Soybean Unsaponifiables: Chromatographic Separations and Characterization[1]

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Abstract

Unsaponifiables extracted from 10 different lots of refined soybean oil were subjected to liquid-liquid chromatographic separations. Three major fractions were obtained. The least polar hydrocarbon fraction constituted 15 to 30% of the unsaponifiables; the most highly polar fraction contained the steroids constituting 35 to 45% of the unsaponifiables. The fraction of intermediate polarity varied in composition from lot to lot, but usually it contained more than 50% of the unsaponifiables. These basic fractions were analyzed by thin layer, gas-liquid chromatography, and by chemical tests for functional groups.

To determine the effects of soybean unsaponifiables on the oxidative and organoleptic stability of edible fats, various concentrations of the extracted and fractionated materials were examined in cottonseed oil. Effects of extraction methods on yields, fractionation characteristics, and composition of the different lots of soybean unsaponifiables are discussed.

Introduction

SOYBEAN OIL REVERSION has been attributed to various factors. Mattil (7) and others (2,4) point out soybean unsaponifiables as the precursor, but the work of Taylor (12), and Sanders (8) indicates that unsaponifiables have no effect in the reversion process. Our investigation was undertaken to determine the true function, if any, of soybean oil unsaponifiables in the reversion process and to learn more about the chemical and physical characteristics of these materials.

Unsaponifiables, as classically defined, are those naturally occurring materials in edible oils, that form no water-soluble soaps when the oil is refluxed with strong alcoholic alkali, but that are soluble in organic solvents such as diethyl ether. However, we question whether this definition is sufficiently broad when the qualification, naturally occurring, is included, since autoxidation appears to increase the unsaponifiable content.

Methods

Soybean oil unsaponifiables were extracted by various techniques; then each lot examined chemically, physically, and organoleptically in search of a better understanding of their function in soybean oil reversion.

The tools utilized in this investigation were liquid-liquid, gas-liquid, and thin layer chromatography along with chemical and spectroscopic analyses. Organoleptic evaluation was employed in the final analysis to test the effect of unsaponifiables on flavor stability.

Experimental

Extraction. Unsaponifiables were obtained from 10 different lots of refined soybean oils by 3 different extraction techniques. The first involved saponification of the oil and subsequent ether extraction of the resulting soaps according to AOCS analytical procedures. In the second, soybean oil was transesterified with methanol in the presence of methoxide catalyst, the bulk of the methyl esters was distilled, and the distillation residues were saponified for extraction of the unsaponifiables. The third technique and most satisfactory employed a modified continuous extraction similar to the method of Hilditch (5).

Continuous extraction was selected since the AOCS method failed to provide enough unsaponifiables for study and, as will be shown later, the saponification-extraction of methyl ester distillation residues provided nonrepresentative unsaponifiables. The continuous extraction method also allowed convenient handling of 1,000 g of oil and produced unsaponifiables of the same composition as those obtained through the AOCS method.

Liquid-Liquid Chromatography. The unsaponifiables obtained by any one of the techniques described were subjected to liquid-liquid chromatography in the manner of Frankel et al. (3). Fifty grams of A. R. 100-mesh silicic acid (labelled "Suitable for chromatographic analysis by the method of Ramsey and Patterson") that had been dried overnight at 110C was used as a support; the mobile phase was 2% by volume methanolic benzene and the immobile phase, 16% methanol (by weight of SiO_2). The samples, 0.1 g, were dissolved in a small amount of mobile phase before chromatographing on this column. Fractions (5 ml) were collected in small preweighed beakers. The solvent was evaporated on a steam plate and the beakers thoroughly dried under vacuum in a desiccator. Weights of the fractions were used to construct distribution plots.

Chromatograms for soybean oil unsaponifiables extracted by the AOCS method (Figure 1) provide a basis for comparing results from the other extraction

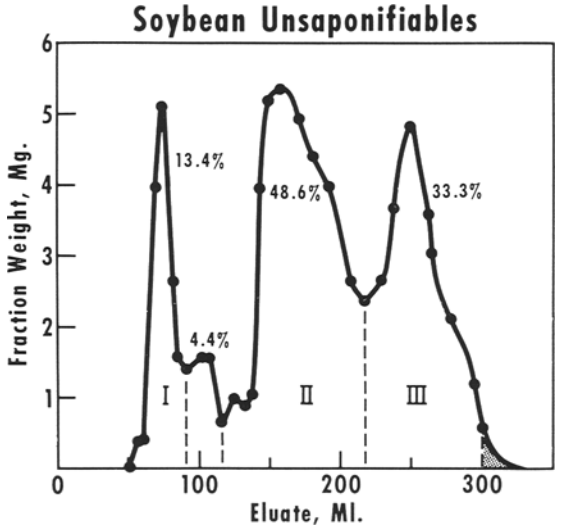

FIG. 1. Liquid-liquid chromatogram of soybean unsaponifiables representative of AOCS and continuous extraction procedures.

[1] Presented at the AOCS meeting in Chicago, 1961.
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TABLE I
Soybean Oil Unsaponifiables

Commercial sample	Extraction code[a]	Unsap. %[b]	Fraction I[c]	Fraction II[c]	Fraction III[c]	Remainder[d] %
A(PV=0)	A[e]	0.44	45.2	18.4	34.2	2.2
A(PV=600)	A	2.25	37.7	23.6	40.0	—1.3
A(PV=2161)	A[f]	2.38	28.8	19.6	23.7	27.9
B	A	1.07	13.0	55.4	25.6	6.0
B	B	1.00	54.1	4.2	31.6	10.1
C	A	1.03	14.6	48.6	33.3	3.5
C	C	1.00	14.0	43.9	34.5	7.6
C	D	1.00	15.4	34.8	45.7	4.1
C	B	1.00	25.4	30.3	31.5	12.8
D	C	0.60	27.2	20.0	49.0	3.8
E	C	1.00	31.3	16.1	46.7	5.9
F	A	0.41	6.6	63.0	22.0	8.4
G	A	0.78	7.6	54.3	26.9	1.2
H	A	1.10	7.7	37.5	41.1	13.7
I	A	0.58	13.4	41.8	28.4	16.4
J	A	1.10	9.4	43.6	34.0	13.0

[a] Extraction code:
A = AOCS procedure.
B = Saponified methyl ester distillation residues.
C = Large-scale continuous extraction.
D = Saponified crude methyl esters.
[b] Per cent based on weight of parent oil.
[c] Per cent of total unsaponifiables.
[d] Per cent of portions between major peaks plus the ether eluate.
[e] Gas-liquid chromatogram of Fraction I, Figure 3.
[f] Gas-liquid chromatogram of Fraction I, Figure 4.

techniques since they were obtained through standard analytical procedures.

All unsaponifiable chromatograms exhibited three major fractions. Fraction I contained the least polar materials, such as hydrocarbons; Fraction II, the components of intermediate polarity; and Fraction III, the most polar portion including those materials eluted only with diethyl ether (indicated by shaded portion of chromatogram in Figure 1). Table I summarizes the percentage distribution of the three major fractions. The unsaponifiable content of each oil is given under the heading "Unsap. %."

Unsaponifiables obtained through saponification and extraction of methyl ester distillation residues were different in composition than those obtained through AOCS extraction of the same oil.

An increase in Fraction I was accompanied by a corresponding loss in Fraction II. Unsaponifiables obtained from the continuous extractor, which is essentially a large-scale AOCS extraction, provided liquid-liquid chromatograms that were the same as those obtained through use of the standard AOCS procedure, and thus Figure 1 stands as representative of both methods.

Figure 2 illustrates the most extreme compositional change that we noted resulting from an unsaponifiable extraction of one lot of oil by the "methyl ester residues" method. Fraction II was all but eliminated,

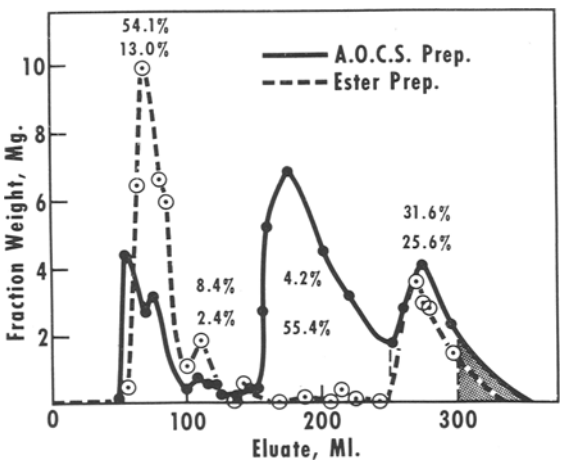

FIG. 2. Liquid-liquid chromatogram of soybean unsaponifiables: AOCS and "Methyl ester distillation residues" methods compared.

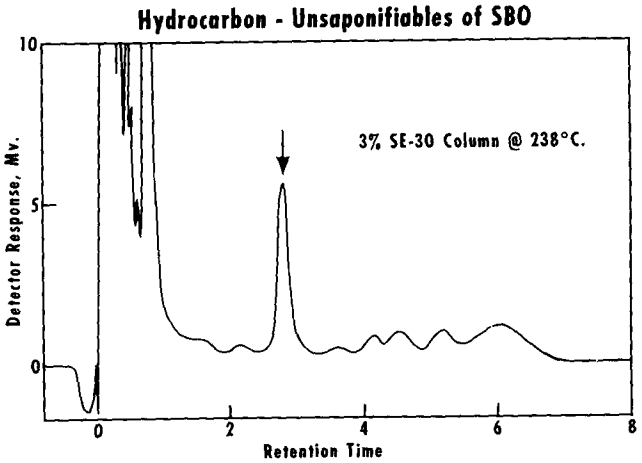

FIG. 3. Gas-liquid chromatogram of Fraction I: Soybean unsaponifiables obtained from unoxidized oil.

while Fraction I increased a proportional amount. The over-all percentage unsaponifiables obtained by this method remained essentially the same as the AOCS method with only a slight increase noted.

Some known materials, considered likely constituents of soybean oil unsaponifiables, were chromatographed. Their elution volumes are as follows:

Squalene, carotene	50– 90 ml
Tocopherol	80–110 ml
Sitosterol, stigmasterol	200–275 ml
Chlorophyll	265–285 ml

Gas-Liquid Chromatography. Unsaponifiables were subjected to high-temperature gas-liquid chromatography on a 4-ft column of 3% General Electric SE-30 Silicone Rubber coated on 60/80 mesh Chromosorb W according to the method of Horning and Sweeley (6,9,11).

Table I, Sample A, shows that oil oxidation followed by decomposition of the hydroperoxides causes a marked increase in unsaponifiable content of the parent oil. While the over-all content of unsaponifiables increases during oxidation, the relative amount of Fraction I decreases. The absolute amount of Fraction I increases because the over-all unsaponifiable content increases so greatly. This change in composition is supported by the gas-liquid chromatograms of unsaponifiables obtained from the same oil at various levels of oxidation. Figures 3 and 4 show chromatograms of Fraction I (Figure 1 and Table I), obtained from liquid-liquid fractionated unsaponifi-

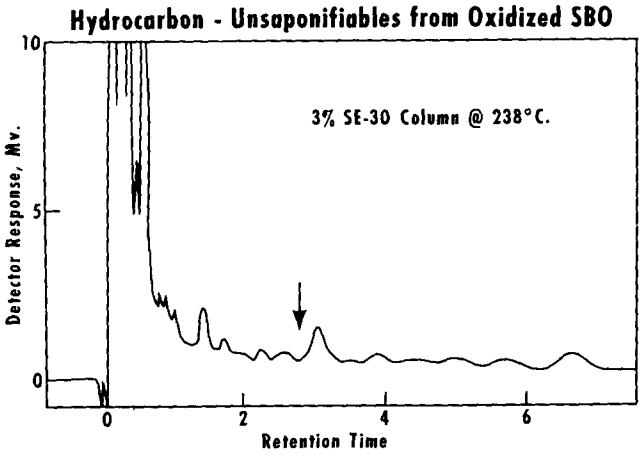

FIG. 4. Gas-liquid chromatogram of Fraction I: Soybean unsaponifiables obtained from oxidized oil.

ables. The peak denoted by the arrow was substantially reduced in quantity as the PV of the parent oil increased.

The semipolar and polar fractions (II and III) of the unsaponifiables failed to chromatograph. When unsaponifiables were acetylated by refluxing with acetic anhydride, followed by cooling and recrystallizing the crude acetates from methanol-water, it was possible to chromatograph Fractions II and III. The acetylated unsaponifiables, when chromatographed, exhibited essentially the same peaks and distribution in the hydrocarbon portion as did the nonacetylated materials. Acetylation also allowed the sterol fraction (III) to be chromatographed effectively. Etherification of unsaponifiables also decreased retention volumes and allowed development of the chromatogram at a more rapid rate than was possible with acetylation.

Thin-Layer Chromatography. Unsaponifiables were subjected to modified thin-layer chromatography according to Stahl (10). The apparatus consisted of U. S. manufactured equipment, silica, and reagents.

The plates (8 x 10 in.) were spread with a slurry of 100-mesh silicic acid (Ramsey and Patterson grade), plaster of Paris, and zinc silicate phosphor, 9:1:0.25 (parts by weight). Zinc phosphor allowed preliminary examination of the plates under ultraviolet radiation. The chromatographed materials when viewed under ultraviolet light appeared as spots of diminished fluorescence against a background of green. The plates were spotted about 1 in. from the lower edge with the materials to be chromatographed and developed in ascending manner in a solvent system consisting of 10% diethyl ether in redistilled pentane-hexane. Figure 5 illustrates the separations achieved with unsaponifiables and related materials. Spots were identified by comparing them with known materials chromatographed simultaneously. A commercial tocopherol concentrate was rich in both saturated and unsaturated hydrocarbons. The hydrocarbons of Figure 5 were obtained from petroleum ether distillation residues (BP above 100C at 0.1 mm pressure). The long polymer trail indicates an almost infinite number of compounds that have varying polarity and distribute themselves along the entire trace. Soybean oils when oxidized and chromatographed exhibited an increase in polymeric materials as the extent of oxidation became greater. Highly oxidized or badly reverted soybean oils showed no increase, and usually a decrease, in hydrocarbon with a considerable increase in polymer content.

Analysis—I. R. Liquid-liquid fractionated unsaponifiables examined by I. R. showed absorption bands indicative of the following:

Fraction I	Fraction II
Unsaturation	Very strong > C=O
Hydrocarbon	Possible ring structure
Trace > C=O and OH	

Fraction III
Strong alcoholic OH
Ring structure possible
Strong > C=O

One unsaponifiable sample showed strong anhydride structure. Only two of five samples showed isolated *trans* in Fraction II (then only a trace). Unsaturation was observed in all fractions.

Qualitative Chemical Analysis. Various qualitative chemical analyses were run on the unsaponifiables. Fraction I contained considerable unsaturation as indicated by bromide uptake. Fraction III contained a small amount of unsaturation, methyl ketone, or secondary alcohol, along with a trace positive test for aromaticity.

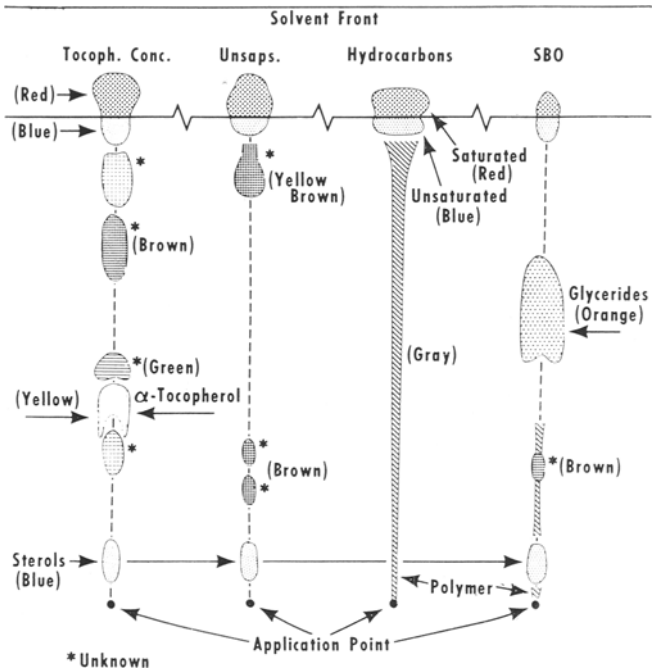

FIG. 5. Thin-layer chromatogram of soybean unsaponifiables and some related materials.

The Liebermann-Burchard test was applied to liquid-liquid chromatographed unsaponifiables. After the fractions were collected, the solvent was removed and each fraction was redissolved in chloroform. The reagent was added to this solution and the colors were allowed to develop. The position of the sterols in Fraction III was readily apparent from the characteristic green produced by the Liebermann-Burchard reagent. Fraction I was bluish-red while Fraction II was a yellowish-brown. A recent report by Capella (1) reveals cycloarthenol as a minor constituent of linseed oil. This material also becomes yellow-brown with Liebermann-Burchard reagent. Further investigation of the yellow-brown fraction of soybean unsaponifiables will be undertaken to determine if a relationship exists between this material and cycloarthenol.

Organoleptic Evaluations. Soybean oil unsaponifiables and known materials considered likely constituents of them were added to various cottonseed oils and evaluated organoleptically. The treated and control samples were scored for odor and flavor on the basis of a 10 to 1 scale with 10 representing "excellent" and 1 "very bad." Flavor responses as listed on the score sheet were: bland, buttery, beany, grassy, rancid, painty, and metallic. After the sample is evaluated on the 10 to 1 scale, it is described as closely as possible by checking the appropriate flavor response.

Known soya sterols added to cottonseed oil did not adversely affect its oxidative or flavor stability (Ta-

TABLE II
Flavor Effect of Sterols in Cottonseed Oil

Days at 60C	Flavor score (P.V.)			Sig.
	Control	Sitosterol	Stigmasterol	
0	8.1 (0.4)[b]	7.7 (0.4)	7.2 (0.5)	†[a]
6	5.2 (6.1)	5.1 (4.4)		†
6	4.7 (6.3)		5.2 (3.3)	†
AOM	31	29	28	

[a] † No significant difference.
[b] Figures in parentheses are peroxide values (P.V.) at time of evaluation.

TABLE III
Flavor Effect of Soybean Oil (SBO) Unsaponifiables in Cottonseed Oil (CSO)

	CSO-A			CSO-B			CSO-C		
	Control	Unsap.	Sig.[a]	Control	Unsap.	Sig.	Control	Unsap.	Sig.
0 Time	7.1 (0.3)[b]	5.9 (0.3)	†	8.1 (0.2)	6.4 (0.2)	*	6.8 (0.3)	7.2 (0.4)	†
3 Days at 60C	5.6 (8.1)	3.6 (2.9)	**	6.0 (3.8)	4.2 (6.5)	**	5.7 (7.4)	5.7 (6.6)	†
AOM	60	324		20	41		52	42	

[a] † No significant difference.
* Significance at the 5% level.
** Significance at the 1% level.
[b] Figures in parentheses are peroxide values (P.V.) at time of evaluation.

ble II). Unsaponifiables were added to various cottonseed oils to determine their effect on the oxidative and flavor stability of the carrier oils. Table III presents a representative sample of the organoleptic data obtained. Table IV presents organoleptic data obtained from cottonseed oils to which unsaponifiables had been added in varying concentrations. Fraction I (Figure 1) of unsaponifiables decreased the oxidative stability of the carrier oils when added at 0.05%. The peroxide values of the oils treated with Fraction I increased greatly over the control samples. The flavor responses obtained from evaluation of unsaponifiables added to cottonseed oil were in most instances similar to reverted soybean oil.

Unsaponifiables obtained from methyl ester distillation residues markedly affected the quality of one cottonseed oil but did not affect the quality of another lot (Table V).

A reconstituted soybean oil was prepared by transesterification of purified soybean oil methyl esters with triacetin. Analysis showed it to contain 95% triglyceride. This oil underwent typical oxidative and flavor degradation although completely free of naturally occurring unsaponifiables. The flavor responses obtained, "grassy, rancid, and painty," are typical of reverted soybean oil.

Results and Discussion

Unsaponifiables affect oxidative and flavor stability of soybean oil. The magnitude of their effect varies from pronounced degradation to no measurable alteration in stability as determined by AOM and organoleptic studies.

When considering oxidative stability alone, the detrimental portion of unsaponifiables was the hydrocarbon nonpolar component (Fraction I, Figure 1). The sterols and components of intermediate polarity were without significant effect. Organoleptic evaluations seemed to depend rather strongly on the cottonseed oils to which the unsaponifiables and their constituents were added. Some oils were more stable to the unsaponifiables than others.

As soybean oil undergoes oxidation, the over-all percentage of unsaponifiables increases and the relative percent of Fraction I decreases. This result was supported by liquid-liquid, gas-liquid, and thin-layer chromatography.

Any hydrocarbons, alcohols, aldehydes, or polymers formed through oxidation would, if sufficiently high in molecular weight, be insoluble in water and form no water-soluble soap when treated with alkali. These materials collecting in the unsaponifiables could give rise to the variable composition noted, depending upon the extent of oil autoxidation. As autoxidation increases, the over-all percentage of unsaponifiables increases. This increase is attributed to the formation and accumulation of these materials. Since Fraction I decreases as oxidation increases (Table I, Sample A), and since Fraction I is known to contain considerable unsaturation, this relative decrease is probably due to the unsaturated nonpolar materials undergoing secondary oxidation.

The ability of these unsaturated hydrocarbons to undergo this secondary oxidation could be responsible for their autocatalytic behavior in the induction of oxidation in either their parent oils or oils to which they were added. Many hydrocarbons that originate in autoxidation are capable of sustaining this process through their ability to form additional hydroperoxides. These alkyl hydroperoxides in turn induce further degradation through their decomposition and yield free radicals, which can undergo fission, polymerize, rearrange, or form other radicals by abstracting a hydrogen atom from a neighboring molecule.

This secondary oxidative degradation could then be responsible for the relative decrease in Fraction I as oxidation of the parent oil increases. End products from oxidation are either volatile (due to low molecular weight) or capable of forming a soap, which can be removed by water washing during the saponification-extraction process.

The methyl ester residue method provided essentially the same amount of unsaponifiable material as did the the AOCS method, although Fraction I increased as Fraction II decreased. This change may be caused by alteration of the natural unsaponifiables during vacuum distillation since in an almost anaerobic environment the ester molecules would be unable to form oxidative products. Any natural unsaponifiable materials present could undergo rearrangement leading to a transformation from polar to less polar structures.

TABLE IV
Concentration Effect of Soybean Oil Unsaponifiables on Flavor of Cottonseed Oil (CSO)

Days at 60C	% Unsap.	CSO-A Flavor score (P.V.)			CSO-B Flavor score (P.V.)		
		Unsap.	Control	Sig.	Unsap.	Control	Sig.
0	0.05	7.7(0.2)[b]	8.2(0.3)	†[a]	7.8(0.1)	7.8(0.3)	†
0	0.3	7.9(0.4)	7.7(0.5)	†			
0	1.0	5.5(0.3)	4.3(1.7)	*			
3	0.05	5.3(6.6)	5.8(8.1)	†	6.1(5.0)	5.6(5.6)	†
3	0.3	5.3(4.7)	6.0(6.4)	*			
3	1.0				4.1(8.2)	5.8(6.3)	**

[a] † No significant difference.
* Significance at the 5% level.
** Significance at the 1% level.
[b] Figures in parentheses are peroxide values (P.V.) at time of evaluation.

TABLE V
Flavor Loss Induced in Cottonseed Oil (CSO) by Soybean Oil (SBO) Unsaponifiables Obtained from Residues of Methyl Ester Preparations

Unsap.	CSO-A (P.V.)	Sig.	CSO-B (P.V.)	Sig.
Initial control	7.8(0.19)[b]		7.0(0.24)	
0.05%	3.3(0.19)	**	7.3(0.20)	†[a]
Aged 3 days at 60C control	5.5(8.74)		6.7(3.1)	
0.05%	3.5(7.35)	**	5.7(4.3)	*

[a] † No significant difference.
* Significance at the 5% level.
** Significance at the 1% level.
[b] Figures in parentheses are peroxide values (P.V.) at time of evaluation.

Since reconstituted soybean oils undergo typical reversion, although completely free of naturally occurring unsaponifiables, the reversion process cannot be attributed wholly to the effect of these materials.

The function of the unsaponifiables, as described here, is minor in soybean oil reversion. Since autoxidation increases unsaponifiable content, the adverse effect noted by previous workers could well have been an effect of materials that had collected in the unsaponifiables resulting from oxidation, not representative of the naturally occurring materials.

Acknowledgment

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The Effect of Germination Upon the Fat of the Soybean[1]

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Abstract

Soybeans of the Chippewa variety of two crops, 1956 and 1957, were germinated in the dark at 25C and the levels of total dry matter and crude fat of both the seedling axis and cotyledons were determined at various periods up to 12 days. The fatty acid content, neutral fat content of the crude fats of the cotyledons, and the fatty acid composition of neutral fat were determined. The fatty acid composition was measured by the ultraviolet spectrophotometric method.

There was a continuous decrease in the total dry matter and crude fat of the cotyledons and whole seedlings of soybeans during 12 days of germination, contrary to observations of some of the earlier workers. Although there was a preferential utilization of the non-fat dry matter during the first two days of germination, there was a slight but significant loss of fat, which gradually increased with the germination time.

Surprisingly little change in the fatty acid composition of the reserve triglycerides occurred even during their most rapid loss from the cotyledons. However, observed changes were statistically significant. No loss of oleic acid occurred until after the second day of germination and its more rapid loss, compared to the other fatty acids, occurred during the period of most rapid fat loss.

The significance of this observation and its relationship to oleic acid as the key intermediary in fat metabolism in plants is discussed.

Introduction

SINCE HELLRIEGEL'S (6) classic work with sunflower seed, the progress of both the fat and the dry matter of oleaginous seeds during germination in the dark has remained uncertain. Increases (2,3,7,11,14, 18) and decreases (16,17,19,20,23) in both these values have been reported with almost equal frequency. Variations in the fat composition as revealed by the iodine value have also been in dispute. Improved techniques enabled Heumann (7) with pumpkin seeds and Combie and Comber (2) with watermelon seeds to show that only minor changes in the fatty acid composition occurred during germination. The most notable change was an increase in the oleic acid content during the early stages.

The object of the present work was to follow the dry matter and fat level of soybeans during their germination in the dark. The fatty acid composition was examined by a combination of the I.V. and spectrophotometric determinations on the refined (chromatographic) fat.

Materials and Methods

A. *Materials.* Representative samples of the Chippewa beans were manually harvested, at maturity, from experimental plots in 1956 and 1957, at Guelph, Ontario. All beans having wrinkled, discolored, or damaged coats were rejected. The average bean weight for both crop years was 140 mg. To minimize and standardize variations in bean weights, the beans selected had individual weight within the range of twice the standard deviation, i.e., 120 mg to 160 mg. Thus 68% of the original material was selected as suitable for experimentation. These beans were placed in sealed bottles and refrigerated until required.

B. *Sterilization.* Prior to germination, the beans were surface sterilized by a detergent wash (1% solution of Tide) followed by successive treatment with aqueous sodium hypochlorite (3.3%) and sorbic acid (0.2%). There was no change in either the viability or the growth rate of treated beans as compared to untreated controls.

C. *Germination.* Weighed lots of beans were germinated upon wet facial tissue in the dark in water saturated atmosphere at 25C for varying periods of time up to 12 days. At least 6 separate determinations were made for each period.

D. *Analytical Methods.* The seedlings were separated into the axis, cotyledons, and seed coats, the latter being discarded.

Dry weights of the plant parts were obtained by the vacuum oven method, A.O.A.C. Official Methods 13.3. Total crude fat was determined by exhaustive extraction of the finely ground, dried material with n-hexane as described in A.O.C.S. Official Method Ba 3.38. During the evaporation of the solvent, the crude

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