PHYSICO-CHEMICAL METHODS FOR THE SEPARATION AND ESTIMATION OF VITAMIN 'A' IN FISH LIVER OILS AND OTHER PHARMACEUTICALS

H. K. KULSHRESHTHA AND J. N. TAYAL

Detachment Defence Research Laboratory (Materials), Gwalior

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A review of the different physico-chemical procedures, including colorimetric methods employed in the determination of vitamin *A* in pharmaceuticals (with greater emphasis on the Carr-Price reaction, involving the antimony-trichloride and glycerol-dichlorohydrin reagent) and the spectrophotometric technique, has been presented. Need for further work with regard to the determination of this vitamin *A* in fish liver oils and multivitamin formulations has been stressed; for its purification and estimation, chromatographic techniques involving column, partition and paper chromatography, have been reviewed. It has been recommended that Indian fish liver oils should be characterised in terms of the biologically active vitamin *A*; collaborative research effort to achieve this has been suggested.

The problem of estimating vitamin *A* content of the essential foods and feeds as well as pharmaceutical preparations including fish liver oils, has made appreciable progress. Estimation of vitaman *A* in foods and feeds presents great difficulty due to interference from substances like carotenoids, xanthophylls, edible fats, and colouring/flavouring materials. However, in the case of pharmaceutical formulations such as multivitamin tablets/drops, concentrate of vitamin *A* and *D*, vitamin *A* and *D* tablets/capsules, fish liver oils etc., the progress has been satisfactory. Regarding the methods of estimating vitamin *A* in these preparations there is, on the one hand, the lengthy and cumbersome, though not altogether dispensable, bioassay procedure and on the other hand, there are the physico-chemical methods based, either on the determination of colour-density of the reaction products involving the different complex substances (with structures not completely understood) or the absorption-maxima characteristics of the vitamin in the ultraviolet range of the spectrum in different solvents. This paper reviews the colorimetric and spectrophotometric procedures for the estimation of vitamin *A*. Special emphasis has been given to the spectrophotometric method of assay as it is cheaper than the bioassay procedure, quicker, easier to handle and more trustworthy. Because of these advantages this method has been officially incorporated in the Pharmacopoeias and other official documents, such as British Pharmacopoeia [1], United States Pharmacopoeia XVI [2], Indian Pharmacopoeia [3] and the methods of the Association of Official Agricultural Chemists [4]. The Pharmacopoeial methods have their own limitations and it has been experienced that the details are not adequate for standardisation of fish liver oils, where chromatographic separation of the different isomers of vitamin *A* becomes indispensable for assessing the correct biological status of the oil. The relevant aspects of chromatographic techniques have also been discussed.

PROPERTIES OF VITAMIN 'A' ISOMERS

General properties—The structure of vitamin *A* (now called retinol), $C_{20}H_{29}OH$ is given in Fig. 1. It is an unsaturated isoprenoid polyene alcohol and is also known as vitamin *A*,

and axerophthol. It is fat-soluble, insoluble in water, sensitive to ultraviolet[5] rays and artificial room illumination[6] when diluted to a few mg/litre. It is unstable in acids, but stable in alkali as also on an acidic or acid-activated adsorbents. It is readily adsorbed on alumina magnesia and other neutral or alkaline adsorbents. Commercially available synthetic vitamin *A* is supplied as palmitate or acetate.

Isomers of vitamin A— Of the five known *cis* isomers of vitamin *A* two are stereochemically hindered and three are unhindered. These are 13, 14 *cis* (neo-vitamin *A*); 9, 10—13, 14 *di-cis*;[7,8] 11, 12 *cis*—; and 7, 8 *cis* vitamin *A*. A brief account of the isomers of vitamin *A* has been given by Oroshnik[7]. Another isomer of vitamin *A* with slightly different benzene ring structure, having an additional double bond in the β—ionone ring, occurs in the liver of some fresh water fishes. The structural formula of this compound[8] is given in Fig. 2.

Out of all these isomers, only the all-trans vitamin *A*, neo-vitamin *A* and vitamin A_2 are of importance for the purpose of this review. In addition, the aldehyde, retinene the dehydration product and anhydrovitamin *A* (which is quite often associated with vitamin *A* as an artefact on chromatography) have also been discussed.

Spectroscopic properties—Vitamin *A* absorbs in the ultraviolet region; the all trans-vitamin *A*, for example, has the absorption maxima in the region of 325—328 mμ. The spectroscopic properties of vitamin *A* have been studied by Cama *et al*[9]. Neovitamin *A*, which was discovered later in fish liver oils is shown to have one third of vitamin *A* activity[10]. Vitamin *A* has a distinctive green fluorescence whereas kitol, anhydrovitamin *A* and vitamin A_2 have different fluorescence. For quantitative estimation of vitamin *A*, no suitable fluorimetric method is available, although Sabotka *et al* [11] have suggested the application of four-fold increase in the fluorescence after irradiation for a few minutes for the distinction of vitamin *A* esters and free alcohols. The spectroscopic properties of vitamin *A* alcohol and acetate in the various solvents as determined by Cama *et al* [9] are given in Table 1, while those of the isomers, as acetate, and vitamin A_2 in Table 2.

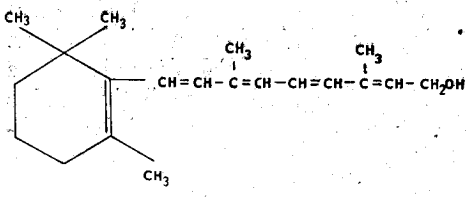

Fig. 1—*Vitamin* A_1, *Retinol*

[all-trans-3, 7-dimethyl-9- (2, 6, 6-tri methyl-1-Cyclohexen-1-yl)-2, 4, 6, 8-nonatetraen-1-ol]

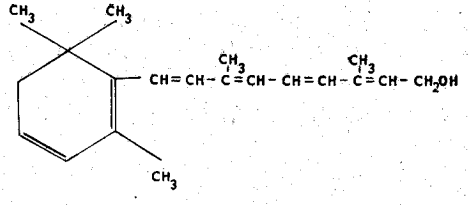

Fig. 2—*Vitamin* A_2.

[all-trans-3, 7-dimethyl-9- (2, 6, 6-trimethyl-1-Cyclohexen-1, 3-yl)-2, 4, 6, 8-nonatetraen-1-ol]

TABLE 1

EXTINCTION VALUES OF VITAMIN A ALCOHOL AND ACETATE IN DIFFERENT SOLVENTS AT WAVE LENGTHS OF MAXIMUM ABSORPTION

	Vitamin A alcohol		Vitamin A acetate	
	Wave length of max. absorption mμ	$E^{1\%}_{1cm}$ max. absorption	Wave length of max. absorption. (mμ)	$E^{1\%}_{1cm}$ maximum absorption
Ethanol	324	1,800	326	1,500
Iso-propanol	325·5	1,835	326	1,535
Cyclo-hexane	326·5	1,745	328	1,515
Light petroleum	324·5	1,830	325	1,595

Antimony-trichloride gives a blue colour with vitamin *A*; the maximum of the colour intensity is obtained at 620 mμ, ($E^{1\%}_{1cm}$ being 5,000) and the amount of colour for other isomers is substantially the same. In the case of vitamin A_2 the maximum of the colour intensity is obtained at 693 mμ. The development of colour with antimony chloride is popularly known as the Carr-Price reaction.[12]

METHODS OF ESTIMATION

The estimation of optical density of the colour produced by addition of antimony-trichloride reagent and the determination of extinction values at the specified wavelength are the two procedures commonly followed for the estimation of vitamin *A*. Important steps concerning these procedures are discussed below :

Preliminary treatment—Often a preliminary treatment necessary for preparing suitable samples for estimating vitamin *A* content of vitaminised preparations is saponification with alkali, usually alcoholic KOH, in an atmosphere of an inert gas (saponification in case of high potency liver oils is not considered essential). The unsaponifiable portion is extracted with diethyl ether, ether removed under suction and the sample prepared by dissolving the residue in a suitable solvent. In the

TABLE 2

EXTINCTIONS VALUES OF DIFFERENT ISOMERS OF VITAMIN 'A' AND VITAMIN 'A'$_2$ AT WAVE LENGTHS OF MAXIMUM ABSORPTION

Name of the Isomer	Wave length of maximum absorption (mμ).	$E^{1\%}_{1cm}$
All-trans Vitamin A	326	1,565
Neo Vitamin A	328	1,435
Vitamin A_2	351; 287	1,460; 820*

*See Shantz[173]

case of vitamin *A* rich oils, such as halibut liver oil, reliable readings are obtained by simple dilution with chloroform, while with cod liver oil saponification is essential. In the latter case, the oil is heated with alcoholic KOH and refluxed for about 1 hour. On cooling, water is added and the vitamin extracted with diethyl ether. The next step comprises evaporating the solvent after carefully drying over anhydrous $Na_2 SO_4$; the residue is immediately dissolved in chloroform for preparing the sample. It would thus appear that, in general, the important steps concerned with the preparation of a sample are: saponification with an alkali followed by extraction with a suitable solvent, removal of the solvent by evaporation, following dehydration of the hitherto incoming moisture and dissolution of the residue in a suitable solvent.

SAPONIFICATION AND EXTRACTION

A number of saponification procedures are reported in literature [13]. However, the United States Pharmacopoeia[2] gives quite a convenient method for fish liver oils and pharmaceutical preparations. Mulder[14] greatly simplified the steps of saponification and extraction by dissolving the alcohol soap solution in benzene prior to addition of water. The theory is that this hinders the enclosure of the hydrophobic nonsap matter by soap micella whose double layer would, otherwise, prevent the action of the extracting agent. A micro-assay method involving the use of methyl alcohol in place of ethyl alcohol and benzene has been developed by Ogawa and Kobayashi[15] who found the method giving higher value by 2—3 % and corrected the value by multiplying with 0·98. Servigne[16] used a method for the removal of sterols and cholesterol from the unsaponified portion of the natural oils by freezing, followed by extraction with alcohol and subsequently by acetonitrile. Vendt[17] eliminated the sterol interference by treatment with digitonin, the latter forming a yellow precipitate; the reaction is sufficiently quantitative but costly for routine analysis. Powell *et al* [18] and Napoli *et al* [19] replaced the four times ether extraction procedure for vitamin *A* as envisaged in the USP XVI[2] by the use of five fold volume of ether so that the time required is short and at the same time there is improvement in the precision by 2—3 % Kulshreshtha & Tayal explored the possibility of such an extraction procedure and confirmed the findings of Napoli *et al* [19]. In a modified analytical procedure for vitamin *A* assay, Tardiff [20] replaced ethyl and isopropyl alcohol by hexane for the extraction of the non-sap portion and preparation of the sample before estimating absorption values (see also Gridgeman [21]). In one of the methods for the extraction of vitamin *A* from cod liver oil phase partition between petroleum ether and 83·0 % aqueous ethanol was used in order to separate the vitamin *A* alcohol from the high molecular weight esters present in fish liver oils [22].

The authors have found that saponification is not essential for pharmaceutical preparations such as vitamin *A* concentrate, vitamin *A* and *D* capsules and particularly some special formulations processed into dosage forms from synthetic vitamin *A*. But in general, saponification becomes necessary, even in cases where the preparation has been made from synthetic vitamin *A*. The problem of removing fat-soluble vitamins from the water-soluble ones is on the whole not difficult; in fact interference in the vitamin *A* determination is due to the presence of available vitamin *E* in many multivitamin formulations [23] and, in addition, to the presence of other fat-soluble vitamins, say *D* and *K*.

Purification by chromatography—Chromatography, both paper and column, has been an indispensible technique in the field of carotenoids, and vitamin *A*[24]. It has been

used for the isolation and purification of the natural pigments as also the separation and estimation of the various isomers of the substances in question[25]. Adsorption analysis has been and is still used for the purpose and is indispensable even in the synthesis of the various vitamin *A* isomers and other derivatives. Prior to submitting the sample to spectrophotometric or colorimetric estimation, sometimes a chromatographic procedure is necessary for the removal of the interfering substances.

Column chromatography—Column Chromatography, using a column of adsorbent, offers a fairly satisfactory method for eliminating irrelevant adsorption in samples of vitamin *A* prepared for colorimetric or ultraviolet absorption estimation[26]. A variety of adsorbents have been used for filling columns for chromatographic purification of vitamin *A* sample. Alumina (Al_2O_3) prepared in various forms, either as aluminium oxide, aluminium hydrate, fibrous alumina, alkaline alumina, acidic alumina or neutral alumina[27], has been very popular with investigators in the field. The advantages with this material are its large capacity, white colour, insolubility in most of the solvents and its chemically inert nature. Hence it can be used for most of the substances; an added advantage of this material is that it can be made to exchange with either cations or anions after suitable preliminary treatment[28]. But there are difficulties: the activity of Al_2O_3 used for the preparation of the column is critical [29-31]. If the adsorbent is more active than the requirement, some of the vitamin *A* may be destroyed; dehydration of the vitamin *A* alcohol to anhydrovitamin *A* also takes place. On the other hand, if it possesses abnormal activity, separation may not take place satisfactorily. Several procedures for the standardisation of alumina have been described in literature.[32—38]. None of the techniques is thoroughly satisfactory and each worker has to carefully standardise his own technique for the purpose [39].

In Hjarde's [40] procedure for purification of vitamin *A* samples, chromatography involving the use of Al_2O_3 provides quite a satisfactory method for preparation of the sample; he also used dibasic calcium phosphate as an adsorbent. The method has been recommended for bulk vitamin *A* preparation[5] and has been reported to be adopted in several European countries, for example, in the Swedish Pharmacopoeia a very judicious combination of Hjarde's procedure of chromotographic purification and spectrophotometry has been specified. To guard against any loss during the chromatographic separation, vitamin *A* reference is also treated in the same way as a preliminary step before spectrophotometric analysis. Another preparation is alumina (Brockmann) mixed with 4 to 8 per cent of its weight of water and allowed to stand for a minimum of twentyfour hours before being used[31]. Other adsorbents employed in the purification and estimation of vitamin *A* are manganese dioxide[41] (not suitable as vitamin *A* is oxidized to retinene) calcium carbonate[42], calcium hydroxide[43] magnesium carbonate, magnesium oxide, magnesium hydroxide[44], sodium carbonate[45], silicic acid[46], diatomaceous earth[47], defatted bonemeal[48—52], etc. The technique for filling the column as also the choice of eluents are highly sepecific and investigators in the field have employed thin layer columns as well *e. g.* Takahashi[53] used a double layer column of superfiltrol (moisture 10·0 %) below and above an alumina column (moisture, 4·0 %) the solvent for elution being hexane-ether (7 : 1); Blattna & Davidek[54] have applied a technique of thin layer chromatography.

APPLICATION OF COLUMN CHROMATOGRAPHY TO THE PREPARATION OF SAMPLES FOR VITAMIN 'A' ESTIMATION

General considerations—A general procedure for applying chromatography for the estimation of vitamin *A* could be outlined as: subject to adherence to the preliminary

precautions for the preparation of the column, the final preparation should be done by pouring a slurry of the adsorbent into the tube and packing by filtration, freely or under mild suction or by tampering successive portions of the adsorbent with a flat tipped rod which is usually supplied with the chromatographic columns[55]. The solvent is always kept within a few centimeters from the top of the column so that the surface of the adsorbent is never dry and exposed to atmospheric air, otherwise oxidative products will be formed, since vitamin *A* is liable to oxidation; keeping the adsorbent always covered with the solvent in the tube will avoid channelling. Vitamin *A* in solution is added preferably in minimum volume of a non-polar solvent, petroleum ether (40°—60°C), followed by small portions of the same solvent; for development of the chromatogram, a mild eluting solvent, usually diethyl ether, may be used. The movement of vitamin *A* band may be followed in two ways[56] (*a*) large number of fractions may be collected and tested colorimetrically by Carr-Price reagent (*b*) the vitamin *A* band may be followed by means of its characteristic light green fluorescence under ultraviolet radiation[57-59]; however, since vitamin *A* is easily destroyed by excessive irradiation, the exposure is carefully done for a short time only.

SEPARATION OF VITAMIN 'A' FROM OTHER VITAMINS, ESPECIALLY VITAMIN 'D'

Separation of vitamin *A* and *D* from each other is important because estimation of either of them presents difficulty if appreciable amount of the other vitamin is present in the sample. Muller[60] employed alumina prepared by his method for the chromatographic separation of vitamin *A* and *D*. A satisfactory method is the use of the earth, Floridin by Wald[41]; a loss of vitamin *A* takes place but the separation is complete and vitamin *D* is quantitatively recovered. Embree and Hawk[61] separated these vitamins by selective adsorption on sodium aluminium silicate; vitamin *A* was eluted with light petroleum and vitamin *D* with acetone. By a double column choromatography of fish liver oils on superfiltrol (activated Bentonite) vitamin *A* was eliminated by Ewing *et al* [62], Bruggeman *et al*[63] used the properties of the antimony trichloride complexes of vitamin *A* and vitamin *D* for the separation of the two vitamins by preparing the complexes on a column of talc; the latter being previously impregnated with the reagent. The vitamin *A* complex was adsorbed and that of vitamin *D* eluted into a starch column. Miwa[64] used a small column (1·5 × 6·0cm) of alumina for the chromatographic separation of vitamin *A* and *D*: rapid chromatographic separation of vitamin *A* from *D* on *bolus alba* (Kaolin) has been described by Tshapka and Plessing[65]. Indigenous bentonite, previously neutralised with HCI and dried at 100°C, was used for the separation of vitamin *A* from *D* by Murea and Bercovici[66]. Arcus and Dunckley[67] used columns of 'Teflon' for the separation of vitamin *A* from vitamin *D*, cholesterol and coprosterol. Takahashi *et al*[53] employed a double layer column of alumina (moisture 4 %) and superfiltrol (moisture, 10 %) for the separation of vitamin *A* from vitamin *D*, the solvent for elution being hexane-ether mixture (7 : 1). Possibility of employing a triple layer column with two layers of super-filtrol above and below the alumina layer was also examined by Takahashi[53]. Castren[68] effected the purification of vitamin *A* from vitamins *D* and *E* in tablets, using thin layer chromatography (*see* also Blattna and Davidek[54]). Mariani and Vicari[69] separated vitamin *A* from *D* in drugs by a chromatographic technique using a column of Filtrol-19, and benzene. Daniels *et al*[70] employed column chromatography for the separation of vitamin *A* from mixture of vitamins on "Celite 545"; the mixture was taken in polyethylene glycol 600 and the mobile phase used was petroleum ether (*see* also Thievagt

and Campbell[71].) A good separation of vitamin A_1 and D_3 was made on 'Fluoropak-80' impregnated with 2,2,4-trimethyl pentane by Chen *et al*[72]; 90% methyl alcohol was employed as the mobile phase.

Separation of vitamin A isomers and retinene from one another by column chromatography—Besides the separation of all-trans vitamin A_1, neo-cis vitamin A, all-trans vitamins A_2 from one another, the separation of vitamin A from retinene or that of vitamin A alcohol from the acetate and the palmitate is of no less importance. Muller[36] and Plack *et al*[73] used an alumina column for the separation of vitamin A from retinene, while Cooley *et al*[45] used sodium carbonate for the purpose. Lederer *et al*[74] and Servigne *et al*[75] used deactivated alumina column for the separation of vitamin A esters from the fish liver oils. Eden[48] has described a micromethod using a small alumina column. Dowler & Laughland[49] modified Eden's procedure, running skelly-solve B solutions of vitamin A oils through a 6×50 mm layer of bone meal. Bro-Rasmussen *et al*[76] have separated and estimated the two vitamin A isomers, *viz.*, neo-vitamin A and all trans-vitamin A_1 and also vitamin A_2 from cod-liver oil on calcium hydrogen phosphate; a loss of 10·0 % potency on this adsorbent has been recorded[40], which could be minimized by careful inactivation of the adsorbent. Pradhan & Mager and Barua & Nayer[77] have reported investigations regarding vitamin A isomers in Indian shark liver oils. Gridgeman *et al*[78], Swann[79], and Barnholdt[80] have also presented the preparation of the column and the elution for separation of different active components of the fish liver oils. Cama *et al*[81], Embree & Shantz[82] and Jansen *et al*[83] have presented details for the separation of vitamin A_1 from vitamin A_2 or the aldehydes; Balasundaram *et al*[84] have described the separation of vitamin A_1, A_2 and anhydrovitamin A_1. Glover *et al*[51] have described the separation of vitamin A esters from vitamin A alcohols on defatted bone-meal; Wilkie and Jones[50] have described a procedure for standardisation of the adsorbent (*see* also Wilkie[85] and Cooley[86]).

Partition and paper chromatography—Partition chromatography or paper chromatography, including reversed phase paper or column chromatography and circular paper chromatography has also been employed for the purification of vitamin A. Datta and Overell[87] were the first to employ alumina treated filter paper chromatography for the separation and detection of vitamin A and its derivatives such as esters, anhydrovitamin A_1 and retinene; small quantities upto 1·0 microgram could be identified by this technique. Brown[88] has described ascending chromatography for separation of common forms of vitamin A from each other and α-tocopherol; he employed silicone impregnated paper strips, to which vitamin solution in n-hexane, chloroform or isopropyl alcohol was transferred, the strips being subsequently dried in CO_2 and examined with ultraviolet irradiation. Boccacci[89] used paper impregnated with mineral or olive oil (3·0 % in petroleum ether) and silicone DC 200/500 (in 5·0 % cyclohexanol) for the separation of vitamin A and its esters from vitamin D, the mobile phase being acetone-water (*see* Subirana *et al*[90]). Chen *et al*[72] used columns of Teflon (poly-tetrafluoroethylene) for the separation of vitamins A and D; Wilkie *et al*[91] used 'Celite 545' in polyethylene glycol 600 and iso-octane as the mobile phase for the chromatographic separation of vitamin A from D (*see* also Theivagt and Campbell[71]). Kaiser and Kagan[92] separated minute amounts of vitamin A alcohol, ester and aldehyde on paper using 1 : 1 isopropanol-water system. Jungalwala and Cama[93] have reported separation of vitamins A_1, A_2 and their derivatives by reverse-phase circular paper chromatography using 90·0 % methyl alcohol as the developer; Mahadevan and Ganguli have recommended silicone impregnated paper and MeOH: BuOH: H_2O system for the separation of higher fatty acids of vitamin A.

Gas chromatography—Dunagin and Olson[94] separated anhydrovitamin *A*, methyl-retinyl ethers and methyl retinoate by gas chromatography at 150°C with columns of Gas Chrome *P* or glass beads coated with SE-30; vitamin *A* alcohol and acetate were separated with a little destruction.

COLORIMETRIC PROCEDURES

Principle—Colorimetric estimation of vitamin *A* is based on the formation of a colour complex, having absorption maxima at certain wave length(s). The major difficulty in such an estimation is that substances other than vitamin *A*, *e.g.*, Cholesterol[95], Carotenoids[96] etc. may also give a colour with the reagent, nevertheless the method has been and is being used for vitamin *A* estimations.

A number of reagents[97-113] for the estimation of vitamin *A* are used. However, the Carr-Price reagent[12] (antimony trichloride) and the glycerol dichlorohydrin[114] are most popular. The Carr-Price procedure is based on the reaction of vitamin *A* with a chloroform solution of antimony trichloride to form a blue colour (peak at 620mμ); a suitable photoelectric colorimeter, or a spectrophotometer may be used for the purpose. A discussion of the mode of complex formation and colour development has been given in literature[113]. The authors have used it as a satisfactory alternative to the ultraviolet absorption procedure for routine estimations of vitaimin *A* in pharmaceutical preparations in spite of the fact that the pharmacopoeias *viz.*, B.P. and USP have not officially recommended this procedure.

Factors governing the validity of the antimony-trichloride method in the estimation of vitamin A-activity—The extinction value of vitamin *A* in $SbCl_3$ colour test represented as $E^{1\%}_{1cm}$ at the wave length 620mμ, is about 5,000 for chloroform; the value is almost the same for the various isomers of vitamin *A* as also for the ester. The colour complex formed during the reaction is not very stable. Various concentrations (from 6% to 25 %) of antimony-chloride in chloroform have been used. For activating the reagent, Cavina[115] used 4·0 % acetyl chloride with 0·4 % to 0·1 % antimony trichloride in chloroform. Similar colour is also produced in other solvents, for example petroleum ether, carbon tetrachloride, cyclohexane[116] and by activated glycerol-dichlorohydrin (GDH) reagent. It has been reported that addition of acetic anyhdride enhances the colour development and also removes slight traces of moisture, if any[117].

Optimum requirements for colour complex formation, for example, choice of solvent and elimination of moisture have been described by Giral[118] as well as Kulshreshtha & Tayal[116]. The distillate of antimony trichloride reagent (20 per cent) containing 2·0 per cent acetyl chloride has been found to be a very good reagent[115]. Evidently the distillate contains very little of antimony trichloride; an interesting hypothesis has been that it is the minute amount of antimony pentachloride which is responsible for colour development. Murata and Nagashima[119] have recently estimated vitamin *A* by development of colour with antimony pentachloride reagent and measurement of optical density at 620 mμ; in this procedure requisite amount of colour was produced by adding 0·01 ml of the $SbCl_5$ reagent (468 mg/ml in chloroform) to 3·5 ml of the sample. Cavina[120] also observed that blue colour changed to a stable reddish purple with absorption maximum at 550 mμ after about one minute; he studied the reaction with various solvents, such as CCl_4, ethylene dichloride, ethylene chlorohydrin, glycerol, petroleum ether, etc., (*see* also McCoord[121]).

Limitations of $SbCl_3$ procedure—The limitations and necessary precautions for the $SbCl_3$ reagent have been discussed by Morton[122], Kofler[123] and Moore[124]. The main disadvantage of the method is the quick fading of the colour; also it is corrosive and stains to glass apparatus. The opaque films formed on account of reaction with moisture on the glass may be conveniently removed by conc. HCl. The staining of glass is insignificant if $SbCl_5$ is present to a smaller extent in $SbCl_3$[119]. The glycerol-dichlorohydrin (GDH) reagent: GDH: (3-dichloro-2-propanol) was introduced by Sobel & Werbin[105] in 1945. The reagent gives a blue colour with vitamin *A*, rapidly changing to violet (maximum absorption at 550 mμ) very similar to the colour of dilute solution of $KMnO_4$; the unstable blue colour initially developed has an absorption of maximum at 625mμ, very near to that seen with the $SbCl_3$ reagent. Feinstein[125] and Sobel & Werbin[114] advocated addition of small quantities of HCl for activating the reagent; with this modification colorimetric measurement gave results agreeing well with the data obtained by UV spectrophotometry[103]. Activation could be achieved by distilling the reagent in the presence of $SbCl_3$; addition of acids other than HCl, for example H_2SO_4[126] and chlorosulphonic acid[127] was studied but the best results were obtained with HCl.

Glycerol-Dichlorohydrin (GDH) vis-a-vis antimony trichloride reagent—The advantages of the GDH reagent over the antimony trichloride reagent are: (*a*) the reagent being non-corrosive in nature can be easily handled (*b*) the reaction is not affected by small amounts of moisture, hence clouding and film formation are absent (*c*) carotene and vitamin *A* can be estimated in the solution simultaneously (*d*) the resultant colour is sufficiently stable so that measurement of optical density could be done within a few minutes, say, two to eight minutes[103].

Some of the disadvantages are : (*i*) there is greater inhibition of colour reaction by other substances than with the antimony trichloride reagent (*ii*) activation is quite critical and cannot be achieved suitably so that the results are not reproducible. It appears that with the GDH reagent the reaction undergoes more changes in producing the 555mμ band rather than the 625mμ band. The development of the two reagents is the result of the search for getting the maximum advantage from either of the two, *viz.*, (*a*) the more sensitive blue or (*b*) its degradation product, the more stable pink. With antimony trichloride, the first product and with GDH the latter is available. The formation of the pink product has been suggested by Dugan *et al*[113] as the result of irreversible oxidative attack by the Lewis bases. Prior to formation of the blue colour complex, if moisture is present, dehydration of vitamin *A* to anhydrovitamin *A* may take place so that the blue complex is not formed[128].

Other colour reactions—Other colour reactions[98, 101, 107, 110–112] have not found much use in the estimation of vitamin *A*. The residue of the non-sap fraction of vitamin *A* sample, dissolved in chloroform, may be used for the purpose. (Jakovljevic[112] has described a new method using phosphotungstic acid in presence of ethyl-acetate). In this procedure, initially the colour is blue but changes to red; the absolute reading may be taken at 538-540 mμ after removing it in the dark for one hour[129]. The method retains all the advantages of the $SbCl_3$ reagent with an added advantage of attainment of colour peak in about one minute. It has been claimed to possess wide applicability and appears to offer a rapid and convenient procedure for determination of vitamin *A* in a variety of substances containing the vitamin. Trifluoroacetic acid TFA (and the anhydride, TFAA) has also been employed for the estimation of vitamin *A*. TFAA might answer the specificity problem in the

colorimetric estimation of vitamin *A* since it is in the correct range to produce the coloured substance with the strong Lewis base, vitamin *A* alcohol, but not with the weaker carotenoid pigments[118]. The coloured species formed with TFA and TFAA are spectoscopically identical to those formed with $SbCl_3$.

PROCEDURE INVOLVING ULTRAVIOLET SPECTROPHOTOMETRY

Historical details regarding development of ultraviolet spectrophotometry for estimation of vitamin *A* have been given by Moore[130]. An earlier work reports the analysis with the results obtained by $SbCl_3$ procedure and the bio-assay data collected by Coward & Morton[131]. The earlier methods necessitated the introduction of a standard for vitamin *A* (β-carotene) by the vitamin *A* sub-committee of the Medical Research Council.

The conversion factor—The current spectrophotometric procedure involves the determination of extinction co-efficient ($E^{1\%}_{1cm}$)* of the characteristic absorption band at 325-328mμ, [132,133]. Evalue or the extinction coefficient can be multiplied by the so called convers on facter to give the potency. The conversion factor was obtained from the biopotency of a typical oil (or standard) divided by its specific ab orbance[134]. Prior to 1950, there was much confusion regarding choice of a suitable conversion factor due to the fact that the different fish liver oils contained extraneous substances in different proportions [132,135,136] In the recent past, considerable work on the subje t, notably, the one reported by Cama *et al*[9] on the extinction co-efficient *vis-a-vis* conver ion factors for crystalline vitamin *A* in different solvents has solved the problem. This has been possible due to three approaches: (*a*) fairly good methods have now become available for e timating the contents of substances causing irrelevant absorption in the region 310—350mμ (*b*) crystalline vitamin *A* of high purity with precise biological and spectrophotometric characteristics is now available and (*c*) the potency of crystalline vitamin *A* has been officially defined as 0·344 μg of vitamin *A* acetate or 0·300 μg. of vitamin *A* alcohol being equivalent to one international unit of vitamin *A*.

Choice of solvent and correction for irrelevant absorption—Regarding solvent, most of the reported work relates to either cyclohexane or isopropyl alcohol[2]; ether, ethyl alcohol and chloroform [9] could also be used. The absorption bands in the different solvents vary both in respect of position and magnitude [137]; also the different isomers of vitamin *A* give different absorption maxima, even in one solvent.

Correction for irrelevant absorption has been possible through the pioneering efforts of Morton and Stubbs [138,139]; a simple approach to this problem has been recorded by Moore [124]. The Morton-Stubbs approach involves measurement of absorption readings at three different wavelengths. The correction procedure is of geometric type[130,140]; the basic assumption is that the irrelevant absorption of most of the oils and concentrates is linear in the region 310–340 mμ. There are points in the same regions on the vitamin *A* absorption curve (wavelength againt absorption) itself whose ordinates bear the relation 6 : 7 : 6, thus making the application of a simple geometric procedure possible; the exact points are chosen and the constants of the correction formula depend upon: (*a*) whether the whole oil or the non-sap is used and (*b*) the solvent is used. The correction formula is of

*$E^{1\%}_{1cm}$ is the absorption value of a solution measured in a 1 cm cell divided by its concentration 1 gm(per 100 ml).

the type

$$E_{(\lambda)} = E \text{ (Vitamin } A) \times C + a + b \times \lambda \quad (1)$$

In this equation, three unknowns are involved, viz., concentration of vitamin A (C) and the constants a and b of the straight line equation of the irrelevant absorption for cyclohexane and isopropyl alcohol respectively. Solving the equation simultaneously at three wavelengths gives an estimate of C[139]. A general correction equation has been derived by Cama *et al* [9] by employing the method of least squares.

Discussion of the official procedures—Morton & Stubbs[139] geometric procedure for eliminating error due to irrelevant absorption, which has been verified for general applicability and validity by many workers[9,140–142], has been officially recognised. The British Pharmacopoeia[143] recommends the use of cyclohexane for extinction measurements at 312·5, 326·5 and 336·5; the correction equation for the non-sap portion is

$$E_{326\cdot5} \text{ (corrected)} = 7 \times (E_{326\cdot5} - 0\cdot422 \times E_{312\cdot5} - 0\cdot578 \times E_{336\cdot5}) \quad (2)$$

(Here E gives the absorbance denoted by the subscript). For examples, where saponification is not essential, the equation is slightly modified viz.,

$$E_{327\cdot5} \text{ (corrected)} = 7 \times (E_{327\cdot5} - 0\cdot405 \times E_{312\cdot5} - 0\cdot595 \times E_{337\cdot5}) \quad (3)$$

(Based on the recommendations of the WHO reports, a conversion factor of 1900 has been adopted in the British Pharmacopoeia).

The United States Pharmacopoeia [2] employs isopropyl alcohol in place of cyclohexane and consequently the extinction readings are to be taken at different wavelengths viz. 310, 325 and 334 $m\mu$, the correction formula being

$$E \text{ corrected } (325 m\mu) = 6\cdot815\, E\,(325 m\mu) - 2\cdot535\, E\,(310\, m\mu) - 4\cdot260\, E\,(334\, m\mu) \quad (4)$$

(Here the conversion factor is 1830)

It is of interest to mention the report of the International Union of Pure and Applied Chemistry on the "Assay[144] of Vitamin A oils"; equally relevant is the publication "Pure All-trans Vitamin A—Acetate and the Assessment of Potency by Spectrophotometry" by Boldingh *et al* [145] (see also Cama *et al*[9]). The report describes the assay of vitamin A in oils, after saponification to be followed by categorising the available samples of the different fish oils into two groups viz (a) where the non-sap fraction of the sample has an absorption maximum lying in the region 323 to 327 $m\mu$ and an extinction ratio E_{300}/E_{325} not exceeding 0·73 and (b) where the unsaponifiable fraction has an absorption maxima lying outside the above region or the extinction ratio E_{300}/E_{325} exceeding 0·73. In category (a) the sample may be assayed by taking absorbance readings at 310, 325 and 334 $m\mu$ and employing the Morton and Stubb correction procedure (USP, 16th edition); for samples falling under category (b) measurement of extinction values at the three wavelengths is preceded by chromatographic purification details for which are described in a recent publication of I.U.P.A.C.[144] The British Pharmacopoeia[1] has stipulated use of isopropyl alcohol similar to USP[2] with certain restrictions. Sometimes, spectrophotometric procedure can be employed on the crude material if the irrelevant absorption can be properly evaluated.

Limitations of the method—Some of the limitations are: (*i*) controversy regarding the conversion factor (*ii*) assumption of linearity regarding the absorption curve and (*iii*) correction procedure for irrelevant absorption. Regarding the conversion factor, the availability of pure crystalline vitamin *A* acetate and other derivatives is indicative of the correct headway in this direction. It has been observed by us and other workers that small departures from the conditions of the experiment may lead to appreciable loss of accuracy.[140, 141, 146, 147] The spectrophotometric procedure assumes that the aliquot of the sample, with little or more amounts of substances causing irrelevant absorption will have the absorption curve identical to that of the pure crystalline vitamin *A* in the same solvent; this assumption is critical, since it may not be correctly so and will not be so, at least on theoretical grounds. The Morton-Stubbs correction procedure assumes the irrelevant absorption to be linear on both sides of absorption maximum leaving the possibility of over-correction by the formula. The latter situation needs rectification by strict adherence to the stipulated conditions.

Estimation of neo-vitamin A—In the fish liver oils, it is desirable to know the contributions of the different isomers of vitamin *A* (all-*trans* and *neo*) and vitamin A_2 (if present) and to express the value in terms of all-transvitamin *A* contribution. In a procedure described by Robeson and Baxter[148], to an aliquot of the sample, taken in an amber coloured flask, benzene maleic anhydride (10·0 per cent w/v) in benzene is added. After mixing, the sample is kept at 25°C for 16 hours; subsequently vitamin *A* may be estimated by the antimony-trichloride colour reaction. The recovery of the all-*trans* variety is 2·0 per-cent only, while that of the neo-isomer is 98·0 per cent; thus the precentage of neo-vitamin *A* originally present in the preparation may be calculated. In another procedure [149], based on Hjardes' method of chromatographic purification[40], a larger column of calcium phosphate is employed; the procedure aims at minimising the contribution of neovitamin *A* and almost eliminating that due to vitamin A_2.

Other methods for the estimation of vitamin A—A technique first introduced by Embree & Shantz[150] and also used by Ames & Harris [151] depends upon the conversion of vitamin *A* to anhydrovitamin *A*. Part of the sample is treated with alcoholic (HCl) to give the anhydrovitamin *A* and the treated sample measured in a spectrophotometer against the untreated sample as control. The difference curve in the region of 370 $m\mu$ gives the amount of anhydrovitamin *A* formed. Budowski & Bondi [152] employed p-toluenesulphonic acid as the catalyst for dehydration; the method could be used for a variety of products, including pharmaceutical formulations and fish liver oils, without the necessity of further purification. Fox & Mueller[153] have employed the partial destruction of vitamin *A* by conc. H_2SO_4 in the treated sample and then using this as a control in the untreated sample; Awapara *et al* [154] have recommended destruction of vitamin *A* by ultraviolet irradiation.

ESTIMATION OF VITAMIN 'A' ACTIVITY OF FISH LIVER OIL

The authors' experience of standardising vitaminised formulations has revealed that a majority of the dosage forms, such as, tablets, injections, capsules and vitamin *A* and *D* concentrates contain synthetic vitamin *A* ester (palmitate or acetate); the vitamin *A* estimation in such preparations does not present much difficulty, if all the stages of the procedure are well standardised, and the extinction values carefully studied and interpreted. The use of the spectrophotometer in experienced hands also contributes to the accuracy of the procedure. The fish liver oils, no doubt present much difficulty[77, 78], which may be attributed to : (*i*) the fish liver oils do not contain the pure vitamin *A* but a mixture of the isomers and vitamin A_2 (*ii*) the oil may

contain vitamins *D* and *E*, hydrocarbons, cholesterol, pigments, fatty alcohols, glycerol-ethers and glycerol fats [155]. Important steps involved in the estimation of vitamin *A* activity of fish liver oils are: (*a*) *saponification*—This involves treatment of the oil by a alcoholic KOH and dissolving the resultant products in water; vitamin *A* and other unsaponifiable matter is extracted in a solvent such as ether and the solvent is removed by evaporation [2, 156]. (*b*) *Molecular distillation*—This is quite effective in removing irrelevant absorption from samples for vitamin *A* assay prepared from fish liver oils [5]. The method separates both vitamins *A* and *E*, but not vitamin *D* and is particularly useful for oils yielding a large per entage of unsaponifiable matter [157]. Omote [158] distilled cod, shark or whale liver oils at 10^{-3} to 10^{-4} mm pressure of *Hg* at 200°C for 5 mts or 150–250°C for 15 minutes, without observing loss of vitamin *A*. Perez & Molero[159] studied different aspects of the molecular distillation procedure such as use of catalyst, *NaOH*, *NaOEt*, concentration of *EtOH*, temperature, reaction time and water content. Morton & Stubbs [139] showed that distilled vitamin *A* concentrates were practically free from irrelevant absorption[160, 151] (*c*) *solvent partition*—Distribution between two immiscible solvents has also been recommended for removal of impurities[161, 162]. (*d*) *chromatographic purification*—Zechmeister *et al* [163] have described chromatographic procedure for purification of the fish liver oils, the adsorbent being $Ca(OH)_2$. Alumina has been commonly used as an adsorbent and required deactivation and standardisation before use. Karrer *et al* [164] applied chromatography in the purification of halibut liver oil. Holmes *et al* [165] adsorbed a pentane solution from the unsaponifiable portion of halibut fish liver oil, first on norit *A* and then on a column of magnesia mixed with 1 part of 'hyflosupercel' to facilitate filtration.

Cod liver oil and low potency fish liver oils—Non-sap fractions from these do not show the characteristic vitamin *A* absorption maximum at 328 $m\mu$ [166]; partially deactivated alumina may be employed for chromatographic purification of the oil as ester [74, 75]. Gridgeman *et al* [167] employed weakly active alumina, the different components of the non-sap fraction of the whale liver oil being absorbed in the order: anhydrovitamin A, vitamin *A*, kitol, sterols and selachyl alcohol. Swann [168] describes *A* similar method for cod liver oil, using alumina, heated for 2 hours at 800°C and cooled with subsequent addition of water. The column was observed by ultraviolet irradiation, and vitamin *A* estimated by estimations in the ultra-violet region. For whale liver oil, Barua & Morton [155] have employed alumina after extraction with 50·0 per cent *EtOH* (removing free vitamin *A* alcohol); vitamin *A* esters were separated from kitol esters with 80·0 per cent alcohol; these could also be separated by chromatography using alumina.

Hjrade [40] introduced a method, using alumina or calcium phosphate, which could be employed for preparations other than fish liver oils. A rapid chromatographic procedure for analysis of the oils, where the chief interfering substance is kitol, present as ester [155] and also anhydrovitamin *A* has been described by Green & Singleton [169]; the method is not so lengthy as the earlier methods of Gridgeman *et al* [167], Barua & Morton [155] and Hjarde [40]. It utilises the property of the acid washed floridin, first neutralised with ammonia, which adsorbs the vitamin *A* quantitatively. The WHO[170] subcommittee on fat soluble vitamins has recommended categorisation of available fish liver oils into two categories according to their spectrophotometric characteristic approaching pure vitamin *A* or not.

RECOMMENDATIONS FOR FUTURE WORK

It is now evident that there is plenty of literature on European fish liver oils on the estimation of vitamin *A* content, while information on the Indian fish liver oils is not

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