having a standard deviation of 0.37. Therefore that estimate of the mean would have a standard error of $0.37/\sqrt{25}=0.074$, and from this we would predict with 95% probability that the true titer of the carload of tallow would be $42.30\pm2(0.074)$ or between 42.15 and 42.45. These limits then are often referred to as the 95% confidence limits, and they provide an estimate of the precision of the observed mean for a sample or group of samples.

Extending the application of these fundamentals, it can be concluded that the analysis of a single sample from the carload of tallow would provide a value having a precision of ±2 (0.37) or ±0.74. If it were decided to analyze two samples, the mean of the two would have a precision of $\pm0.74/\sqrt{2}$ or ±0.52. And using this relationship of precision to sample size, it would be possible to arrive at the number of samples which would be needed to give a precision of any desired magnitude.

The uncomplicated sampling illustration selected for this discussion appears to reflect errors of sampling, or of analysis, or both. The sampling plan was not designed to isolate the variation associated with these two sources of variation. However, with an appreciation for the application of probability statistics and an understanding of the few fundamental relationships detailed herein, it is a relatively simple matter to design investigations in such a manner that the known sources of variation may be isolated from any system of variables.

Development of new analytical methods frequently necessitates quantitative estimation of the errors associated with the various manipulations involved (*i.e.*, weighing, pipetting, titration, instrument reading, etc.). In collaborative studies of analytical methods it may be desired to obtain estimates of the magnitude of the variations between duplicate analyses on a single day, the day-to-day variation, the variation among analysts within a laboratory, and the variation among laboratories. From such comprehensive studies it is possible to make critical and objective evaluations of methods for use in research or control work.

In studies of manufacturing processes related statistical designs may often be useful. Isolating the sources of variation in an operation and estimating their magnitude is an essential step in the inauguration of a control program; and the formulation of sampling, analysis, and acceptance plans requires a characterization of the variables which cannot be obtained effectively without the aid of the statistical approach.

There are in rather widespread practice more or less classical designs for such investigations, many of which have been described in the technical journals in a variety of fields. The computational techniques are somewhat more extensive than the ones described here but are within easy mastery by nonmathematicians and may be found clearly described in many introductory statistical tests.

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Measurement of Chain Length

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PLASTIC FATS AND edible oils are made up almost exclusively of three units of fatty acid and one unit of glycerol; the combination is called a triglyceride. Whether the combination is a fat or an oil at room temperature depends on the chain length and the degree of unsaturation of the fatty acids which are present in the triglyceride. If the fatty acid chain contains only four carbon atoms, as in butyric acid, the melting point of the fatty acid is much lower than one which contains eighteen carbon atoms as in stearic acid. A change in the degree of unsaturation seems to influence the melting point of a fatty acid even more than a change in the length of the carbon chain. For example, an increase in one double bond decreases the melting point 18°C. as compared with a decrease of only 9°C. between the C_{14} myristic and the C_{16} palmitic acid. Therefore if glycerol is esterified with more than two unsaturated fatty acids, the resulting triglyceride is a liquid or an "oil" at room temperature. If, on the other hand, glycerol is esterified with only long chain saturated fatty acids or only one mole of oleic and two moles of palmitic or stearic acid, the resulting triglyceride is a solid or "fat" at room temperature.

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Natural fats and oils have been found to contain mixtures of triglycerides which are uniquely characteristic of a specific fat. Lard and beef fats contain from 2 to 15% trisaturated glycerides (GS_3) while cottonseed or soybean oil do not contain any trisaturated glycerides (1). Butterfat, which contains a larger proportion of lower saturated fatty acid than any other fat, usually contains less than 1% triunsaturated glycerides although variations up to 7% GU_3 have been reported.

The plastic fats or shortenings actually contain from 15 to 35% of the solid phase; the remainder is in a liquid phase incorporated into the solid phase to improve shortening performance and to keep the melting point of the mixture of triglycerides below body temperature. A shortening can be formulated from vegetable oils by two different methods—one, which

is no longer used, has involved a simple blending of a hard fat, such as beef tallow, with cottonseed oil; the other has involved hydrogenation of vegetable oils in order to convert linolenic and linoleic to oleic and stearic acids. The hydrogenated fats are then blended in order to obtain the proper consistency and degree of unsaturation.

That the conversion of oleic to stearic acid increases the melting point of a triglyceride is shown in Table 1.

TABLE I

Effect of Unsaturated Fatty Acids on the Melting Point of Triglycerides

Glyceride	M.P.° C.
S-O-S	41
S-O-O	23
S-S-O	38
P-O-P	35
M-O-M	26

S—Stearic, P—Palmitic, M—Myristic, O—Oleic.

Distearin monolein has a melting point 18°C. higher than monostearin diolein, or 41 and 23°C. respectively. The oil is therefore hydrogenated sufficiently to produce enough of the solid phase to convert the oil into a plastic fat at room temperature. The triglycerides in this solid phase have melting points well above body temperature, but the presence of the liquid phase acts as a solvent so that the plastic fats are liquids at 37°C. or 98.6°F.

The nutritional value of a plastic shortening or an edible oil has usually been determined by the rate of growth which can be maintained in white rats fed the fresh fats for varying lengths of time. It has been shown that essential fatty acids are necessary for reproduction and possibly other vital functions such as sterol metabolism (2). However in fats which are subjected to excessive heating before they are consumed, the presence of a large amount of essential fatty acids is undesirable.

IN THE HUMAN DIET only one-third of the edible fats are consumed in the fresh untreated form. The remainder are subjected to various treatments which can promote oxidatively or thermally induced changes in the unsaturated fatty acids before they are consumed. For example, in 1953 over 172 million lbs. of corn and cottonseed oil were heated to 340-400°F. for long periods in the production of potato chips (3). Fats used in doughnut fryers and in deep fat fryers in restaurants are also exposed to similar treatment. The temperatures cited are those at which the processes are supposedly conducted. However, because of poor heat transfer and lack of proper agitation (4), certain zones within a commercial frying unit reach considerably higher temperatures.

As saturated fatty acids do not polymerize easily, it would seem that fats which contain a maximum amount of saturated fatty acids would be more desirable for frying purposes than corn or cottonseed oil. The hardening of vegetable oils through hydrogenation has thus served as an important way to preserve the nutritional value and increase the stability of vegetable oils to heat treatment and possible polymerization. However the complete saturation of trilinolein to tristearin would increase the melting point from −13°C. to 73°C., which is well beyond the body temperature of 37°C., and therefore also undesirable. Furthermore hydrogenated fats do not drain as readily or give as well browned a product as corn or cottonseed oil.

Another way to produce frying fats of minimum unsaturation and proper melting point is through a decrease in chain length of the fatty acid. A decrease of two carbon atoms in a fatty acid chain lowers the melting point of a triglyceride approximately 4°C. (Table 2). The relative positions of the fatty acids

TABLE II

Effect of the Length of the Chain on the Melting Point of a Triglyceride

Glyceride	M.P.° C.	Glyceride	M.P.° C.
16-18-16	68	12-18-18	54
18-16-16	62	10-18-18	49
18-16-14	58	8-18-18	47
16-18-18	65	6-18-18	44

are also important. If stearic acid is in a beta position, as in monostearin dipalmitin, the melting point is higher than if it is in an *alpha* position, or 68 and 62°C., respectively. The presence of even one short chain fatty acid lowers the melting point of the triglyceride significantly (5). For example, the presence of caproic acid, as in caproin distearin, lowers the melting point of tristearin 29°C. or from 73 to 44°C., respectively. Butterfat is a good example of how the presence of short chain fatty acids affects the triglyceride composition of an edible fat.

Accurate methods for determining the proportion of the various glycerides present and the chain lengths of the fatty acids in these glycerides must be available in order to produce better frying fats. Hilditch (6) has developed a method, which has recently been modified by Kartha (7), for determining the proportion of various triglycerides in a fat or an oil. This method is based on the oxidation of the unsaturated fatty acids with potassium permanganate in acetone and removal of the mono- and di-carboxyl acid fragments by washing them out of the azelaoglycerides. The sodium salts of the latter are then separated from the unoxidized trisaturated glycerides in ether solution. Another method based on the partial separation of the triglycerides from solvents at room temperature has recently been developed by Bhalerao (8). In this method the triglycerides are dissolved in absolute ethanol, and the alcohol insoluble fraction is further fractionated from acetone.

The approximate chain length of the fatty acids in a triglyceride or mixture of triglycerides can most easily be determined by the saponification or "sap" number (Table 3). As the length of the fatty acid

TABLE III

The Saponification Values of the Methyl, Ethyl, and Glyceryl Esters of the Saturated Fatty Acids

Carbon Atoms	Acid	Methyl Ester	Ethyl Ester	Glyceryl Ester
C_4	Butyric	549.3	483.0	556.6
C_6	Caproic	431.0	389.0	435.5
C_8	Caprylic	354.6	325.7	357.6
C_{10}	Capric	301.2	280.1	303.4
C_{12}	Lauric	261.8	245.7	263.4
C_{14}	Myristic	231.5	218.8	232.8
C_{16}	Palmitic	207.5	197.2	208.5
C_{18}	Stearic	188.0	179.5	188.8

chain increases, the number of ester groups per gram of fat decreases and fewer carboxyl groups are available for saponification. This is best shown by the decrease in the saponification numbers of the methyl, ethyl, and glyceryl esters of the saturated fatty acids as the chain length increases from C_2 to C_{18} carbon atoms.

It is obvious that the saponification number of a mixture of esters would give only an approximate

TABLE IV

Changes in Reichert Meissl Number of Butter Fat Due to Addition of Vegetable Fat

Fat	Reichert Meissl No.
Butter fat	28.0
Butter fat + 10% veg. fat	25.6
Butter fat + 30% veg. fat	19.8
Variation from Hunziker	20-34

value for the length of the carbon chain of the fatty acids in the mixture. In order to determine the chain length of the individual acids Hilditch fractionated mixtures of methyl esters by high vacuum distillation. The saponification value of each fraction was then determined, and on the assumption that no more than three fatty acids were present in each fraction, the approximate percentage composition of each fraction was calculated. However when long chain saturated and unsaturated fatty acids were present, it was difficult to separate the fatty acids quantitatively. Hilditch therefore separated the saturated from the unsaturated fatty acids by forming the lead or lithium salts of the acids and by taking advantage of their difference in solubility in ethanol. Barium, magnesium, thalium, and other metallic soaps have been prepared and fractionated in a similar manner. However none of these methods gave an absolutely clear-cut separation of the unsaturated and saturated fatty acids. In fact, it is possible to separate the free saturated and unsaturated fatty acids more easily by low temperature fractional crystallization from acetone than by the lead salt procedure (9) .

The unsaturated have also been removed from the saturated fatty acids through the formation of urea complexes and by countercurrent distribution. With urea complexes the best separations occurred between stearic and linoleic acids (10). It became more difficult to separate the saturated fatty acids from each other. The countercurrent distribution method (11) developed by Craig has been used to separate the lower saturated fatty acids as well as the saturated and unsaturated fatty acids.

Oxidation with performic acid as a means of forming water-soluble hydroxy acid derivatives of the unsaturated fatty acids (12) and oxidation with potassium permanganate as a means of their complete removal from the saturated fatty acids (13) has not proven satisfactory. In both cases the presence of saturated fatty acids of short chain length would produce errors in the determinations. Furthermore oxidation with permanganate produces water-soluble breakdown products which would increase the difficulty in determining the percentage composition of the short chain fatty acids which may have been present.

The latter may be determined most easily by their removal from a fatty acid mixture through steam distillation. This method has been used successfully for many years by dairy chemists as butterfat contains a higher percentage of short chain saturated fatty acids than any other edible fat. In fact, the presence of butyric and caproic acid as determined by the Reichert Meissl method has served as an index of purity for butterfat. This value is defined as the ml. of 0.1 NaOH required to neutralize the fatty acids obtained from a 5-g. sample under the specific conditions of the method (14). Hunziker (15) has stated that the Reichert Meissl value of genuine butter has been reported to vary from 20-34. Actual adulturation of butterfat with 10% vegetable fat (Table 4) decreased the Reichert Meissl value from 28.0 to 25.6. The Reichert Meissl value did not drop to below 20 until 30% of vegetable fat was present in the adulterated sample. On the other hand, a recent survey (16) has indicated that normally the Reichert Meissl value varies only 2.5 units or from 26 to 28.5. The Kirschner or Jensen-Kirschner value has also been used as a means of identifying acids of short chain length. This value is indicative of the amount of butyric acid in a fat. The steam-volatile, water-soluble acids other than butyric acid are removed as silver salts, and the amount of butyric acid present is determined by titration with N/10 alkali.

Butyric acid has also been removed from fatty acids of longer chain length by partition chromatography between ethylene glycol and hexane on a silicic acid column (17). The butyric acid was washed through the column and titrated against alcoholic potassium hydroxide. When this method was applied to adulterated samples of butter fat in our laboratory, no improvement in accuracy over the Reichert Meissl value was noted (Table 5). A sample which contained 10% coconut oil seemed to contain as much butyric acid as the unadulterated butter fat.

TABLE V

The Butyric Acid Content of Butterfat Deliberately Adulterated with 10% of Various Fats

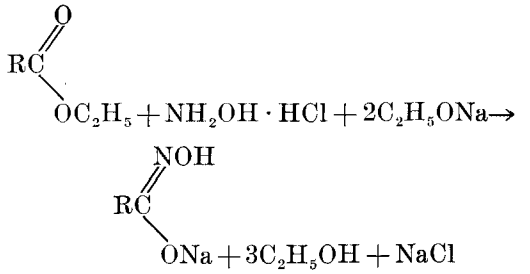
Butter Fat	Butyric Acid (% molar)
None	9.6-11.3
Coconut Oil	9.3
Hydrogenated Fat	9.2
Hydrogenated Fat	9.0
Oleo Oil	9.0
Mixture (75% coconut oil)	9.3
Mixture (25% coconut oil)	8.9
Rearranged Lard	10.1
Synthetic Fat Mixture	10.0

Coconut oil has served as an adulterant of butter fat because of its small effect on the Reichert Meissl number. However coconut oil can be easily identified by means of the Polenske number. This value is an index of the content of caprylic, capric, and lauric acids which are steam-volatile but water-insoluble fatty acids. The Hehner number or non-volatile water-insoluble acids is due to the presence of myristic and fatty acids of longer chain lengths. Taufel (18) has recently combined these values with the saponification value. In this method a 5-g. sample was saponified and titrated to determine the saponification value. The alcohol was removed on a boiling water bath, water added, and the soaps neutralized with dilute H_2SO_4. The fatty acids were then subjected to steam distillation, and the Reichert Meissl and Polenske values of the distillate were determined.

PAPER CHROMATOGRAPHY and silicic acid columns have recently been used as methods for the measurement of chain length. In paper chromatography, filter paper coated with paraffin oil or latex served as a supporting medium in reversed-phase chromatography (19). The fatty acids were recognized as brown spots of lead sulfide or purple spots of lead rhodizonate at the fatty acid sites. Fatty acids from C_2 to C_{16} in chain length have been separated and identified with the aid of a water-jacketed, silicic acid column and methyl cellosolve and Skellysolve B as solvents (20).

The hydroxamic acid derivatives of fatty acids have also been separated and identified by paper chromatography (21). The hydroxamic acid derivatives can

be prepared from the ethyl esters of aliphatic acids by treating them with hydroxylamine hydrochloride and sodium ethylate:



The descending method has been used with either n-butanol or ethyl acetate as a developer and ferric chloride as an indicator (Table 6).

TABLE VI

The Rf Values of the Hydroxamic Acid Derivatives of the Saturated Fatty Acids

Hydroxamic acid	n-butanol		Ethyl acetate	
	Rf	Color	Rf	Color
Butyric	0.84	Purple	0.58	Purple
Caproic	0.93	Purple	0.88	Purple
Caprylic	0.94	Reddish purple	0.91	Reddish purple
Capric	0.95	Reddish purple	0.93	Reddish purple
Lauric	0.95	Reddish brown	0.93	Reddish brown
Myristic	0.96	Reddish brown	0.93	Reddish brown
Palmitic	0.96	Brown	0.93	Brown
Stearic	0.97	Brown	0.94	Brown

The physical methods which have been used to determine chain length have involved x-ray spectroscopy, ultraviolet, and infrared spectrophotometry. The application of x-ray spectroscopy to fatty acids and related compounds has helped considerably in elucidating their structures and has been used extensively by Piper *et al.* (22, 23, 24) in the identification of fatty acids derived from natural sources. In the case of long chain aliphatic compounds of an odd or even homologous series, the addition of each pair of CH_2 groups increases the cell length or C-axis by 4.6 Å. It is therefore possible to identify any member of the series by a single crystal measurement. The long spacings measured by x-ray diffraction are due to repetition of like planes occupied by chain ends (25).

In interpreting the composition of the long chain fatty acids in natural waxes, Piper, Chibnall, and associates have made use of a series of curves of mixed melting points, and their corresponding spacing data. The standard curves for the acids show a large depression, and those for the alcohols show a small depression. It is evident that the melting point data alone are insufficient to fix the composition of a binary mixture. It is therefore necessary to seek further confirmation from the spacing data. Methods have been devised by these workers (26, 27) for extending the observations made on a limited number of synthetic mixtures to cover a much larger number which have not been specifically prepared. The same process has been applied to a limited number of mixtures containing three acids, but here interpretations are more difficult. If the values of the x-ray spacings for the same polymorphic modifications of the various homologous series of long chain aliphatic compounds are plotted against the number of carbon atoms in the chain, a series of straight lines is obtained.

The ultraviolet absorption spectrum is associated with specific chromophores in a molecule rather than with the molecule as a whole. The saturated hydrocarbons, alcohols, and ethers are extremely transparent in the region above 200 mμ, and hence they are normally used as solvents. The introduction of an ethylenic linkage or a carboxyl group produces a specific absorption, but these absorptions do not seem to reflect greatly on differences in chain length of the molecule. There has been very little work reported in the far ultraviolet region. Russoff *et al.* (28) investigated the absorption spectra of various fatty acids in the Schumann region and have found that acetic, myristic, and caprylic exhibit a broad band at 205 mμ and a strong absorption beginning at 185 mμ. There has been no quantitative data presented however.

The type of absorption in the infrared is associated with simple atomic groupings, involving vibrational or rotational energies. The absorption bands at 3.3 microns and 6.9 microns are apparently the only ones that are generally related to the chain length of a fatty acid molecule (Table 7). However certain discrepancies encountered make it rather difficult to apply the absorption in these regions in a straightforward manner.

TABLE VII

Infrared Absorption Spectral Data for Chloroform Solutions of Specified Saturated Fatty Acids

Fatty Acid	Wavelength of Absorption Bands					
	3.3 μ			6.9 μ		
	λ	ε	ε per C-H	λ	ε	ε per C-H
Caproic	3.30	126.60	7.05	6.98	46.46	2.59
Caprylic	3.30	158.63	7.27	6.98	56.24	2.31
Capric	3.32	185.60	7.23	6.95	58.55	2.28
Lauric	3.30	214.33	7.22	6.90	64.10	2.16
Myristic	3.30	248.91	7.40	6.90	68.51	2.04
Palmitic	3.32	274.37	7.30	6.92	79.49	2.11
Stearic	3.32	304.38	7.32	5.80	79.65	1.92

The absorption at 3.3 microns arises from the C-H stretching vibration. The studies of O'Connor *et al.* (29) with fatty acids and their methyl and ethyl esters reveal that the partial extinction coefficient per C-H group is reasonably constant. However, because of the unresolved O—H . . . . . O bands, the absorption in this region fails to obey Beer's Law.

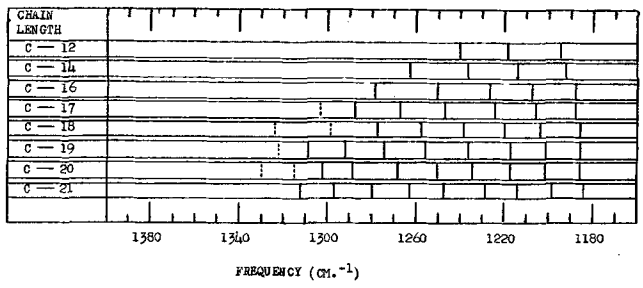
The band at 6.9 microns is normally attributable to the C-H bending vibration. As before, the partial extinction coefficient per C-H group is approximately constant for the three homologous series studied by these investigators though they are considerably weaker in intensity. The deviation of this band is slight compared to the band at 3.3 microns. From such studies as these it is definitely possible to establish chain length in single component systems and, in the case of binary or ternary mixtures, to arrive at a fairly accurate composition indicative of chain length.

More recently Sinclair *et al.* (30) have observed that the methylene rocking vibration at 13.8 microns is due to saturated fatty acids of varying chain length. These vibrations are qualitatively independent of the chain length, but the relative intensity increases progressively as the chain length increases. The solid phase spectra offers better prospects for the identification of individual acids and mixtures, provided the complications caused by polymorphism can be controlled. The apparent extinction coefficient increases by 3 units for each methylene group added, from 37 for myristic acid to 60 for heneicosanoic acid.

The infrared absorption spectra of crystalline films and Nujol mulls of fatty acids have also been studied by Sinclair *et al.*, who have shown a progression of absorption bands between 8.47 and 7.4 microns, which are apparently due to the rocking and/or twisting

vibrations of the methylene group. Furthermore these bands are not seen in the spectra of the fatty acids in CS_2 solution. As the carbon chain increases in length, the number of recognizable progression bands increases from 3 in the case of lauric acid to 9 in the case of heneicosanoic acid (Table 8). The increase in the number of bands with chain lengths, the regularity of the spacings, and the displacement to lower frequencies with increasing chain lengths are clearly depicted in Table 8. With more quantitative data along the same lines it should be possible to establish the chain length of binary, ternary, or polynary mixtures (31).

TABLE VIII



Both chemical and physical means are therefore available for the measurement of chain lengths. The accuracy of these measurements is however dependent on the purity of the fatty acids examined. As it is difficult to completely separate fatty acids of varying chain lengths from each other, it is apparent that accuracy is more dependent on a method for isolating them than on accurate methods of analysis. In spite of these difficulties it is apparent from this presentation that the measurement of chain length will become an important factor in the development of better shortenings and frying oils.

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Determination of Unsaturation

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ONE OF THE most important analytical determinations that an oil chemist must make is the measurement of unsaturation of an oil. Most manufacturing processes involve this determination to some extent as well as does the classification of fats and oils for trade and use. In spite of the importance of this determination there is as yet no one simple, rapid, and accurate method which will give consistent results on all types of oils. Much research time and effort has been expended to develop a good method, but the problem is yet unsolved. However, there are methods in use that do give a more or less quantitative measure of unsaturation; it is the purpose of this paper to discuss some of these methods.

The generally accepted method of expressing the degree of carbon to carbon unsaturation of a fat, oil, or derivative is by the term iodine value or iodine number. This value is defined as the grams of iodine which add to 100 g. of the sample, or in other words, the weight percentage of iodine based on the weight of the sample which adds to the sample. The results are expressed in terms of iodine whether iodine, or some other reagent, is actually used. Either of the terms, iodine value or iodine number, is correct; iodine value is preferred by the author and will be used in this discussion.

Since halogens will add to carbon-carbon double bonds, most of the methods make use of this property. In its simplest terms the determination consists of adding a halogen to a weighed quantity of sample and measuring in some way the amount of halogen which reacts with the unsaturation. The actual procedure for the determination is very simple. A small sample is weighed into a flask fitted with a ground glass stopper and is then dissolved in chloroform or carbon tetrachloride. A quantity of the halogen solution, usually iodine bromide or chloride in acetic acid, is pipetted into the flask containing the sample and also into a similar flask containing no sample (the blank). The flasks are stoppered and allowed to stand in the dark for at least 30 min. to allow the halogenation reaction to go to completion. An aqueous solution of potassium iodide is added and the halogen which failed to react with the sample reacts with the KI liberating iodine.

$$X_2 + 2KI = 2KX + I_2$$

The iodine is then titrated with standard sodium thiosulfate solution, using starch as an indicator.

$$I_2 + 2Na_2S_2O_3 = 2NaI + Na_2S_4O_6$$

$$I \rightleftharpoons Na_2S_2O_3$$