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Determination of vitamin A in cod liver oil and vitamin concentrates by an improved spectrophotometric method

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Determination of vitamin A in cod-liver oil and vitamin concentrates by an improved spectrophotometric method

By

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Of the multidude of methods for determining vitamin A (2,11,22), mainly the two following methods have been widely accepted in practice:

(1) Colorimetric determination according to CARR-PRICE

(2) UV spectrophotometric determination

In spite of numerous suggestions of improvements and changes, the colour reaction according to CARR-PRICE (5) has remained almost unchanged for more than 4 decades, and on account of its high sensitivity it is used mainly for analyses of foodstuffs and feeds.

Spectrophotometric determination by measuring the extinction in ultra-violet light between 325 nm and 330 nm is widely popular

for the quantitative determination of vitamin A in cod-liver oil, fish oils and vitamin A concentrates, and has been incorporated in many pharmacopoeias.

Both methods, however, are not very specific since interferences occur easily on account of impurities of vitamin A (e.g. decomposition products of vitamin A, various sterenes, carotenes and other polyenes). Moreover, a comparative study by M.E.CHILCOTE et al. (7) with 28 different fish oils and thorough studies by other authors (12,17) have shown that deviations between results of chemical and chemico-physical methods and between these results and those of biological methods may be considerably greater than admissible. Chromatographic methods, which might eliminate these shortcomings and have proved useful during the last few years for the determination of D vitamins(e.g.3,20,22), are unsatisfactory because of the high instability of vitamin A. According to E.LUDWIG and U.FREIMUTH (13), the deviations may be as great as 10-25%.

Another method, reported by P.BUDOWSKI and A.BONDI (4), is of interest; it is based on the conversion of vitamin A into its anhydro compound. It is highly specific, but unfortunately very sensitive to interferences and less well reproducible (2). It has recently been modified by K.HARASHIMA et al. (9) and by A.HÄRTEL (8) to suit the purpose of determining vitamin A in

biological material and of examining feeds, but even its modified version is not altogether satisfactory, especially if determination is to be carried out in coloured extracts.

By systematically studying the reaction processes and selecting the most favourable reaction conditions, we have succeeded (23) in modifying and improving the method to the effect that it fulfills the requirements of universal application. It is now easy to apply and involves little time and labour, provided it is carried out with great accuracy. In particular, cumbersome chromatographic purification methods, which experience has shown to be accompanied by uncontrollable losses in vitamin A, are in general not necessary.

After appropriate preparation of the test material by saponification and extraction of the unsaponifiable components, vitamin A can be determined in all pharmaceutical preparations, to a large extent also in foodstuffs and feeds. In cod-liver oil and similar substances, determination is possible within 2 hours (range of error [standard deviation of 24 samples] ± 2.0%).

If vitamin A is determined, however, according to the regulation 6/3 of the DAB (translator's note: German Pharmacopoeia), Supplement 1959, deviations of more than ± 20% must be expected between individual values. Therefore the new method seems to be specially suitable as a pharmacopoeial determination method of

the vitamin A content of cod-liver oil.

Dehydration of vitamin A

Dehydration of vitamin A consists in the conversion of a molecule with "normal" structure (I,IIa) into a "retro" compound* (IIb,III). According to P.MEUNIER et al. (15), this conversion takes place over a mesomeric carbonium compound (IIa,IIb) and results in an increase of the number of double bonds in the molecule from 5 to 6. It is accompanied also by the development of a fine structure of the absorption spectrum in the manner of the carotene spectrum and by a displacement of absorption to longer wave-lengths. (Fig.1).

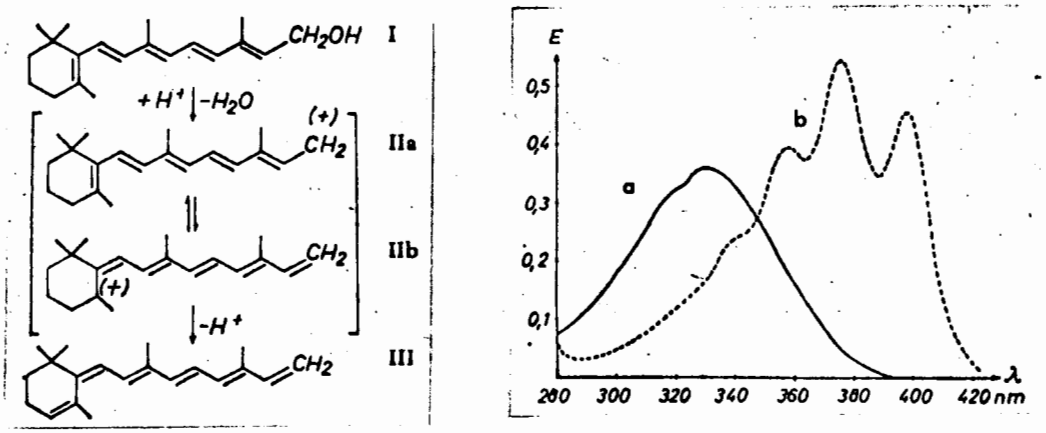


Fig.1. Absorption spectra of all-trans-vitamin A and its anhydro compound (b)

Dehydration is a time reaction the speed of which depends

* According to W.OROSHNIK et al., (16), "retro" compounds are "isomeric polyenes of the ß-Ionone series in which the conjugated double bond system is displaced into the ring by one C-atom".

on the reaction conditions, in particular on the solvent. In benzene, the solvent used by P.BUDOWSKI and A.BONDI and since then generally accepted, it takes place very rapidly and yields different results, depending on the "activity" of the dehydration reagent**. Chlorinated hydrocarbons, however, are more suitable dehydration media. Among them, chloroform and dichloromethane have proved particularly suitable. By adding alcohol or other polar solvents, the speed of the reaction can be sufficiently reduced to let dehydration take place under controllable conditions. A solution of 50 mg. of p-toluenesulfonic acid in 100 ml. of dichloromethane, with 0.5 per cent by volume of ethanol, has proved the most satisfactory dehydration reagent. Its preparation is easy, it is stable and highly insusceptible to humidity. 1452

Dehydration of the all-trans-vitamin A*** to the anhydro compound can be illustrated by the change in the absorption spectrum (Fig.2a and b), and its reaction kinetics can be closely observed by means of the extinction change at 397 nm.

As Fig.3 shows, the extinction rises steeply at first, reaches its maximum after 2.5 minutes, and gradually decreases again on account of the decomposition of the anhydro vitamin(see Fig.2b).

** P.BUDOWSKI and A.BONDI use a solution of p-toluenesulfonic acid in benzene which is "activated" (dehydrated and oversaturated) before use by partial distillation of the solvent. This oversaturated solution is stable only for a short time and very sensitive to even traces of humidity.

*** We are indebted to the firm Deutsche Hoffmann-La Roche AG, Grenzach/Baden for so generously supplying us with vitamin A compounds.

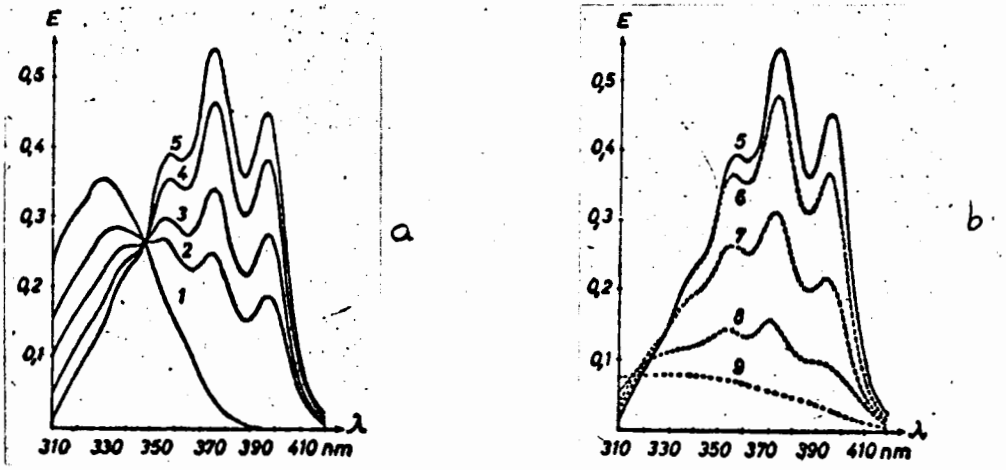


Fig.2. Dehydration of all-trans-vitamin A by p-toluene-sulfonic acid (0.025%) in ethanol-dichloromethane (0.5:99.5 V/V)

Curve 1: before dehydration
Curve 2: after 15 seconds
Curve 3: after 30 seconds
Curve 4: after 1 minute
Curve 5: after 2.5 minutes
Curve 6: after 10 minutes
Curve 7: after 40 minutes
Curve 8: after 2 hours
Curve 9: after 5 hours

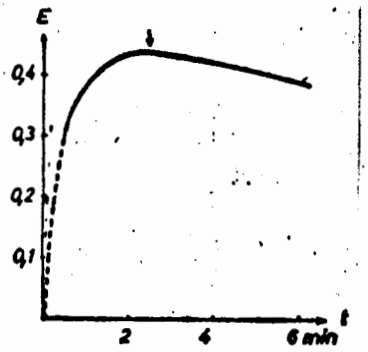


Fig.3. Change of extinction at 397 nm during dehydration of all-trans-vitamin A (23)

Contrary to the method by P.BUDOWSKI and A.BONDI in which the extinction maximum is reached a few seconds after the reagent has been added, it is possible with the new dehydration medium to interrupt the reaction after 2.5 minutes, at the extinction maximum, by adding diethylamine or another base soluble in

dichloromethane. By reducing the H^+ concentration in the reaction mixture, further conversion of the anhydro vitamin formed is immediately stopped. Also, neutralization of the dehydration agent makes it possible to practically eliminate interferences by UV absorbing impurities by means of a blank test and to considerably improve reproducibility.

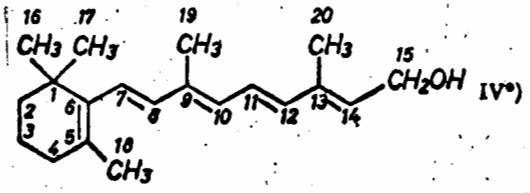
In the blank test, the dehydration agent is neutralized with base right at the beginning, before being added to the vitamin-containing test solution, so that dehydration cannot take place. Both test solution and blank value contain the unsaponifiable components and all reagents in identical concentrations, they differ only with regard to the time when the neutralizing agent is added. Another advantage over the method by P.BUDOWSKI and A.BONDI is the fact that it is not necessary to remove the toluenesulfonic acid required for dehydration by extraction or other manipulations from the solution to be measured. This eliminates another operation which might give rise to errors and prevents distortion of the measured results by different treatment of test solution and blank value.

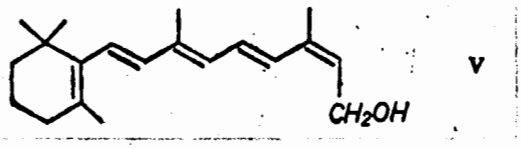
The maximum of the anhydro compound at 397 nm is particularly suitable for measuring the extinction values because there vitamin A shows practically no absorption (cf.Fig.1).

In addition to the all-trans form of vitamin A (IV)*,

* Numbering in the manner usual for carotenes

the most important and biologically most active compound which today is generally used as international standard, the 13-cis isomer, the neovitamin A (V), is also highly significant.





V

Together with the all-trans form, it occurs in almost all natural and synthetic products, so that "vitamin A" in the physiological sense is a mixture of these two geometrical isomers (6,10). In fish-liver oils and vitamin A concentrates, C.D. ROBESON and J.G. BAXTER (21) found neovitamin A contents of 32-39% of the entire vitamin A content.

Biologically, the 13-cis component is less active than the all-trans vitamin. According to S.R. AMES et al.(1), the ratio between the activities of the two components is 0.75:1.

Since the extinctions of the two isomers, however, at 1453
397 nm have, according to Table 1, a ratio of 1865:2410 (i.e. 0.77:1), the results of spectrophotometric determinations for both vitamins at the same time agree well with their biological activities. Considering that approximately one third of natural

"vitamin A" consists of neovitamin A, and that with a dehydration period of 2.5 minutes only 91% of the extinction maximum of anhydro neovitamin A is reached, 1 part of the total content of vitamin, determined by spectrophotometry and related to the all-trans form, corresponds to the activity of 1.02 parts of all-trans vitamin A.

Table 1. Spectral properties and dehydration periods of all-trans and neo-vitamin A in ethanol-dichloromethane (0.5:99.5 V/V)

	Absorptions-maxima	$E^{1\%}_{1\,cm}$	Dehydr.-Zeit (Dehydration period)
all-trans-Vitamin A	330,5 nm	1800	2,5 min
Anhydro-verbindung (compound)	357, 375, 397 nm	2100, 2875, 2410	—
Neo-vitamin A	333,5 nm	1665	5,0 min
Anhydro-verbindung (compound)	357, 375, 397 nm	1840, 2410, 1865	—

Saponification of the test material

Since vitamin A occurs mostly in esterified form in combined preparations and in natural products, it is necessary with almost all methods to liberate the vitamin from its ester compounds before determination. This is done by saponification which simultaneously results in the separation of ballast material and in the accumulation of the vitamin to be determined. Saponification is carried out with ethanolic potassium hydroxide solution, and it is expedient to

conduct it in the presence of nitrogen to prevent oxidation of the vitamin with atmospheric oxygen. An addition of hydroquinone is recommended as additional protection against oxidation, while for the separation of the liberated vitamin ether is frequently used. Weighty arguments, however, speak against both these agents. By oxidation, hydroquinone very easily forms p-quinone which may react with vitamin·A in the form of a "Diels Alder reaction", thereby making it impossible to determine the latter. To prevent this disturbance, we have replaced hydroquinone with the more suitable butylhydroxyanisole.

The following factors speak against the use of ether:

(1) There is the danger of ether peroxides being formed which react with the oxidation-sensitive vitamin A and destroy it.

(2) With most saponification solutions, emulsions are formed which are separated only with difficulty and therefore involve a great amount of time and labour.

(3) The fair solubility of ether in water may also lead to considerable losses of vitamin A (up to 5%) when the ether extracts are washed with water.

Therefore, an indifferent solvent which is practically immiscible with water, such as hexane or petrol ether (Pharmacopée Francais VIII, Pharmacop.Helv. V/Suppl.II, and German Pharmacopoeia 7/DDR 1964), should be used for vitamin extraction after saponification.

According to our experiences, pentane is even more suitable.

F.R. PREUSS and A. KNECHT (19),and A. MARIANI and C.VICARI (14) point out the advantages of cold saponification which, however, does not find the approval of R. STROHECKER and H.M. HENNING (22). In experiments of our own with purest vitamin A (all-trans vitamin A alcohol) which underwent hot saponification and then cold saponification (in the presence of nitrogen and butylhydroxyanisole) and was examined thin-layer chromatographically after extraction with pentane, we did not observe any changes either by isomerization processes or by decomposition products. In no case was the recovered substance different from the pure, untreated original substance. Also, the vitamin A used was recovered quantitatively in both cases by spectrophotometric determination. For reasons of time, hot saponification should therefore be preferred, especially when preparations containing carbohydrates are examined.

Furthermore, enzymatic fusion is recommended (11,24) as preliminary treatment of dry vitamin A preparations. Since it always involves losses of vitamin A, however, this treatment cannot be approved. The method proposed by the Analytical Methods Committee (2) (triple saponification) is preferable.

On the basis of these findings, the procedure described below has proved to be particularly satisfactory.

Procedure

Since the dehydration time and the extinction maximum depend on the reaction temperature, on the p-toluenesulfonic acid content of the solution and on the ethanol content of the dichloromethane, strict adherence to the following procedure is imperative.

All operations must be carried out as quickly as possible, the presence of atmospheric oxygen and direct daylight should be minimized, and brown-glass tubes/should be used throughout.
and vessels

(a) Saponification

30 ml. of absolute ethanol, 0.1 g. of butylhydroxyanisole and a solution of 1.0 g. of potassium hydroxide in 2 ml. of water are added to 1-2 g. of the material to be tested; the latter should be weighed accurately. The mixture is boiled in the presence of nitrogen for 30 minutes on a water-bath on the reflux condenser. With preparations rich in carbohydrates and proteins, a second saponification in 50% ethanol and possible a third one in aqueous solution (2) follow. After rapid cooling under running water, the mixture is transferred into a separatory funnel with 30 ml. of water and extracted 4 times with 40 ml. of pentane each under moderate agitation. The combined pentane extracts are washed twice with 100 ml. of n/2 potassium hydroxide solution each and twice with 100 ml. of water each. The organic phase is dried over sodium

sulfate and quickly filtered through cotton wool into a distillation flask. Then it is washed with 10 ml. of pentane and concentrated to 3-5 ml. in the vacuum rotation evaporator below 35°. The remaining solvent is expelled without heat and vacuum by introducing nitrogen. The residue on evaporation is immediately absorbed in ethanol-containing dichloromethane and diluted so much that the solution does not contain more than 20 I.E. vitamin A per ml. (test solution).

(b) Dehydration

Main sample: 5.00 ml. of dehydration agent are added to 5.00 ml. of test solution at 20° (± 1°). The mixture is allowed to stand for exactly 2.5 minutes at 20°, and 0.05 ml. of diethylamine is mixed in quickly.

Blank sample: 0.05 ml. of diethylamine is added to 5.00 ml. of dehydration reagent. After mixing them well, 5.00 ml. of test solution are added.

(c) Spectrophotometric determination

The extinction of the main sample is compared with that of the blank sample at 397 nm in a 1-cm-vessel with stopper. From the extinction measured, the vitamin A content is computed by means 1454 of the calibration curve (Fig.4) or by the formula (23)

$$29.46 \cdot \frac{v}{e} \cdot E_{397} = \text{I.E. vitamin A per gram of material tested}$$

v = volume of test solution (in ml)
e = test material weighed in (in g)

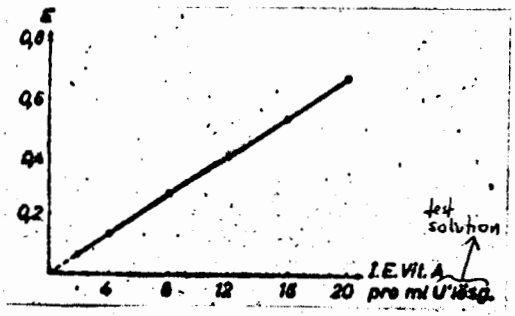


Fig.4.
Calibration curve for spectrophotometric determination of vitamin A

(d) Reagents:

(1) Nitrogen: Compressed nitrogen is used which is conducted through an alkaline solution of pyrogallol and subsequently through concentrated sulfuric acid to remove traces of oxygen and humidity.

(2) Pentane: (boiling point 34-36°; d = 0.625)

(3) Dichloromethane, containing ethanol: 500 ml. of dichloromethane (for analysis) are washed 4 times with 250 ml. of water each, dried over potassium carbonate and filtered. Then the solvent is distilled, the first and the last runnings are thrown out, and 0.50 ml. of ethanol (absolute) is added to 100.0 ml. of distillate each.

(4) Dehydration reagent: After drying over P_2O_5, 50.0 mg. of p-toluenesulfonic acid f.Chrom. "Merck"* are dissolved in 100.0 ml. of ethanol-containing dichloromethane (reagent No.3) under vigorous agitation.

* Translator's note: The abbreviation "f.Chrom." presumably means "für Chromatographie" = for chromatography

Examination of oily vitamin A solutions

Determination was carried out in a self-prepared solution of 10.1 mg. of all-trans vitamin A acetate in 30.0 g. of Ol.Arachidis DAB 6 (German Pharmacopoeia). The unsaponifiable fraction obtained after saponification is absorbed in 50 ml. of ethanol-containing dichloromethane, and the vitamin A content is determined as directed.

Computation: $\frac{1473}{e} \cdot E_{397}$ I.E. vitamin A pro gram

Theoretical content: 969 I.E. pro gram of oil

Summary

An improved specific method for the determination of the vitamin A content in drugs and other products is described which is based on the conversion of the vitamin A alcohol into its anhydro compound.

The improvement of the procedure is based on the selection of the most favourable reaction conditions for dehydration. The method is suitable both for the examination of raw and finished products used in pharmaceutical industry and for the analysis of foodstuffs and feeds; it has proved highly satisfactory in the examination of several hundred preparations.

With the determination of vitamin A in cod-liver oil and in oily solutions, the range of error, saponification included,

is ± 2.0% (standard deviation of 24 samples each). Special emphasis is placed on the suitability of this method for pharmacopoeial methods.

Table 2. Determination of vitamin A in arachis oil (self-prepared)

1 Einwaage an Öle (g)	2 Extinktion E	3 Einzelwerte c (I. E./g)	4 Mittelwerte $\bar{c}$ (I. E./g)	5 Wirkstoff gefunden Standardabweichung 6 s (I. E.)	$\frac{s}{c}100$ (%)
0,512	0,340	976			
0,495	0,317	942			
0,502	0,322	942			
0,488	0,328	988			
0,512	0,350	1006			
0,500	0,333	980	964	±18,2	1,9
0,496	0,319	946			
0,482	0,314	958			
0,514	0,333	952			
0,494	0,321	956			
0,509	0,336	970			
0,516	0,335	956			
0,906	0,587	953			
0,988	0,648	964			
1,020	0,669	947			
1,142	0,732	942			
0,915	0,622	1000			
0,850	0,574	993	966	±18,8	1,9
1,100	0,737	935			
1,202	0,777	950			
1,111	0,728	964			
1,042	0,682	962			
0,925	0,615	978			
0,872	0,567	956			

1 - weighed-in amount of oil
2 - extinction
3 - individual values
4 - mean values
5 - amount of active substance recovered
6 - standard deviation

Same legend for Table 3

Table 3. Determination of vitamin A in cod-liver oil

1 Einwaage c (g)	2 Extinktion E	3 Einzelwerte c (I. E./g)	4 Wirkstoff gefunden Mittelwerte $\bar{c}$ (I. E./g)	5 Standardabweichung s (I. E.)	6 $\frac{s}{c}$ 100 (%)
0,492	0,288	870			
0,486	0,280	848			
0,512	0,305	876			
0,505	0,284	828			
0,488	0,273	824			
0,499	0,291	858			
0,498	0,286	852	852	±16,4	1,9
0,515	0,303	864			
0,508	0,296	858			
0,491	0,290	868			
0,498	0,284	838			
0,507	0,293	850			
0,992	0,561	832			
0,933	0,550	823			
1,002	0,589	865			
0,971	0,571	865			
0,989	0,584	868			
1,105	0,639	850	850	±16,7	2,0
0,936	0,574	860			
1,003	0,597	875			
1,123	0,631	826			
1,091	0,623	841			
0,987	0,577	860			
1,159	0,661	839			

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