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LIPOXIDASE AND FLAVOR FORMATION

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During the evolution of biological life forms, the lipoxidase enzymes were incorporated into the matrix of ordered complex biochemical pathways for the express purpose of creating flavor problems for food processors. Fortunately, this enzyme occurs predominantly in the plant kingdom and within this realm its importance to food processors in a practical sense is relegated to only a few species.

What is most unfortunate is that it occurs in highest concentration with appropriate substrates in the legumes and notably *Glycine max*, or the soybean. This oilseed can contribute significantly to alleviating the present worldwide protein malnutrition problem, since it is a significant source of plant protein of high biological value in comparison to other plant species and offers a much more economical protein source than that derived from most animal sources.

While protein malnutrition on a worldwide basis is a popular topic, caloric intake does not take a back seat in the evaluation of the over-all world feeding problem. The soybean with its high oil content serves a dual function of providing both protein and calories.

One of the simplest processing methods for converting soybeans to an edible food is to extract the soybean with water via a grinding operation to produce a beverage known as soy milk. This product is not a revolutionary discovery as the Chinese have been producing soy milk for centuries. However, the typical soy milk as produced in the traditional Chinese method leaves much to be desired for the western taste or as a new product to be introduced to areas suffering from malnutrition, since the typical rancidity of this product is beyond the tolerance point of most tastes. Our research on lipoxidase and its relation to rancidity in the production of soy milk encompasses what some might consider strictly academic interests as well as practical approaches.

Model system studies of the lipoxidase catalyzed oxidation of cis,cis-1,4-pentadiene moieties as typified by linoleic and linolenic acids have provided us with some basic information on the parameters influencing this reaction. From a flavor standpoint, the oxidation of polyunsaturated acids by lipoxidase catalysis results in a complex mixture, predominantly aldehydes of various degrees of unsaturation and chain length, some of which are agreeable and others very disagreeable in odor, which in total may be described as oxidative rancidity.

For the enzyme to function, oxygen and water must be present in the system as well as substrate. In addition, there are certain limiting parameters of pH, temperature, and ionic strength of various inorganic and organic mole-

cules. Alteration of any of the above factors will usually change the rate of enzyme catalyzed hydroperoxidation of the substrate and its subsequent decomposition, but it has been assumed that the course of the reaction remains the same. Stated another way, the enzyme controlled hydroperoxidation proceeds by the same energy transformations, the same conformational and configurational intermediates, and produces the same end products regardless of variation in the environmental parameters. Most evidence supports this assumption.

Evidence has been presented by Dolev *et al* (1), using a lipoxidase isolate of soybean, that in the hydroperoxidation of linoleic acid the optically active 13-hydroperoxyoctadeca-9,11-dienoic acid (cis-trans) is produced quantitatively to the exclusion of other products. A study *in vivo* on a natural product such as the soybean introduces complicating factors in the interpretation of data from lipoxidase catalyzed autoxidation of the polyunsaturated fats that are native to the soybean. One cannot be certain that only a single lipoxidase is operative in soybean. Several different lipoxidases have been partially characterized to exist in various plant species. The procedure for isolation of the commercially available soybean lipoxidase may indeed result in a single lipoxidase, but there is no proof of the homogeneity of soybean lipoxidase in structure and function within the intact soybean.

Indirect evidence has been presented for a soybean lipodehydroperoxidase by Gini and Koch (2) which purportedly catalyzes the decomposition of the hydroperoxides formed by lipoxidase catalyzed oxidation of linoleic and linolenic acid equivalents. Such a system would lead to a rapid formation of primary decomposition products of the hydroperoxides to form volatile off-flavor components.

Ames and King (3) have shown that the variation in lipoxidase activity with the ionic strength of the reaction can result in an alteration of the pH profile given by lipoxidase with linoleic acid. Koch *et al* (4) have shown that with increasing ionic strength at a constant pH of 7.4 there is a steady increase in lipoxidase activity. In one of our model system studies with increasing NaCl concentration the effect is of the opposite nature as shown in Figure 1. The pH is nearly constant for the system over a range of concentrations and is near the isoelectric pH reported for soybean lipoxidase. The components of this model system were commercial soybean lipoxidase, linoleic acid, salt, and water at 28°C.

We have attempted to study the lipoxidase activity-ionic strength relationship as it may pertain to flavor development. Initially, model systems were used to follow conjugated diene development at 234 mμ. The ionic strength of the reaction medium was altered with NaCl (Fig. 1), $NaHCO_3$, and $KHCO_3$ with concentrations between .005 to .5 molar. Commercial preparations of lipoxidase were reacted with linoleic and linolenic acid and methyl linoleate. With $KHCO_3$ and linoleic acid the reaction rate increased steadily with increasing $KHCO_3$ concentration with a few minor inflections in the curve. With $KHCO_3$ and methyl linoleate very low activity was obtained over the entire range of concentrations; substrate availability may have been limiting. The pH of the reaction mixture varied between 8.6 to 8.8 over the range of concentrations since it was an unbuffered system. The reaction rates shown in Figure 2 are for $KHCO_3$ and linolenic acid with the pH range between 8.6 and 8.9. The data points are connected in this graph and a dotted line shown for the average reaction rate.

The rapid increase in reaction rate between 0.000 and 0.005 molar $KHCO_3$

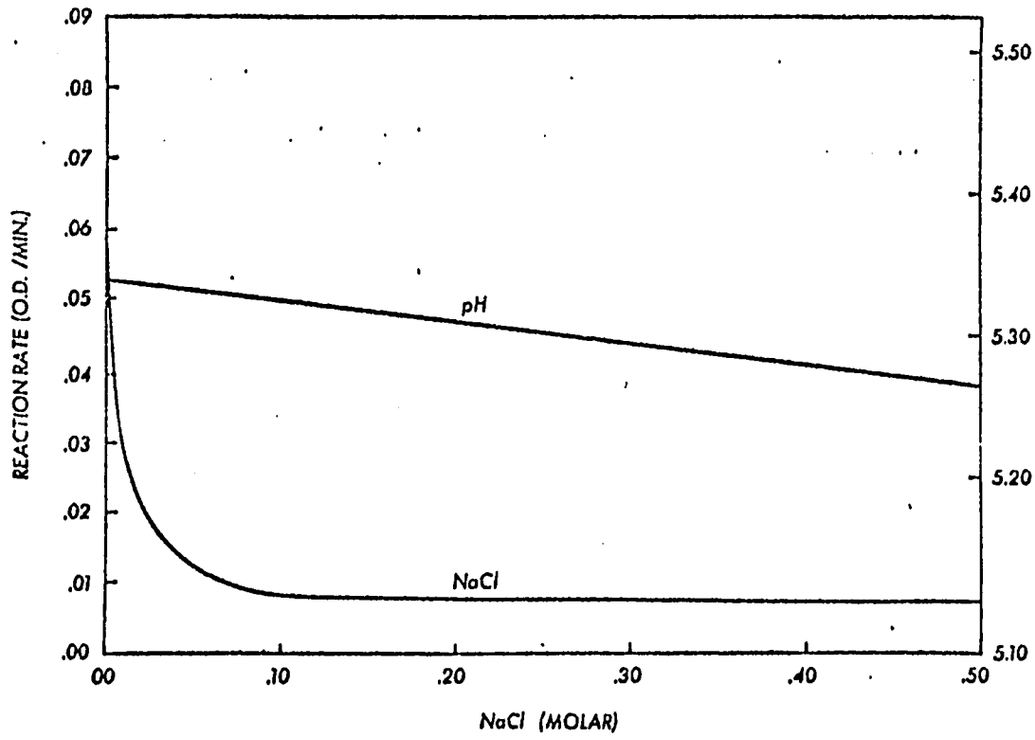


Fig. 1.--Effect of NaCl concentration on the reaction rate of lipoxidase on linoleic acid as measured by conjugated diene formation (234 mu).

is probably due to increased substrate availability through the potassium linolenate salt formation. At .005 m $KHCO_3$ all of the linolenic acid present can theoretically be converted to the potassium salt. Little importance is attributed to the minor variations in reaction rate since this is not a replicated experiment.

Two replicates were run on the reaction rate for $NaHCO_3$ and linolenic acid. Figure 3 shows the average point data as well as the dotted line depicting the reaction rate average. Despite insufficient statistical proof, the possibility that the minor variations in reaction rate over the range of ionic strengths are of real consequence can not be dispelled.

Experiments were conducted to relate ionic strength of the reaction medium of a food system with flavor development as measured by gas chromatography. Soy milk lends itself readily to this type of study with high concentrations of native lipoxidase and what might be considered as an unlimited supply of substrate in the form of linoleic and linolenic acid equivalents.

Concentrations of $NaHCO_3$ of 0.0, 0.005, 0.025, 0.040, 0.070, 0.100, 0.250, and 0.500 molar were chosen for the grinding medium in the preparation of a soy milk extracted at low temperatures. All conditions of the soy milk extraction were identically maintained with $NaHCO_3$ concentration as the only variable. The volatiles were distilled under vacuum, condensed, and extracted with CS_2. The CS_2 extract was concentrated to a standard volume for gas chromatographic analysis. Individual components of the gas chromatographic pro-

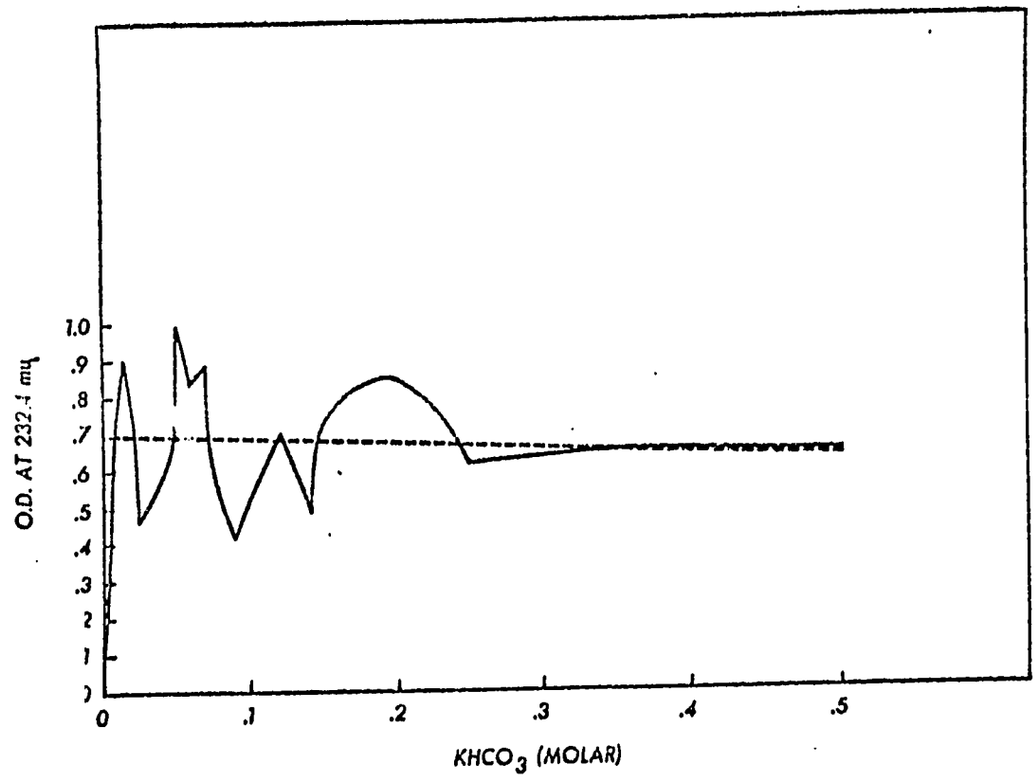


Fig. 2.--Effect of $KHCO_3$ concentration on the reaction rate of lipoxidase on linolenic acid as measured by conjugated diene formation (234 mu). Solid line represents data points. Dotted line is averaged reaction rate.

file showed variation by simple inspection over the range of concentrations of $NaHCO_3$.

Approximately 20 of these peaks were subjected to quantitation. A plot of the relative quantitative variation of each peak with ionic strength provided us with several groups of curves as shown in Figures 4 and 5. Some of these curves represent only one or two component peaks while others represent up to five peaks.

With slight reservation the majority of these peaks arise as a result of lipoxidase catalyzed oxidation of linoleic and linolenic acid equivalents. There is no doubt that the ionic strength of the solution bears a relation to the formation of the off-flavor volatiles. The exact cause and effect relationship is open to further research. We cannot say that any particular component of the volatile mixture is a major contributer to off-flavor. Many of the components in themselves possess pleasant fruity-type aromas. Others are rather objectionable. It is the blend of these volatile components and their relative and absolute concentration that affects the particular flavor or off-flavor sensation that is perceived.

If we assume that a component or components that follow a pattern such as evidenced by Type 6 in Figure 5 were of a very undesirable nature, then we can envision a processing condition for the extraction of soy milk that permits a degree of flavor control through modification of the $NaHCO_3$ concentration of the grinding medium.

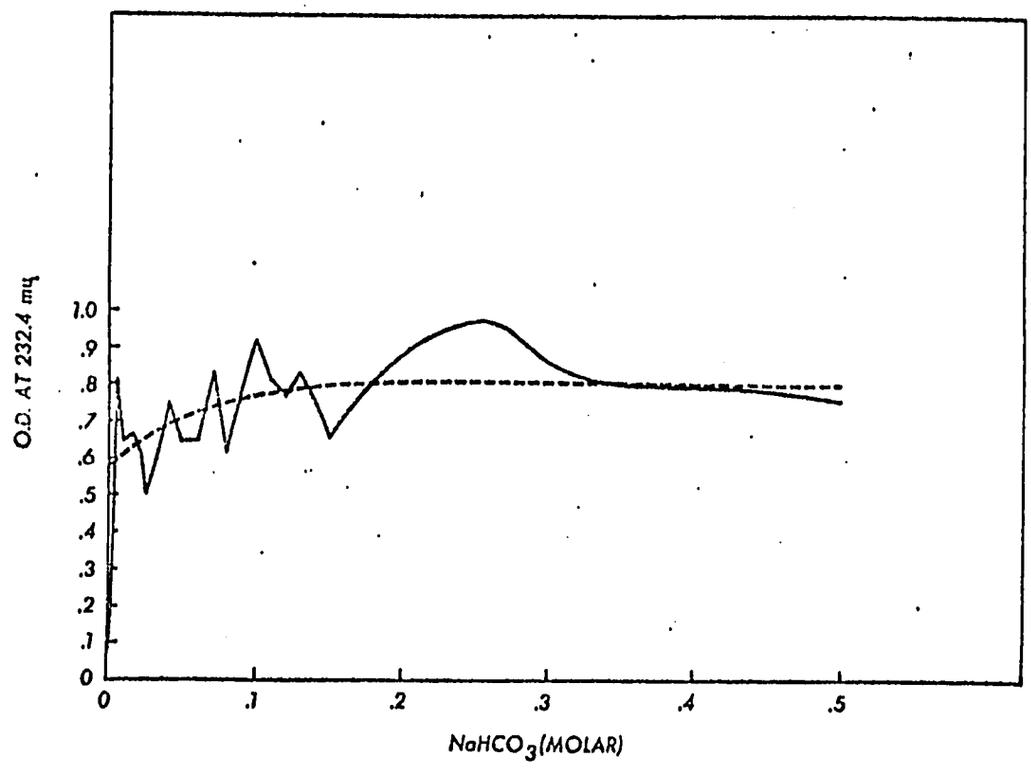
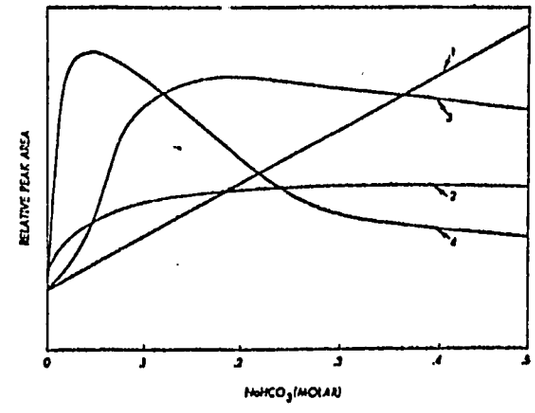


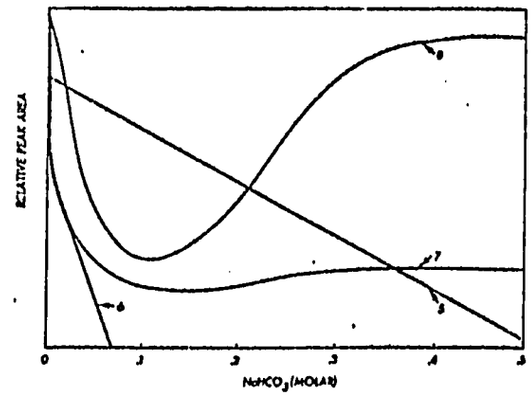
Fig. 3.--Effect of $NaHCO_3$ concentration on the reaction rate of lipoxidase on linolenic acid as measured by conjugated diene formation (234 mu). Solid line represents data points. Dotted line is averaged reaction rate.

Fig. 4.--Effect of $NaHCO_3$ concentration on the formation of volatiles in soy milk. Initial ascending relationships. The curves represent relative peak area over the concentration range and are not intended to imply quantitative relationships between types of curves.



The relation between lipoxidase activity and off-flavor formation can be further evidenced by analysis of the lipid fraction of soy milk through gas chromatography of the methyl ester derivatives, and this can, in turn, be related to conjugated diene formation in soy milk. Under conditions of a cold water extraction of the soybeans in a Waring blendor with 100 grams of soybeans and 1,000 ml water, all of the linoleic and linolenic acid equivalent is completely oxidized within 40 minutes after initiation of the grinding pro-

Fig. 5.--Effect of $NaHCO_3$ concentration on the formation of volatiles in soy milk. Initial descending relationships. The curves represent relative peak area over the concentration range and are not intended to imply quantitative relationships between types of curves.



vided air is continuously pumped into the system. This amounts to 10 grams of polyunsaturated fat oxidized per liter of soy milk in 20 minutes.

This relation of lipoxidase activity can be extended to other legume seeds as shown by the volatile profiles (Fig. 6) of extracts prepared in a similar manner to soy milk. The largest component in the case of all these preparations is hexanal which elutes early (5 minutes) in the chromatogram. This would support the evidence of Dolev *et al* (1) that the reaction is essentially stereospecific for the formation of the 13- hydroperoxy form of linoleic acid, although other lipoxidases of different stereospecificity may be present in one or all of legume seeds investigated.

If a soy milk is prepared under conditions of extreme oxygen deficiency, the gas chromatographic profile is practically devoid of volatile components with the exception of one component which arises only if soaking of the soybeans is used as a pre-processing step. This component optically active is 1-octen-3-ol which possesses a mushroom odor. The optical activity indicates an enzyme directed mechanism with linoleic acid as a probable precursor. It might be suggested that lipoxidase is involved in this reaction, but such proof of a mechanism involving lipoxidase remains for future investigation. Formation of this component appears to be pH dependent with maximal production near neutrality. At soak temperatures above 50°C the amount of 1-octen-3-ol formed is considerably reduced which supports the contention for a biologically directed mechanism. This compound has a threshold near 1 ppm in soy milk which can be obtained by soaking the beans for 2 to 3 hours in 50°C water prior to a hot water extraction.

The rancid flavor of soy milk resides in the volatile fraction. This defect can be corrected either by removing volatiles as a final step in processing or by preventing their formation. The latter is a more economically feasible solution. As shown by Figure 7 a decrease in formation of volatiles with higher water temperatures at grinding indicated thermal inhibition of the oxidative rancidity flavor mechanism attributable to lipoxidase enzyme activity. With consideration of the extremely high rate of enzyme catalyzed oxidation of the soy milk lipids at low temperatures, Wilkens *et al* (5) have shown that heat inactivation of lipoxidase must be accomplished concomitant with the grinding operation with water at a minimum temperature of 80°C. Heating at a minute or two after a cold water grinding cannot overcome the flavor damage that occurs during and immediately following a cold water grinding operation. Even though the enzyme may then be inactivated with only a portion of the lip-

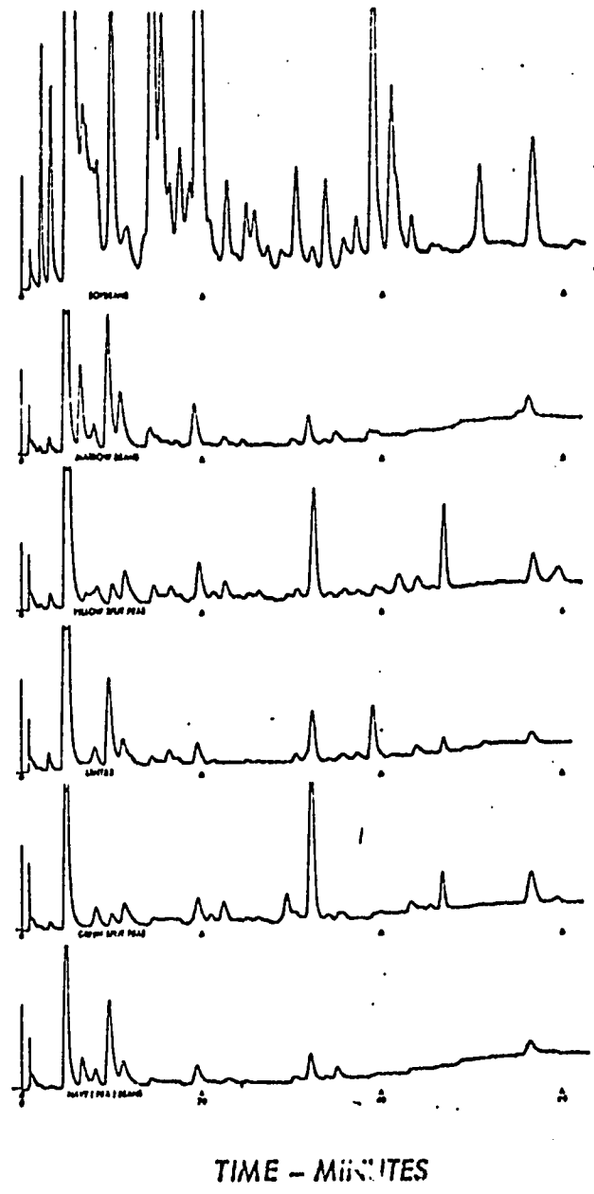


Fig: 6.--Gas chromatographic volatile profiles of legume seeds extracted with cold water.

id oxidized and a small amount of volatiles formed, the free radical mechanism for autoxidation has been triggered for the formation of a more complex array of volatile compounds than would be possible by lipoxidase activity alone.

The stability of soy milk to autoxidation after a hot water extraction process is rather remarkable. What is more impressive is that the addition of up to 50 mg of purified lipoxidase per liter of properly processed soy milk resulted in no off-flavor development. Only upon addition of 100 ppm ferrous sulfate did rancidity commence in a typical autoxidative mechanism. It can be assumed that the natural content of tocopherols in soy milk provides a

powerful natural antioxidant effect, provided proper processing conditions have been employed to prevent the initiation of oxidative rancidity by lipoxidase or the autoxidative mechanism.

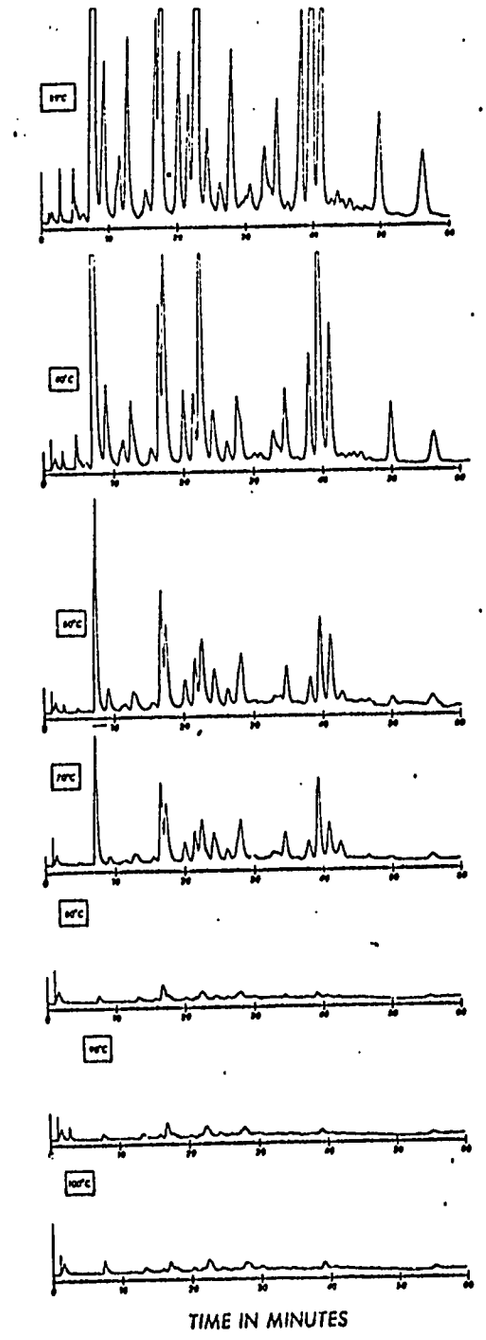


Fig. 7.--Volatile composition of soymilks, produced with a rapid-hydration grinding process from dry, dehulled soybeans, as influenced by temperature of water during grinding.

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