PROBLEMS RELATED TO THE DETERMINATION OF ANTIOXIDANTS AND FAT-SOLUBLE VITAMINS IN PREMIXES

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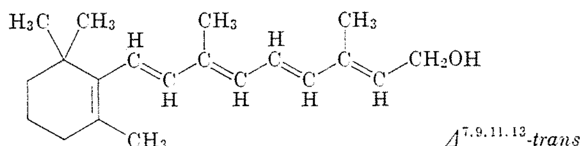
Our procedures developed for the determination of vitamins A, E and K_3, choline chloride and antioxidants in premixes depend on the composition of the latter. Vitamin premixes have simpler compositions and contain 10 to 12 constituents, viz. various fat- and water-soluble vitamins, an antioxidant (usually EMQ), occasionally glypondine and some antibiotics. More complex premixes contain additional constituents like zinc bacithracine, ardinone, dl-methionine, furidine etc. The analytical procedure is also affected by the manufacturer of the individual constituents: e.g. EMQ made by Monsanto differs largely from EMQ made by Material KTSz regarding its impurities. The elimination of the interfering effect of the relatively high metal salt content (iron, zinc, copper carbonate, cobalt and magnesium sulphate, manganese oxide etc.) in so-called mixed premixes and the use of BHT as antioxidant in the place of EMQ is also a problem. A mixed premix averagely contains around 20 constituents. The carrier of the premix is also essential. In our studies we used bran and alfalfa flour for this purpose. Since the carrier also contains several constituents, these may also affect the analytical procedure.

In order to eliminate the numerous interfering effects due to the complex nature of the samples as rapidly and reliably as possible, we developed the procedures for determining individual vitamins and antioxidants by means of the following method: we selected 2 to 3 representatives of vitamin premixes, mixed premixes and premixes containing BHT suited to study the interfering effect of all constituents of the premix type in question on the determination of vitamins A, E and K_3, choline chloride and antioxidant. Special samples of the selected premixes were then prepared by the Phylaxia Vaccine and Nutriment Co. that contained no vitamin A, E, K_3, choline chloride and antioxidant, resp. The constituent to be determined was then added to the sample in known amounts, in different phases of the analysis; based on the experimental results, changes were made in the analytical procedure. It is a precondition of satisfactory accuracy that the constituent to be determined should be separated from the accompanying substances adequately. Methods for purification must be so chosen that the sum of vitamin

losses in the individual purification stages should cause an error in the result of the analysis that lies within a reasonably narrow range. The experimental parameters of the methods selected for vitamin A, E, K_3 etc. determinations in the adequately purified solutions were established by means of stock solutions to ensure satisfactory accuracy of the analysis. Relatively large premix samples (5.00—8.00 g) must be used for analysis, since the error caused by the inhomogeneity of the premix is also included in the total error.

Determination of vitamin A

The biologically most active form of vitamin A is vitamin A_1 or retinol, namely its all-*trans* form:



Neoretinol is the Δ^{13}-*cis*-$\Delta^{7,9,11}$-*trans* form. In natural retinol sources these two forms are present in a ratio of 65 : 35. The two other isomers can only be obtained by synthesis.

Vitamin A is introduced into premixes in the form of "Mikrovit A", oily vitamin A acetate (or palmitate) in small gelatine capsules.

From the analytical view, the following properties of vitamin A should be borne in mind: it is soluble in fats, various organic solvents like ethanol, *iso*-propanol, cyclohexane, petroleum ether, hexane, diethyl ether, chloroform, and insoluble in water. It is decomposed by the action of light and air. Decomposition is enhanced by heat and UV light, by the presence of certain metal ions and by peroxides occurring in oils and some organic solvents. The vitamin A solution can be stabilized by using antioxidants, e.g. tocopherols, hydroquinone, butylhydroxyanisols, n-propylgallate etc.

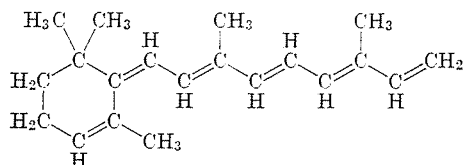
Known methods for the determination of vitamin A

Vitamin A can be determined by extinction measurement in the UV range at the appropriate wave length, by the method determining anhydrovitamin A, and by spectrophotometry based on colour reactions, e.g. the Carr—Price reaction or the colour reaction with activated glycerin-dichlorohydrine.

Spectrophotometry in the UV range is feasible only when the solution does not contain other constituents absorbing UV light in the 300 to 350 nm range. However, this is not the case even with vitamin premixes having the

simplest compositions. For this reason, UV absorption was only used for checking the correctness of reference standards (e.g. U.S.P. Reference Standard) applied in preparing the calibration curve of the colour reaction.

The essence of the method of BUDOWSKI and BONDI [1] is that vitamin A dissolved in anhydrous benzene loses one molecule of water by the action of p-toluene sulphonic acid, whereby the alcohol is converted into anhydrovitamin A_1:



This highly unsaturated compound shows sharp absorption maxima at 377 and 399 nm. The advantage of this method is that — on the one hand — anhydrovitamin A_1 gives substantially greater extinction at 399 nm than vitamin A at its absorption maximum (325—328 nm, depending on the solvent), and — on the other hand — the interfering effect of impurities is much smaller at 400 nm than at 325 nm. A further advantage of the method might consist in the fact that oxidation products of vitamin A do not interfere with the measurement (this could be very important in stability studies of premixes). However, in the case of premixes, even the solutions purified with column chromatography on Al_2O_3 have a slight yellow colour, therefore the error caused by the "background" in the range around 400 nm would be intolerable.

The most frequently used spectrophotometric method is that of CARR—PRICE [2]. Vitamin A dissolved in chloroform gives a blue colour reaction with antimony(III) chloride. Extinction is measured at 620 nm. The advantage of this method as compared to the above-listed methods is that less interfering effects must be taken into account. Its disadvantage is that the blue colour fades away rapidly, so that extinction measurement must be performed within 5 to 10 seconds after the addition of the reagent. Development and fading of the colour are affected — in addition to time and temperature — by traces of water and alcohol. To avoid hydrolysis of the reagent, the analysis must be carried out in closed cuvettes, using perfectly water-free solvents and glass tools.

The colour reaction originally proposed by SOBEL and WERBIN [3] using activated glycerin dichlorohydrine is very well suited for determining vitamin A alcohols and esters.

The reaction is carried out in chloroform. A relatively stable blue product that slowly changes into mauvish pink is formed. Although this method is less sensitive than the CARR—PRICE reaction, it has the important advantage

that the colour remains stable for a longer time and traces of water and alcohol interfere to a lesser extent. We therefore chose this method for determining the vitamin A content in premixes, after having previously studied the factors affecting the accuracy of the colour reaction. These factors include the amount of the activator acetyl chloride, the period between the preparation and use of the reagent, the amount of reagent, the time necessary for the colour to develop. If proper experimental conditions are observed, the standard deviation of the method is $\pm 2.2\%$. Special studies were consacrated to establish the interfering effect of EMQ, petroleum ether and ethanol traces which may remain in the solution prepared for spectrophotometration. Trace amounts of petroleum ether and ethanol already reduce extinction. EMQ behaves similarly to fat-soluble vitamins as to solubility in the course of purification processes. A preliminary wash-out of EMQ from the premix with petroleum ether is connected with the risk of vitamin A losses in the case of the gelatine capsules being damaged, due e.g. to the moisture content of the premix. It is expedient to separate EMQ from vitamin A by column chromatography. Up to concentrations of 0.5 μg/ml EMQ does not affect the colour reaction of vitamin A. Higher concentrations, however, cause an increasing negative error, while very high amounts cause a positive error.

Separation of vitamin A from interfering constituents of the premix

Before determining the vitamin A content in synthetic mixtures, the sample must be saponified in all cases. This operation is carried out with alcoholic potassium hydroxide solution, in the presence of different protective substances, depending on the composition of the premix. In the saponification process, vitamin A esters are converted into the corresponding alcohol. The latter is extracted with petroleum ether and the extracts are submitted to alkaline, eventually acid and aqueous washes for further purification.

Saponification and petroleum ether extraction give a certain purification degree. However, our experiments revealed that — except in the case of a few vitamin premixes with very simple compositions — many interfering constituents still remain in the vitamin A solution which cause a great negative error in the colour reaction. The generally applied and internationally acknowledged method for eliminating the interfering effects is purification by column chromatography. The column is filled with basic Al_2O_3 activated by heating at 400 °C and setting the required activity by exposing the compound to (moisture adsorption) a water vapour atmosphere. Basic Al_2O_3 grades (products of Merck and Woeln) release sorbed water in one stage around 100 °C as shown on the derivatogram of a basic Al_2O_3 manufactured by Woeln and con-

taining 9% of water (Fig. 1). The water extract of suitable grades has a pH value of 9.0 to 10.0. Acid or neutral Al_2O_3 should not be used. These products release sorbed water in two stages during heating (cf. Fig. 2 showing the derivatogram of an acid Al_2O_3 manufactured by Reanal).

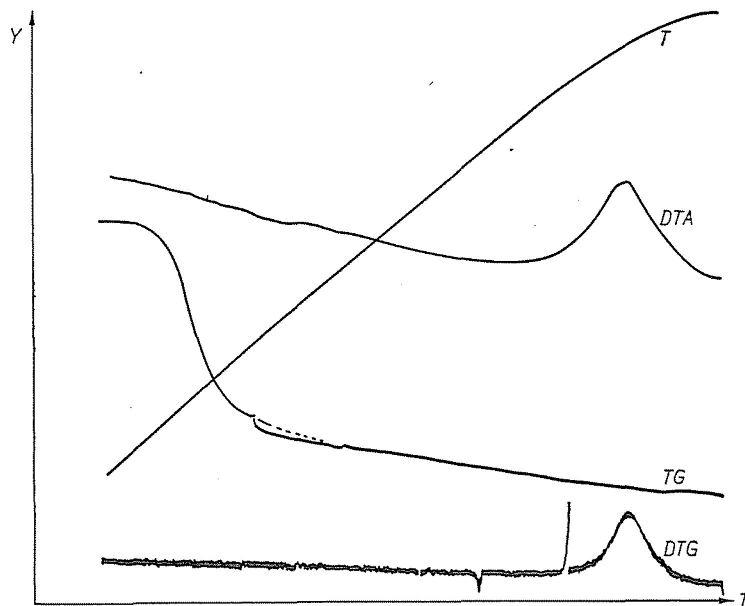


Fig. 1. Derivatogram of basic Al_2O_3 manufactured by Woeln

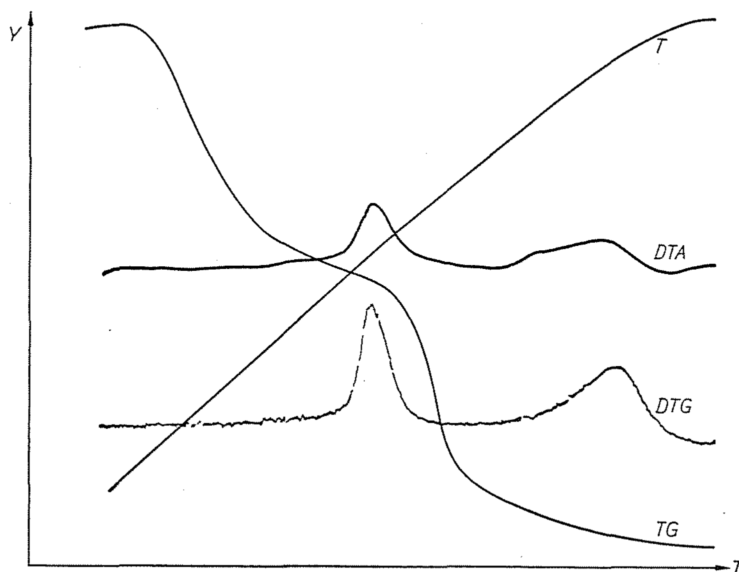


Fig. 2. Derivatogram of acid Al_2O_3 manufactured by REANAL

In column chromatography, first the constituents interfering with the colour reaction of vitamin A are successively removed by using appropriate eluents, and finally pure vitamin A is collected as a separate fraction.

The activity of the Al_2O_3, the compositions of the eluents and the required volumes must be chosen so as to correspond to the composition of the samples. The purification by column chromatography causes a $\sim 5\%$ loss in vitamin A.

Determination of vitamin A in mixed premixes

According to our assumption and to literature data [4] it was expected that the hydrolysis operation in the presence of large amounts of metal hydroxides or even of the metal constituents bound in the form of EDTA complexes would cause substantial vitamin A losses when mixed premixes will be analyzed. In fact, model hydrolysis experiments carried out in the presence of the metal constituents indicated errors of -10%. Three methods were tried to remove the metal components interfering already with the hydrolysis operation:

(i) dissolution of the interfering metal constituents of the mixed premix prior to hydrolysis,

(ii) dissolution of the gelatine capsules and separation of vitamin A from the inorganic substances prior to hydrolysis,

(iii) modification of the hydrolysis process for the untreated mixed premixes.

The preliminary dissolution of the metal constituents was attempted with ethanol or methyl-*iso*-butylketone adjusted to pH 2 with hydrochloric acid. With acidified ethanol we succeeded to remove the metal constituents to such an extent that when a known amount of vitamin A was added to the mixed premix after the removal of metals with acidified ethanol, this amount was recovered with an error of -10%. However, in spite of the promising preliminary experiments, this method did not prove feasible, since the acidified ethanol dissolves about 20 to 25% vitamin A from the gelatine capsules and thus results in an inadmissibly high error.

Next we attempted the enzymatic digestion of the gelatine capsules using diastase and papaine, followed by the extraction of vitamin A from the aqueous ethanolic suspension of the digested material with a mixture of diethyl ether and petroleum ether. Subsequently the extract was hydrolyzed in the usual manner and purified by column chromatography. Vitamin A was recovered with an error of -25 to -30%, owing partly to incomplete digestion and partly to the substantial adsorption of vitamin A on the carrier.

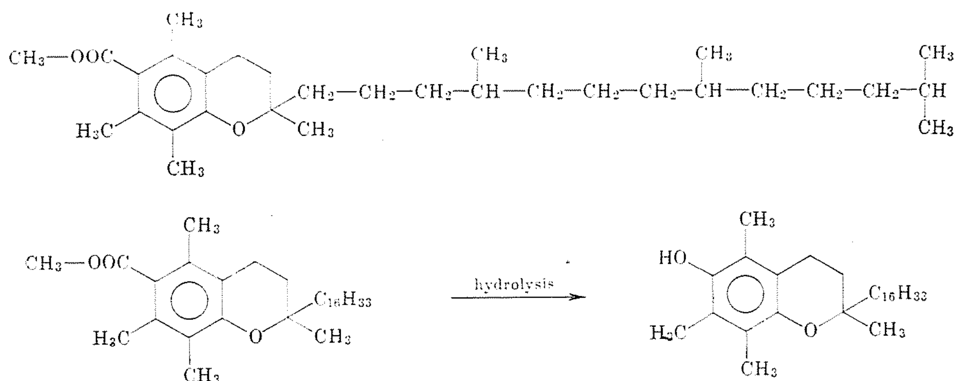
These negative results indicated that only a modified hydrolysis operation can be successful. We carried out model experiments using synthetic

mixtures that consisted of the inorganic constituents of an average mixed premix and known amounts of Mikrovit-A. In these experiments, we applied increasing amounts of hydroquinone as antioxidant and largely increased amounts of sodium sulphide to neutralize the effect of the metal salts. Hydrolysis was followed by the usual extraction with petroleum ether, wash of the extract, change of the solvent and determination of the vitamin A content.

When hydrolysis was carried out in the presence of sufficient amounts of the protective substances, vitamin A was recovered with an error of $\pm 3\%$.

Determination of vitamin E

The biologically most active member of the vitamin E family is racemic α-tocopherol acetate:



In vegetable and animal substances, natural d-α-tocopherol is accompanied by a number of other tocopherols. Vitamin E is introduced into premixes in the form of "Mikrovit-E" which is oily α-tocopherol acetate in small gelatine capsules. From this product, tocopherol acetate has to be separated and converted to alcohol, because the method found best suited for determining vitamin E, viz. the EMMERIE—ENGEL reaction is applicable only to the alcohol.

From the analytical viewpoint, the following properties of vitamin E should be borne in mind: vitamin E, and particularly the free alcohol, is sensitive to heat, air oxygen, light and strong chemical actions. Inorganic salts enhance the decomposition of vitamin E.

Known methods for the determination of vitamin E

Tocopherols show a characteristic absorption maximum in the UV range. However, UV spectrophotometry is only applicable for checking pure

vitamin products. The absorption maximum of dl-α-tocopherol in ethanol is found at 292 nm, that of dl-α-tocopherol acetate in ethanol at 285 nm.

One of the fluorometric determinations [5] is based on the condensation of α-tocopheryl quinone (obtained from vitamin E by oxidation with nitric acid) with o-phenylene diamine. The phenazine derivative obtained in this reaction shows yellowish-green fluorescence in methanol. We found that the method yielded reliable results only when it was applied to pure vitamin products. In premix analyses, it is unsuited, because after purification with floridine (which causes least vitamin E losses and was therefore applied in our studies) there still remain such constituents in the solution which were found to falsify fluorometric measurements.

Probably the most widespread method for determining vitamin E is the spectrophotometric method developed by EMMERIE and ENGEL [6], based on the reducing property of tocopherol alcohols. In the presence of excess amounts of iron(III) chloride and α, α′-dipyridyl, an amount, equivalent to the tocopherol alcohol, of iron(II) ions, or better, their intensely red complex is formed. The intensity of the colour is measured at 520 nm. Our preliminary experiments indicated that this was the most suitable method for determining vitamin E in premixes. Impurities affect the results to a lesser degree than in the fluorometric or cerimetric procedure, and thus relatively simple purification operations are satisfactory. Also, as a spectrophotometric method, it is advantageous for determining the relatively small vitamin E content in premixes.

The polarographic method [7—9] is based on the measurement of the anodic oxidation of α-tocopherol on the dropping mercury electrode, or of the cathodic reduction wave obtained after a previous oxidation to tocopheryl quinone. In the two-electron irreversible anodic oxidation, tocopherols are converted to the corresponding quinone and the chromane ring is split. If the second variant is to be applied, the oxidation prior to polarography is carried out in alcoholic medium with iron(III) chloride. The standard deviation of both procedures is $\pm 5\%$. However, since the polarographic method is only applicable for larger amounts of vitamin E, this method is out of question for the analysis of premixes.

Oxidimetric titration with a solution of cerium(IV) sulphate in the presence of diphenylamine as indicator [10] is also only suited for determining pure α-tocopherol or tocopherol esters in relatively high concentrations, in the presence of indifferent, non-oxidizing accompanying substances. The latter condition must even be taken into account when Mikrovit E is being analyzed. By way of example, we wish to mention that the vitamin E content of a Mikrovit E sample appeared unrealistically high (32.9%) when determined by UV spectrophotometry and cerimetry. The colour reaction of EMMERIE and ENGEL gave a result of 20.2%. After shaking the sample with sulphuric acid

and thereby removing the impurities, the mean value of the results obtained by the mentioned three procedures was 19.7%. This finding indicated that the presence of impurities did not affect the result of the colour reaction.

Separation of vitamin E from interfering constituents

Similarly to the determination of vitamin A in premixes, the first step in vitamin E determination is also alkaline hydrolysis. In this operation the gelatine capsules are dissolved and tocopherol acetate is converted to the alcohol. From the alcoholic alkali solution, vitamin E is extracted with petroleum ether, diethyl ether, chloroform etc. Our experiments indicated that petroleum ether (the least polar among the above solvents) suits the purpose best of all, since it dissolves metal complexes least and extracts — besides vitamin E — only the fat-soluble accompanying substances, thus this extraction is already a purification in some extent.

For further separation of the interfering constituents, column chromatography with floridine earth appeared suitable. Floridine was first separated into granulometric fractions. The 125—400 μ fraction proved the most satisfactory. Floridine activated with tin(II) chloride binds vitamin A and EMQ which — owing to their reducing nature — would appear in the EMMERIE—ENGEL reaction as vitamin E, while vitamin E can be eluted with benzene. The column chromatography of vitamin E causes a negligible loss of about 1%.

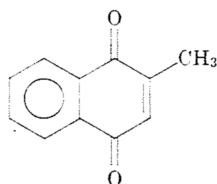
The determination of vitamin E after hydrolysis, extraction with petroleum ether, alkaline and aqueous wash of the extract and purification with floridine earth still yielded results 20% (rel.) higher than the actual percentage. This large positive error can only be due to reducing impurities. Their identification would have been a very difficult task, owing to the great number of constituents in the system. Fortunately the identification proved unnecessary: by inserting a sulphuric acid 1 : 1 wash after the alkaline wash of the petroleum ether extract, the error of the determination was reduced to less than $\pm 10\%$. The vitamin E losses occurring during alkaline and acid washes are negligible.

Purification with floridine can also be carried out as a rapid method without making use of a chromatographic column. Activated floridine is shaken with the solution containing vitamin E and subsequently the solid phase and the organic phase are separated by centrifugage and decantation. Of course, however, the efficiency of column purification is much higher.

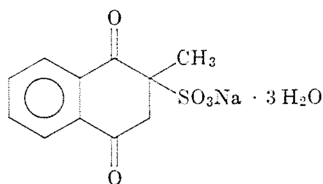
The separation of vitamin E from BHT can be achieved only by column chromatography on basic Al_2O_3. First BHT is eluted with petroleum ether, and subsequently vitamin E with petroleum ether containing 10% diethyl ether.

Determination of vitamin K_3

Vitamin K_3, i.e. 2-methyl-1,4-naphthoquinone belongs to the group of fat-soluble vitamins. It is introduced into premixes in water-soluble form, namely as menadion-bisulphite-sodium:



menadion



hydrated menadion-bisulphite-sodium

The property of menadion-bisulphite-sodium that it is converted to menadion soluble in organic solvents both by the action of alkali and acid can be utilized for its separation. It should also be borne in mind that naphthoquinones readily undergo photolytic decomposition. For this reason, solutions containing vitamin K_3 should be protected from light as far as possible.

Methods for the determination of vitamin K_3

The absorption spectrum of menadion corresponds to the characteristic absorption spectra of quinones. Its absorption maximum in ethanol is found at 332 nm. However, UV spectrophotometry is applicable only for checking the concentration of stock solutions used for calibration.

Larger amounts of vitamin K_3 can be determined by polarography. Similarly to other quinones, 2-methyl-1,4-naphthoquinone yields a reversible cathodic reduction wave on the dropping mercury electrode. The value of the half-wave potential depends on the pH value of the solution.

ONRUST and WÖSTMANN [11, 12] determined the vitamin K_3 content of poultry feed after extraction with petroleum ether, in the presence of a suitable conducting salt and *iso*-propanol. According to HRDÝ [13] the sulphite compound can also be directly reduced on the dropping mercury electrode. For determining the vitamin K_3 content in premixes, the petroleum ether extracts, after changing the solvent to ethanol, are polarographed in the presence of ammonia-ammonium chloride buffer under nitrogen. The relationship wave height *vs.* concentration is linear in the 10 to 60 $\mu g/cm^3$ concentration range. Thus, the polarographic method can be applied favourably when the menadion bisulphite content of the premix is 1 to 5 mg/g.

For determining small amounts of vitamin K_3 MENOTTI [14] proposed the colour reaction with 2,4-dinitrophenylhydrazine. By heating it with this reagent in ethanol containing hydrochloric acid, menadion is converted to

the corresponding hydrazone. After saturation with ammonia, a coloured product is obtained, with absorption maximum at 635 nm. The extinction *vs.* concentration relationship is linear in the 1 to 10 $\mu g/cm^3$ concentration range. Extinction values change little even after standing for 24 hours. Our experiments indicated a standard deviation of the method within $\pm 5\%$. Thus, the method is suited for determining the vitamin K_3 content in premixes.

The spectrophotometric method of KOFLER [15] should also be mentioned. This is based on the blue colouring arising in alcoholic menadion solutions by the action of ethyl cyanoacetate and ammonia. The absorption maximum is at 570 nm and the intensity of the colour is relatively stable.

Separation of vitamin K_3 from accompanying constituents

Our first attempt to recover vitamin K_3 from premixes was extraction with ethanol in a Soxhlet apparatus. Ethanol dissolves menadion bisulphite-sodium, but also dissolves EMQ (BHT) and a number of other interfering constituents, as indicated by the brown colour of the ethanolic extract. A polarographic determination of vitamin K_3 in such solutions showed that the polarographic wave was shifted along the potential axis and its height substantially reduced by the effect of impurities. The error of the determination was -20 to -30%.

We then experimented with floridine and basic Al_2O_3 to remove the impurities. Experiments with stock solutions showed that floridine activated with tin(II) chloride and basic Al_2O_3 bound vitamin K_3 from its petroleum ether solution, whereas activated floridine let it through when it was dissolved in benzene. We therefore made the ethanolic extract of the premix alkaline and extracted menadion from it by shaking with benzene. This solution was then purified by column chromatography on floridine and polarographed after changing the solvent. However, this purification proved unsuccessful: the polarogram was uninterpretable. The method of washing with sulphuric acid which was satisfactory in the separation of vitamin E from impurities did not work either. By washing the solution of vitamin K_3 in petroleum ether with sulphuric acid 1 : 1, 2/3 of the vitamin was lost, as indicated by spectrophotometric measurement.

Finally, we succeeded to recover vitamin K_3 from premixes and to separate it from accompanying substances that interfered with both the polarographic and spectrophotometric determination by utilizing the solubility of menadion-bisulphite-sodium and menadion in different solvents. The procedure consists in the extraction of menadion-bisulphite-sodium with water from the premix. The aqueous filtrate is then made alkaline with sodium hydroxide and menadion is extracted with petroleum ether. The vitamin K_3

content of the latter solution can then be determined without any further purification both by spectrophotometry and polarography.

Determination of the antioxidant EMQ

One antioxidant added to premixes is 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline polymer, abbreviated EMQ. This is a brown, viscous liquid, soluble in oil and various organic solvents like petroleum ether, acetone, ethanol etc. Trade names are Santoquin (Monsanto Corp., U.S.A.), Niflex D (Nitrokémia, Fűzfő, Hungary), Matechin I and II (Material KTSz, Hungary) etc.

Routine determination methods for EMQ

EMQ is not homogeneous. One of its contaminants originating in the manufacturing process may be p-phenetidine. According to the classical analytical procedure, total basicity is determined by titration with perchloric acid in glacial acetic acid, while the p-phenetidine content is determined by acetylation from a separate sample.

Choy and co-workers [16] found that the solution of EMQ in ethanol containing hydrochloric acid had a sharp absorption maximum in the UV range at 296 nm, with a linear relationship between extinction and concentration in the 1 to 100 $\mu g/cm^3$ concentration range. However, UV spectrophotometry can be applied at best for comparing EMQ grades manufactured by different companies, and moreover only if the individual products are also submitted to qualitative analysis, e.g. by paper, thin layer or gas chromatography [17, 18]. In the case of premixes so many interfering effects must be reckoned with that UV spectrophotometry cannot be applied even after purification, e.g. by column chromatography on Al_2O_3.

As routine methods for determining the EMQ content in premixes, the Emmerie—Engel colour reaction and the fluorometric method were applied.

The Emmerie—Engel colour reaction is based on the reducing action of EMQ. In an alcoholic solution, EMQ rapidly and quantitatively reduces iron(III) ions to iron(II) ions. The latter yield the well-known bright red complex with α,α'-dipyridyl. This complex has an absorption maximum at 520 nm and the relationship between extinction and concentration is linear in the 1 to 12 $\mu g/cm^3$ range. However, all constituents that reduce iron(III) ions to iron(II) ions interfere with the Emmerie—Engel colour reaction. The reducing capacity of twice-ground bran, measured after extraction with petroleum ether and solvent change, and expressed as EMQ was found to be negligible (maximum 0.1 mg/g). On the other hand, the p-phenetidine content in Niflex D samples (which was readily detectable and identifiable by gas chromatography) may cause important errors, since it is measured in the Emmerie—Engel reaction as EMQ.

The fluorometric procedure described in the literature [19 to 21] appeared more promising for routine analyses. The fluorometric spectrum of an EMQ sample manufactured by Monsanto is shown in Fig. 3. The spectrum was taken with a "Spekol" spectrophotometer fitted with a fluorometric device, in an acetonic solution containing 4 μg EMQ per cm^3. The exciting light supplied by a mercury vapour lamp is monochromatised by the apparatus. Thus the spectrum represents the intensity of the emitted fluorescent light as a

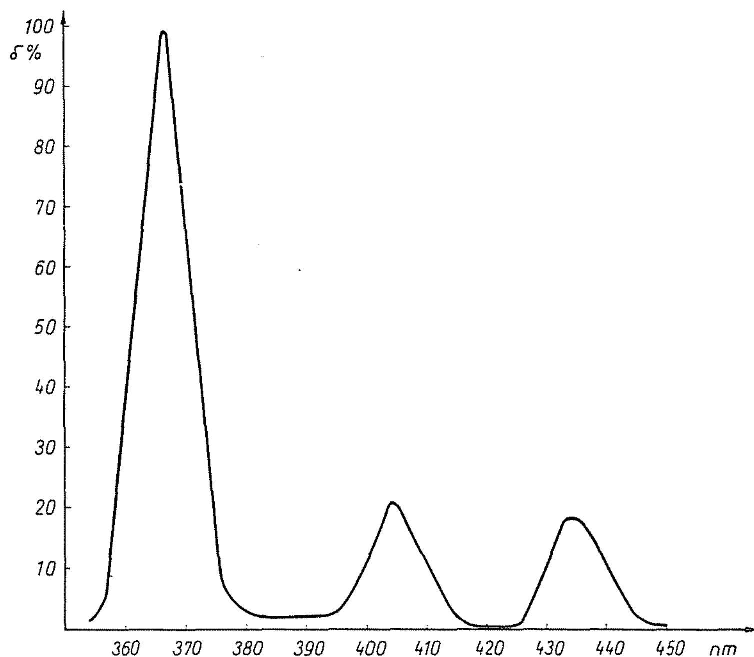


Fig. 3. Fluorometric spectrum of EMQ manufactured by Monsanto

function of the wave-length of the exciting light. EMQ exhibits three fluorescence maxima at 366, 404 and 434 nm exciting wave-lengths, with relative percentages of 100, 21 and 18. Therefore the sensitivity is greatest when measurements are made at 366 nm. This wave-length was also used to prepare the calibration curve, using stock solutions in acetone. The relationship between the intensity of the fluorescent light and EMQ concentration is linear in the 1 to 10 $\mu g/cm^3$ concentration range. Before measurement, it is necessary to thermostat the solutions for a certain period. The fluorometer is adjusted by means of a fluorometric glass standard, with a yellow glass filter placed before the slit of the detector.

Our experiments indicated that p-phenetidine impurities practically did not affect the fluorometric results, since the fluorescence intensity of this compound at 366 nm was substantially lower.

EMQ reference standard

A critical revision of analytical procedures for EMQ, as well as the development of new methods is largely impeded by the fact that up to the present no pure, stable 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline has been produced. Our studies showed that Santoquin (product of Monsanto) which has been recommended in the literature as reference standard was also composed of several constituents. The product was separated into three fractions by molecular distillation at a pressure of 0.4 torr. The "active agent" percentages (expressed as EMQ) of the fractions, related to the original product, are listed in Table 1. All three fractions were snow-white as long as they were kept in sealed ampoules in solid carbon dioxide. At room temperature, however, the colour of the fractions in the sealed ampoules rapidly turned yellow and then brown. This might be the consequence of a rapid polymerization process. In all three fractions several constituents were detected by gas chromatography. We did not, however, attempt their identification. Since no homogeneous commercial product is available, and the synthesis — and even more so, the storage — of such a product would meet with great difficulties, we used Santoquin as reference standard in our premix studies.

Extraction of EMQ from the premix

EMQ was separated from the interfering constituents of the premix by solvent extraction. From the extract, an acetonic solution with the required concentration is prepared and its EMQ content is determined by fluorometry.

In the case of vitamin premixes, extraction with acetone proved satisfactory. In the case of mixed premixes, however, acetone also dissolves other constituents that increase fluorescence intensity, so that EMQ percentages exceeding factual values by about 30% were measured. We therefore use

Table 1

EMQ content in fractions obtained by molecular distillation of Santoquin (Monsanto) in percentages of the original sample

Fraction	EMQ %, determined by	
	colour reaction with α,α'-dipyridyl	fluorometry
Fraction I Boiling at 117—125 °C	90.9	90.0
Fraction II Boiling at 135—146 °C	108.0	107.9
Fraction III Distillation residue	114.2	112.0

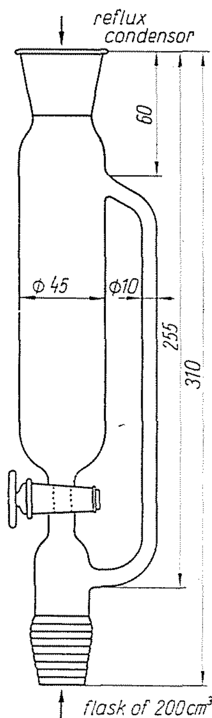


Fig. 4. Modified Soxhlet apparatus

petroleum ether to extract EMQ from mixed premixes. This solvent is then evaporated in a rotating vacuum distilling apparatus under nitrogen and the oily residue is dissolved in acetone.

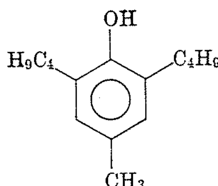
EMQ extraction yields depend on extraction time and temperature. Preliminary experiments indicated that quantitative EMQ extraction can be obtained in the modified Soxhlet apparatus shown in Fig. 4 after 30 minutes (pure solvent droplets fall down at that time). The advantage of the modified Soxhlet apparatus is that relatively small solvent volumes completely cover the sample that is in contact with pure solvent all the time. The sample is weighed into a sample holder made of G3 sintered glass from which EMQ is readily washed out.

Under the given experimental conditions, no fluorescent matter was extracted by petroleum ether from EMQ-free premix samples.

The described method, i.e. extraction with petroleum ether and fluorometric measurement in acetone gave a standard deviation of $\pm 1.5\%$ with vitamin premixes and mixed premixes varying in composition. The deviation of the mean experimental value from the amount factually added to the premix did not exceed $\pm 2\%$. The EMQ content added to premixes is usually 25 mg/g.

Determination of the antioxidant BHT

2,6-Di-*tert*.butyl-4-hydroxytoluene (abbreviated BHT):



is a white crystalline substance soluble in various organic solvents like chloroform, *iso*-octane, methanol, ethanol etc. Added to foods and nutriments, it is a powerful antioxidant. In mixed premixes it is applied in concentrations of 25 mg/g.

Current methods for determining BHT

The detection and determination of BHT has been discussed in numerous papers. However, the proposed methods refer to BHT determination in fats and simple fat-containing foods.

The UV absorption spectrum of BHT shows a characteristic maximum between 270 and 280 nm. However, the method can only be applied for determining BHT separated from accompanying substances by e.g. column chromatography on Al_2O_3 [22].

SZALKOWSKI and GARBER [23] separate BHT from fats and oils by water vapour distillation and bring it into reaction with the diazonium compound of dianisidine. The coloured product formed in the coupling reaction is extracted with chloroform and measured spectrophotometrically. This method has the advantage to be highly specific: other antioxidants react with a colour intensity weaker by two orders of magnitude, and/or the coloured product's absorption maximum is at another wave-length.

The complexometric procedure developed by SEDLÁCEK [24] is based on the reducing action of BHT on silver ions in ethanol containing sodium acetate. The metallic silver is filtrated, dissolved in nitric acid and brought into reaction with $K_2Ni(CN)_4$. The nickel ions released in an equivalent amount to the silver ions are then converted into their complex with EDTA and excess EDTA is titrated with zinc sulphate in the presence of Eriochrome black T as indicator.

SCHWECKE and NELSON [25] report a gas chromatographic procedure for determining antioxidant content in foods and packaging materials. Making use of a column filled with impregnated chromosorbe and a flame ionisation detector, they were able to determine BHT in amounts of 5 to 50 ppm.

In studies on antioxidants in foods, the main objective is identification, since numerous antioxidants are commercially available. Several paper and thin-layer chromatographic methods have been developed for the separation of various antioxidants from the sample and from each other [26—29].

Determination of BHT in mixed premixes

We first tested the feasibility of determining BHT in mixed premixes by UV spectrophotometry. The samples were extracted with chloroform and the extinction of the extract was measured at 283 nm. Owing to the moisture content of the samples, the chloroform extracts were rather opalescent. We therefore dried the extract with anhydrous sodium sulphate. However, even after this operation the measured extinction values were much higher than expected, owing to the extraction of different interfering substances from the sample. An error of about +10% was found. In the case of stale premixes the error occasionally was as high as +50%. Experiments with BHT-free stale premix showed that the decomposition products assumably being formed during storage caused high extinction at 283 nm. To separate the decomposition products, we evaporated the chloroform extracts to dryness, dissolved the residue in petroleum ether and purified the solution by column chromatography using basic Al_2O_3 that contained 10% water eluting BHT with petroleum ether. After changing the solvent to chloroform, extinction was measured at 283 nm. The error was —20%.

These unfavourable results moved us to change over to the spectrophotometric determination utilizing the colour reaction with dianisidine. The absorption curve shown in Fig. 5 was obtained by the following procedure: the solution of BHT in methanol : water 1 : 1 was brought into contact with a solution of 3,3'-dimethoxybenzidine (dianisidine) in methanol containing hydrochloric acid and with a freshly prepared aqueous sodium nitrite solution. After 10 minutes the orange-coloured solution was transferred to a separatory funnel and shaken with chloroform. The red product extracted with chloroform has an absorption maximum at 520 nm. The specific extinction of the solution in chloroform is thrice that of the aqueous solution. Chlorinated solvents, e.g. chloroform, methylene chloride, ethylene dichloride are suited for extraction. The position of the absorption maximum slightly depends on the solvent. The colour intensity of solutions in chloroform is stable for about 1 hour if the solution is protected from light. We tested the effect of nitrite, dianisidine and hydrochloric acid concentrations on extinction. Similarly to literature data, we found that colour intensity is most affected by the nitrite concentration. Maximum colour intensity can only be obtained by applying defined $NaNO_2$, dianisidine and acid concentrations. The colour reaction follows the Lambert—Beer law in the concentration range of 1 to 8 μg BHT per cm^3.

For determining BHT in premixes, we extract the sample with chloroform. After filtration and changing the solvent to methanol the colour reaction is carried out without any further purification. For evaluation 2 to 3 calibration points are taken each time and under identical conditions, using a freshly prepared BHT stock solution.

More detailed information on our analytical procedures, also including the determination of bound choline, choline chloride and trimethylamine will be given in subsequent papers.

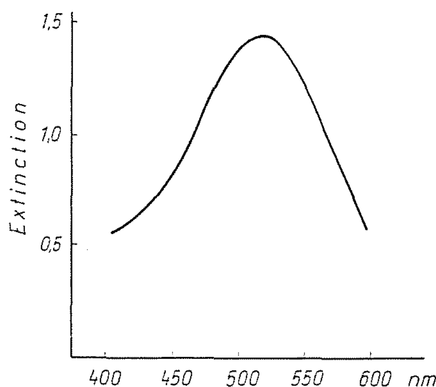


Fig. 5. Absorption curve of the coloured product obtained in the reaction between BHT, dianisidine and sodium nitrite. Solvent: chloroform.

Summary

Procedures have been elaborated to determine vitamins A, E, K_3, choline chloride as well as EMQ and BHT antioxidant content of vitamin and mixed premixes. The way of determinations depends on the composition of the premixes, the origin of the individual constituents, on the carrier and in the case of mixed premixes, on the metal salt content, too. Therefore, our procedures have been developed so that the constituent to be determined has been added to premixes free from vitamins A, E, K_3, choline chloride and antioxidant, in known amounts at different phases of the analysis. Then the analytical procedure was changed according to the experimental results. It is a precondition of satisfactory accuracy that the experimental parameters of the base reaction chosen for the determination of individual constituents are reliably maintained. Other important factor of the accuracy is to apply chemical procedures of digestion and purification likely to facilitate separation of vitamins A, E, K_3, choline chloride and the antioxidants from the accompanying substances disturbing the base reaction with a loss as little as possible.

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