A NEW METHOD FOR THE DETERMINATION OF CHOLESTEROL
AND ITS APPLICATION TO THE ESTIMATION OF THE
EGG CONTENT OF ALIMENTARY PASTES

By

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This dissertation is presented with the permission of the Chief of the Food and Drug Administration.

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INTRODUCTION

In the United States, definitions and standards of identity for alimentary pastes to serve as a guide for officials charged with the enforcement of the Food and Drugs Act of 1906 first issued as Food Inspection Decisions No. 162¹ and No. 171² in 1916 and 1917 respectively. They were re-issued in 1919 in U. S. D. A. Circular No. 136³. These advisory standards established egg alimentary pastes as those pastes containing not less than 5 per cent of the solids of whole egg and plain alimentary pastes as those containing less than that amount. No moisture limit was set up. In 1927 Food Inspection Decision No. 206⁴ provided revised standards for alimentary pastes which were re-issued in U. S. D. A. Service and Regulatory Announcements, Food and Drug No. 2⁵, in 1927 and appeared in the same form in Revisions 1⁶, 2⁷ and 3⁸ of the latter published in 1928, 1931 and 1932 respectively. Revisions 4⁹ and 5¹⁰ in 1933 and 1936 respectively retained the same limits of composition as the immediately preceding revisions but changed the form of the standards. The general term "alimentary pastes" was dropped in favor of the classification "macaroni and noodles". These advisory standards are still guiding pending the promulgation of legal definitions and standards of identity for such products as authorized in the Federal Food, Drug, and Cosmetic Act of 1938. Such legal standards are in the process of formulation at the present time.

The standards¹⁰ now applicable are set forth as follows:

2. Noodles, Egg Noodles. The shaped and dried doughs prepared from wheat flour and eggs, with or without water and with or without salt. The egg ingredient may be whole egg and/or egg yolk. In the finished product the moisture content does not exceed 13 percent and the egg-solids content upon the moisture-free basis is not less than 8.5 percent. Noodles

are commonly ribbon-shaped.

3. Plain Noodles. The shaped and dried doughs prepared from wheat flour and water, with or without salt. In the finished product the moisture content does not exceed 13 percent. Plain noodles are commonly ribbon-shaped.

The problem of determining the egg content of alimentary pastes is one which has engaged the attention of food chemists for many years. European workers, especially in Germany and Italy, where these products constitute a more significant part of the diet than in the United States, have devoted particularly much time and effort to the problem. Strohecker and Vaubel¹¹ have published recently an excellent review of the problem as a whole and of the work of the European investigators. Tillmans, Riffart and Kuhn¹² also discuss the work. Buchanan¹³, in the only comprehensive report in English on the analysis of alimentary pastes of known composition, has also briefly reviewed the field, including work done in this country. It is therefore unnecessary at this point to do more than summarize briefly the deficiencies of the only method which gained any significant attention (prior to the advent of the cholesterol method), namely, that based on the determination of the lipoid (lipid) phosphorus. This is the term used to designate the organically bound phosphorus extracted by lipid solvents. In the first place, referring to the basic values for the lipoid phosphorus in flours and eggs reported by Hertwig¹⁴ even this average value for flour (0.055 per cent on the dry basis) constitutes a correction to be applied which approaches in magnitude the amount of lipoid phosphorus contributed by the egg component in a noodle containing 5.5 per cent of whole egg solids on the dry basis. In view of the fact that flours vary considerably in lipoid phosphorus content (0.041 per cent to 0.072 per cent according to unpublished work by V. E. Munsey, U. S. Food and Drug Administration) and the chief objective is to detect noodles containing less than the 5.5

per cent of egg solids specified in the standard, this is a serious disadvantage in the method. Even more important is the fact that the lipid phosphorus content of alimentary pastes frequently decreases markedly on storage as reported in the above reviews. Hertwig¹⁵ also observed a decrease in lipid phosphorus in the course of the manufacturing process and suggested that the empirical factor 1.1 be used to correct the determined lipid phosphorus value for such loss. Bornmann¹⁶ and Mitchell¹⁷ subsequently reported that the lipid phosphorus in eggs may decrease very rapidly under certain conditions due to an enzymatic hydrolysis. In view of the foregoing it is obvious that the lipid phosphorus method has serious forensic disabilities and the enforcement of standards for egg alimentary pastes has been greatly hampered as a consequence. It was the purpose of the work here reported to develop a method for the estimation of the egg content of alimentary pastes which would be suitable for use in regulatory operations on such products. Alfond¹⁸, who has contributed much to the development of analytical methods in this field, suggested that study of the sterol content of eggs, flour and alimentary pastes be undertaken. As Tillmans, Riffart and Kuhn¹² had shown that the sterol determination was of value in this problem and that the sterol content of alimentary pastes remained relatively constant during storage, this investigation of the problem was made.

For the purpose of clarity in presentation, the development of the method adopted for the determination of cholesterol itself is discussed before consideration is given to its application to alimentary pastes and their ingredients. Actually the development of the method and its application constituted concurrent studies, the experience gained in one phase of the problem leading to modifications in the approach to the other. Moreover, though this investigation began in 1934, several unavoidable interruptions, one of two years' duration, impeded progress and made impossible

complete execution of the work which had been planned. Keeping these facts in mind will contribute to an understanding of some phases of the work, the purpose of which might not otherwise be clear.

THE DETERMINATION OF CHOLESTEROL

The Choice of a Method

If a method is to be of practical value in regulatory work it is most important that it should yield reasonably accurate and reproducible results, not only in the hands of different analysts but also in different laboratories. The attainment of such an end is not to be expected if the method requires undue attention to technique, too strict control of conditions or a degree of experience which can be acquired only by constant use of the procedure. The food analysts engaged in regulatory work must deal with a great variety of foods and normally will have only occasional use for any one method. These are essential factors which must be borne in mind in considering adoption of a method for the present purpose.

A noodle containing 5.5 per cent of egg solids on the dry basis will have a sterol content of about 0.15 per cent to 0.20 per cent. Since the need is for a method particularly designed to detect the presence of less than 5.5 per cent of egg solids, it is obvious that a suitable cholesterol determination on the macro scale requires an inordinately large sample. As the content of unsaponifiable matter (a gravimetric determination) may be expected to be of some interpretive value in conjunction with the sterol content in the detection of adulterated elementary pastes, it is not desirable to use a sample of less than 10 grams. A smaller sample would also be less representative. The procedure originally developed in the present work was, in fact, based on the use of a 20 gram sample. The amount of cholesterol in such samples comes within the semi-micro range. This means that a micro method associated with a rather large aliquot factor or a semi-micro procedure must be adopted.

It is well known that quantitative organic microanalytical procedures require considerable experience and unusual attention to manipulative technique on the part of the analyst. It is therefore not surprising to find such lack of agreement among various workers regarding the reliability of the various microanalytical methods for cholesterol. Abelin¹⁹ and Wasitzky²⁰ have published comprehensive reviews of the methods and Ruiz and Torres²¹ have made a critical study of a number of them. These methods, particularly the very sensitive colorimetric ones, are, of course, necessarily used for clinical and many other biological analyses. The colorimetric methods are based on unknown concurrent reactions which make slight variations in technique the source of considerable differences in results. Such methods require a close control of the time of reaction, temperature, concentration and purity of reagents and illumination, among other factors. They are, moreover, subject to interference by non-sterol substances. Lampert²² used a colorimetric method for estimating the egg content of ice cream based on the cholesterol present. He noted the effects of interfering substances in some samples. Tillmans, Riffart and Kuhn¹² were unsuccessful in attempting to apply colorimetric methods to the determination of sterols in alimentary pastes due to traces of interfering substances in the extracts. Though the Schönheimer and Sperry²³ procedure eliminated such non-sterol interference by precipitation of the sterols with digitonin before application of the color reaction, the other sources of error remain. The method reported by Sobel, Dreker and Natelson²⁴ involves precipitation of the sterols as pyridine sterol sulfates before application of the color reaction. Riffart and Keller²⁵ report the application of a colorimetric method using a spectrophotometric measurement to the determination of sterol in alimentary pastes but again it is noted that "nur genaues Einhalten der einmal aufgestellten Arbeitsvorschrift ergibt bei gleicher

Cholesterinkonzentration den gleichen Farbton und die gleiche Farbstärke". It has therefore seemed highly improbable that these colorimetric methods would satisfy the requirements. The other type of micro method which has been applied to a considerable extent depends upon precipitation of the sterols as digitonides and oxidation of the latter under controlled conditions with standard dichromate, the excess being determined iodimetrically. An empirical factor is determined for the relation of the volume of standard thiosulfate solution used per milligram of cholesterol. The method is applicable to not more than two milligrams. Such procedures were described by Szent-Györgyi²⁶ and by Okey²⁷ and modified by Turner²⁸ and Yusada²⁹. The Szent-Györgyi procedure was first adapted to the sterol determination in alimentary pastes and eggs by Tillmans, Riffart and Kuhn¹². They again emphasize the importance of the most exact adherence to their procedure. It is noted that their duplicate determinations of cholesterol in eggs and noodles show differences of 10 per cent or more in some instances. Riffart and Keller²⁵ considerably improved the results by application of Yusada's procedure²⁹ but it is still subject to the general objections raised above. The micro methods which depend on the precipitation of the sterols with digitonin are also subject to the objections discussed below with respect to this reagent.

Turning to a consideration of semi-micro methods for cholesterol the only available methods were found to be based on the precipitation of the sterol with digitonin, a method introduced by Windaus in 1908³⁰. This depends upon the fact that digitonin combines mole for mole with cholesterol in alcoholic solution and forms a very sparingly soluble molecular compound, cholesterol digitonide, probably of the indicated formula:

The high molecular weight of the compound and its sparing solubility in many lipid solvents make it a satisfactory substance for the gravimetric determination of cholesterol. It forms similar compounds with practically all naturally occurring sterols. Unfortunately digitonin is an extremely expensive reagent and one of variable purity. In a study of the application of the digitonin method to the determination of cholesterol on a semi-micro scale, Dam³¹ found that the weight of the precipitate depended upon the amount of excess digitonin used, making necessary the use of a correction curve to determine the quantity of cholesterol. Then Schonheimer and Dam³² found that the amount of excess digitonin required in the precipitation varied not only with different sterols but with the particular sample of digitonin used. Ewert³³ confirmed this and showed that both the amount and the concentration of digitonin affect the weight of the precipitate obtained. These facts mean that the analyst must establish a correction curve for each new sample of digitonin used, a most unsatisfactory procedure. Breusch³⁴ more recently reaffirmed this. Terrier³⁵ attempted to apply the digitonin method to the present problem but obtained unsatisfactory results. In a later publication³⁶ he described an inconvenient procedure which depends on weighing the digitonide precipitate, decomposing it by boiling with xylene, reweighing the insoluble residue and obtaining the cholesterol by difference. This method loses the chief advantage of application of the digitonin, that is, the high gravimetric factor, and is based on the unsound principle of making the result depend on a small difference between two weights, each of which is determined by a procedure involving experimental error. The fact that the expensive digitonin is recovered is offset by its failure to react quantitatively like the original reagent. The recovered product is recommended only for approximate determinations.

The report by Windaus³⁷ in 1906 showed that cholesterol could be precipitated almost quantitatively from its ether solution as the dibromide by addition of a solution of bromine in glacial acetic acid, while phytosterol bromide precipitated only upon addition of considerable water. In this brief report Windaus described several experiments of a semi quantitative character in which the procedure was applied to relatively large amounts of cholesterol-phytosterol mixtures. Holde³⁸ in the same year reported briefly that an attempt to apply the method to the detection of small amounts of animal fats in vegetable fats was unsuccessful. Lewkowitsch³⁹ stated that the method had served to his satisfaction for the same purpose, but neither of these investigators gave any data on the subject. It was also found that Popp⁴⁰ had applied the procedure to the detection of egg in alimentary pastes apparently in a more or less qualitative way, but made only brief mention of his work and submitted no data. A survey of the literature did not reveal that any adequate study of this method had been made with respect to its possibilities as a quantitative method. At the same time the labile character of the bromine in cholesterol dibromide (5-6 dibromocholestanol) suggested that a semi-micro procedure based on the titration of the bromine might be developed. The necessary reagents are inexpensive and readily available. The extraordinary precautions as to purity of reagents specified in other available methods did not appear necessary and the use of special apparatus which cannot be readily assembled in any food analysis laboratory was not required. Moreover the possibility existed that the correction factor necessary due to the phytosterols of the flour in other procedures might be made negligible, if not eliminated. The investigation of the possibilities of a cholesterol dibromide precipitation method was therefore undertaken.

The Cholesterol Dibromide Method

Review of the Literature. Cholesterol dibromide was prepared as long ago as 1868 by Wislicenus and Moldenhauer⁴¹. It was also described by Liebermann⁴² and Cloez⁴³ prior to publication of the Windaus method³⁷. Windaus and Hauth⁴⁴ found that the tetrabromide of stigmasterol and of its acetate also precipitate under the same conditions as cholesterol dibromide. The Windaus procedure has been widely used for the detection of cholesterol and stigmasterol, for the preparation of cholesterol dibromide in a number of syntheses and in the purification of cholesterol. Although Windaus³⁷ stated that the pure dibromide precipitated almost quantitatively and referred to the melting point and crystal form as means of identification, he did not report an analysis or the melting point. Windaus and Lüders⁴⁵ in 1920 reported the melting point as 122°, but still gave no analysis. In fact no analysis of the product just as precipitated in the Windaus method appears to have been published. Lifschütz⁴⁶ asserted that the product obtained by the Windaus procedure is not pure, but holds acetic acid which it slowly loses on standing. He reported a melting point of 110°-111° for the compound prepared according to Windaus, which dropped to 94° after crystallization from alcohol. The latter temperature is the same Lifschütz⁴⁷ found for the melting point of "pure" cholesterol dibromide prepared by bromination in ether and precipitation with 90 per cent alcohol, a product which contained 28.64 per cent bromine as compared with 29.26 per cent required for the dibromide, $C_{27}H_{45}OBr_2$. Lifschütz found that the bromine was quantitatively removed from cholesterol dibromide simply by heating the dibromide with alkali in alcohol. Schönheimer⁴⁸, in purifying cholesterol, debrominated the dibromide by boiling it with sodium iodide in alcoholic solution. Pirrone⁴⁹ described several bromination products of cholesterol obtained by the Windaus³⁷ and the Cloez⁴³

methods. In a more comprehensive article on the same work Pirrone⁵⁰ reported that the bromine content of cholesterol dibromide could be determined with sufficient accuracy by the Volhard procedure in acetone solutions or by titration of the iodine liberated when sodium iodide was added to the acetone solution. The analyses which were made on macro quantities yielded results slightly below the theoretical by the argentometric method and about 5 per cent low by the iodometric one. The low result was attributed to the excessive amount of starch solution required to develop the blue end point. De Fazi and Pirrone⁵¹ described various products from the application of a number of methods to the bromination of different cholesterol samples. They concluded that the bromo-derivatives obtained depended on the bromination method used and the origin of the cholesterol. According to their report the product as precipitated by the Winkler³⁷ method is not pure cholesterol dibromide. All of the cited investigators have noted the gradual decomposition with evolution of hydrogen bromide which cholesterol dibromide undergoes on exposure to moisture and light. Balls⁵² in a study of the factors giving rise to the abnormal iodine absorption number of cholesterol found that chloroform, glacial acetic acid or pyridine should not be used as solvents for the purpose of halogenating cholesterol. He reported that by the use of carbon tetrachloride as solvent and iodine monobromide as halogenating agent at 0° the theoretical iodine number was obtained and all of the consumed halogen was organically bound. He did not isolate the bromination products, but determined the organically bound halogen by the difference between the total halogen consumed and that inorganically bound. At 25° reactions other than direct addition to the double bond occurred.

The Precipitation of Cholesterol Dibromide. The chief purpose of the work now to be described was to establish whether, in the light of the

investigations reported above, cholesterol dibromide could be precipitated in such a way as to permit quantitative application of the procedure on a semi-micro scale. The cholesterol used was the product of melting point 147° - 148° sold by the Pfennig Chemical Company, Naukegen, Illinois. Lampert²² had reported this product to be of good quality. In a number of preliminary orientation experiments which are not to be reported in detail it was found that the use of carbon tetrachloride as the sole solvent for the bromination at 0° was not feasible, because the solubility losses were too great with the precipitants which could be used. The initial experiments disclosed that by using ether as the solvent, a solution of bromine in carbon tetrachloride as the brominating agent, and a mixture of four volumes of glacial acetic acid with one of water as the precipitating agent, with both bromination and precipitation at 0° , a good recovery of cholesterol was possible. For this purpose the micro crystalline precipitates were filtered on tared sintered glass crucibles and after washing were dried first in vacuo over H_2SO_4 , then to constant weight by aspiration with dry air. For use in developing a rapid titrimetric method cholesterol dibromide was prepared according to Lifschütz⁴⁷. The product did not contain the theoretical bromine content for cholesterol dibromide. However its bromine content was established on the macro scale by the Duin⁵³ modification of the Stepanow⁵⁴ sodium reduction method. An argentometric determination of the bromine in semi-micro quantities of the dibromide was developed involving removal of the bromine by evaporation with alcoholic potassium hydroxide solution, slight acidification of the residue, and titration of the bromide ion with 0.01 N silver nitrate, using eosin as indicator, as recommended by Kolthoff and Furman⁵⁵.

Since the report of De Fazi and Pirrone⁵¹ suggested cholesterol of different origins might behave differently in bromination procedures, it was

felt necessary to demonstrate that egg cholesterol would react in the present method just as did the Pfanstiehl cholesterol used to study the method. Accordingly the cholesterol was isolated from about a pound of fresh egg yolk. Moreover Schönheimer⁴⁸ reported that cholesterol frequently contained small amounts of saturated sterols and described a method of removing these and a procedure for determining the amount of saturated sterols in cholesterol. The purification involves precipitation of the cholesterol as the dibromide by the Winkaus method, regeneration of the cholesterol by boiling the dibromide with sodium iodide in alcohol and repetition of the process on the regenerated product. Schönheimer reported yields of 10 per cent to 15 per cent. The Pfanstiehl cholesterol was "purified" by this process, only one precipitation being made because of the small yield obtained. Schönheimer's method for determining the saturated sterols in cholesterol is based on the fact that cholesterol dibromide does not precipitate with digitonin. The determination involves precipitating the cholesterol as dibromide from cold alcohol, filtering, adding digitonin to the filtrate and allowing the solution to stand in the cold for 48 hours, whereupon any saturated sterol present precipitates as the digitonide. When this method was applied to the original Pfanstiehl cholesterol and the "purified" product, the former was indicated to contain 2.3 per cent and the latter 2.2 per cent of saturated sterol. At about this time the report of Gardner and Gainsborough⁵⁶ was noted in which it was stated that cholesterol dibromide itself decomposed on standing in alcoholic solution under conditions of the method, yielding 2 per cent to 3 per cent of digitonin-precipitable sterol. They concluded that the Schönheimer procedure was not accurate for small quantities of saturated sterols.

The cholesterol dibromide precipitation method was applied at -12° to 20 mg. samples of the egg cholesterol, the original Pfanstiehl cholesterol and the "purified" Pfanstiehl cholesterol. The results shown in Table I indicate no significant differences between these samples.

TABLE I

The Precipitation of Various Cholesterol Samples

Description of Sample	Precipitate	
	Weight	Bromine Content
	mg.	per cent
Egg cholesterol	26.0	28.1
Egg cholesterol dried in vacuo at 100°	25.9	28.1
Pfanstiehl cholesterol	26.2	28.1
"Purified" Pfanstiehl cholesterol dried in vacuo at 100°	25.5	28.0

The theoretical yield is 28.2 mg. of cholesterol dibromide containing 29.26 per cent of bromine. The composition of the precipitate will be discussed later.

To determine the effect of the bromine concentration, 20 mg. samples of Pfanstiehl cholesterol were brominated and precipitated at 0° , varying only the bromine content of the carbon tetrachloride. The results in Table II show that the precipitation is not at all sensitive to the bromine concentration, the only noticeable effect being a small decrease in the weight of precipitate with the higher concentrations of bromine.

TABLE II

The Effect of Bromine Concentration on the Precipitation

Bromine Content of the Carbon Tetrachloride Solution		Precipitate	
		Weight	Bromine Content
g. per ml.		mg.	per cent
0.1	:	25.4	27.9
0.2	:	25.4	27.8
0.4	:	24.8	27.9
0.6	:	24.8	28.0
0.8	:	24.3	27.8

A concentration of 0.2 g. of bromine per ml. of carbon tetrachloride is ample for the purposes for which the present method is designed.

That the close control of temperature is not a critical factor in the bromination and precipitation is indicated by the results in Table III obtained by applying the method to 20 mg. samples of Pfanstiehl cholesterol at different temperatures. The only significant differences represent the expected small increase in the loss due to solubility with increase in temperature.

TABLE III

The Effect of Temperature on the Precipitation

Temperature		Precipitate	
		Weight	Bromine Content
°C		mg.	per cent
-12	:	26.0	28.2
0	:	25.3	28.4
+10	:	24.7	28.3

Since a temperature of 0° is most conveniently maintained this was adopted as the temperature to be used.

The procedure was now applied to various amounts of cholesterol. The results in Table IV show the composition of the precipitate and the deviation of the individual results from the weight of the precipitate calculated from the linear function

$$\text{mg. of precipitate} = 1.359 (\text{mg. of cholesterol}) - 1.6$$

determined by the least squares method.

TABLE IV

The Relation Between the Precipitate Obtained
and the Weight of Cholesterol Used

Cholesterol	:	Precipitate			
:	:	Weight		Deviation	Bromine
:	:	Observed	Calculated	:	Content
:	:	:			
mg.	:	mg.	mg.	mg.	per cent
:	:	:			
10	:	12.2	12.0	+0.2	:
20	:	25.1	25.6	-0.5	23.0
30	:	39.2	39.2	0.0	27.9
40	:	53.4	52.8	+0.6	27.6
50	:	66.0	66.3	-0.3	28.2
:	:	:			
Average deviation				0.3	

The conditions of the bromination and precipitation now having been established, a more rapid filtering device which could be conveniently used to maintain the low temperature during filtration was developed. In the gravimetric studies filtrations on the sintered glass crucibles were quite tedious, as a filter of fine porosity (Jena G4) was required to retain the micro crystalline precipitate. The method was applied to semi-micro quantities of egg cholesterol and the bromine in the precipitates titrated

with 0.01 N silver nitrate according to the established procedure. The results gave the relation

$$\text{mg. of cholesterol} = 1.5 + 2.09 (\text{ml. of } 0.01 \text{ N AgNO}_3).$$

The argentometric titration had given satisfactory results in the bromine determinations, as it has a sharp end point. However the end point involves a change from an orange-pink to a red-violet color, and considerable experience with the titration is necessary to attain good results. Accordingly attention was given to the iodometric method based on the reaction between alkali iodide and cholesterol dibromide in acetone which Pirrone⁵⁰ and De Fazi and Pirrone⁵¹ had reported to be fairly satisfactory on the macro scale.

The Iodometric Estimation of Cholesterol Based on the Cholesterol Dibromide-Sodium Iodide Reaction. In the articles last cited the Italian investigators gave no details of their procedure other than the fact that they used a 20 per cent aqueous solution of potassium iodide and found 20 to 25 ml. of starch solution necessary to develop the starch-iodide color. It appears that the method was applied to the analysis of only one cholesterol dibromide sample, a single result being reported. This value of 28.04 per cent bromine is quite low compared with the theoretical bromine content (29.26 per cent) and the values reported by other methods (28.93 per cent and 29.08 per cent). In the presence of some organic solvents the starch-iodide color is by far less sensitive than the yellow color of the iodine itself as indicated by Kolthoff and Furman⁵⁵. Therefore in the present work the iodine liberated in the cholesterol-sodium iodide reaction in acetone and alcohol was titrated with sodium thiosulfate solution just to disappearance of the iodine color. In the concentrations of solutions used 0.03 ml. of 0.01 N iodine solution produced a perceptible yellow color and 0.05 ml. a quite distinct color, so that practically no correc-

tion for that factor is necessary. In the initial work on this procedure the reaction between cholesterol dibromide and sodium iodide was tried in acetone and also in alcohol. However the reaction was so slow in the latter solvent that its use was not practicable. Attempts to obtain cholesterol dibromide containing the theoretical quantity of bromine had been unsuccessful. The accuracy of the method was therefore judged by comparison with the argentometric method, using four different preparations of cholesterol dibromide. The results are shown in Table V.

TABLE V
Comparison of the Argentometric
and Iodometric Methods

Cholesterol Dibromide	Iodometric Method				Argentometric Method 0.01 N AgNO ₃
	Acetone	NaI	Reaction Time	0.01 N Na ₂ S ₂ O ₃	
mg.	ml.	g.	hours	ml.	ml.
26.0	20	0.5	1	9.01	9.09
26.0	20	0.5	2	9.00	
25.0	40	0.5	0.5	8.46	8.67
25.0	40	0.5	1	8.45	
25.0	40	1.0	1	8.46	
25.0	40	0.5	1	8.46	8.66
10.0	40	0.5	1	3.37	3.51
25	40	0.5	1	8.68	8.83

These results indicated that the iodometric method could be employed in this work. The bromination and precipitation of semi-micro quantities of cholesterol by the procedure as now developed (Appendix, page 62) was undertaken, the precipitates being dissolved in acetone, treated with

sodium iodide and the liberated iodine titrated with 0.01 N sodium thio-sulfate. Pfanstiehl cholesterol and egg cholesterol were used. The results are shown in Table VI, with the deviations from the weights of cholesterol calculated by the linear relation

$$\text{mg. of cholesterol} = 1.0 + 2.16 (\text{ml. of } 0.01 \text{ N Na}_2\text{S}_2\text{O}_3)$$

as obtained by the least squares method.

TABLE VI

The Iodometric Cholesterol Determination Based on
the Cholesterol Dibromide-Sodium Iodide Reaction

Description of Sample	Weight Sample	Titer 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$	Weight Calculated	Deviation
	mg.	ml.	mg.	mg.
Egg cholesterol	5.0	1.82	4.9	+0.1
" "	10.0	4.10	9.9	+0.1
" "	15.0	6.60	15.3	-0.3
" "	20.0	8.95	20.3	-0.3
Pfanstiehl cholesterol	5.0	1.82	4.9	+0.1
" "	5.0	1.85	5.0	0.0
" "	10.0	4.10	9.9	+0.1
" "	10.0	4.08	9.8	+0.2
" "	15.0	6.46	15.0	0.0
" "	15.0	6.57	15.2	-0.2
" "	20.0	8.86	20.1	-0.1
" "	20.0	8.76	19.9	+0.1
" "	40.0	17.92	39.7	+0.3
Average deviation				0.15

These data indicated that the method would yield results of sufficient accuracy for the intended purpose. It was applied to the analysis of eggs, flours and noodles of known composition. The results will be considered later.

Under the conditions of the method the acetone solution of the precipitate contains some water. It was found that this amounted to not more

than 1.5 ml. A number of experiments was undertaken to determine the effect of this and other factors on the titration. These showed that the presence of 1.5 ml. or less of water decreased the titer to some extent, while appreciably greater amounts, e.g., 3 ml., caused a significant decrease. These experiments also indicated that the titer was independent of the volume of acetone within reasonable limits, was not affected by exposure to light and reached its maximum in less than an hour even in the presence of water.

Prior to the development of the oxidative iodometric procedure, the cholesterol dibromide-sodium iodide reaction was used as a convenient method of testing the comparative purity of various fractions in the study of the preparation of pure cholesterol dibromide. It was found that the pure dibromide containing 29.26 per cent of bromine was indicated to contain 28.6 per cent of bromine by the sodium iodide method. This method therefore gives results about 2 per cent low in the absence of water. A few experiments involving titration of 0.01 N iodine solutions in acetone under conditions comparable to those used in the method indicated that the deficiency is not due to reaction of the liberated iodine with the acetone.

The iodometric procedure based on the cholesterol dibromide-sodium iodide reaction in acetone has definite advantages over the argentometric method. It is more rapid and more precise. Moreover, since, according to Schönheimer⁵⁷, only sterol dibromides having the added bromine in the nuclear ring system give this reaction with sodium iodide readily, the interference due to sterols like stigmasterol having a double bond in the side chain might be expected to be less. The titration does not require as much experience on the part of the analyst as does the argentometric

titration. On the other hand, this iodometric method yields somewhat low results in the bromine determination. The slight uncertainty due to variations in the amount of water in the acetone solution of the precipitates is a disadvantage which could be overcome at the sacrifice of convenience and simplicity through drying the precipitate and filter by long aspiration with dry air. The chief disadvantage in practice is the interference with observation of the end point caused by the frequent presence of some color in the acetone solution of precipitates obtained in applying the method to flours and alimentary pastes. This interference was considerably reduced by a purification of the sterol extracts before precipitation through adsorption on alumina as described later.

The Interference of Wheat Phytosterols in the Determination of Cholesterol. In a preliminary application of the cholesterol dibromide precipitation method the sterol recovered in a mixture of flour and egg was greater than that found in the flour and eggs separately. Also a flour extract was found to have a sterol content greater as determined in the presence of cholesterol than in its absence. Therefore a study of the precipitation of cholesterol in the presence of wheat phytosterols was made.

There is but meager information available on the identity of the sterols of flour. Anderson and Nabenhauer⁵⁸ in a brief study concluded that sitosterol and dihydrositosterol were present. Balls⁵⁹ reported the presence in flour oil of an ester, believed to be a sitosterol ester. Anderson and Nabenhauer⁶⁰ state that sitosterol and dihydrositosterol occur throughout the wheat grain, but the germ contains mostly sitosterol while the endosperm is richer in dihydrositosterol and the bran contains even more of the latter. The very low sterol content of flour makes

difficult the isolation of adequate amounts with which to work. Wheat germ oil, however, contains a very complex mixture of sterols, identification of which has been the object of several investigations. Anderson, Shriner and Burr⁶¹ found dihydrositosterol and a mixture of unsaturated sterols which were called α -, β - and γ -sitosterols. Wallis and Fernholz⁶² found α -sitosterol to be a mixture of two doubly unsaturated sterols, α_1 -sitosterol being an isomer of stigmasterol and α_2 -sitosterol probably a homolog. They deduced from Bengtsson's⁶³ discovery of the identity of stigmasterol and sitosterol that β -sitosterol must be 22 - 23 dihydrostigmasterol. Bernstein and Wallis⁶⁴ then confirmed that fact. Ichiba⁶⁵ reported the presence of dihydrositosterol and four isomeric sitosterols in wheat germ oil. Karrer and Salomon⁶⁶ found α - and β -tritosterols in the oil, but point out that these have not yet been shown to be chemical individuals.

In view of the complexity of the wheat germ oil sterols it was believed that the interference of these compounds in the cholesterol procedure would give a rigorous test of the method with sterols representative of those that might be found in farinaceous products. Accordingly the sterol fraction was isolated from wheat germ oil by a procedure similar to that of Anderson, Shriner and Burr⁶¹. Mixtures of this product with cholesterol were brominated, precipitated and the amount precipitated (calculated as cholesterol) was determined by the iodometric method with sodium iodide in acetone. The calculation was according to the relation

$$\text{mg. precipitated (as cholesterol)} = 2.16 (\text{ml. of } 0.01 \text{ N Na}_2\text{S}_2\text{O}_3).$$

The first experiments showed that there was coprecipitation of the cholesterol and phytosterol dibromides when the precipitant was four

volumes of glacial acetic acid plus one volume of water, even though small amounts of phytosterol itself did not give a precipitate. When the precipitant was changed to nine volumes of glacial acetic acid plus one of water, the precipitation of a mixture of 20 mg. of cholesterol and 5 mg. of phytosterol yielded several milligrams less than did 20 mg. of cholesterol alone. Addition of 10 and 15 mg. of the phytosterol to 20 mg. of cholesterol yielded regularly increasing quantities of precipitate above the minimum observed when 5 mg. were used. A re-examination of the precipitation using the first precipitant suggested that in that case too there was a minimum in the precipitation occurring when only a few tenths of a milligram of phytosterol was present. It was found that the slight increase in water content of the precipitant when it was made by diluting four volumes of glacial acetic acid to five volumes with water sufficed to eliminate the minimum. This is the precipitant which was used in the study of the iodometric method above. The results of numerous experiments involving use of this precipitant with phytosterol alone and with mixtures of cholesterol and phytosterol are plotted in the graph, Figure 1. The abscissa represents the weight of phytosterol used; the ordinate is the weight of precipitate calculated as cholesterol, which is determined by titration as described above. The amounts of cholesterol used in the series were 0, 5, 10 and 20 mg. respectively, beginning with the first series from the bottom.

Within the limits of experimental error the curves for the mixtures containing cholesterol have the same initial and final slopes, which also closely approach the slope of the phytosterol line. It is interesting to note that when the ratio of the phytosterol to cholesterol approximates 1.25 to 1 there is a deflection in the curves for the mixtures containing the smaller amounts of cholesterol. It is recognized that

Figure 1
The Coprecipitation of Cholesterol
and Phytosterol Dibromides

caution must be exercised in interpreting the results in view of the complexity of the phytosterol mixture. However, it is worth noting that one or more of the constituents of the mixture forms a far more soluble bromide than cholesterol dibromide, yet so long as sufficient cholesterol is present this more soluble bromide precipitates quantitatively with the other. Moreover it is indicated that only about 80 per cent of the phytosterol is precipitated as a bromide. The preparation contains about 3 per cent of water of crystallization and assuming that the formula given by Anderson and Nebenbauer⁶⁰ for determining from the specific rotation the amount of sitosterol in sitosterol-dihydro-sitosterol mixtures is applicable for approximate purposes to this phytosterol preparation, it is concluded that the latter does contain about 80 per cent of sitosterol. This means that the deflection noted in the curves occurs when the ratio of cholesterol to sitosterol is 1 to 1. As shown by Lettre⁶⁷ the formation of molecular compounds is a characteristic of the sterols and is not excluded between sterols as closely related in structure as cholesterol and γ -sitosterol are considered to be. The formation of such compounds between the sterol dibromides does not seem to have been studied and it is not claimed to have been demonstrated in this work as far as circumstances permitted the study to be carried on. However, it is pointed out that the formation of such a molecular compound between cholesterol dibromide and sitosterol dibromide would account very satisfactorily for the results obtained. Thus the compound dibromide might be expected to be more soluble than cholesterol dibromide in ether-acetic acid mixtures containing little water. This difference would decrease rapidly with increase in water content until the mixtures resulting from the precipitant used in the present method might dissolve the same amount of the two bromides within limits of the deter-

mination. After the compound dibromide present had precipitated, any excess of sitosterol dibromide would remain in solution up to the point where its solubility was also exceeded, accounting for the deflection in the curves for mixtures containing small amounts of cholesterol. With an amount of cholesterol exceeding considerably the amount of phytosterol which would precipitate in the absence of cholesterol no deflection in the curve for such cholesterol-phytosterol mixtures would be expected.

Although it was not believed desirable to introduce an acetylation step into the quantitative procedure, if this would have overcome the coprecipitation difficulty its feasibility would have been considered. However, a few experiments with mixtures of cholesterol acetate and phytosterol acetate demonstrated that coprecipitation of the dibromides also occurred with these compounds.

From the standpoint of the cholesterol determination the significant fact is that the amount of sitosterol dibromide which precipitates with cholesterol dibromide is independent of the quantity of cholesterol present so long as this equals or exceeds the sitosterol content of the sterol mixture. Hence, in the analysis of flour, plain noodles or noodles of very low egg content sufficient cholesterol must be added to the unsaponifiable matter to assure that an excess of cholesterol is present.

Preparation of Pure Cholesterol Dibromide and Pure Cholesterol.

It was noted above that precipitates obtained in the study of conditions for the cholesterol dibromide precipitation method contained consistently about 28 per cent of bromine instead of the 29.26 per cent required by the pure dibromide, $C_{27}H_{46}OBr_2$. The same composition of the precipitate was found when as much as a gram of cholesterol was brominated and pre-

precipitated under similar conditions. It will be recalled that these conditions represent a modification of the Windaus method to adapt it to the present purpose while at the same time observing, insofar as possible, the important factors noted by Halls⁵² in studying the abnormal iodine absorption number of cholesterol, namely, halogenation at 0°, use of carbon tetrachloride as solvent and the absence of acetic acid during the halogenation. It was desired to obtain some of the pure dibromide for unequivocal testing of the bromine determination. Though the dibromide is not isolated as such in the cholesterol determination, evidence that it is quantitatively formed under those conditions will be presented in the following section.

Lifschütz⁴⁶ criticized the Windaus method on the ground that the precipitate was not pure but held loosely bound acetic acid. He reported that by brominating cholesterol in ether and precipitating it with 90 per cent alcohol pure cholesterol dibromide was obtained. He also stated that this pure dibromide, when recrystallized from glacial acetic acid, had the properties of the Windaus product and on recrystallization from alcohol reverted to the pure state. No analysis of the Windaus product is reported and a single analysis of Lifschütz' product indicated a bromine content of 28.64 per cent. In the present work pure cholesterol dibromide was not obtained by the original Windaus procedure³⁷ nor by a somewhat modified form described by Windaus and Lüders⁴⁵. Difficulty in completely removing acetic acid from the precipitates was also noted. The products when recrystallized from alcohol contained even less bromine and the occurrence of decomposition was apparent in the solutions. Recrystallization of the Windaus product from acetone as recommended by De Fazi and Pirrone⁵¹ also failed to yield the pure

dibromide. The sensitivity of the compound to heat, moisture and solvents readily attacked by the labile bromine causes the difficulty in obtaining the pure product. From the present work there is reason to believe that molecular compounds of the cholesterol dibromide with some of its decomposition products may occur. This would account for the observed formation of products having different compositions and different melting points on recrystallization of impure cholesterol dibromide to constant melting point from acetone, alcohol or ether. On the basis of the experience with this compound it appeared that the preparation should be obtained pure without the necessity for recrystallization and in a dry state if possible. Accordingly, a procedure was developed involving bromination with a slight excess of bromine in carbon tetrachloride solution with chilling, precipitation of the dibromide with petroleum ether at -10° to -15° and rapid filtration and drying first by aspiration with dry air until room temperature is attained, then in vacuo over phosphorus pentoxide to constant weight. The product melts to a clear colorless liquid at 114.4° - 114.8° , followed by effervescent decomposition with browning. All previously obtained preparations had exhibited such decomposition before or on melting. The pure dibromide contained the theoretical bromine content (29.26 per cent) as determined by the Volhard method and by the iodometric van der Meulen⁶⁸ method, according to Kolthoff and Yutzy⁶⁹ described in the following section. By the iodometric method based on the sodium iodide reaction in acetone the pure dibromide contained 28.6 per cent of bromine, which is the highest bromine content by this method observed in any of the previously obtained preparations.

Since analytically pure cholesterol dibromide could be thus pre-

pared, cholesterol of unquestionable purity is obtainable therefrom by debromination under mild conditions. Schönheimer⁴⁸ had purified cholesterol by precipitating the dibromide (Winkaus method), recrystallizing it from alcohol and debrominating by boiling it with sodium iodide in alcohol. This process is repeated with the regenerated cholesterol. The yields reported are 10 per cent to 15 per cent. Even lower yields have been obtained by this method in the present work. Moreover, in view of the instability of cholesterol dibromide (noted also by Schönheimer), it does not seem good practice to heat the substance in alcohol during a purification process. However, cholesterol dibromide is debrominated smoothly and rapidly at room temperature by the action of sodium iodide in acetone. Accordingly, the pure cholesterol dibromide prepared as described was allowed to stand over-night in acetone solution with excess sodium iodide, the liberated iodine was reduced with sodium thiosulfate solution and the precipitated cholesterol was recrystallized to constant melting point from alcohol (two crystallizations). The yield was 63 per cent.

It is emphasized that the Pfanstiehl cholesterol used in the purification process is evidently free of saturated sterols within the limits of the Schönheimer determination, as previously discussed. An opportunity has not yet been afforded to test whether the precipitation process will remove saturated sterols from cholesterol, which was the primary purpose of Schönheimer's process. However, it is believed that the debromination is preferably carried out in either process by the sodium iodide reaction at room temperature.

The Iodometric Estimation of Cholesterol by the van der Meulen Method as Modified by Kolthoff and Yutzy. Subsequent to the development of the iodometric method based on the cholesterol dibromide-sodium iodide reaction and its application to the analysis of alimentary pastes, there

appeared the rapid and accurate modification of the van der Meulen⁶⁸ method for determining small quantities of bromides described by Kolthoff and Yutzy⁶⁹. This method is based on the oxidation of the bromide ion to bromate with sodium hypochlorite solution, reduction of the excess hypochlorite with sodium formate and iodometric determination of the bromate. The procedure as published is applied to quantities up to 8 milligrams of bromine. Drake and Busbey⁷⁰ had applied a similar procedure to the determination of methyl bromide in air. It is apparent that such a method would eliminate the chief disadvantages of the other iodometric method, namely, the uncertain end point sometimes noted due to colored extracts and the effect of water on the titration. Furthermore, since the van der Meulen method yields for the titration six equivalents of iodine for each equivalent of bromine the increased accuracy would permit use of a smaller sample. By some adjustment of the reagents it was found that the Kolthoff and Yutzy procedure gave very satisfactory results for the determination of up to at least 40 milligrams of bromine. The revised procedure was then applied to samples of pure cholesterol dibromide following evaporation of ether-alcohol solutions of these with potassium hydroxide and neutralization with hydrochloric acid. The results are shown in Table VII.

TABLE VII

Analyses of Cholesterol Dibromide by
the Oxidative Iodometric Method

Cholesterol Dibromide	Titer 0.02056 N $\text{Na}_2\text{S}_2\text{O}_3$ (corrected for blank)	Bromine
mg.	ml.	per cent
5.65	6.06	29.37
10.15	10.82	29.19
14.95	16.01	29.33
20.05	21.42	29.26
20.25	32.31	29.25
36.65	39.09	<u>29.21</u>
Average		29.27

The revised procedure was now used to determine the relation between the cholesterol and the titer when applied to the cholesterol dibromide precipitation method (Appendix, page 86). The purified cholesterol was used. The results indicated the relation

$$\text{mg. of cholesterol} = 0.55 + 0.688 (\text{ml. of } 0.02 \text{ N } \text{Na}_2\text{S}_2\text{O}_3).$$

In Table VIII are shown the individual results and the deviations from the values calculated by the above relation. The correction for the blank determination on the reagents amounted to 0.64 ml.

TABLE VIII

Iodometric Cholesterol Determination
by the Oxidative Method

Cholesterol Used	Titer 0.02 N $\text{Na}_2\text{S}_2\text{O}_3$ (corrected for blank)	Cholesterol Calculated	Deviation
mg.	ml.	mg.	mg.
5.40	7.24	5.55	-0.15
4.90	6.39	4.95	-0.05
5.20	6.92	5.30	-0.10
9.95	13.64	9.95	0.00
10.20	13.90	10.10	+0.10
9.75	13.33	9.70	+0.05
15.40	21.80	15.55	-0.15
14.80	20.46	14.60	+0.20
15.10	21.02	15.00	+0.10
20.40	28.93	20.45	-0.05
20.00	28.32	20.00	0.00
19.95	27.86	19.70	+0.25
29.95	43.01	30.10	-0.15
30.30	43.43	30.20	+0.10
30.45	43.32	30.35	+0.10
39.95	57.01	39.75	+0.20
39.80	57.10	39.60	0.00
40.15	57.63	40.15	0.00
50.10	72.20	50.20	-0.10
Average deviation			0.10

The theoretical factor in this determination for converting the 0.02 N sodium thiosulfate titer to cholesterol is 0.6444 instead of 0.688 as found in the above equation from the experimental data. This would indicate that only 93.7 per cent of the theoretical amount of cholesterol dibromide was actually formed and precipitated. Since a side-reaction involving oxidation of the secondary hydroxyl group might account for the discrepancy the method was applied to cholesterol acetate. The results agree closely with the relation

$$\text{mg. of cholesterol acetate} = 0.85 + 0.757 (\text{ml. of } 0.02 \text{ N } \text{Na}_2\text{S}_2\text{O}_3)$$

as shown in Table IX. The theoretical factor in this determination for cholesterol acetate is 0.7144, indicating only 94.4 per cent of the dibromide was precipitated. This is in very good agreement with the value obtained with cholesterol, considering the small number of samples of cholesterol acetate used.

TABLE IX

Application of the Cholesterol Method
to Cholesterol Acetate

Cholesterol Acetate Used	Titer 0.02 N $\text{Na}_2\text{S}_2\text{O}_3$ (corrected for blank)	Cholesterol Acetate Calculated	Deviation
mg.	ml.	mg.	mg.
10.10	12.41	10.25	-0.15
20.15	25.25	19.95	+0.20
29.95	38.52	30.00	-0.05
39.95	51.74	40.00	-0.05

In order to determine if the lacking bromine was still present organically bound in the filtrates, a series of cholesterol samples was brominated and precipitated as usual, the filtrates and washings kept at 0° , being collected in 100 ml. volumetric flasks. These mixtures were diluted to the mark with water at 0° , filtered again and the bromine determined in the second precipitate. The cholesterol was calculated from the titer using the theoretical conversion factor 0.6444. The results in Table X show that the theoretically required amount of bromine must still be organically bound, evidently as cholesterol dibromide. The titer has been corrected for a blank on the reagents of 0.64 ml.

TABLE X
Total Cholesterol Recovery

Cholesterol Used	Precipitate		Precipitate from Filtrate		Total Cholesterol Recovered	
	Titer	Cholesterol	Titer	Cholesterol		
	0.02 N	Calculated	0.02 N	Calculated		
	$\text{Na}_2\text{S}_2\text{O}_3$		$\text{Na}_2\text{S}_2\text{O}_3$			
mg.	ml.	mg.	ml.	mg.	mg.	per cent
10.05	13.35	8.60	2.46	1.58	10.18	101.3
19.95	27.85	17.94	3.46	2.23	20.17	101.1
30.00	42.35	27.29	4.56	2.94	30.23	100.8
39.90	56.84	36.63	5.33	3.43	40.06	100.4

It was also found that if the precipitating agent was diluted to about 50 per cent acetic acid instead of the approximately 80 per cent used normally in the method, all but a few tenths of a milligram of the cholesterol was recovered in the precipitate. These results suggest that an equilibrium is established under the bromination conditions between two forms of cholesterol dibromide, one of which is much less soluble than the other. Since epimerization of the hydroxyl group is not likely under these conditions, it seems probable that the formation of isomers arising from cis and trans addition to the double bond may account for the observed results. Although the investigations by Pirrone^{49, 50} and De Fazi and Pirrone⁵¹ indicate the existence of four cholesterol dibromides melting at about 97°, 106°, 114° and 122° respectively, they have succeeded in obtaining only cholesterol by debromination of these products. The limitations of time have not permitted a further investigation of the relation of the two forms evidently occurring in the present case.

The iodometric method for the cholesterol determination based on the

oxidative procedure is believed to be the most suitable one from the standpoint of the objectives of the present work. As shown, the method is sufficiently precise and accurate. The titration end point is one with which all analysts are familiar and the titer is not affected by the presence of water in the precipitates. As compared with the sodium iodide method the chief disadvantages are that the oxidative method requires more time and may be expected to be affected more by any non-sterol bromides which might precipitate.

THE ESTIMATION OF THE EGG CONTENT OF ALIMENTARY PASTES BY MEANS OF THE CHOLESTEROL DETERMINATION

The Cholesterol Content of Hen's Eggs

Review of the Literature. The cholesterol of the egg occurs practically entirely in the yolk. Dam⁷¹ and Kusui⁷² found only a trace in the entire white of an egg. Tillmans, Riffart and Kuhn¹² concluded from a limited number of analyses by their rather unsatisfactory method that all of the cholesterol was present in the free form. However, studies by Mueller⁷³, Thannhauser and Scheber⁷⁴, Dam⁷⁵ and Kusui⁷² indicate 10 per cent or more of the sterol in fresh eggs is esterified. Dam⁷¹ found from 0.468 per cent ~~to~~ 0.610 per cent of total cholesterol in individual eggs. Tillmans, Riffart and Kuhn¹² report an average of 1.49 per cent of free cholesterol in eight samples of egg yolk. Lampert²² found an average of 1.36 per cent of total cholesterol in the yolk. Perlman⁷⁶ reported from 0.54 per cent to 0.72 per cent of free cholesterol in twelve individual eggs, the average being 0.60 per cent. Riffart and Keller²⁵ found the average free cholesterol in three samples of yolk to be 1.49 per cent (titrimetric method) and 1.50 per cent (colorimetric method).

The Cholesterol Content of Eggs by the Precipitation Method Based on the Cholesterol Dibromide-Sodium Iodide Reaction. The cholesterol dibromide precipitation method is designed for application to the unsaponifiable matter, that is, for the determination of the total cholesterol content. Dam⁷⁷ showed that drying of lipid extracts containing cholesterol at 109° caused some decrease of the free sterol content. He found that the saponification of such extracts with sodium

ethylate in the presence of air led to some loss of cholesterol. He also reported that the total cholesterol in eggs could be extracted by heating the egg with 60 per cent potassium hydroxide solution for two to three hours and extracting the mixture with ether. This method was adapted to the present work. The egg is heated three hours on the steam bath with the alkali and the unsaponifiable matter extracted by a procedure developed from the Kerr-Sorber method⁷⁸ as modified by Hertwig, Jamieson, Baughman and Bailey⁷⁹. The cholesterol dibromide precipitation method was then applied to the unsaponifiable matter for the determination of the cholesterol.

In Table XI are the results obtained by the original method (Appendix, page 70) on the eggs used in the preparation of alimentary pastes of known composition. The latter will be referred to as authentic alimentary pastes. Since the method developed for use on alimentary pastes involved an acid hydrolysis before the saponification step, a sample of eggs was also subjected to acid hydrolysis prior to the alkali treatment. The results indicate that the acid hydrolysis does not affect the cholesterol determination in eggs materially.

These results by the cholesterol dibromide-sodium iodide method were not so satisfactory as desired, in considerable part due to the appearance of some color in the acetone extract which obscured the end point. The great variation in the amount of unsaponifiable matter also contributed to the lack of precision. In some earlier work it had been found that a very efficient aluminum oxide adsorbent for cholesterol could be prepared by ignition of basic aluminum acetate powder. Accordingly, the unsaponifiable matter was purified by adsorption on alumina from petroleum ether solution and elution of the adsorbed matter with ether. It was apparent that all of the adsorbed

matter was not eluted by the ether, but the sterol was recovered free of most of the colored substances which interfered with the titration. For convenience of reference the substances eluted by the ether have been called the adsorbed unsaponifiable matter. The results obtained by this method (Appendix, page 72), when applied to the eggs previously analyzed, are also shown in Table XI.

TABLE XI

The Cholesterol Content of Eggs Used in Authentic
Alimentary Pastes by the Cholesterol
Dibromide-Sodium Iodide Procedure

Description: of Sample	Original Method			Adsorption Method		
	Total	Unsaponi- fiable	Cholesterol in the Solids	Total	Absorbed Unsaponi- fiable	Cholesterol in the Solids
	per cent	per cent	per cent	per cent	per cent	per cent
Frozen whole eggs	27.47	3.14*	2.19*	27.49	2.36	2.26
	27.30	3.14*	2.30*		2.36	2.26
		3.10	2.23			
		2.85	2.34			
Frozen yolk	46.53	3.87	2.97	46.67	3.12	2.98
	46.54	4.30	3.03		3.07	3.00
		3.93	3.01			
Chinese dried yolk	97.32	3.36	2.89	97.55	3.95	2.87
	97.37	3.63	2.85		2.97	2.86

*Subjected to acid hydrolysis before saponification.

It is evident that the adsorption procedure greatly improved the precision of the unsaponifiable matter determination, though the latter does not include all of the unsaponifiable matter. The precision of

the cholesterol determination is also markedly improved.

The above analyses were made in the spring of 1936. The frozen eggs were kept at about -10° C., while the dried yolk was stored at about 5° C. until subsequent analyses were made in the spring of 1940.

The Cholesterol Content of Eggs by the Precipitation Method with the Oxidative Iodometric Procedure. One of the purposes in the development of the oxidative iodometric procedure was to eliminate the difficulty experienced in the former procedure due to the color in the acetone solutions of the precipitates. The adsorption technique reduced this interference considerably but included certain features to which objection could be raised. The adsorption step was therefore eliminated. It was noted (Table XI) in the results for the unsaponifiable matter by the original method that the pair which had been obtained by an acid hydrolysis before saponification agreed perfectly, while the other results varied considerably. The acid hydrolysis step was therefore included in applying the new procedure to the eggs previously examined, with the results shown in Table XII under "Acid Hydrolysis Method".

Although the results were very good for the cholesterol determination, the unsaponifiable matter method was still unsatisfactory. In the course of these determinations it was noted that in some cases finely divided calcium soaps suspended in the ether extracts were not entirely removed by the filtration through cotton. These were removed by filtration through anhydrous sodium sulfite, however, and the direct saponification procedure was tried with this method of filtration. The results obtained on the same eggs used in the previous analyses are shown in Table XII under the heading "Revised Method". Between each

series of analyses the thawed eggs were refrozen, so the variations in results as between series may be taken as including over all sampling errors. On the basis of these results this procedure (Appendix, page 83) was adopted for the determination of the cholesterol content of eggs.

TABLE XII

The Cholesterol Content of Eggs Used in Authentic
Alimentary Pastes by the
Oxidative Iodometric Procedure

Description of Sample	Total Solids	Acid Hydrolysis Method		Revised Method	
		Unsaponifi- able Matter in Solids	Cholester- ol in Solids	Unsaponifi- able Matter in Solids	Cholester- ol in Solids
	per cent	per cent	per cent	per cent	per cent
Frozen	26.91	2.66	2.27		
whole eggs:	26.91	2.77	2.26		
		2.82	2.26		
				2.66	2.20
	26.87			2.84	2.19
				2.88	2.19
				2.78	2.25
	26.73			2.79	2.26
				2.77	2.24
Frozen	46.11	3.56	2.94		
yolk	46.09	3.82	2.98		
		3.59	2.97		
				3.43	2.97
	46.15			3.45	2.97
				3.50	2.96
Chinese	96.94	3.62	2.84		
dried yolk:	96.96	3.57	2.79		
				3.45	2.81
	96.97			3.39	2.80

The Cholesterol Content of Commercial Frozen Eggs and Dried Eggs.

During 1936 samples of commercial frozen eggs were collected at egg packing plants in various parts of the country by inspectors on the field staff of the Food and Drug Administration, United States Department of Agriculture. While maintained in the frozen condition these samples were sent to Washington, D. C., and were stored at about -10° C. Due to unavoidable circumstances analyses of these samples could not be made at the time. Since the results obtained on the eggs used in the preparation of the authentic alimentary pastes showed no significant differences under like storage conditions for the same period, a number of the commercial samples were analyzed by the new method. In addition, a few samples collected recently from storage lots of the 1939 pack have been analyzed, as have also several samples of dried egg yolk. The amount of cholesterol found in the solids of commercially broken out eggs will depend to a considerable extent on the breaking and particularly on the separating practices of the various plants. Since practically all of the fat and the cholesterol occur in the yolk, the amount of cholesterol in the fat is a better index of the variation in the cholesterol content of eggs from various regions than is the amount of cholesterol in the solids. Therefore the fat by acid hydrolysis has also been determined on the commercial samples by the A. O. A. C. method⁸⁰. The results of these analyses are tabulated in Tables XIII, XIV and XV. The figures in parentheses are duplicate results obtained after the original sample had been re-frozen following the first analysis. They are not included in the averages. Sample No. 5 of Table XIII has also been omitted from the averages in view of its abnormally high solids content for whole egg. This value (29.46 per cent) is far outside the range (25.97 per cent to

28.33 per cent) observed by Mitchell, Alfend and McFall⁹¹ in seventy-four samples of commercially broken out whole eggs. Otherwise the variation in the cholesterol content is essentially the same as was observed by the last-named workers for other egg yolk constituents.

The accumulation of data on the cholesterol content of commercial egg products has not been carried far in the present work, partly because it is only since the 1939 season closed that the legal definitions and standards of identity for the various products as promulgated last year became effective. Operations under these standards may significantly affect the composition of the commercial egg products and it has therefore been considered inadvisable to complete analysis of all the old samples on hand.

The results obtained, however, demonstrate several important facts. It is evident that the cholesterol content of commercially broken out whole eggs and yolks does not vary too much to permit use of the cholesterol content of foods as an index of the egg content. Moreover, the cholesterol content of frozen eggs and dried eggs shows no change on storage over long periods. It is likewise shown that the cholesterol content of eggs does not change when the eggs are dried.

TABLE XIII

The Cholesterol Content of Commercial Frozen Whole Eggs

No.:	Year	Source of : Packed:Shell Eggs: (State)	Total : Solids	Fat by Acid:Hydrolysis : in Solids	Unsaponi-:fiable : Matter : in Solids:	Cholester-:ol in : Solids	Cholester-:ol in : Fat
:	:	:	per cent:	per cent	per cent:	per cent	per cent
1 :	1936 :	Neb.-Ia. :	26.84 :	45.12	2.75 :	2.13 :	4.72
2 :	" :	? :	27.26 :	45.67	3.17 :	2.23 :	4.89
3 :	" :	Calif. :	27.73 :	48.21	2.69 :	2.13 :	4.42
4 :	" :	Mo.-Kans.:	25.81 :	43.63	2.60 :	2.08 :	4.77
:	:	:	(25.65):	---	(2.61) :	(2.06) :	--
5 :	" :	Ide. :	(29.46):	(47.18)	(3.01) :	(2.47) :	(5.24)
:	:	:	(29.29):	---	(3.04) :	(2.46) :	--
6 :	" :	Mo.-Kans.:	26.85 :	45.51	2.77 :	2.13 :	4.69
7 :	" :	Calif. :	26.50 :	46.19	2.65 :	2.03 :	4.39
:	:	:	(26.63):	---	(2.48) :	(1.99) :	--
8 :	" :	Colo. :	26.26 :	45.54	2.51 :	2.02 :	4.44
:	:	:	(26.51):	---	(2.55) :	(2.01) :	--
9 :	" :	Kans. :	26.51 :	44.93	2.74 :	2.20 :	4.90
10 :	" :	Wash. :	26.09 :	45.15	2.69 :	2.06 :	4.56
:	:	:	(26.11):	---	(2.52) :	(2.03) :	--
11 :	1939 :	? :	27.27 :	44.30	2.44 :	2.05 :	4.63
12 :	" :	? :	26.73 :	45.45	2.76 :	2.10 :	4.82
13 :	" :	? :	25.46 :	44.15	2.76 :	2.14 :	4.85
:	:	:	:	:	:	:	:
		Maximum :	27.73 :	48.21	3.17 :	2.23 :	4.90
		Minimum :	25.46 :	43.63	2.44 :	2.02 :	4.39
		Average :	26.61 :	45.32	2.71 :	2.11 :	4.66
		:	:	:	:	:	:

TABLE XIV

The Cholesterol Content of Commercial
Frozen Egg Yolks

No.:	Year	Source of	Total	Fat by Acid	Unsaponi-	Cholesterol-	Cholesterol-
:	:	Packed:Shell Eggs:	Solids:	Hydrolysis	fiabile	ol in	ol in
:	:	(State)	:	in Solids	Matter	Solids	Fat
:	:	:	:	in Solids:	:	:	:
:	:	:	per cent	per cent	per cent	per cent	per cent
:	:	:	:	:	:	:	:
1	1936	Tex.	43.54	59.92	3.59	2.88	4.81
2	"	Neb.-Ia.	45.50	61.54	3.77	2.93	4.76
3	"	Calif.	45.80	61.29	3.55	2.86	4.67
4	"	Kans.	46.07	61.23	4.00	3.15	5.12
			(46.24)	---	(3.74)	(3.11)	--
			:	:	:	:	:
5	"	?	44.10	60.61	3.51	2.80	4.62
6	"	Ia.	43.51	61.16	3.70	2.88	4.71
7	"	Mo.-Kans.	40.43	57.98	3.39	2.77	4.73
8	"	Calif.	42.49	59.54	3.49	2.69	4.52
			(42.37)	---	(3.26)	(2.68)	--
			:	:	:	:	:
9	"	Wash.	41.54	58.76	3.39	2.66	4.53
			(41.63)	---	(3.38)	(2.73)	--
			:	:	:	:	:
10	"	Ida.	42.63	59.10	3.88	3.00	5.08
			(42.69)	---	(3.43)	(2.96)	--
			:	:	:	:	:
11	"	Colo.	46.10	60.60	3.54	2.86	4.73
12	"	?	44.67	60.53	3.88	2.89	4.77
13	1939	?	44.41	---	3.52	2.95	--
14	"	?	46.11	61.05	3.46	2.96	4.85
15	"	?	45.78	60.35	3.48	2.89	4.79
			:	:	:	:	:
		Maximum	46.11	61.54	4.00	3.15	5.12
		Minimum	40.43	57.98	3.39	2.66	4.52
		Average	44.18	60.25	3.61	2.83	4.77
			:	:	:	:	:

TABLE XV

The Cholesterol Content of Commercial Dried Eggs

No.:	Description	Year	Source	Total	Fat by	Unsepon-	Choles-	Choles-
:	of Sample	Pre-	of	Solids	Acid	ifiable	terol	terol
:	:	pared:	Shell	:	Hydroly-	Matter	in	in
:	:	:	Eggs	:	sis in	in	Solids	Fat
:	:	:	(State):	:	Solids	Solids	:	:
:	:	:	:	per cent:	per cent:	per cent:	per cent	per cent
:	:	:	:	:	:	:	:	:
1	Dried whole	1936:	Mo.-Kan.	97.94	45.73	2.66	2.20	4.81
:	egg*	:	:	:	:	:	:	:
2	Dried yolk**	"	Mo.-Kan.	97.70	57.83	3.48	2.78	4.81
3	"	1939:	?	97.20	61.56	3.45	2.94	4.78
4	"	"	?	95.58	60.99	3.48	2.91	4.77
5	"	"	?	95.59	60.68	3.48	2.84	4.68
:	:	:	:	:	:	:	:	:
		Maximum	:	97.70	61.56	3.48	2.94	4.81
		Minimum	:	95.58	57.83	3.45	2.78	4.68
		Average	:	96.82	60.26	3.47	2.87	4.76
:	:	:	:	:	:	:	:	:

*Prepared from the same lot of liquid egg as Sample No. 4, Table XIII

**Prepared from the same lot of liquid yolk as Sample No. 7, Table XIV

The Sterol Content of Farinaceous Ingredients
of Alimentary Pastes

Review of the Literature. There are but a few published investigations regarding the quantity of sterols in flour and similar wheat products. Tillmans, Riffart and Kuhn¹² found from 0.0108 per cent to 0.0256 per cent of free sterol calculated as cholesterol on the dry basis in wheat flour and grits (farina?). Leulier and Crevat⁸² report 0.039 per cent of sterol in a soft wheat flour. Keding⁸³ obtained 0.099 per cent of sterol by ether extraction and 0.115 per cent by hydrolysis and then extraction. Riffart and Keller²⁵ give the free sterol content on the dry basis in wheat flour and grits as from 0.0111 per cent to 0.0253 per cent calculated as cholesterol. Costa⁸⁴ found from 0.012 per cent to 0.015 per cent of sterol calculated

as cholesterol on the dry basis in four Italian semolinas. Hagemann⁶⁵ was cited as having found 0.034 per cent of total sterol in hard flour and Sullivan and Howe⁶⁶ obtained 0.018 per cent of total sterol by petroleum ether extraction of a straight hard wheat flour.

The Sterol Content of Farinaceous Ingredients by the Precipitation Method Based on the Cholesterol Dibromide-Sodium Iodide Reaction. The cholesterol method was originally designed for application to 20 g. samples of alimentary pastes. It seemed possible that the unsaponifiable matter might be extracted from such samples after direct saponification without prior extraction of the fat, which is a time-consuming process and requires the use of an automatic extractor. The saponification could not be made directly but after a short acid hydrolysis which liquefied the sample the alkaline hydrolysis was applicable. By adding the alkali in the form of pellets of potassium hydroxide, the acid was neutralized and excess alkali sufficient to produce about a 60 per cent concentration in the liquid phase was introduced and the mixture heated on the steam bath for three hours, as in the procedure on eggs. The unsaponifiable matter was then extracted by a procedure based on the modified Kerr-Sorber method. To the unsaponifiable matter was added 30 mg. of cholesterol to provide the excess required as indicated by the coprecipitation studies and the usual cholesterol dibromide precipitation procedure was applied. The difference between the cholesterol found and that added represented the sterol present in the flour. This procedure (Appendix, page 80) was applied to the flour used in the preparation of the authentic alimentary pastes and to several other wheat products, with the results shown in Table XVI under "Original Method". There are also included under "Adsorption Method" the results obtained by application of the adsorption process

(Appendix, page 81) described in connection with the methods for the determination of the cholesterol content of eggs.

It is evident from the data that the adsorption method yields the same results as the original method for the sterol content, though, as is the case with eggs, the adsorbed unsaponifiable matter is much lower than the total unsaponifiable matter. These preliminary results also indicated that the sterol content of the various farinaceous ingredients did not show great variation.

TABLE XVI

The Sterol Content of Farinaceous Ingredients
of Alimentary Pastes by the Cholesterol
Dibromide-Sodium Iodide Procedure

Description of Sample	:	:	Original Method		:	Adsorption Method	
			Unsaponi- fiable Matter in Solids	Sterol(as Cholesterol) in Solids		Adsorbed Unsaponi- fiable Matter in Solids	Sterol(as Cholesterol) in Solids
	:	:	per cent	per cent	:	per cent	per cent
Durum Flour*	:	:			:		
Batch No. 1	:	:	86.13	0.132	:	--	--
Batch No. 3	:	:	86.15	0.131	:	--	--
Batch No. 6	:	:	86.15	0.130	:	--	--
Batch No. 10	:	:	86.13	0.131	:	0.080	0.036
No. 1 Semolina	:	:	85.48	0.131	:	--	--
Standard Semolina	:	:	85.56	0.137	:	--	--
Texas Farina	:	:	86.68	0.106	:	--	--
Kansas Hard Flour	:	:	86.93	0.113	:	--	--
Hard Spring Flour	:	:	88.65	0.102	:	0.058	0.033

*Flour used in the preparation of authentic alimentary pastes

The Sterol Content of Farinaceous Ingredients of Authentic Alimentary Pastes by the Precipitation Method Based on the Oxidative Iodometric Procedure. With the development of the oxidative iodometric method which permits satisfactory cholesterol determinations on smaller samples the procedure was revised to apply to 10 g. of flour or alimentary pastes instead of the 20 g. previously used. The sample size might be reduced even more so far as the cholesterol determination is concerned but the weight of unsaponifiable matter would be too small to permit reasonably accurate determinations with an ordinary analytical balance. The unsaponifiable matter determination should be retained in this method since it is likely to be of value in detecting the adulteration of alimentary pastes.

The samples of the durum flour used in the authentic alimentary pastes and previously analyzed in 1936 as reported above in Table XVI had been stored since that time in tightly closed Mason jars at a temperature of about 5° C. The earlier analyses indicated the samples to be identical. When the new procedure was applied to this flour in 1940 using 10 g. samples with the adsorption technique, slightly lower results were obtained than in 1936. That the difference was not due to the method of determining the bromine was shown by applying the cholesterol dibromide-sodium iodide method. The same results were obtained when the new procedure was applied without the adsorption step, with the addition of either 10 mg. or 20 mg. of cholesterol to the unsaponifiable matter before the bromination and precipitation. The results obtained in these studies are shown in Table XVII.

TABLE XVII

The Sterol Content of Flour After Storage
at 5° C. from 1936 to 1940

Analytical Method	: :Cholesterol: : added to :Unsaponifi- :able Matter:	: : Unsaponifiable : Matter : in the Solids : Adsorbed Total	: : Sterol (as :Cholesterol): : in the : Solids	: : Decrease : in Sterol : Content : 1936-1940
	: : mg. :	: : per cent :	: : per cent :	: : per cent :
Cholesterol dibromide- sodium iodide	: : 10.4 :	: : 0.075 :	: : -- :	: : 0.031 :
Cholesterol dibromide- sodium iodide	: : 9.9 :	: : 0.075 :	: : -- :	: : 0.034 :
Oxidative iodometric	: : 10.0 :	: : 0.075 :	: : -- :	: : 0.031 :
" "	: : 10.4 :	: : 0.075 :	: : -- :	: : 0.032 :
" "	: : 10.1 :	: : -- :	: : 0.115 :	: : 0.032 :
" "	: : 20.0 :	: : -- :	: : 0.114 :	: : 0.030 :

The decrease is a very slight one but still may be significant, particularly in view of the fact that a slight decrease in the sterol content of the alimentary pastes of about the same order was observed under similar storage conditions. In developing the revised procedure for the cholesterol determination a study of the effect of the various conditions (acid hydrolysis, saponification, drying, etc.) on cholesterol alone indicated that the presence of alcohol during the hydrolysis and saponification as in the original method led to some loss of the cholesterol. The alcohol was originally added to decrease the tendency of the flour to lump on addition of the acid. However the lumps are readily broken up by occasional shaking of the mixture during the hydrolysis. Though the omission of alcohol did not produce any noticeable difference in the sterol content found by the method in alimentary pastes, it was considered advisable to omit it from the revised method.

The Recovery of Cholesterol Added to Flour by the Revised Method.

The revised method (Appendix, page 89) was tested for the recovery of cholesterol added to flour. For this purpose the sterol found in 10 g. samples of flour to which cholesterol had been added was corrected for the sterol found in the flour by the usual method of adding cholesterol to the unsaponifiable matter extracted from 10 g. of the flour. The results are shown in Table XVIII. The slight amount of cholesterol not recovered is probably lost in the washing out of the soaps from the ether extract of the saponification mixture. It was found that the unsaponifiable matter in the saponification mixture itself is completely extracted by the procedure used.

TABLE XVIII

Recovery of Cholesterol Added to Flour

Cholesterol Added		:	:	:	:	:
To Flour	To Unsaponifiable Matter	:	Sterol (as Cholesterol)	:	Sterol in the Flour	: Cholesterol Recovered
		:	Found	:		
mg.	mg.	:	mg.	:	mg.	mg. per cent
0	10.0	:	12.7	:	2.7	-- --
0	20.0	:	22.7	:	2.7	-- --
10.0	0	:	12.3	:	2.7	9.6 96
20.0	0	:	22.1	:	2.7	19.4 97

The Sterol Content of Farinaceous Ingredients Commercially Used in Alimentary Pastes by the Revised Method. The revised method was applied to the determination of the sterol content of samples of flour, durum flour and semolina actually in use commercially in the manufacture of alimentary pastes. These samples were collected at factories in various parts of the United States by inspectors on the field staff of the Food and Drug Admin-

istration. The results are tabulated in Table XIX. The values for the fat by acid hydrolysis on these samples are taken from reports by analysts in the field stations of the Food and Drug Administration.

The sterol content of the farinaceous ingredients shows little variation and the average is quite low, thus decreasing the uncertainty in applying a correction factor in the calculation of the egg content of alimentary pastes.

TABLE XIX

The Sterol Content of Farinaceous Ingredients
of Commercial Alimentary Pastes

No.:	Description of Sample	Total Solids	Unseponi- fiable Matter	Sterol(as Cholesterol): in Solids	Fat by Acid: Hydrolysis	Sterol in Fat
:	:	per cent	per cent	per cent	per cent	per cent
1 :	Durum flour	87.95	0.10	0.022	2.22	.99
2 :	"	86.83	0.11	0.023	1.82	1.26
3 :	"	87.65	0.11	0.025	2.50	1.00
4 :	"	88.59	0.11	0.027	2.47	1.09
5 :	"	86.85	0.10	0.022	1.82	1.21
6 :	"	89.02	0.10	0.021	--	--
7 :	"	88.74	0.12	0.026	2.25	1.16
8 :	"	88.94	0.10	0.021	1.93	1.09
9 :	"	89.12	0.10	0.021	1.59	1.32
10 :	"	88.14	0.12	0.025	2.09	1.20
11 :	"	89.64	0.10	0.022	1.84	1.20
12 :	"	89.34	0.12	0.031	2.44	1.27
13 :	Semolina	88.83	0.10	0.021	--	--
14 :	"	88.61	0.10	0.023	1.81	1.27
15 :	"	88.23	0.11	0.023	1.45	1.61
16 :	"	89.67	0.11	0.023	--	--
17 :	"	89.96	0.10	0.022	2.40	.92
18 :	"	87.74	0.10	0.023	1.76	1.31
19 :	Flour	88.97	0.10	0.025	1.98	1.26
20 :	"	88.40	0.14	0.044	2.15	2.05
21 :	"	87.92	0.10	0.024	1.76	1.36
22 :	"	88.24	0.10	0.025	2.05	1.22
23 :	"	88.55	0.10	0.028	1.91	1.47
24 :	"	89.31	0.10	0.021	1.73	1.21
Maximum		89.96	0.14	0.044	2.50	2.05
Minimum		86.83	0.10	0.021	1.45	.92
Average		88.54	0.11	0.0245	2.00	1.26

The Sterol Content of Alimentary Pastes as an Index of the Egg Content

Review of the Literature. Popp⁴⁰ reported briefly on the possibility of using Winclaus' cholesterol dibromide reaction for estimating the egg content of alimentary pastes but gave no data. Cappenberg⁶⁷ published a note describing a procedure which involved weighing the cholesterol extracted from 400 to 500 g. samples of noodles but also failed to give any data. No further work on this subject appeared until 1930 when Tillmans, Riffart and Kuhn¹² published the micro method based on an adaptation of the Szent-Györgyi²⁶ procedure. This method was discussed above. Soldi and Testore⁸⁸ reported a colorimetric method applicable to approximate estimation of the egg content of alimentary pastes. Riffart and Keller²⁵ considerably improved the oxidative digitonin procedure as applied to noodles by adapting the Yusada²⁹ modification to these products. They also devised a colorimetric procedure which gave good results in their hands, but is handicapped by an inconvenient color measurement which must be made at frequent intervals over periods up to an hour to determine the maximum. Kluge⁸⁹ and Costa⁸⁴ have applied a combination of the sterol and lipid phosphorus determinations to alimentary pastes for detecting adulteration of the latter with plant lecithins. Terrier³⁵ discussed difficulties encountered in applying a gravimetric digitonin method to the estimation of the cholesterol in egg pastes. Subsequently Terrier³⁶ described the method previously commented upon which depends on the loss in weight of the digitonide precipitate observed on decomposing the molecular compound with boiling xylene, this loss representing cholesterol.

The Sterol Content and Egg Content of Authentic Alimentary Pastes by the Precipitation Method Based on the Cholesterol Dibromide-Sodium Iodide Reaction. In 1936 H. A. Lepper of the Food and Drug Administration personally supervised the preparation of a series of alimentary

pastes of various known egg contents by usual commercial methods at the plant of A. Zerega's Sons, Inc., Brooklyn, New York. A summary of his report of the preparation of these noodles is given in the Appendix, page 91.

. The actual egg solids content of the noodles was calculated from the solids content of the egg products and flour used as given above in Tables XI and XVI respectively. The various products were identified by batch numbers. The liquid eggs used were freshly broken. Samples of these for analysis were frozen for preservation and are so designated in Table XI.

The sterol content of the authentic alimentary pastes was determined by the original method (Appendix, page 80) and for a few samples by the adsorption method (Appendix, page 81). The results of the analysis are shown in Table XX. In Table XXI are the percentages of egg solids found as calculated from the general formula

$$E = \frac{(S - f)100}{e - f}$$

in which E = the percentage of egg solids in the total solids of the sample

S = the percentage of sterol in the total solids of the sample

e = the average percentage of cholesterol in the egg solids used

f = the average percentage of sterol in the total solids of flour.

The values used in the present calculations are the average values found by the respective methods in the eggs and flour, Tables XI and XVI respectively.

TABLE IX

The Sterol Content of Authentic Alimentary Pastes by the
Cholesterol Dibromide-Sodium Iodide Procedure

Batch:	Total	Original Method		Adsorption Method	
No. :	Solids	Unsaponi-	Sterol (as	Adsorbed Unsaponi-	Sterol (as
:	:	fiabile	Cholesterol)	fiabile Matter	Cholesterol)
:	:	Matter	in Solids	in Solids	in Solids
:	:	in Solids	:	:	:
:	per cent	per cent	per cent	per cent	per cent
:	:	:	:	:	:
1	88.79	0.13	0.035	--	--
2	88.82	0.23	0.123	--	--
3	89.62	0.30	0.197	0.249	0.197
4	89.72	0.45	0.331	0.389	0.330
5	89.21	0.50	0.377	--	--
5*	89.21	0.58	0.377	--	--
6	88.85	0.27	0.160	0.216	0.160
7	88.19	0.39	0.276	0.329	0.276
8**	88.39	0.31	0.191	--	--
9**	89.33	0.14	0.040	--	--
10***	89.29	0.15	0.036	--	--
11	90.77	0.29	0.186	0.239	0.185
:	:	:	:	:	:

*10 gram sample used instead of 50 gram sample used in other cases

**Contains added carotene in oil

***Contains added Vita-Pro (commercial carotene in oil preparation)

TABLE XXI

Egg Content of Authentic Alimentary Pastes by the
Cholesterol Dibromide-Sodium Iodide Procedure

Batch No.	Description of Eggs Used	Egg Solids Present in Total Solids	Original Method		Adsorption Method	
			Egg Solids in Total Solids (found)	Egg Solids Recovered per cent	Egg Solids in Total Solids (found)	Egg Solids Recovered per cent
1	--	0.0	0.0	--	--	--
2	Fresh yolk	3.1	2.9	94	--	--
3	" "	5.6	5.4	96	5.4	96
4	" "	10.3	9.9	96	10.0	97
5	" "	12.2	11.5	94	--	--
6	Fresh whole egg	5.6	5.5	98	5.6	100
7	Fresh whole egg	11.3	10.7	95	10.9	96
8*	Fresh yolk	5.2	5.2	100	--	--
9*	--	0.0	0.1	--	--	--
10**	--	0.0	0.0	--	--	--
11	Chinese dried yolk	5.3	5.3	96	5.3	96

* Contains added carotene in oil

** Contains added Vite-Pro (commercial carotene in oil preparation)

It is evident that the method yields quite satisfactory results for the determination of the egg solids content of alimentary pastes over a wide range of composition.

The present tentative method for determining whether an egg noodle contains whole egg solids or yolk solids is given in the Methods of Analysis of the Association of Official Agricultural Chemists, Third Edition⁹⁰. This method has not been entirely satisfactory and progress has been made on the improvement of the method. However this work has not been completed and is a subject for later reporting.

The Sterol Content and Egg Content of Authentic Alimentary Pastes

by the Revised Method. The authentic alimentary pastes prepared in 1936 have been stored since that time in cardboard cartons in a constant temperature room at 5° C. Although it will be necessary eventually to test the revised method (Appendix, page 89) using a series of freshly prepared noodles of known composition, it has not been considered advisable to go to the necessary expense at this time, in view of the fact that new legal standards for noodles are now under consideration. It is also desirable that work be completed on other determinations, such as the crude albumen nitrogen method, before such a series of authentic noodles is prepared. However the application of the revised method to the authentic alimentary pastes prepared in 1936 can be used to test its accuracy in the estimation of the egg solids. The method was applied to these samples, with the results shown in Table XXII. It was shown above that a slight decrease in the sterol content of the flour used in these samples had occurred under like conditions of storage. For the purpose of determining the recovery of egg solids by the revised method, the calculations by the general formula given above were made, taking the sterol content of 0.031 per cent for the flour as determined by the revised method and the average sterol content of the eggs used as determined by the same method. The recovery of egg solids appears to be somewhat better by the revised method. In an alimentary paste of low egg content prepared with a flour of higher than average sterol content (the case in the present series) the cholesterol content may approach the minimum one to one ratio of cholesterol to sitosterol required according to the coprecipitation studies. It is therefore advisable in the case of samples below the standard in egg content to add 10 mg. of cholesterol to the unsaponifiable matter before the dibrom-

ide precipitation. In the application of the method practically to samples of unknown composition the advisability of adding the cholesterol can be judged from the content of unsaponifiable matter. The presence of less than the 0.27 per cent of unsaponifiable matter found in a standard whole egg noodle may be taken as a guide. The sterol content obtained in the case of Batch Number 2 in this series is noted to be somewhat higher when cholesterol is added before the precipitation, though the effect on the indicated egg content is not of serious consequence from a practical standpoint.

TABLE XXII

The Sterol Content and Egg Content of Authentic
Alimentary Pastes by the Revised Method

Batch No.	Description of Eggs Used	Total Solids	Egg Solids Present in Total Solids	Unsaponifiable Matter in Total Solids	Sterol (as Cholesterol) in Total Solids	Egg Solids in Total Solids	Egg Solids Recovered
		per cent	per cent	per cent	per cent	percent	per cent
1	--	87.65	0.0	0.12	0.029	0.0	--
2	Fresh yolk	87.37	5.1	0.22	0.112	2.8	90
2*	" "	87.37	5.1	0.22	0.113	3.0	97
3	" "	87.83	5.6	0.31	0.192	5.5	98
4	" "	87.62	10.3	0.47	0.331	10.2	99
5	" "	88.07	12.2	0.55	0.406	12.8	105
6	Fresh whole egg	87.06	5.6	0.27	0.154	5.6	100
7	" " "	87.13	11.3	0.40	0.267	10.7	95
8**	Fresh yolk	86.92	5.2	0.32	0.191	5.5	106
9**	--	87.08	0.0	0.15	0.040	0.3	--
10***	--	87.25	0.0	0.13	0.033	0.1	--
11	Chinese dried yolk	87.44	5.5	0.30	0.181	5.4	98

* 10 mg. of cholesterol added to the unsaponifiable matter before precipitation

** Contains added carotene in oil

*** Contains added Vita-Pro (commercial carotene in oil preparation)

The above results are sufficient to show that the revised method gives a good recovery of the egg solids in alimentary pastes over a wide range of composition. This method does not require any reagents which are unduly difficult or expensive to obtain in a satisfactory state of purity nor any apparatus not normally available in the food laboratory. Moreover, since the determination is not sensitive to slight variations in conditions and involves no unusual manipulative operations, it is believed that the method will give good results not only in the hands of analysts inexperienced in its use but also in different laboratories. Tentative formulas for the calculation of the egg content from the sterol content of alimentary pastes are given in the Appendix, page 92.

The Effect of Storage on the Sterol Content and Indicated Egg Content of Alimentary Pastes

It was pointed out above that one of the chief disadvantages in the use of the lipid phosphorus as an index of the egg content of noodles was the decrease in the lipid phosphorus content on storage. The results given in Tables XX and XXII are an indication that the sterol content of alimentary pastes does not decrease to a serious extent on four years' storage at 3° C. An even more severe test which may be expected to have a far more drastic effect on the noodles than any storage conditions conceivably to be encountered in practice has also been made. The ground samples of noodles prepared for analysis in April, 1936, were kept in closed Mason jars under laboratory conditions until March, 1940, during which time they had been exposed to the high temperatures prevailing in Washington for four summers. These samples had developed a strong, musty odor. When analyzed at the end of this time by the revised method, the samples showed the sterol contents indicated in Table XXIII.

TABLE XXIII

The Sterol Content and Indicated Egg Content of Authentic
Alimentary Pastes After Four Years' Storage
at Room Temperature

Batch No.	Description of Eggs Used	Total Solids	Egg Solids Present in Total Solids	Unsaponifiable Matter in Total Solids	Sterol (as Cholesterol) in Total Solids	Egg Solids in Total Solids (found)	Egg Solids Recovered
		per cent	percent	per cent	per cent	percent	per cent
1	--	90.53	0.0	0.12	0.025	0.0	--
2	Fresh yolks	90.49	3.1	0.22	0.107	2.4	77
2*	" "	90.49	3.1	0.22	0.116	2.7	87
3	" "	90.57	5.6	0.32	0.190	5.2	93
4	" "	89.22	10.3	0.47	0.328	9.9	96
5	" "	91.06	12.2	0.53	0.391	12.1	99
6	Fresh whole egg	90.30	5.6	0.27	0.151	5.2	93
7	Fresh whole egg	89.19	11.3	0.40	0.267	10.5	93
8**	Fresh yolk	90.26	5.2	0.32	0.188	5.1	98
9**	--	89.70	0.0	0.14	0.036	0.0	--
10***	--	89.85	0.0	0.13	0.029	0.0	--
11	Chinese dried yolk	91.04	5.5	0.29	0.177	5.1	93

* 10 mg. of cholesterol added to the unsaponifiable matter before precipitation

** Contains added carotene in oil

*** Contains added Vita-Pro (commercial carotene in oil preparation)

The indicated egg content has been calculated by the general formula, taking the average sterol content of the eggs used as determined by the revised method but taking for the sterol content of the flour the original value of 0.037 percent found in 1936. This gives the minimum egg solids content on the basis of the sterol content. It is noted that even under these extreme conditions practically all of the sterol originally present can be recovered. It is worthy of note that in both series of determinations made in 1940 the chief loss of sterol seems to be due to the sterols

of the flour.

It was shown above that the sterol content of frozen eggs does not change on prolonged storage and that the sterol content of liquid eggs does not change on drying. From the standpoint of regulatory operations, therefore, it can be concluded that the sterol content of a sample of alimentary paste is essentially independent of the type of eggs used, fresh, frozen or dried, and of the conditions of storage.

EXPERIMENTAL PART

The Procedure Used in the Gravimetric and Argentometric Studies of the Cholesterol Dibromide Precipitation

The Bromination and Precipitation Procedure. The cholesterol in a 25 x 150 mm. test tube was dissolved in 2 ml. of anhydrous ether and the solution was cooled in a bath to the desired temperature. To the solution was added 0.3 ml. of a solution of bromine in carbon tetrachloride. After the solution had been in the bath 10 min. there was added 10 ml. of acetic acid solution (4 vol. of glacial acetic acid + 1 vol. of water), also cooled to the bath temperature, and the mixture was vigorously stirred. After 5 min. the precipitate was filtered with suction, washed once with 5 ml. of the acetic acid solution at the bath temperature and then with water until the washings were neutral to litmus. For the gravimetric determinations the filtration was made on a tared Jena G4 sintered glass crucible; the precipitate was dried first in vacuo over sulfuric acid and then by aspiration with dry air to constant weight.

The Argentometric Method. In the initial development of the argentometric method a sample of cholesterol dibromide was used which had been prepared according to Lifschütz⁴⁷ and found to contain 27.42 per cent and 27.47 per cent of bromine in duplicate determinations on 0.5 g. samples by the Duin⁵³ modification of the Stepanow⁵⁴ sodium reduction method. In the first argentometric procedure used on the semi-micro scale samples of the above preparation were dissolved in ether-alcohol mixtures and evaporated after addition of 0.3 ml. of 50 per cent potassium hydroxide solution. The residue was treated with 2 ml. of 20 per cent potassium bicarbonate solution and the residue after evaporation was ignited for 30 min. at 500°.

The ash was taken up in water, the solution filtered and after acidifying slightly with acetic acid the bromide in the filtrate was titrated with 0.01 silver nitrate, using 1 drop of 0.5 per cent eosin as indicator. The bromine found in the above sample was 27.37 per cent. It was later found unnecessary to ignite or filter the residue from the alkaline hydrolysis and the simplified procedure as described below was adopted.

The cholesterol dibromide precipitate obtained as described above was dissolved from the filter with ether and alcohol and the solution received in a 125 ml. Erlenmeyer flask. After addition of 0.3 ml. of 50 per cent potassium hydroxide the mixture was evaporated on the steam bath. The residue was taken up in 40 ml. of water and 4 ml. of 15 per cent acetic acid was added. To the solution was added 5 ml. of 0.01 N potassium bromide solution and the bromide was titrated with 0.01 N silver nitrate using 1 drop of 0.5 per cent eosin solution as indicator. The titer was corrected for the added bromide and a blank determination on the reagents.

The Experimental Data Used in the Construction of Figure 1

The Phytosterols Used in the Coprecipitation Studies. The phytosterols were isolated from wheat germ oil kindly furnished by Dr. George F. Jamieson, of the United States Department of Agriculture, using essentially the procedure of Anderson, Shriner and Surr⁶¹. Two preparations had respectively melting points of 137°-139° and 138°-139°, specific rotations (chloroform, 20°) of -27° and -27.7°, and contained 2.50 per cent and 3.07 per cent of water. In spite of the slight differences in properties these two phytosterol preparations showed no significant differences in the coprecipitation studies. Assuming the validity of the Anderson and Nabenhauer⁶⁰ formula, these phytosterols contained approximately 15 per

cent of dihydrositosterol.

The Coprecipitation of Cholesterol and Phytosterol Dibromides.

Mixtures of cholesterol and phytosterol were brominated, the dibromide precipitated and the bromine in the precipitates determined by the procedure described in the Appendix, page 76. The weight of the sterol precipitate was calculated as cholesterol from the titer using the factor 2.16. The data used in the construction of Figure 1 are shown in Table XXIV.

TABLE XXIV

Data Used in the Construction of Figure 1

Cholesterol	:	Phytosterol	:	Titer	:	Sterol Precipitated
	:		:	0.01 N $\text{Na}_2\text{S}_2\text{O}_3$	:	Calculated as Cholesterol
	:		:		:	(2.16 x Titer)
mg.	:	mg.	:	ml.	:	mg.
5	:	0	:	1.76	:	3.8
"	:	"	:	1.77	:	3.8
5	:	5	:	3.23	:	7.0
"	:	"	:	3.34	:	7.2
5	:	10	:	4.63	:	10.0
"	:	"	:	4.35	:	9.4
5	:	15	:	5.45	:	11.8
"	:	"	:	5.42	:	11.7
5	:	20	:	6.85	:	14.8
"	:	"	:	7.05	:	15.2
10	:	0	:	4.10	:	8.9
"	:	"	:	4.11	:	8.9
10	:	5	:	5.79	:	12.5
10	:	10	:	7.48	:	16.2
"	:	"	:	7.48	:	16.2
10	:	15	:	8.96	:	19.4
"	:	"	:	9.01	:	19.4
"	:	"	:	8.88	:	19.2
10	:	20	:	10.24	:	22.1
"	:	"	:	10.40	:	22.4
10	:	25	:	10.96	:	23.6
10	:	30	:	12.74	:	27.5
20	:	0	:	8.63	:	18.6
20	:	10	:	11.95	:	25.8
20	:	20	:	15.42	:	33.3
20	:	30	:	18.78	:	40.5
0	:	15	:	3.26	:	4.9
0	:	20	:	4.18	:	9.0
0	:	25	:	5.05	:	13.1
0	:	30	:	8.03	:	17.3

The Procedure for Preparing Pure Cholesterol Dibromide
and Pure Cholesterol

Preparation of Pure Cholesterol Dibromide. The procedure is described as applied to 2 g. of cholesterol. The cholesterol in 10 ml. of carbon tetrachloride is cooled in ice until the solution starts to congeal. There

is added slowly from a burette while the mixture liquefies but is kept cool the solution of bromine in carbon tetrachloride (0.3 to 0.4 g. of bromine per ml., determined by titration) until an excess of 0.2 ml. over the calculated amount theoretically required has been added. The clear solution is cooled in ice water for 15 min. and 70 to 80 ml. of petroleum ether at -10° to -15° is added. The mixture is vigorously stirred for about 3 min. while kept at -10° , filtered rapidly with suction on a Jena G3 filter and the precipitate washed until white with petroleum ether at about -10° . The precipitate is pressed down hard to remove excess solvent and dry air is drawn through until the filter and precipitate have attained approximately room temperature. The precipitate is broken up and dried to constant weight in a vacuum over phosphorus pentoxide. It is kept over phosphorus pentoxide in a desiccator protected from the light. On the above scale the yield is about 1.5 g. of cholesterol dibromide melting clear at 114.4° - 114.8° (corrected) followed by decomposition. This product was found to contain 29.26 per cent, 29.26 per cent, 29.29 per cent of bromine in triplicate determinations on macro quantities by ashing with potassium hydroxide and applying the Volhard method. By the oxidative iodometric method on the macro scale quadruplicate determinations indicated 29.12 per cent, 29.25 per cent, 29.25 per cent and 29.20 per cent of bromine. Results obtained on semi-micro quantities were given in Table VII.

Preparation of Pure Cholesterol. Pure cholesterol was prepared from 13 g. of the pure cholesterol dibromide. The dibromide was dissolved in a liter of acetone at room temperature, 50 g. of sodium iodide was added and the solution was allowed to stand over-night. The iodine was reduced with a slight excess of sodium thiosulphate solution and the mixture was diluted

to about 2.5 l. with water. The white precipitate was filtered off and washed copiously with water. The product was recrystallized from alcohol (twice) to constant melting point and dried to constant weight over phosphorus pentoxide in an Abderhalden drier with boiling toluene in the heating chamber. The yield was 8.0 g. melting at 148.0° - 148.8° (corrected), corresponding to 87 per cent on the basis of the dibromide used and 53 per cent on the basis of the original cholesterol (15 g.) used in preparing the dibromide.

SUMMARY

The need for a method for the determination of the egg content of alimentary pastes has been discussed.

The factors involved in the adoption of a method based on a cholesterol determination have been stated and available methods have been considered in the light of such factors.

A study of the conditions necessary for the bromination of cholesterol and precipitation of cholesterol dibromide as the basis of a quantitative determination of cholesterol has been made.

An iodometric method for the determination of cholesterol based on the cholesterol dibromide precipitation and the reaction between cholesterol dibromide and sodium iodide in acetone has been developed and applied to the estimation of the sterol content of eggs, flour and alimentary pastes.

The interference of wheat phytosterols in the determination of cholesterol by the cholesterol dibromide precipitation method has been studied.

A method for the preparation of pure cholesterol dibromide has been developed and cholesterol has been purified thereby.

An iodometric method for the determination of cholesterol based on the cholesterol dibromide precipitation and the application of the van der Meulen oxidative bromine determination has been developed. This method has been applied to the determination of the sterol content of eggs, flour and alimentary pastes.

It has been shown that the sterol content of the solids is not changed by long storage of eggs in the frozen condition nor by the com-

mercial process of preparing dried eggs.

The sterol content of a number of samples of commercially prepared frozen eggs and dried eggs and of farinaceous ingredients used commercially in alimentary pastes has been determined.

The sterol content of alimentary pastes has been shown to be a useful index of the egg content.

It has been established that the sterol content of alimentary pastes does not decrease materially on long storage at ordinary temperatures.

APPENDIX

The Determination of the Sterol Content of Eggs Based on the
Cholesterol Dibromide-Sodium Iodide ReactionOriginal Method.

Unaponifiable Matter

Reagents

Concentrated potassium hydroxide solution. Dissolve 60 g. of reagent-quality potassium hydroxide in 40 g. of water.

Alcohol. Reagent-quality ethyl alcohol, approximately 95 per cent by volume.

Ether. Ether U. S. P.

Dilute potassium hydroxide solution. Dissolve 11.2 g. of reagent-quality potassium hydroxide in 1 l. of water.

Dilute hydrochloric acid. Dilute one volume of hydrochloric acid U. S. P. with four volumes of water.

Phenolphthalein solution. A 1 per cent solution of phenolphthalein in alcohol.

Apparatus

Separatory funnel. A 300 ml. separatory funnel, ether-tight when the stopcock is lubricated with water.

Erlenmeyer flasks. One of 125 ml. capacity and one of 300 ml. capacity.

Test tubes. Two, 25 x 150 mm.

Centrifuge bottles. Two 8 oz. nursing bottles.

Pressure siphon. A two-hole rubber stopper to fit the 8 oz. nursing bottles, equipped with a pressure bulb and a siphon tube of 3 mm.

bore.

Determination

(a) Dried Eggs. Weigh accurately into a 125 ml. Erlenmeyer flask approximately 1 g. of yolk or 1.5 g. of whole egg. Moisten with 1 ml. of water and add 5 ml. of concentrated potassium hydroxide solution. Cover with a small watch glass and heat 3 hours on the steam bath, swirling occasionally to disintegrate any lumps. Cool to about 30°, add 30 ml. of alcohol and swirl until the insoluble matter is finely dispersed. Add 50 ml. of ether, mix thoroughly and transfer to the separatory funnel. Wash the flask with two more 50 ml. portions of ether, add to the separatory funnel and thoroughly mix the ether solutions by swirling. Wash the saponification flask with 100 ml. of dilute potassium hydroxide solution and pour into the separatory funnel in a slow, steady stream while swirling the liquid gently. Allow the liquids to separate (about 10 min.) and slowly draw off as much of the soap solution as possible, including any small quantity of emulsion, into a nursing bottle. Add 50 ml. of ether to the soap solution, close the bottle tightly with a soft moistened cork and shake vigorously. Centrifuge and transfer the ether layer to the separatory funnel as completely as possible using the pressure siphon without blowing over an excessive quantity of soap solution. Wash the ether solution with successive 100 ml. portions of the dilute potassium hydroxide solution as before until the washings become not more than slightly turbid on acidifying with hydrochloric acid. Wash the ether solution free of alkali in like manner with 30 ml. portions of water (test with phenolphthalein solution). The total volume of ether should be maintained above 150 ml. by adding more ether if necessary. Transfer the ether solution quantitatively through

a pledget of cotton in the stem of the funnel to a 300 ml. Erlenmeyer flask containing a small piece of porcelain, washing the funnel with 10, 5, 5 ml. portions of ether. Evaporate or distil the ether to dryness on the steam bath (caution!) and dry the residue for 30 min. at 100° - 105° . After the flask has cooled transfer the residue to the weighed test tube using four 10 ml. portions of ether. (For satisfactory results the drying and weighing of the tube must be done as follows: dry the clean test tube and a similar one to be used as a counterpoise at 100° - 105° for 1 hour; remove from the oven and place near the balance with the balance case open for 30 min.; weigh the tube using the counterpoise.) Place the tube in a 300 ml. Erlenmeyer flask full of water at an initial temperature of 45° - 50° on the steam bath while directing a stream of clean air upon the surface of the ether so that it is rapidly rotated and evaporate to apparent dryness. Remove the tube from the water, wipe with a clean towel and place in the oven with the counterpoise at 100° - 105° for 1 hour. Cool and weigh in the same way as before. Deduct from the weight of the unsaponifiable matter any blank obtained from the reagents used determined by the same procedure described in the method. Determine the sterol in the unsaponifiable matter as described under "Cholesterol", page 76.

(b) Liquid Eggs. Weigh accurately into a 125 ml. Erlenmeyer flask approximately 4 g. of whole egg or 2 g. of yolk; add to the whole egg 10 ml., to the yolk 5 ml., of concentrated potassium hydroxide solution and proceed as described under "Dried Eggs", page 71.

Adsorption Method.

Adsorbed Unsaponifiable Matter

Reagents

Aluminum oxide adsorbent. Prepare as follows from basic aluminum

acetate powder (approximately 90 per cent of this salt should pass a 100-mesh sieve): heat slowly at first, then ignite in a platinum dish at a bright red heat over a Meker burner, stirring occasionally to facilitate oxidation, until the powder is pure white. About 3 hours' heating is required. As soon as the material has cooled enough to handle conveniently, pass the powder through a 100-mesh sieve and immediately transfer to a container having a tight cover.

Asbestos. Digest asbestos of the amphibole variety with hydrochloric acid solution (one volume of concentrated hydrochloric acid + three volumes of water) for two to three days. Wash free from acid and digest for a similar period with 10 per cent sodium hydroxide solution. Wash the asbestos free from alkali and digest for several hours with nitric acid solution (one volume of concentrated nitric acid + three volumes of water). Wash the asbestos free from acid and shake with water to a fine pulp.

Sand. Pass clean sand through a 60-mesh sieve and digest with concentrated hydrochloric acid until the extracts are practically colorless. Wash out the acid, dry the sand and ignite at 500°.

Petroleum ether. Reagent-quality petroleum ether with boiling range 30°-65°.

The other reagents used are those described under "Unsaponifiable Matter", page 70.

Apparatus

Filter. Prepare a filter from a Knorr extraction tube with the stem lengthened to about 10 cm., using a layer of asbestos about 3 mm. thick covered by a layer of sand of about 5 mm.

Filtration bell jar. A bell jar of sufficient size to accommodate

a 25 x 150 mm. test tube, connected to a vacuum through a two-way stop-cock.

The other apparatus used is that described under "Unsaponifiable Matter", page 70.

Determination

(a) Dried eggs. Weigh accurately into a 125 ml. Erlenmeyer flask approximately 1 g. of yolk or 1.5 g. of whole egg. Moisten with 1 ml. of water and add 5 ml. of concentrated potassium hydroxide solution. Cover with a small watch glass and heat 3 hours on the steam bath, swirling occasionally to disintegrate any lumps. Cool to about 30°, add 30 ml. of alcohol and swirl until the insoluble matter is finely dispersed. Add 30 ml. of ether, mix thoroughly and transfer to the separatory funnel. Wash the flask with two more 30 ml. portions of ether, add to the separatory funnel and thoroughly mix the ether solutions by swirling. Wash the saponification flask with 100 ml. of dilute potassium hydroxide solution and pour into the separatory funnel in a slow steady stream while swirling the liquid gently. Allow the liquids to separate (about 10 min.) and slowly draw off as much of the soap solution as possible into a nursing bottle, including any small quantity of emulsion. Add 50 ml. of ether to the soap solution, stopper the bottle tightly with a soft moistened cork and shake vigorously. Centrifuge and transfer the ether layer to the separatory funnel as completely as possible, using the pressure siphon for the purpose without blowing over an excessive quantity of the soap solution. Wash the ether solution with two successive 100 ml. portions of the dilute potassium hydroxide solution as before and then with 30 ml. portions of water until free of alkali (test with phenolphthalein solution). Transfer the ether solution quantitatively through a pledget

of cotton in the stem of the funnel to a 300 ml. Erlenmeyer flask containing a small piece of porcelain, washing the funnel with 10, 5, 5 ml. portions of ether. Evaporate or distil the ether to dryness on the steam bath (caution!) and dry the residue for 30 min. at 100°-105°. After the flask has cooled, treat the residue with 20 ml. of petroleum ether, swirling the flask and freeing the porcelain chip if it adheres to the glass. Add 1 g. of the aluminum oxide adsorbent, swirl the liquid with the adsorbent for a minute, wash down the sides of the flask with a few ml. of the petroleum ether and swirl again. Allow the adsorbent to settle, supporting the flask on its side across the mouth of a 600 ml. beaker so that the aluminum oxide forms a compact mass in the angle at the bottom of the flask. Decant the petroleum ether with minimum transfer of adsorbent through the filter previously washed successively with water, alcohol, ether and petroleum ether. Wash down the sides of the flask and extract the adsorbent as above with three successive 10 ml. portions of petroleum ether, finally washing the lip of the flask, the sides and tip of the filter tube with a few ml. of the same. Discard the petroleum ether extracts. Extract the adsorbent in exactly the same way as before, using ether in place of petroleum ether; collect the ether in the weighed test tube, evaporating some of the ether while the adsorbent is settling during the last extractions. (The last extract must be colorless, although the aluminum oxide may still retain some color.) For satisfactory results the drying and weighing of the tube must be done as follows: dry the clean test tube and an exactly similar one to be used as a counterpoise at 100°-105° for one hour, remove from the oven and place near the balance, with the balance case open, for one-half hour; weigh the tube using the counterpoise. Place the tube in a 300 ml. Erlenmeyer flask full of water

at an initial temperature of 45° - 50° on the steam bath while directing a stream of clean air upon the surface of the ether so that it is rapidly rotated and evaporate to apparent dryness. Remove the tube from the water, wipe with a clean towel and place in the oven with the counterpoise at 100° - 105° for 1 hour. Cool and weigh in the same way as before. Deduct from the weight of the adsorbed unsaponifiable matter any blank obtained from the reagents used, determined by the same procedure described in the method. Determine the sterol in the adsorbed unsaponifiable matter as described under "Cholesterol", page 76.

(b) Liquid Eggs. Weigh accurately into a 125 ml. Erlenmeyer flask approximately 4 g. of whole egg or 2 g. of yolk; add to the whole egg 10 ml., to the yolk 5 ml., of concentrated potassium hydroxide solution and proceed as described under "Dried Eggs", page 74.

Cholesterol

Reagents

Ice. Prepare at least a 3-gallon pail full of crushed ice.

Bromine reagent. Weigh to 0.1 g. a narrow-mouth glass-stoppered flask of about 25 ml. capacity containing 5 ml. of reagent-quality carbon tetrachloride. Add 4 to 5 g. of reagent-quality bromine, weigh again and dilute with carbon tetrachloride to a final concentration of 0.22 ± 0.02 g. of bromine per ml.

Acetic acid solution. Pipet 200 ml. of reagent-quality glacial acetic acid into a 250 ml. glass-stoppered volumetric flask; dilute to 250 ml. with water, mix cautiously, dilute to the mark and mix again.

Anhydrous ether. Reagent-quality diethyl ether distilled from sodium.

Asbestos. Prepare asbestos as described on page 73.

Sand. Prepare sand as described on page 73.

Acetone. Reagent-quality acetone.

Sodium iodide. Reagent-quality granular sodium iodide.

0.01 N Sodium thiosulphate solution. Prepare by dilution of standard 0.1 N sodium thiosulphate solution with freshly boiled and cooled distilled water.

Apparatus

Ice bath. A container of about 4 l. capacity and 10 to 15 cm. depth filled with crushed ice.

Graduated cylinders. One cylinder of 25 ml. capacity; one cylinder of 10 ml. capacity.

Test tubes. One 18 x 150 mm. test tube; four 25 x 150 mm. test tubes.

Stirring rods. Glass rods about 4 mm. in diameter and about 19 cm. in length.

Mohr pipettes. One Mohr pipette graduated to 0.01 ml.; one Mohr pipette graduated to 0.1 ml.

Filtration bell jar. A bell jar of sufficient size to accommodate a 125 ml. Erlenmeyer flask, connected to a vacuum by a two-way stopcock.

Device for filtering at 0°. Remove the stem at the apex from a 60° Bunsen funnel 11 cm. in diameter. Enlarge the opening at the apex to about 1 cm. diameter by grinding or grating off the glass. Cut an approximately 1 cm. length from the end of a one-hole rubber stopper of a size that fits snugly in the opening of the funnel. Pass the stem (lengthened to about 10 cm.) of a Knorr extraction tube through the stopper in the funnel apex and then through a stopper to fit the bell jar. Prepare in the filter tube a mat of asbestos about 6 to 8 mm. thick and cover with an approximately 12 mm. layer of sand.

Determination

It is convenient to make four determinations at the same time. Pack the bromine reagent, the 25 ml. graduated cylinder and the 18 x 150 mm. test tube containing the four stirring rods in the ice. Pack ice about the tubes in the filtering device, taking care none gets into the filters. Cool the acetic acid solution to about -5° in an ice-salt mixture.

Wash down the sides of the 25 x 150 mm. test tubes containing the sterol with 2.0 ml. of anhydrous ether delivered from a Mohr pipette; stopper with a cork and pack the tubes in the ice bath for 10 min. To one of the tubes add 0.20 ml. of the cold bromine reagent from a Mohr pipette, mix the contents by swirling, stopper and replace in the ice bath. (Start this procedure at 3 min. intervals with the other samples.) After 10 min. add rapidly 15 ml. of the acetic acid solution measured in the cold 25 ml. graduated cylinder, stir well for 5 min. and allow the mixture to stand in the ice bath for 10 min. With the suction on, pour the mixture rapidly into the filter tube. Wash down the sides of the test tube with 5 ml. of the cold acetic acid solution and replace in the ice bath. When the liquid in the filter just recedes below the surface of the sand add the acetic acid from the test tube. Repeat the washing in like manner with 5 ml. of cold acetic acid solution and suck the filter free of excess liquid. Wash the test tube and filter repeatedly with approximately 5 ml. portions of cold water until the washings are neutral to blue litmus. Thoroughly drain the test tube and apply suction to the filter until drops of water no longer fall from the stem. Remove the ice pack from around the filter tube and discard the filtrate and washings. Place a 125 ml. glass-stoppered Erlenmeyer flask under the filter so that the stem projects well into the flask. Wash the test tube and filter with

four successive 10 ml. portions of acetone, stirring up the sand and allowing the solvent to stand for about a minute before sucking the filter free of excess liquid. Wash down the stem of the filter and sides of the flask with a 5 ml. portion of acetone. (The solution should be practically colorless; any color should be noted in order to guard against passing the end point in the titration.) Add approximately 0.5 g. of sodium iodide, stopper, dissolve the salt by gentle swirling of the liquid and set aside in a dark place for one hour. Titrate with 0.01 N sodium thiosulphate solution against a white background in daylight to the complete disappearance of the yellow color or, if the original solution of the precipitate was colored somewhat, until there is no change of color. The detection of the end point is facilitated by comparing the titrated solution with an equal volume of acetone in the same type of flask, presuming the original solution was colorless. With quantities of cholesterol exceeding about 60 mg., a precipitate will form in the flask during the titration. However, this readily flocculates and does not interfere with the observance of the end point if allowed to settle for a short time.

$$\text{mg. of cholesterol} = 1.0 + 2.16 (\text{ml. of } 0.01 \text{ N Na}_2\text{S}_2\text{O}_3).$$

The Determination of the Sterol Content of Alimentary Pastes
and Farinaceous Ingredients Based on the
Cholesterol Dibromide-Sodium Iodide Reaction

Original Method.

Unsaponifiable Matter

Reagents

Cholesterol. Highest quality cholesterol obtainable of melting point not less than 147° . Test for purity by the cholesterol method given on page 76.

Potassium hydroxide. Pellets of reagent-quality potassium hydroxide.

Concentrated hydrochloric acid solution. Dilute 3 volumes of hydrochloric acid U. S. P. with 2 volumes of water.

The other reagents used are those described under "Unsaponifiable Matter", page 70, omitting the concentrated potassium hydroxide and dilute hydrochloric acid solutions.

Apparatus

The apparatus used is that described under "Unsaponifiable Matter", page 70, in addition to a 500 ml. Erlenmeyer flask.

Determination

Weigh 20 g. of the sample (ground to pass a 20 mesh sieve) into a 500 ml. Erlenmeyer flask, add 20 ml. of alcohol and agitate so that all portions are moistened. Add 50 ml. of the concentrated hydrochloric acid solution and heat on the steam bath for 30 min. with occasional swirling to disintegrate all lumps. While cooling the inclined flask under the tap add carefully 65 gm. of potassium hydroxide pellets at such a rate that the liquid may boil but not so violently as to cause loss by spurling.

While it is still hot place the flask on the steam bath, cover with a small watch glass and heat for 3 hours with occasional swirling of the mixture to carry down any material adhering to the sides. Cool to about 30°, add 35 ml. of alcohol and mix thoroughly. Add 100 ml. of ether, swirl the mixture vigorously for a minute and transfer to the separatory funnel, washing the flask with two 50 ml. portions of ether. Wash the flask with 50 ml. of water, pouring this into the funnel in a slow stream while the mixture is gently rotated. After the ether layer has separated sharply at the upper liquid interface (about 10 min.) draw off the lower layers into a nursing bottle. Proceed from this point as described under "Dried eggs", page 71, beginning with the directions "add 50 ml. of ether to the soap solution **". In the analysis of farinaceous ingredients of alimentary pastes add 50 mg. of cholesterol to the unsaponifiable matter before applying the cholesterol method and correct the result accordingly.

Adsorption Method.

Adsorbed Unsaponifiable Matter

Reagents

Cholesterol. Highest quality cholesterol obtainable of melting point not less than 147°. Test for purity by the cholesterol method, page 76.

Potassium hydroxide. Pellets of reagent-quality potassium hydroxide.

Concentrated hydrochloric acid solution. Dilute 3 volumes of hydrochloric acid U. S. P. with 2 volumes of water.

The other reagents used are those described under "Adsorbed Unsaponifiable Matter", page 72, omitting the concentrated potassium hydroxide

and dilute hydrochloric acid solutions.

Apparatus

The apparatus used is that described under "Adsorbed Unsaponifiable Matter", page 72, in addition to a 500 ml. Erlenmeyer flask.

Determination

Proceed as described under "Unsaponifiable Matter", page 80, to the point where the soap solution is drawn off into a nursing bottle. Proceed from this point as described under "Dried Eggs", page 74, beginning with the directions "add 50 ml. of ether to the soap solution, ** ". In the analysis of farinaceous ingredients of alimentary pastes add 20 mg. of cholesterol to the adsorbed unsaponifiable matter before applying the cholesterol method and correct the result accordingly.

The Determination of the Sterol Content of Eggs Based on
the Oxidative Iodometric Procedure

Revised Method.

Unsaponifiable Matter

Reagents

Concentrated potassium hydroxide solution. Dissolve 60 g. of reagent-quality potassium hydroxide in 40 g. of water.

Alcohol. Reagent-quality ethyl alcohol approximately 95 per cent by volume.

Ether. Ether U. S. P.

Dilute potassium hydroxide solution. Dissolve 11.2 g. of reagent-quality potassium hydroxide in 1 l. of water.

Dilute hydrochloric acid. Dilute one volume of hydrochloric acid U. S. P. with four volumes of water.

Dried ether. Immediately before use shake ether U. S. P. with an excess of anhydrous calcium chloride and filter.

Anhydrous sodium sulphate. Reagent-quality granulated anhydrous sodium sulphate.

Phenolphthalein solution. A 1 per cent solution of phenolphthalein in alcohol.

Apparatus

Separatory funnel. A 500 ml. separatory funnel, ether-tight when the stopcock is lubricated with water.

Erlenmeyer flasks. One of 125 ml. capacity and one of 300 ml. capacity.

Filtration bell jar. A bell jar of sufficient size to accommodate a 300 ml. Erlenmeyer flask connected to a vacuum by a two-way stopcock.

Test tubes. Two, 25 x 150 mm.

Centrifuge bottles. Two 8 oz. nursing bottles.

Pressure siphon. A two-hole rubber stopper to fit the 8 oz. nursing bottles, equipped with a pressure bulb and a siphon tube of 2 mm. bore.

Sintered glass filter. A Jena 11 G3 sintered glass filter or its equivalent.

Determination

Weigh accurately into a 125 ml. Erlenmeyer flask approximately 2.5 g. of whole egg, 1.5 g. of yolk, 1 g. of dried whole egg or 0.7 g. of dried yolk and add 10 ml. of concentrated potassium hydroxide solution. Cover with a small watch glass and heat 3 hours on the steam bath, swirling occasionally to disintegrate any lumps. Cool to about 30°, add 30 ml. of alcohol and swirl until the insoluble matter is finely dispersed. Add 50 ml. of ether, mix thoroughly and transfer to the separatory funnel. Wash the flask with two more 50 ml. portions of ether and thoroughly mix the ether solutions by swirling. Wash the saponification flask with 100 ml. of dilute potassium hydroxide solution and pour into the separatory funnel in a slow steady stream while swirling the liquid gently. Allow the liquids to separate (about 10 min.) and slowly draw off the soap solution, including any small quantity of emulsion, into a nursing bottle. Rinse down the sides of the funnel with 10 ml. of dilute potassium hydroxide and draw this off into the bottle. Add 50 ml. of ether to the soap solution, close the bottle tightly with a soft moistened cork and shake vigorously. Centrifuge and pour dilute potassium hydroxide in a gentle stream down the

side of the bottle until the ether is brought almost up to the neck. Transfer the ether layer to the separatory funnel as completely as possible, using the pressure siphon. Rinse the portion of the siphon tube in the bottle and the sides of the bottle with a 10 ml. portion of ether and transfer the ether to the separatory funnel through the siphon. Wash the ether solution with successive 100 ml. portions of dilute potassium hydroxide, swirling the liquid as before, until the washings become not more than faintly turbid on acidifying with dilute hydrochloric acid. Wash the ether solution free of alkali with 30 ml. portions of water (test with phenolphthalein solution). The volume of the ether should be maintained above 150 ml. by addition of more ether if necessary. Filter the ether solution into a 300 ml. Erlenmeyer flask with gentle suction through a 3 mm. layer of anhydrous sodium sulphate on the sintered glass filter, rinsing the separatory funnel and filter with 10, 5 and 5 ml. portions of ether. Rinse the stem of the filter with dried ether, add a porcelain chip to the flask and evaporate the ether on the steam bath (caution!) to a volume of about 30 ml. Transfer the ether solution to the weighed test tube using 10, 10, 5 ml. portions of dried ether. (For satisfactory results the drying and weighing of the tube must be done as follows: dry the clean test tube and a similar one to be used as a counterpoise at 100°-105° for 1 hour; remove from the oven and place near the balance with the balance case open for 30 min.; weigh the tube using the counterpoise.) Place the tube in a 300 ml. Erlenmeyer flask full of water at an initial temperature of 45°-50° on the steam bath while directing a stream of clean air upon the surface of the ether so that it is rapidly rotated and evaporate to apparent dryness. Remove the tube from the water, wipe with a clean towel and place in the oven with the counterpoise at 100°-105° for 1 hour.

Cool and weigh in the same way as before. Deduct from the weight of the unsaponifiable matter any blank obtained from the reagents used, determined by the same procedure described in the method. Determine the sterol in the unsaponifiable matter as described below under "Cholesterol".

Cholesterol

Reagents

Alcohol. Reagent-quality ethyl alcohol approximately 95 per cent by volume.

Ether. Ether U. S. P.

Monobasic sodium phosphate. Reagent-quality $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$.

Sodium hypochlorite solution. Dissolve 88 g. of reagent-quality sodium hydroxide in 200 ml. of water; add about 1500 ml. of crushed ice and pass in chlorine until 71 g. is absorbed; dilute to 2 l. and store in dark bottles in the refrigerator. Discard if the concentration becomes less than 0.93 N sodium hypochlorite.

Sodium formate solution. An aqueous solution of reagent-quality sodium formate containing 0.5 g. per ml.

Hydrochloric acid solution. An approximately 6 N solution of hydrochloric acid U. S. P.

Sodium chloride. Reagent-quality sodium chloride.

Methyl red indicator. Dissolve 0.5 g. of methyl red in 50 ml. of alcohol, dilute to 100 ml. with water and filter.

0.02 N sodium thiosulphate solution. Prepare from reagent-quality sodium thiosulphate and freshly boiled and cooled distilled water, to which 1 per cent of amyl alcohol has been added. Standardize against pure potassium iodate. This solution, if protected from evaporation and light, will

retain its titer almost indefinitely.

Starch solution. A 1 per cent solution of soluble starch.

Concentrated potassium hydroxide solution. A 50 per cent solution of reagent-quality potassium hydroxide.

Potassium iodide solution. A 20 per cent solution of reagent quality potassium iodide.

Ammonium molybdate solution. A 5 per cent solution of ammonium molybdate.

The other reagents used are those described under "Cholesterol", page 76, omitting the acetone, sodium iodide and sodium thiosulphate solution.

Apparatus

The apparatus used is the same as described under "Cholesterol", page 76.

Determination

With the exception that the time of stirring the precipitation mixture is reduced from 5 min. to 3 min., the procedure is as described under "Cholesterol", page 76, down to the point where the precipitate on the filter has been washed free of acid. Place a 300 ml. Erlenmeyer flask under the filter so that the stem projects well into the flask. Wash the test tube and filter with 10 ml. of alcohol, 10, 5, 5 ml. portions of ether and finally with 10 ml. of alcohol, stirring up the same with each portion of solvent and allowing the mixture to stand for about a minute before applying the suction. Wash down the stem of the filter with 2 ml. of ether, add 1 ml. of concentrated potassium hydroxide solution, mix and wash down the sides of the flask with 5 ml. of ether. Evaporate

the ether and alcohol completely on the steam bath, using a stream of clean air to remove the final alcohol vapors. Add 40 ml. of hot water to the residue, mix, and neutralize the alkali with 6 N hydrochloric acid, using 1 drop of the methyl red indicator. Add 10 g. of sodium chloride, 3 g. of monobasic sodium phosphate and 20 ml. of sodium hypochlorite solution. Bring the solution just to vigorous boiling, remove from the heat and add immediately, with care, 5 ml. of sodium formate solution. Cool and dilute to about 150 ml. with water. Add 5 ml. of potassium iodide solution, a drop or two of ammonium molybdate solution and 25 ml. of 6 N hydrochloric acid. Titrate at once with 0.02 N sodium thiosulphate solution, using starch solution as indicator. Correct the titer for a blank determination on the reagents.

$$\text{mg. of cholesterol} = 0.55 + 0.688 (\text{ml. of } 0.02 \text{ N Na}_2\text{S}_2\text{O}_3).$$

The Determination of the Sterol Content of Alimentary Pastes
and Farinaceous Ingredients Based on the
Oxidative Iodometric Procedure

Revised Method.

Unsaponifiable Matter

Reagents

Cholesterol. Highest quality cholesterol obtainable of melting point not less than 147° . Test for purity by the cholesterol method given on page 86.

Potassium hydroxide. Pellets of reagent-quality potassium hydroxide.

Concentrated hydrochloric acid. Dilute hydrochloric acid U. S. P. with an equal volume of water.

The other reagents used are those described under "Unsaponifiable Matter", page 83, omitting the concentrated potassium hydroxide solution.

Apparatus

The apparatus used is that described under "Unsaponifiable Matter", page 83, in addition to a 500 ml. Erlenmeyer flask.

Determination

Weigh a 10 g. sample (ground to pass a 20-mesh sieve) into a 500 ml. Erlenmeyer flask and add with shaking 50 ml. of the concentrated hydrochloric solution. Heat on the steam bath 30 min. with frequent shaking to disintegrate any lumps. While cooling the inclined flask under the tap

add carefully 30 g. of potassium hydroxide pellets at such a rate that the liquid may boil but not so violently as to cause loss by spurting. While it is still hot place the flask on the steam bath, cover with a small watch glass and heat for 3 hours with occasional swirling of the mixture to carry down any material adhering to the sides. Cool to about 30°, add 30 ml. of alcohol and 50 ml. of water and mix well. Add 100 ml. of ether, swirl vigorously for a minute and transfer the mixture to the separatory funnel, washing the flask with 50 ml. and 25 ml. portions of ether. Wash the saponification flask with 50 ml. of dilute potassium hydroxide solution, pouring this into the funnel in a slow stream while the mixture is gently rotated. After the ether layer has separated sharply at the upper liquid interface (about 10 min.) draw off the lower layers into a nursing bottle, including any small quantity of emulsion. Proceed from this point as described under "Determination", page 87, beginning with the directions "rinse down the sides of the funnel with 10 ml. of dilute potassium hydroxide ***". If the unseaponifiable matter amounts to less than 0.27 per cent on the dry basis add 10 mg. of cholesterol to it before applying the cholesterol method and correct the result accordingly.

The Preparation of the Authentic Alimentary Pastes

The alimentary pastes were prepared during March, 1936, at the plant of A. Zeraga's Sons, Inc., Brooklyn, New York, under the personal supervision of H. A. Lepper of the Food and Drug Administration, United States Department of Agriculture, by the usual commercial process.

The flour used in all batches was from the same carload lot of Durum Fancy Patent, H. H. King Flour Mills, Minneapolis, Minn.

The liquid egg yolk and whole egg used were freshly prepared from Missouri shell eggs of very good quality at the egg breaking establishment of Marshall and Kirby, New York, N. Y. Samples of the churned eggs for analytical purposes were immediately placed in a sharp freezer and maintained in the frozen condition until analyzed. The dried egg yolk used was from China.

The "Vita-Pro" was a commercial carotenized flour preparation obtained from the National Kream Company, Brooklyn, New York.

Carotene mix "A" was made from 5 lbs. of Ceresota flour and 298 g. of oil containing 2000 p.p.m. of carotenoids. The oil was obtained from the American Chlorophyll Company, Washington, D. C. Carotene mix "B" was prepared from 5 lbs. of Ceresota flour and 290 g. of the same oil.

Samples of other farinaceous products taken included No. 1 semolina, standard semolina, Texas farina and Kansas hard flour.

The weighings were made on a scale sensitive to one-half ounce.

The composition of the mixes used in preparing the alimentary pastes is shown in Table XXV.

TABLE XXV

The Ingredients Used in the Preparation of the
Authentic Alimentary Pastes

Batch:	Flour:	Eggs Used		Water:	Other Ingredients Used	
No. :	Used :	Kind	Weight:	Used :	Kind	Weight
:	:	:	:	:	:	:
:	lbs.:		lbs.:	lbs.:		lbs.
:	:	:	:	:	:	:
1 :	200 :	--	-- :	49.75:	--	--
2 :	200 :	Fresh yolk	11.69 :	42.75:	--	--
3 :	200 :	" "	22.00 :	38.5 :	--	--
4 :	200 :	" "	42.37 :	28.5 :	--	--
5 :	200 :	" "	31.56 :	23.0 :	--	--
6 :	200 :	Fresh whole	37.50 :	23.75:	--	--
:	:	egg	:	:	:	:
7 :	200 :	Fresh whole	80.00 :	-- :	--	--
:	:	egg	:	:	:	:
8 :	194.4:	Fresh yolk	20.53 :	37.75:	Carotene mix "B"	5.56
9 :	194.4:	--	-- :	52.25:	Carotene mix "A"	5.56
10 :	182.4:	--	-- :	51.5 :	"Vita-Pro"	12.00
11 :	200 :	Chinese dried	10.50 :	55.0 :	Salt	4.00
:	:	yolk	:	:	:	:
:	:	:	:	:	:	:

Tentative Formulas for the Calculation of the Content
of Egg Yolk and of Whole Egg in Alimentary Pastes
from the Sterol Content

Formula for the Egg Yolk Content of Alimentary Pastes. The following formula is proposed tentatively, pending the accumulation of more data, for the calculation of the amount of commercial egg yolk solids in an alimentary paste:

$$Y = \frac{(C - 0.024)100}{2.88 - 0.024} = (C - 0.024)35$$

Y = the percentage of commercial egg yolk solids in the sample
(moisture-free basis)

C = the percentage of sterol (calculated as cholesterol) in the
sample (moisture-free basis).

Formula for the Whole Egg Content of Alimentary Pastes. The following formula is proposed tentatively, pending the accumulation of more data, for the calculation of the amount of commercial whole egg solids in an alimentary paste:

$$E = \frac{(C - 0.024)100}{2.11 - 0.024} = (C - 0.024)48$$

E = the percentage of commercial whole egg solids in the sample (moisture-free basis)

C = the percentage of sterol (calculated as cholesterol) in the sample (moisture-free basis).

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