.2	3.1
Arachidic	0.9	0.9	1.0	1.3	0.9	0.9
Free fatty acids						
Palmitic	26.6	27.2	31.2	27.8	26.3	29.3
Stearic	46.2	26.9	47.9	25.4	33.6	28.1
Oleic	22.8	35.0	17.6	36.5	30.2	33.5
Linoleic	3.0	9.8	1.7	8.9	8.7	8.2
Arachidic	1.5	1.2	1.5	1.5	1.3	1.0

[a] Expressed as percentages of the total area under the GLC peaks corresponding to the five listed acids.
[b] No significant differences were observed for fat samples extracted with hexane, ether or methanol-chloroform (1:4 v/v).

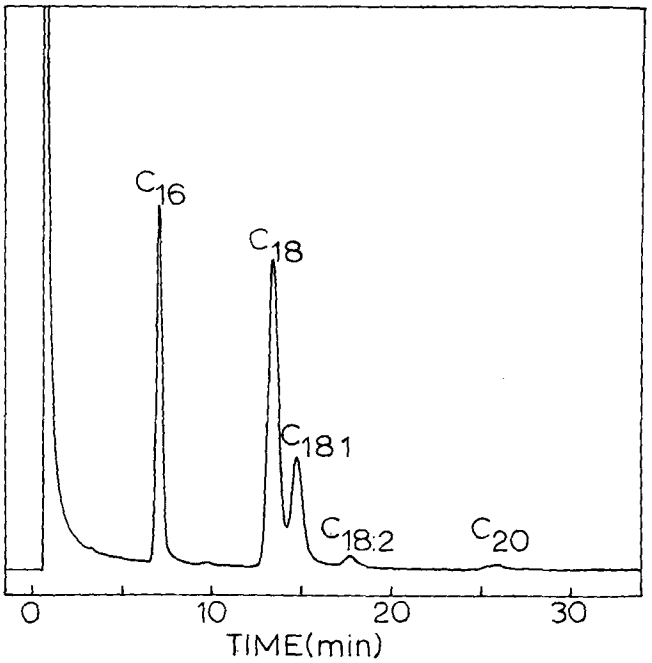

FIG. 2. Gas chromatographic analyses of the free fatty acids (methyl esters) present in moldy cacao beans.

composition of fat from moldy beans, thus showing that the mold was active in fat utilization.

While being far from conclusive, indirect evidence indicates that an oxidative mechanism is involved in the observed changes in percentages of fatty acids. Clearly the increases in methyl ketones and unsaturated aldehydes noted by Hansen and Keeney (2,3) are readily explained in these terms. The peroxide numbers of fat samples, which might be expected to parallel the carbonyl content of the fat, showed little relationship to the origin of the sample. Freshly pressed fat from both moldy and non-moldy beans showed peroxide numbers not exceeding 3 meq./1000 g fat, too low to be indicative of significant oxidation. It would thus seem that, if the formation of carbonyls from the fat occurs via peroxide formation, the rate of decomposition of the peroxides must be rapid.

It should be noted that the results of Smith and Alford (6,7) suggested that relatively few microorganisms have the ability to cause the oxidative reactions normally associated with rancidity, namely the formation of peroxides and their subsequent decomposition into carbonyls. However, some microorganisms such as *Aspergillus flavus* did exhibit the ability not only to remove peroxides from rancid lard, but also to cause significant increases in 2,4-dienals, alkanals and methyl ketones. Since molds of the genus, *Aspergillus,* are commonly observed on cacao beans, the low peroxide numbers of fat samples from moldy beans should not be considered inconsistent with mold activity.

When the fat was subsequently separated into the free acid and neutral lipid fractions, the analyses (Table II), showed two significant features. First, only minor differences exist in the compositions of the neutral lipid fractions (mono-, di- and triglycerides) of fat from moldy and non-moldy beans. Second, a significant decrease in the proportion of unsaturated acids is evident in the free fatty acid fraction of moldy beans (Table II). The former would seem to preclude the possibility of preferential lipolysis being the cause of the decreased unsaturated acid content of the free fatty acid fraction. Most microbial lipases attack the 1,3 positions of triglycerides. Since approximately 90% of the oleic acid of cacao fat is esterified at the 2 position this explanation initially seemed satisfactory. However, the required proportionate increase in the oleic acid content of the neutral lipid fraction of moldy beans was not observed. The latter clearly reflects the earlier observation of decreased unsaturated acid content in the total fatty acid analysis of moldy beans. These observations indicate that the presumed conversion of fatty acids to carbonyls must occur after lipolysis since chemical changes prior to this step would be shown in the neutral lipid analyses. Furthermore, while there may be differences in the rate, it is reasonable to suggest that both saturated and unsaturated acids are involved in the formation of carbonyls. The observed increase in carbonyls could result in part from the conversion of saturated acids via the pathway suggested by Hawke (4), which involves lipolysis of the fat, oxidation of the acids thus formed to β-keto acids and finally decarboxylation of the β-keto acids to form the corresponding methyl ketones. Shorter chain methyl ketones may be produced by a similar mechanism from acids formed from the autoxidation of unsaturated acids. The susceptibility of unsaturated acids to autoxidation is well known and certainly will be enhanced if lipoxidase is produced by the mold. The autoxidation of oleic, linoleic and linolenic (trace amounts only), acids could then give rise to the increases in 2-enals and 2,4-dienals observed by Hansen and Keeney (2,3).

The absence of a consistent increase in palmitic acid (similar to that for stearic acid) in the free fatty acid fraction of moldy fat is difficult to explain. Since some increase in both palmitic and stearic acids is observed in the total fatty acid analysis of moldy beans, it is difficult to see why this should not also be shown in the free fatty acid analysis, particularly in view of the similarities in the reactivities of these two acids. One explanation may, of course, be sample variation. There remains, however, some possibility of preferential metabolism of palmitic acid and it is hoped that further radiochemical experiments using the labeled acids will resolve this question.

ACKNOWLEDGMENT

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