VITAMINS

Ch.11

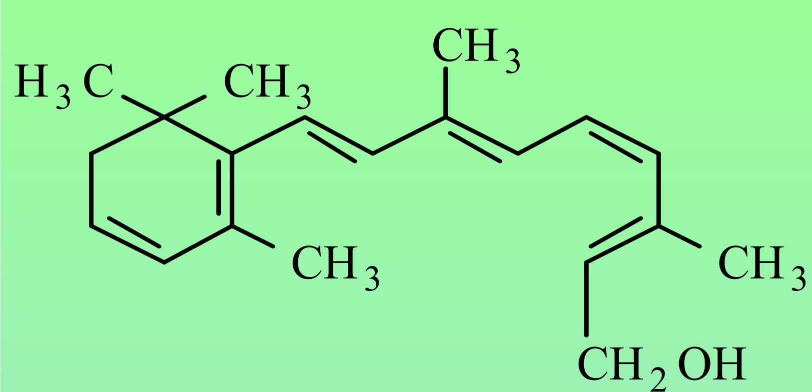
Definition and importance

- Minor food components (with low M.W.)that are required in small quantities(catalytic amounts) for normal metabolism.

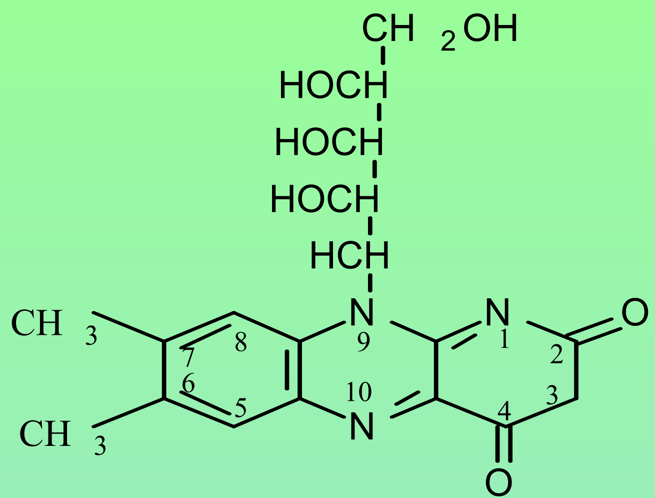
Concentration ranges of Vitamins in 100 gr food:

- mcg(Vit A,D,B12, folic acid..)
- mg(Vit Cand E)

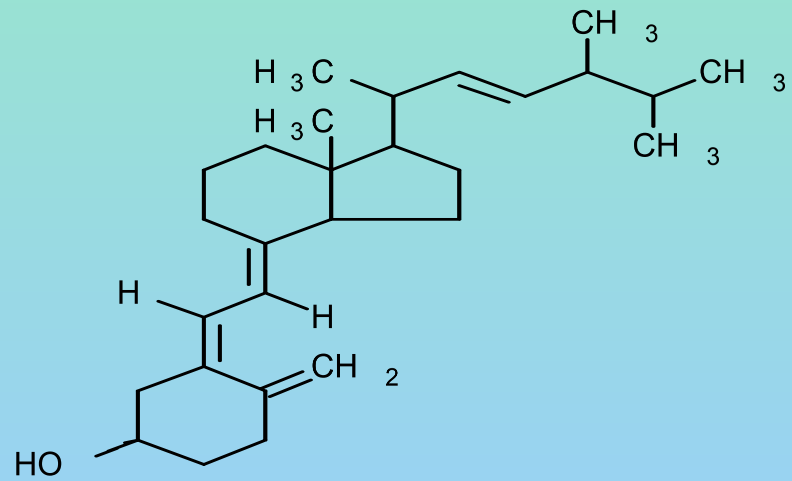
- Cannot be synthesized in human body
- Their insufficiencies cause diseases
- Required for defining accurate food composition information for nutritional labelling



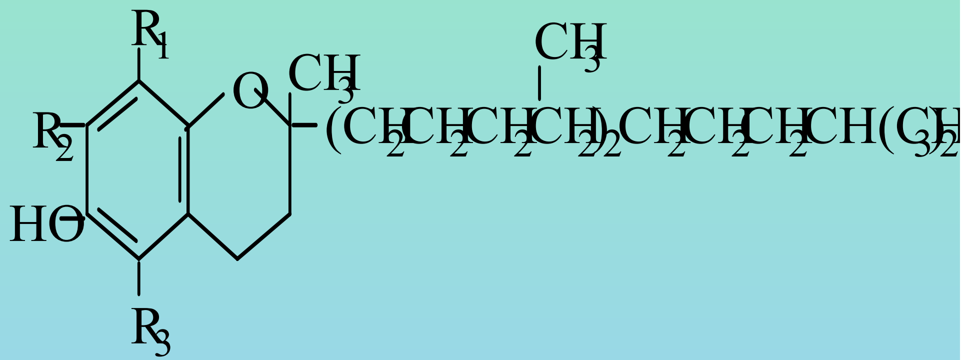
Vitamin A



Vitamin B2

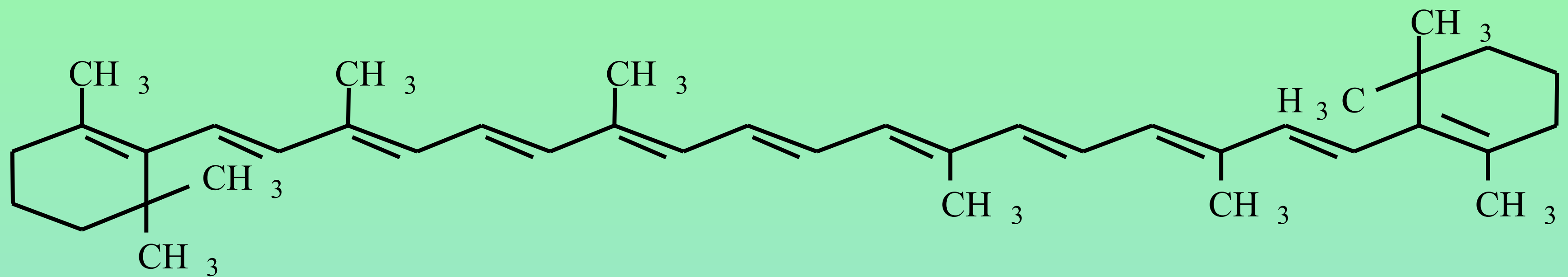


Vitamin D2

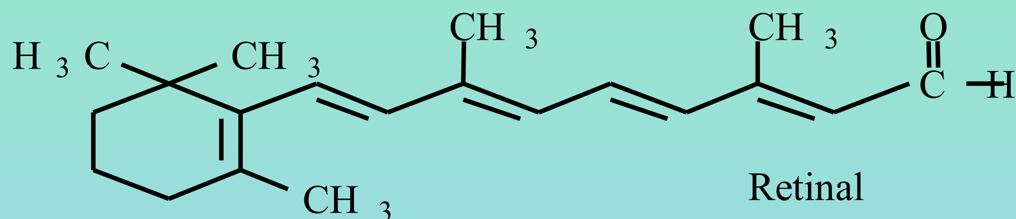


Vitamin E

β - Carotene

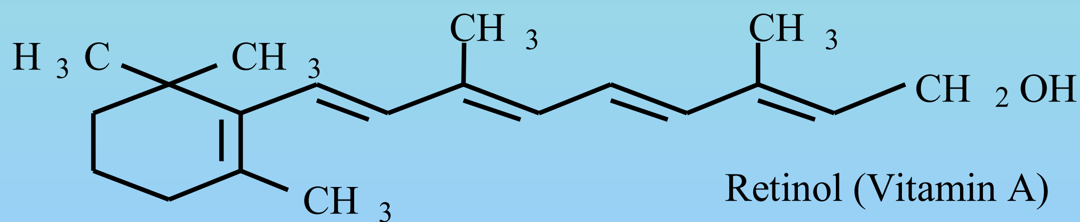


Oxidation



Retinal

- 2H



Retinol (Vitamin A)

Vitamins –vitamers-in Foods

Compounds showing the same vitaminic activity are called “vitamers”.

<u>Vitamers of Vitamin A</u>:

- **Retinol**
- **Retinyl acetate**
- **Retinyl palmitate**

.β-carotene

<u>Vitamers of Vitamin D</u>:

- **Ergocalciferol (D2)**
- **Cholecalciferol(D3)**

Vitamers of Vitamin C:

Ascorbic acid

Dehydroascorbic acid

Ascorbyl palmitate

Vitamers of nicotinamide:

- **Nicotinic acid**
- **Nicotinamide adenin dinucleotid**

Vitamers of pyridoxine:

- **Pyridoxal**
- **Pyridoxamine**

Vitamers of Vitamin E:

- **α-Tocopherol, β-Tocopherol,γ- Tocopherol, δ-Tocopherol**
- **α-Tocotrienol, β-Tocotrienol,γ- Tocotrienol, δ-Tocotrienol**

Comparative biological activity of vitamers:

1 IU of VitA = 0.3 mcg retinol
= 0.344 mcg of retinyl palmitate
=1.8 mcg β-carotene

1mcg retinol=6mcg β-carotene

1 IU of VitD = 0.025 mcg $VitD_3$

1 mcg cholecalciferol = 40 IU VitD

1mg pyridoxine=1.012mg pyridoxal=1.008mg pyridoxamine

1α-TE= α-Tocopherol Equivalent=βToc.*X*0.5
γToc*X0.1; δTocX0.03

"BIOAVAILABILITY" : Sometimes, even if the vitamin is present in the food commodity, it is not "bio-available".

Ex: Part of Niacin in cereals is "bound" and not available. Nutritionists are more interested in bioavailability of vitamins .

Sample Preparation Hints

1. Vitamins are very labile (easily decompose). Most of them are sensitive to light, so you should always try to work in subdued light, protection from light source with a film barrier which does not permit the sunlight passing. They are also sensitive to heat and oxidation. Therefore,they have to be preserved at cold temperatures and with minimum surface area exposed to air: sample must preferably be stored at -20°C and under nitrogen gas before analysis.

2. Remove inedible portions since results are expressed in mg/100 g edible portion.

3. Homogenize sample just before analysis(For VitC, aqueous metaphosphoric acid solution should be added for preservation upto 3 months).

4. Sample size should be adequate, so before the analysis you should have an idea of aproximate concentration range (check from Food Composition Tables).

5. Fat soluble vitamins require saponification prior to analyses.

EXTRACTION of VITAMINS

The vitamin has to be extracted from its biological matrix before assay. Typical extraction methods for vitamins:

1. For Vit.C:Cold extraction with water-acetic acid-metaphosphoric acid.

2. For Vit. $B_1$and B_2: Boiling or autoclaving in acid plus enzyme treatment

3.For Vit.A+D+E:Organic solvent extraction, saponification, re-extraction with organic solvent.

Classification of Vitamin Assays:

1. Physico-chemical Methods
2. Microbiological assays using bacteria-yeasts etc.
3. Bioassays involving humans and laboratory animals

I.Physicochemical Methods for Vitamin Analyses

A. Titrimetric Methods (Vit.C)

B. Spectroscopic Methods

B.1. UV Visible spectrophotometry
(Vit A,D,β- carotene, Vit E)

B.2. Fluorimetry (Vit B_1)

C. Chromatographic methods:i.e. Vit. E and vitamers by HPLC :
(simultaneous multi-vitamin analyses: water solubles/oil solubles:will require removal of interfering substances)

A. Titrimetric analysis (Vitamin C)

Ex: Use of a <u>redox</u> dye (DCIP) (blue-colored 2,6-Dichloroindophenol reagent)

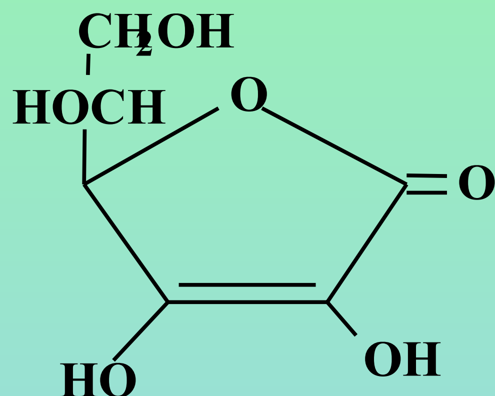
The analysis involves oxidation of ascorbic acid to L-dehydroascorbic acid by the dye.
At the endpoint, the rose-pink color in acid solution should last for at least 10 seconds.

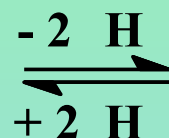
Procedure:

- **Extract Vit C from sample using metaphosphoric acid; remove interfering macroconstituents by precipitation withPb acetate, add EDTA to chelate Fe and Cu**
- **pH adjustment to 3.5 with NaAc. buffer solution**
- **Titration with dichloroindophenol reagent (to faint pink color).**

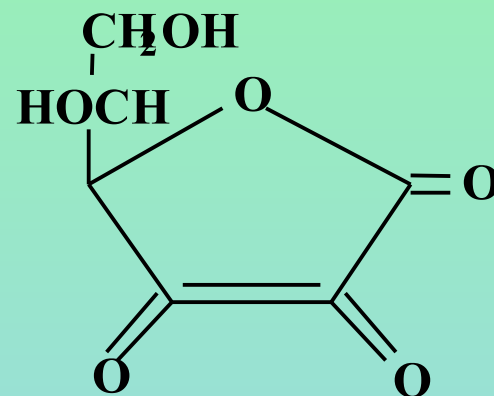
VITAMIN C

Ascorbic Acid





Dehydroascorbic Acid



Use HSCH2CH2(SH)CH2OH (2,3-DIMETHYLPROPANOL) as reducing agent for converting dehydroascorbic acid to ascorbic acid

Calculation for Reduced Vitamin C

$$\text{mg Ascorbic Acid/g} = (A-B) \times \frac{F}{E} \times \frac{V}{X}$$

A = **ml of 2,6-dichloroindophenol for sample titration**

B = **ml of 2,6-dichloroindophenol for blank**

F = **mg of ascorbic acid equivalent to 1 ml of indophenol standard solution**

V = **Initial assay solution volume**

E = **Number of grams**

X = **Volume sample aliquot titrated**

B. Spectroscopy

B.1. UV-Visible Spectrophotometric Methods

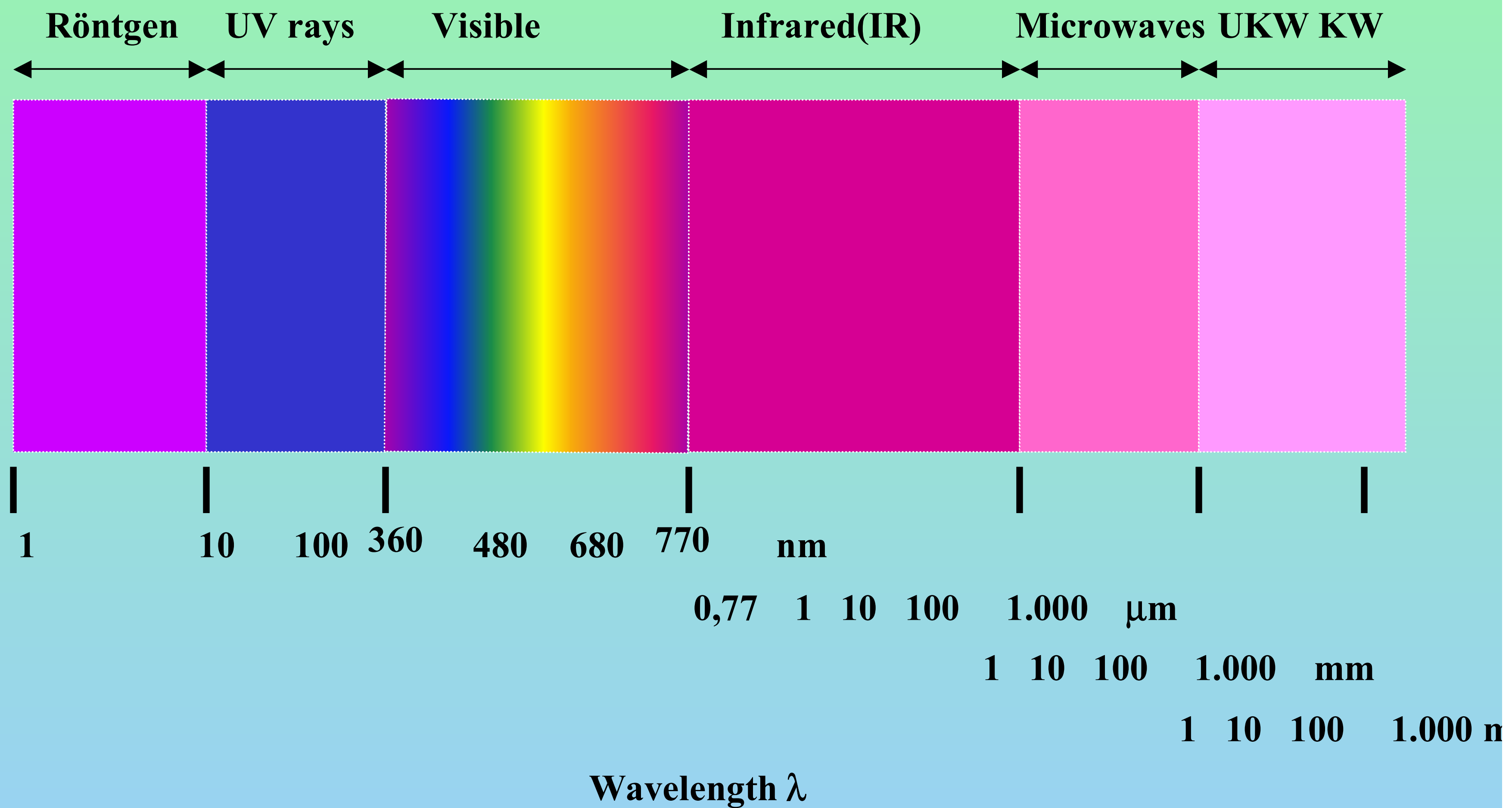
Principle: The amount of the radiation transmitted by a solution depends on its concentration, as stated in Beer-Lambert Law :

$A = \alpha . b . C$

Where C is concentration in mg/ml,

α: molar absorbtivity (the absorbance of 1 mol subs)

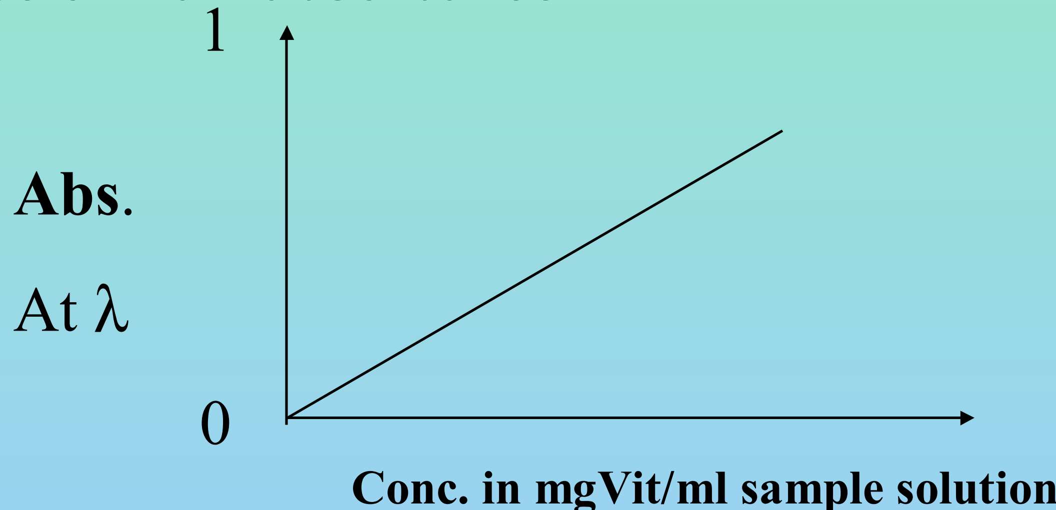
ELECTRMAGNETIC SPECTRUM



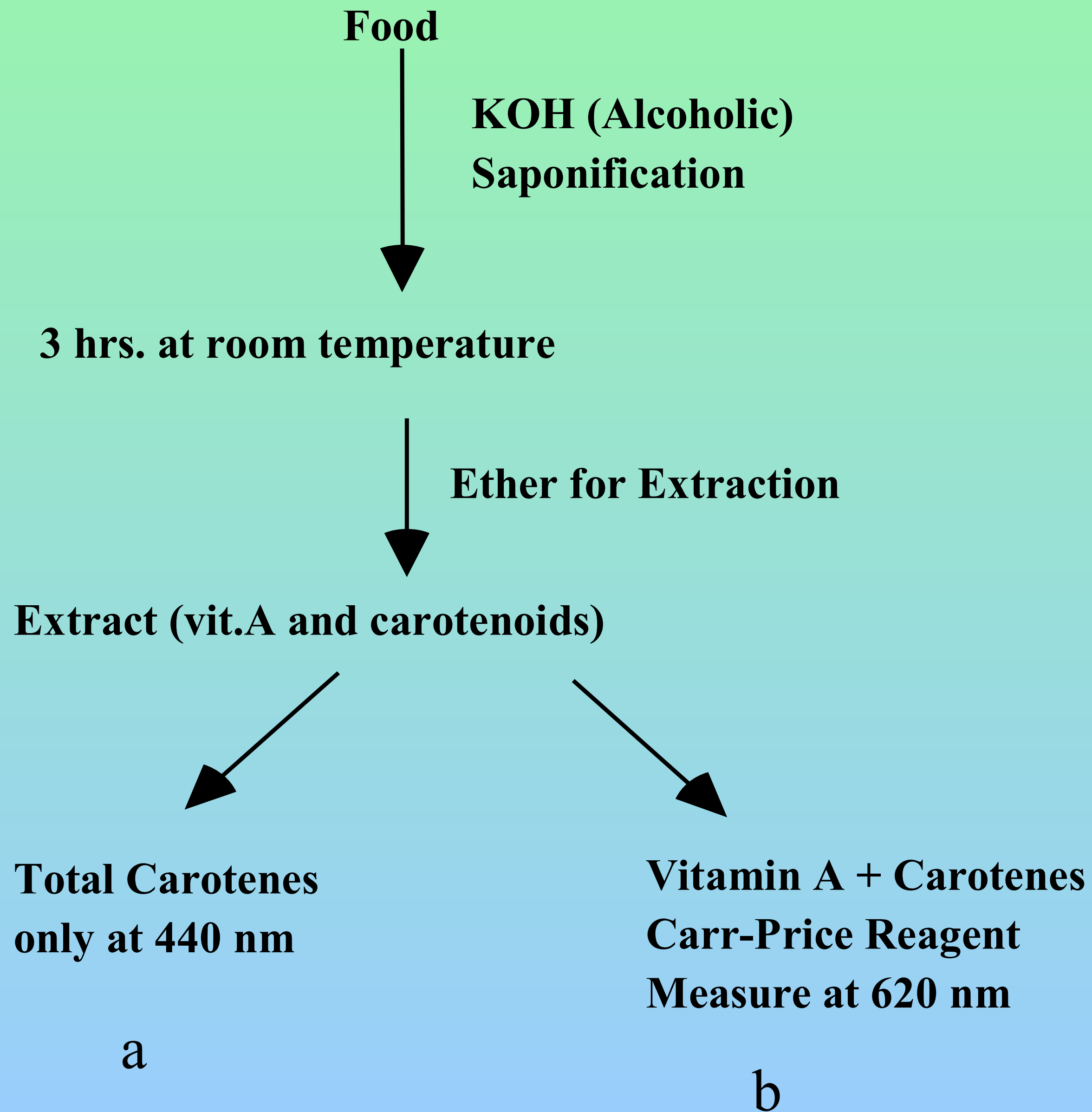
Relating Absorbance to Concentration

The graph showing (Concentration in mg st/ml solution) versus absorption is linear inside the absorbance range (0-1.0).

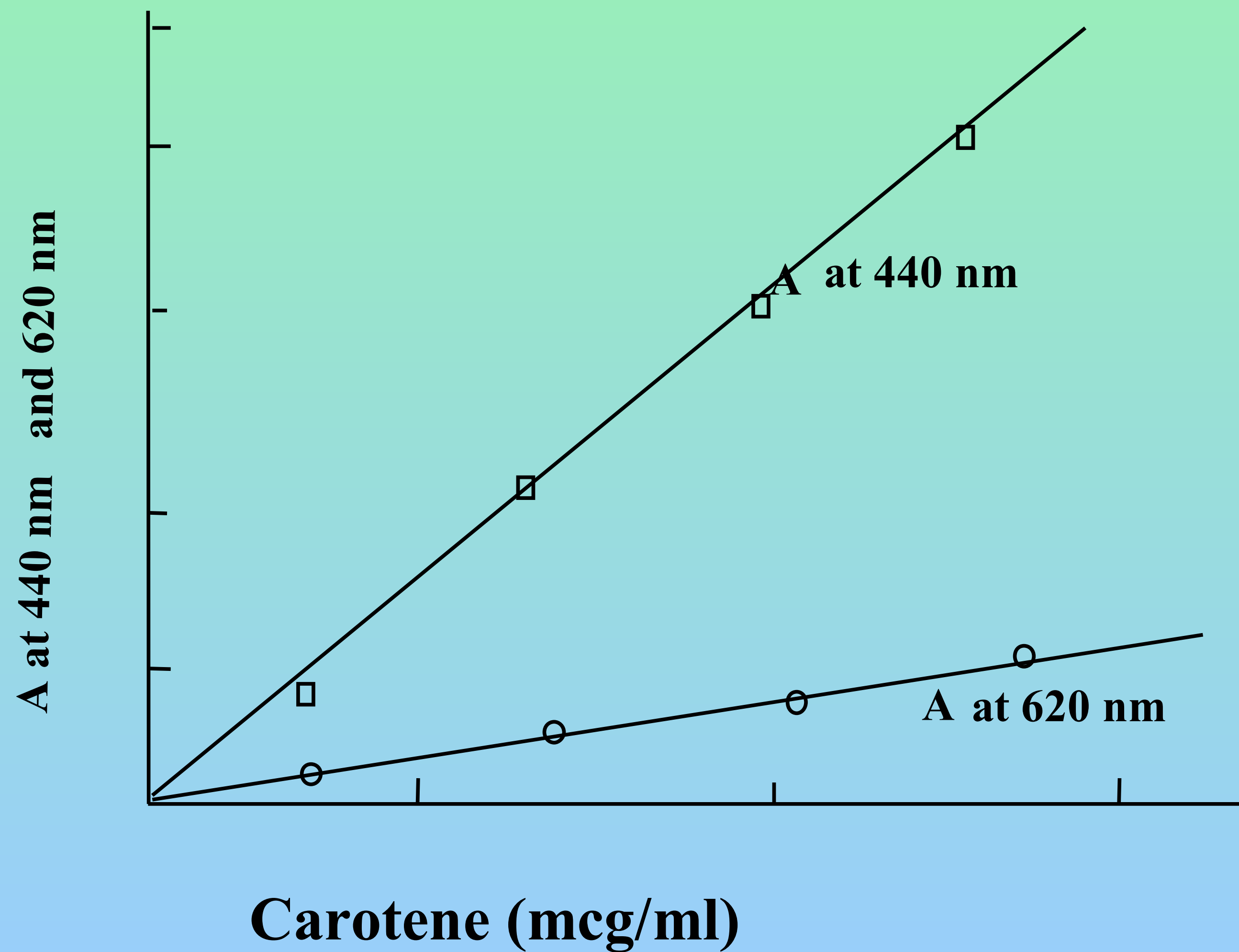
For this purpose, standard vitamin solutions in gradually increasing concentrations are prepared; their Abs values are recorded. Necessary dilutions should be made to fit in the linearity range of calibration graph(Abs 0.1-1). You can scan the solution in instrument from 380-700 nm to determine the real A max values from the scan-graph, which indicate to you the wavelength(λ) where you should measure the absorbance values of sample and stds, since the sensitiviy of instrument is greatest at wavelength with min. transmittance or max. absorbance.



Example 1: Vitamin A and β - Carotene Determination

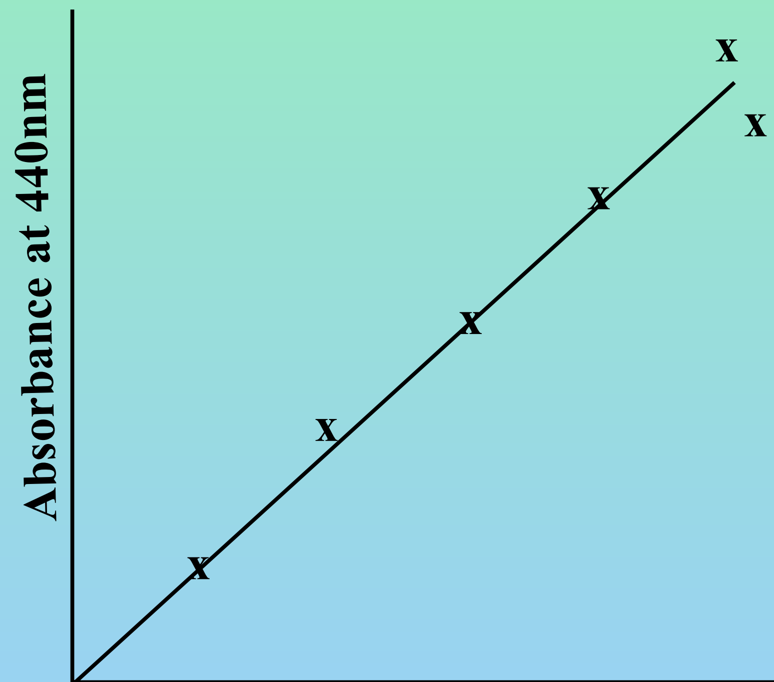


β-CAROTENE STANDARD ABSORBANCES AT 440 AND 620 nm

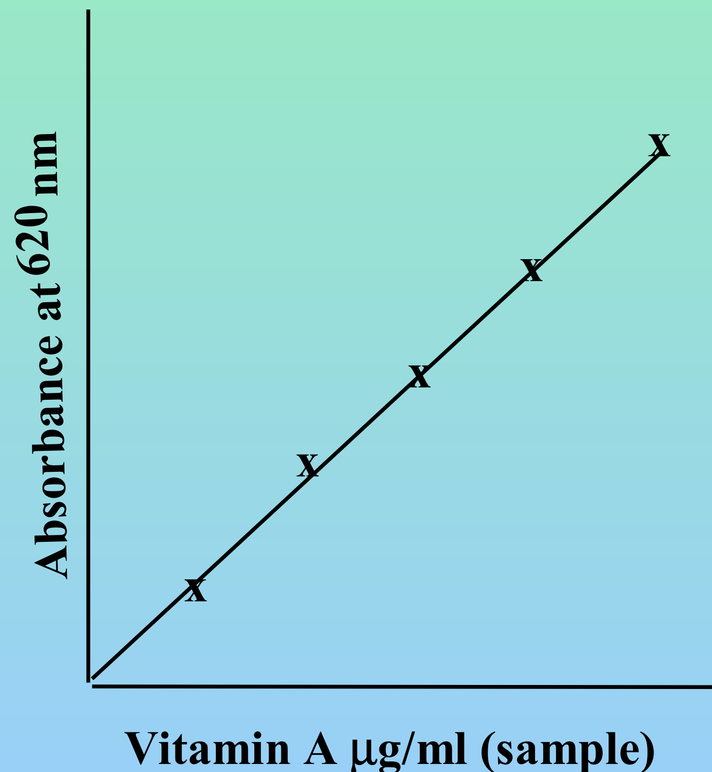


Use carotene absorbance a at 440 nm and then convert this to its absorbance at 620 nm and subtract it from the absorbance at 620 nm of b to determine the absorbance at 620 due to Vitamin A.

Carotene Absorbance at 440nm

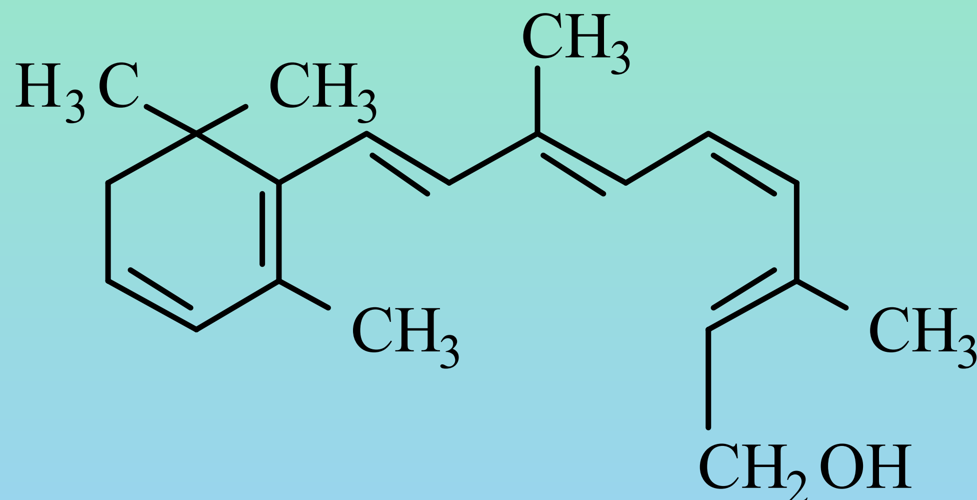


Vitamin A Absorbance at 620 nm



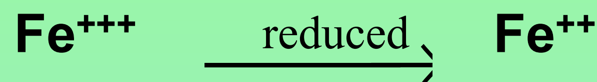
VITAMIN A ANALYSIS BY [CARR-PRICE METHOD]:

Principle: Carr-Price reagent, $SbCl_3$ in $CHCl_3$ gives a blue coloured complex with Vit A .Intensity of absorbance of the blue colour formed(at 610nm) is correlated with conc. of VitA. Prepare the calibration graph with standard VitA solns. From abs. value, you can determine the concentration. If however, carotenes are also present, they should be separated from VitA by column chromatograthy on aluminum oxide.Carotenes should be eluted first with diethyl ether and determined at 450nm.

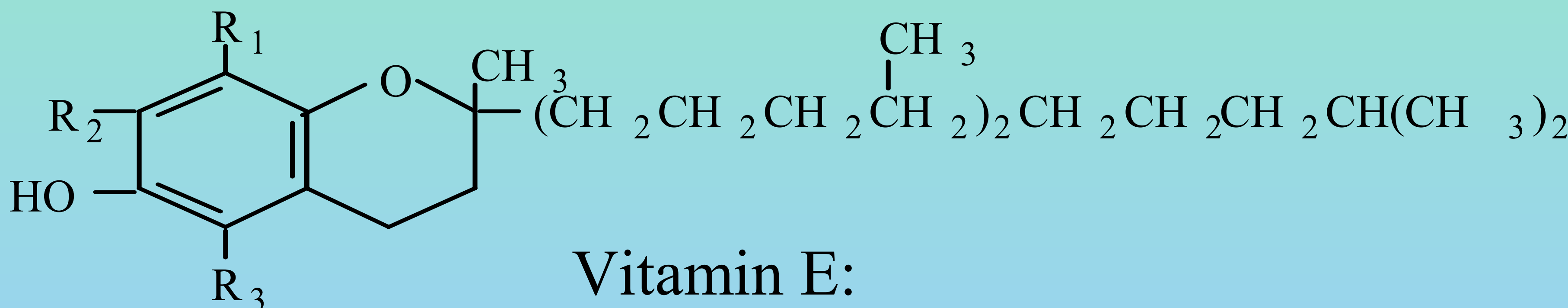


Vitamin A

Ex 2: VITAMIN E ANALYSIS by (EMMERIE-ENGEL METHOD):

Fe^{+++}  Fe^{++}

Principle: Tocopherols are oxidized by $FeCl_3$(at the same time, Fe^{+++} is reduced to Fe^{++}) which forms a red-colored complex with (α-α dipiridyl reagent). You should also run a sample blank to get rid of other interferences . Concentration is correlated to the intensity of red color formed.



Example 3:Niacin Determination

1. Digestion an Hydrolysis

Sample + $Ca(OH)_2$

2. Precipitate protein with (NH4)2SO4

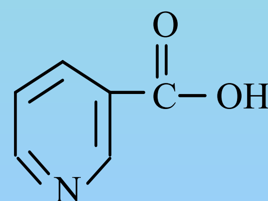
3. Centrifuge and Filter

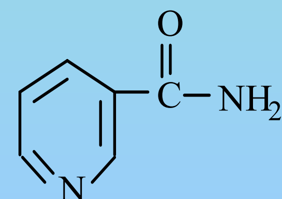
4. Rupture of Pyridine Ring with CNBR (Cyanogen Bromide)

5. Color Formation with Sulfanilic Acid

6. Determine the Absorption at 470 nm

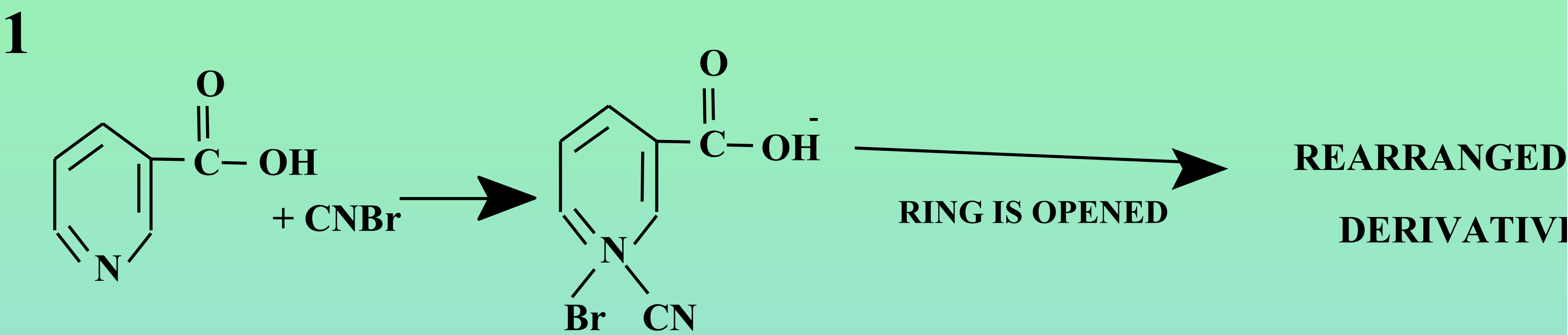
7. Plot the Standard Curve of Niacin vs. Concentration



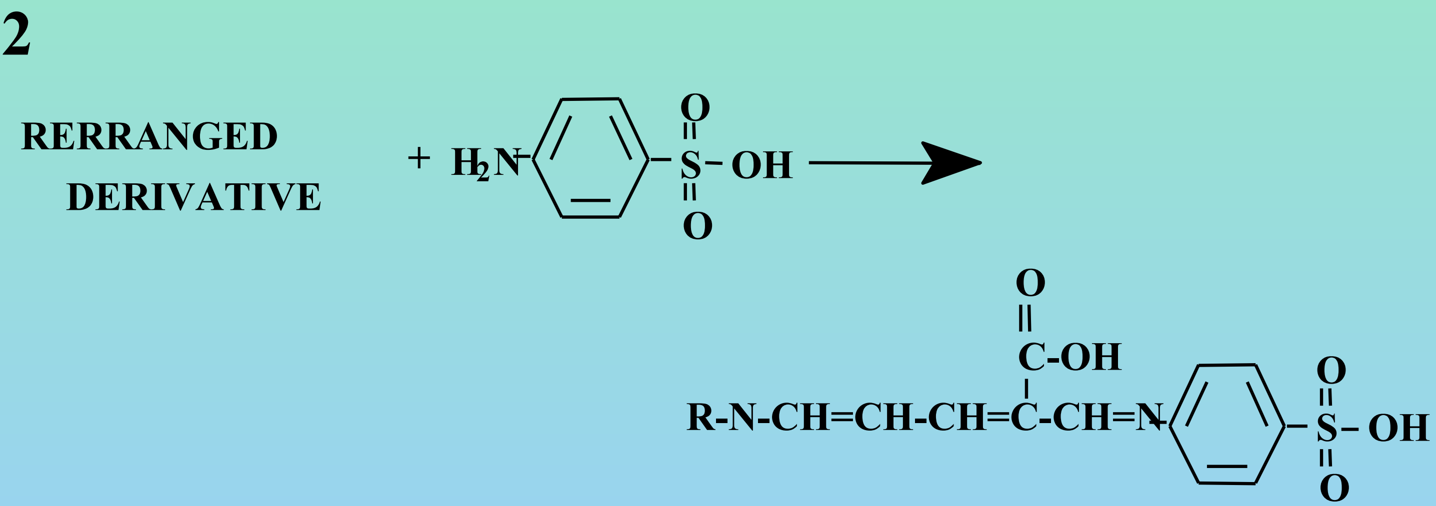


Steps in Niacine Determination

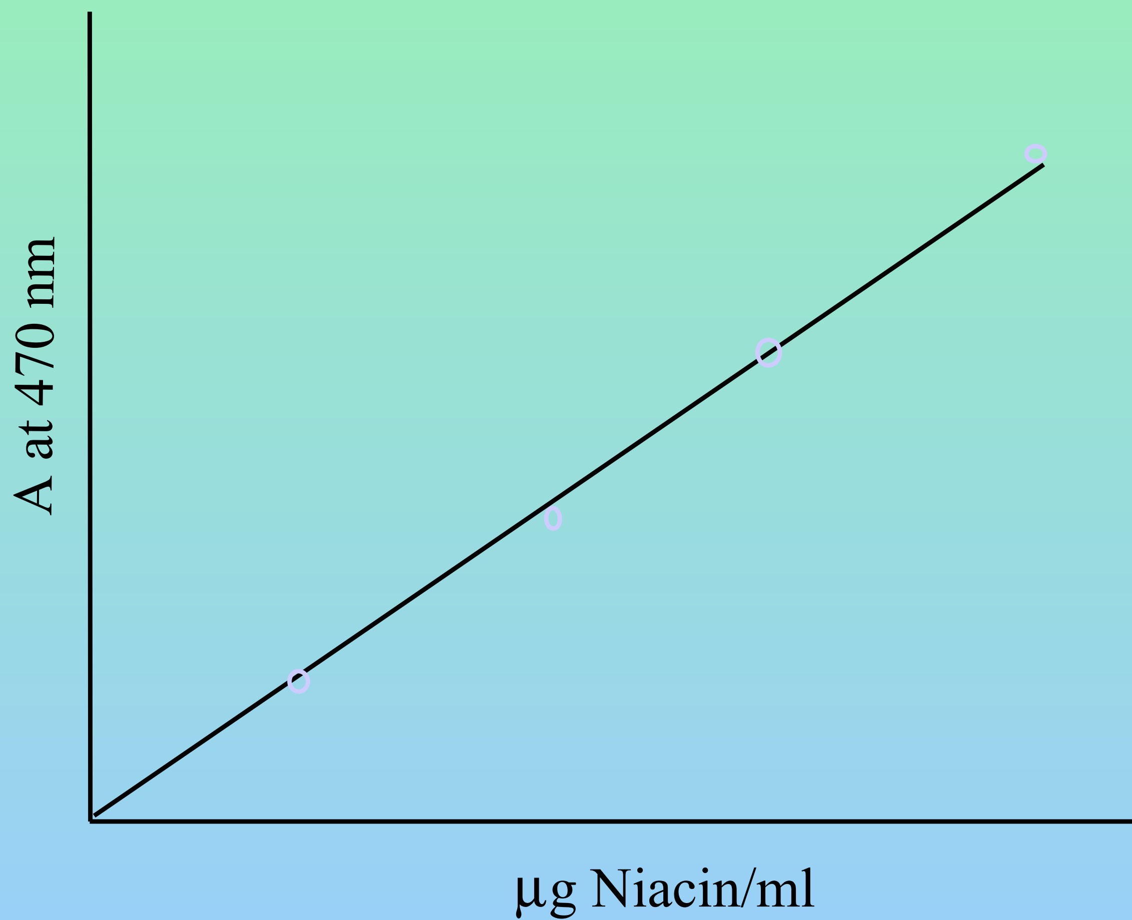
1



2



NIACINE STANDARD CURVE



B.2. FLUORIMETRY

Measures luminesence(which means systems that can be made to glow) . There are two types of glow: “Fluorescence” and “Phosphorescence”. Such substances contain either an electron-donating group (ex:amines), or multiple conjugated double bond-aromatic rings. Presence of groups that can withdraw electrons(carboxyl or azo groups) destroy fluorescence.

Quantitative analysis based on fluorimetry involve initial fluorescence measurement, treatment with an oxidative reagent, and final measurement of fluorescence destroyed by electron-withdrawing group analysed.

Ex: VITAMIN B_1 (Thiamine) ANALYSIS BY FLUOROMETRIC METHOD

Method is based on the fluorescence measurement of the oxidized form of thiamin(thiochrome)following extraction and enzymatic hydrolysis of the phosphate esters of thiamin and chromatographic cleanup. In other words, thiamine in sample is oxidized to "thiochrome" which gives fluorescence by phosphatase enzyme.Measurements made at 365nm/435nm*Ex*/Em*.* A standard curve is used again for quantitating samples.

**Ex*: Excitation wavelength

**Em*: emission wavelength

Thiamin Determination in Foods

Sample extraction:

5 g enriched flour

75 ml 0.1 N HCl

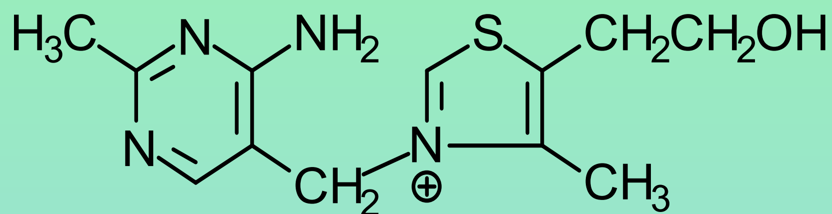
Make volume to 100 ml

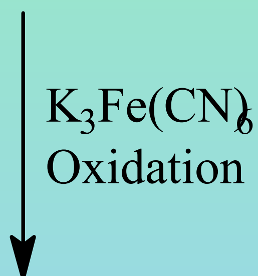
Digest at 100^0C for 30 min

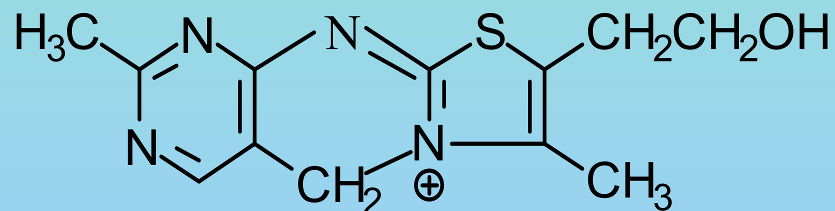
Centrifuge and filter

THIAMIN DETERMINATION

pyrimidine **thiazole**







THIOCHROME (Fluorescent)

Excite thiochrome at 365 nm and measure the absorbance at 435 nm

Oxidation of Thiamine

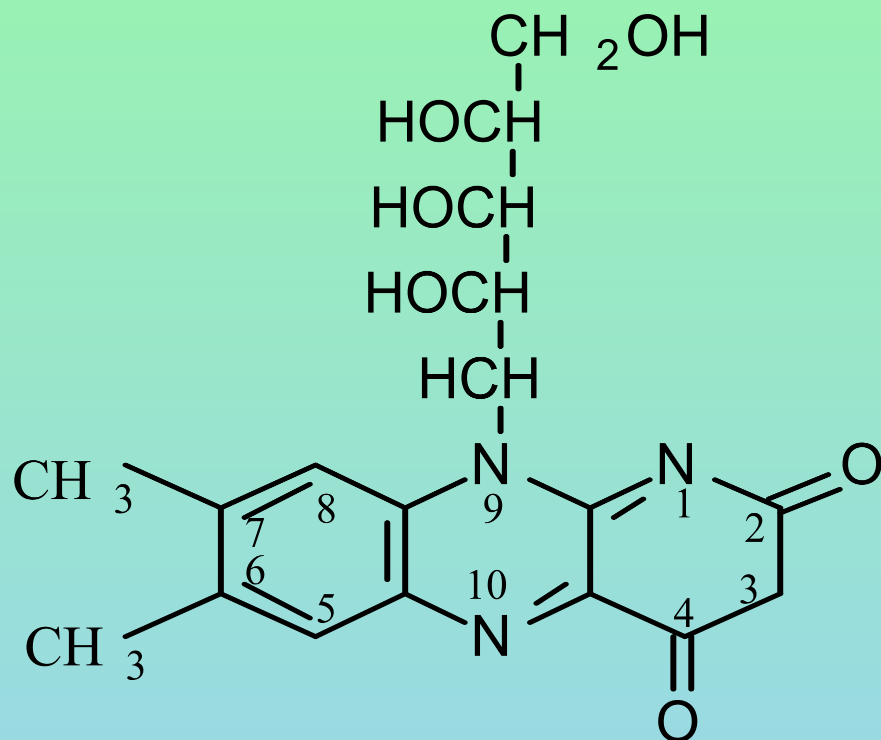
Tube 1	**Tube 2(sample blank)**
5 ml Sample	**5 ml Sample**
2.5 g NaCl	**2.5 g NaCl**
3 ml K3FeCN6	**3 ml NaOH**
13 ml Isobutanol	**13 ml Isobutanol**
Centrifuge	**Centrifuge**

Tube 3	**Tube 4(standard blank)**
5 ml Standard (thiamin)	**5 ml Standard**
2.5 g NaCl	**2.5 g NaCl**
3 ml K3FeCN6	**3 ml NaOH**
13 ml Isobutanol	**13 ml Isobutanol**
Centrifuge	**Centrifuge**

Thiamin Standard Solution = 0.2 μg/ml

Calculation of B_1(μg of B_1/5 ml assay sample)=(Tube 1 – Tube) /(Tube 3 - Tube 4)

Example: RIBOFLAVIN(Vit.B2) DETERMINATION



6,7 Dimethyl-9-D-1-Ribitylisoalloxazine

Fluorometric Method

Autoclave in 0.1 N HCl for 30 min

Adjust pH to isoelectric point to precipitate proteins

Filter

Determination: In 2Test Tubes, add:

1)10 ml sample+ 1 ml of riboflavin standard

2)Blank: 10 ml sample + 1 ml H_2O

+ 1 ml acetic acid, + 0.5 ml $KMnO_4$ (4%)

+ 0.5 ml 3% H_2O_2

($KMnO_4$ color should disappear in 10 seconds)

+ 20 mg powdered $Na_2S_2O_4$. Measure fluorescence value(F)

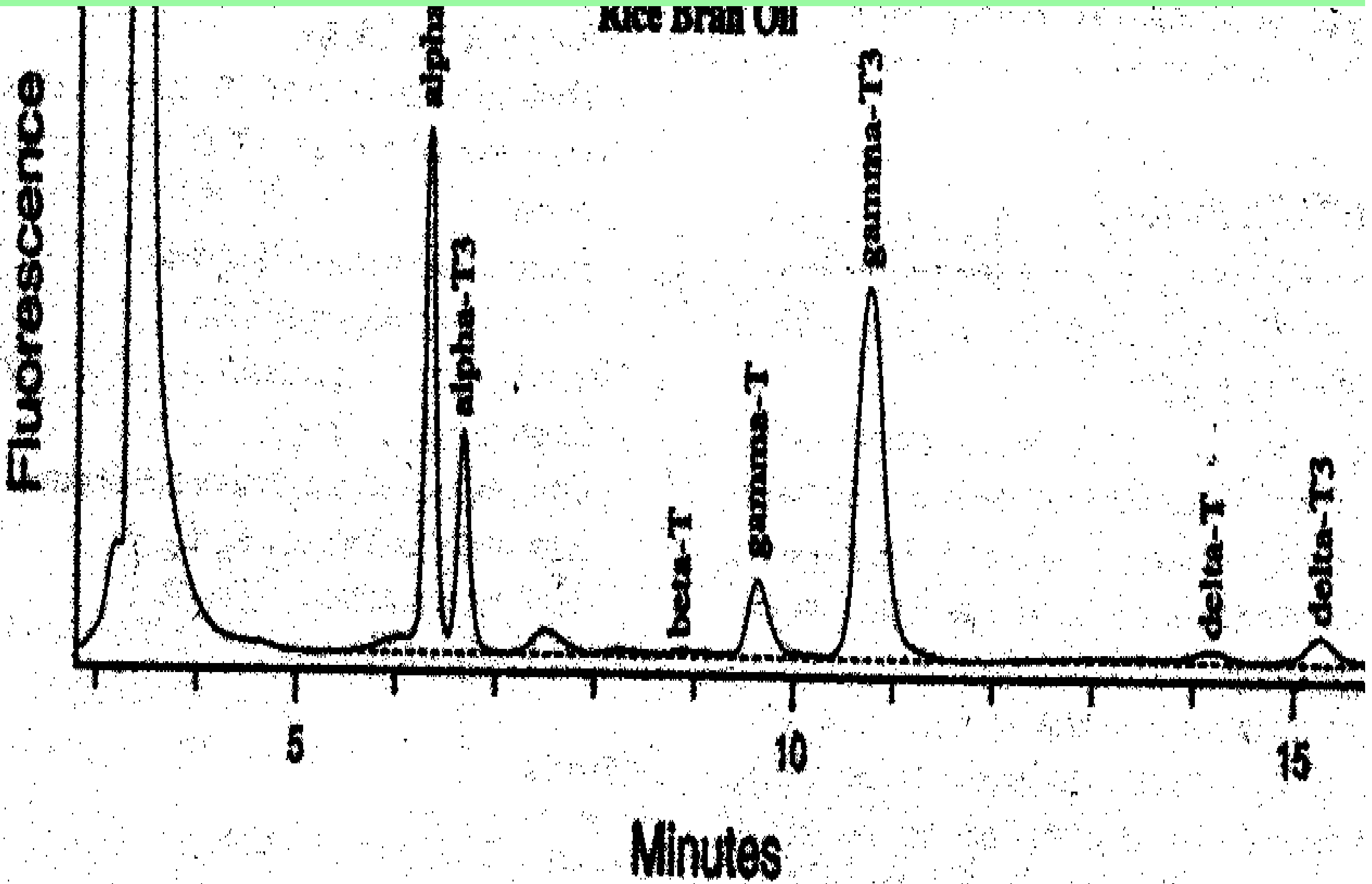
Calculation: mg Riboflavin/ml Final Sample Solution=

$$\frac{[F(sample) - F(blank)] \times 0.1 \times 0.001}{F(sample + standard) - F(sample)}$$

C. CHROMATOGRAPHIC METHODS:

HPLC is now the method of choice for multi- vitamin analyses, yielding simultaneous results for both water solubles and oil solubles.
Especially for Vitamin E with 8 known vitamers, HPLCcan estimate all these multiple forms simultaneously. For this purpose, for food products, the sample is saponified under reflux , extracted with hexane, and injected onto HPLC column. For margarines, $MgSO_4$ is added to remove water phase, the filtered extract being assayed directly. Different vitamers of Vit E (α, β, γ, ΔT_{oc}) can also be seperated on a mobile phase of (isopropanol and hexane) or (dioxane and hexane) and quantitated by using external standards and by linear regression from respective peak areas. Also a "recovery" test should be carried using an internal standard(adding known amount of standard Vitamin E to sample and extracting likewise).The detector used is preferrably Fluorescence detector at 290nm/330nm Ex/Em.

G.C. can not be used in vitamin analyses, because when you volatilize vitamins, they will decompose.



18-7 Chromatogram of rice bran oil showing tocopherols and tocotrienols.

II. Microbiological Methods:

These are limited to analysis of some water soluble vitamins and are very sensitive and specific for each vitamin. They involve analytical microbiology,meaning that here <u>microorganisms</u> are being used as “reagents”instead of chemicals.

Principle: In the presence of limiting amounts of essential micronutrients, the amount of microbial growth is a function of the concentration of micronutrient.

These methods are time-consuming and strict adherance to analytical protocol is critical for accurate results.

They are specific microorganisms which are used as indicators like *Saccharomyces uvarum* for pyridoxine analyses, *Lactobacillus leichmanii* for Vit B_{12} , *Lactobacillus plantarum* for niacin. These microorganisms are specified with their ATCC(American Type Culture Collection) numbers, since these are very specific as to their growth response. In order to express the results quantitatively, there should either be a direct measurement of growth, using (Turbidimetry at 600 nm); Or you can measure a specific metabolic response . For example, for lactobacilli growth, measuring acidity, CO_2 production, respiration-O_2 uptake, gravimetry, can give an indication of m.o. growth, which in turn is a function of the Vitamin content. However,any slight detail may alter the results.

ESSENTIAL STEPS in ANALYSES:

1. **Preparation of media for stock cultures and maintenance of cultures.**
2. **Preparation of nutrient -deficient media (i.e. casein, a.a., vitamins, mineral salts]**

Ex: Vitamin B_6 deficient media

3. **Preparation of your innoculum culture**
4. **Extraction of nutrient (vit.) from sample**
5. **Setting up of the assay**
6. **Sterilization of the assay tubes and media**
7. **Innoculation with test organism (ie.*Lactobacillus plantarum*)**
8. **Incubation for growth of m.o.**
9. **Determining the metabolic response**
10. **Calculation of results**

You have to make sure the test m.o. are sensitive to nutrient and media. All above procedures have to be done under very careful conditions for not destroying the character of m/o.Produce a gradation between no growth and maximum possible growth.

Ex= Determination of niacin in pudding

- **You start with sample solution preparation:**
- **Take 50g pudding+160 ml (acidified H_2O); (H_2SO_4+H_2O),**
- **Autoclave 30 min at 121°C**
- **adjust pH to 6-6.5 with NaOH then to 4.5 with H_2SO_4.**
- **Transfer to 1 liter flask and bring to 1000 ml with H_2O.**
- **Filter and pipette 10 ml into 50 ml beaker; adjust pH to 6.8, transfer to 200 ml flask and fill to volume.**

NIACIN MICROBIOLOGICAL ASSAY PROCEDURE

Sample Preparation

Weigh out enough sample to contain ca. 0.1 mg of niacin, add 1 *N* H_2SO_4, macerate, autoclave 1 hr at 121°C, and cool. Adjust pH to 6.8, dilute to volume (ca. 0.1 g niacin/ml), mix, and filter.

Assay Tube Preparation

In at least duplicate use 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml sample filtrate and make up the difference to 5.0 ml with H_2O, then add 5.0 ml of Difco Basal Medium for Niacin Assay broth to each tube, autoclave 10 min at 121°C, and cool.

Standard Preparation

Prepare assay tube in at least duplicate using 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml standard solution (0.1 µl/ml niacin), make up difference to 5.0 ml with H_2O, then add 5.0 ml of assay broth and treat identically to sample tubes.

Inoculation and Incubation

Prepare inoculum using *Lactobacillus plantarum*, ATCC 8014 in Bacto Lactobacilli Broth AOAC (Difco). Add one drop of inoculum to each tube, cover tubes, and then incubate at 37°C for 16–18 hr, i.e., until maximum turbidity is reached in tubes containing the highest concentration of niacin.

Determination

Measure %T or absorbance at any wavelength between 540 and 660 nm.

figure 18-3 The microbiological assay of niacin, AOAC Method 944.13, 45.2.04 (1).

Prepare the Sample Solution:

$$\frac{50\,\text{gr pudding}}{1000\,\text{ml}} \times \frac{10\,\text{m}}{200\,\text{ml}} \rightarrow \text{concentration of sample} = 0.0025\,\text{g pudding}/1\,ml\ \text{solution}$$

Prepare the Standard Solution:

$$\frac{100\,\text{ml niacin}}{1000\,\text{ml}} \times \frac{5\,\text{ml}}{500\,\text{ml}} \times \frac{5\,\text{ml}}{100\,\text{ml}}$$

Concentration of st.sol’n= 0.05 mg niacin/ml solution

A- Sample tubes

	U	U	U	U	U	U
Sample Volume	0	1	2	3	4	5
H_2O volume	5	4	3	2	1	0
Total volume	5	5	5	5	5	5
Sample #	1	2	3	4	5	6

1: Sample blank
6: Uninnoculated color blank, no innoculum

B. Standard Tubes:

	U	U	U	U	U	U	U
Niacin st. vol.	0	0	1	2	3	4	5
H_2O volume	5	5	4	3	2	1	0
Mcg niacin	0	0	0.05	0.1	0.15	0.20	0.25
St. #	1	2	3	4	5	6	7

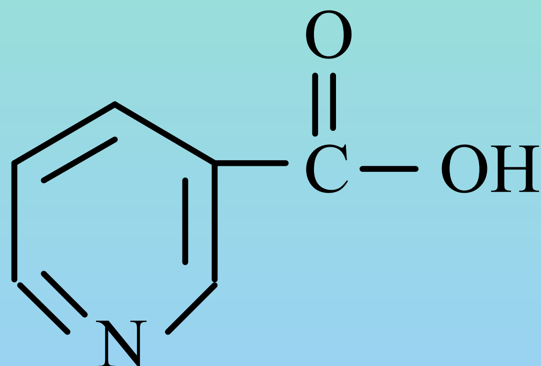
\#

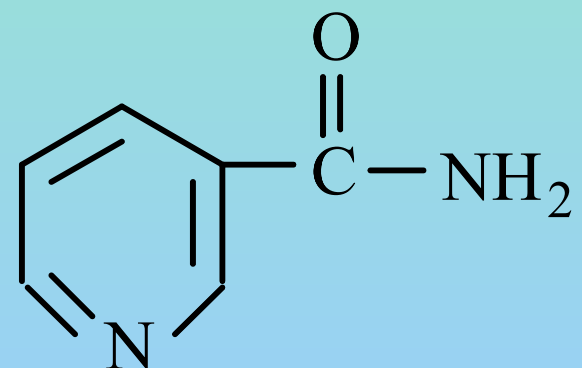
1: Uninnoculated color blank, no innoculum

2: Inoculated std. Blank

7: 0.25 mg niacin, so you expect highest growth of m/o

- Contents of all tubes are diluted with water to the same volume, and an equal volume of basic medium is added to each tube, then plugged wwith cotton, sterilized by autoclaving.
- On to each tube,except uninoculated blanks, add 1 drop of *Lactobacillus plantarum* inoculum,
- Incubate 18 hours at 37°C, cool, read turbidity of tubes at 660 nm.
- Plot readings on semilogarithmic paper.
- These types of methods have recently been automated. Also, bioassays with chicks or rats are used for niacin.





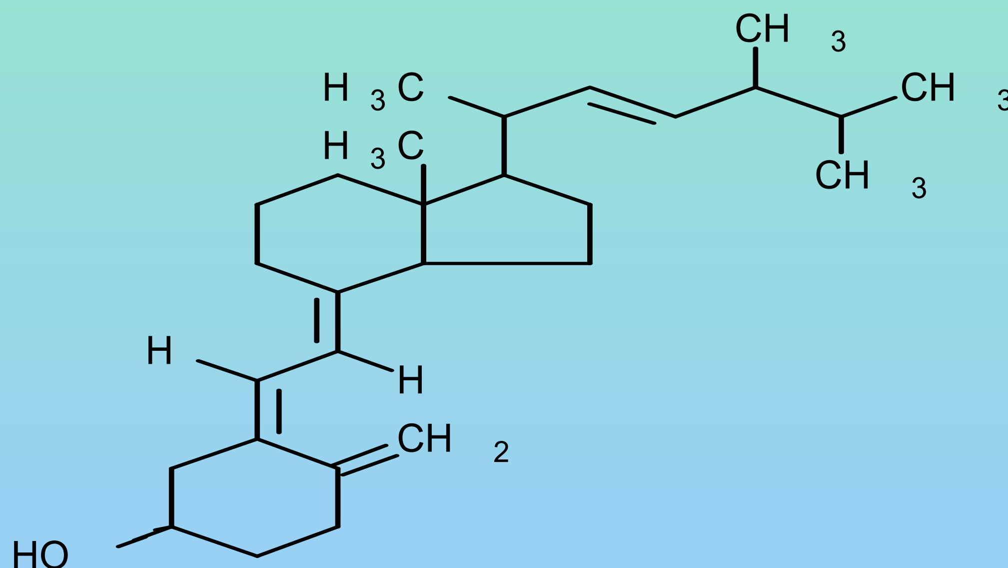
III. Bioassays

Bioassays are used only for Vitamins B_{12} and D.

Example: Vitamin D Analysis

This test, based on bone calcification, involves Vitamin D depletion and sacrifice of test organism. Therefore it is limited to use of animals rather than humans as test organism.

Vitamin D2



VITAMIN D BIOASSAY PROCEDURE

Sample Preparation

AOAC International provides specific instructions for preparation of various matrices for the bioassay. In some cases, saponification is used.

Depletion Period

Rats are suitable for depletion at age ≤30 days with body weight of ≥ 44 g but ≤ 60 g. Rachitogenic diet is fed for 18–25 days.

Assay Period

The assay period is the interval of life of the rat between the last day of the depletion period and the eighth or eleventh day thereafter. Feeding protocols are specified. During the assay, depleted rats are fed known and unknown amounts of Vitamin D from standards and samples, respectively.

Potency of Sample

Vitamin D in the sample is determined by the line test from staining of the proximal end of the tibia or distal end of the radius or ulna.

18-2 figure

The bioassay of Vitamin D by the line test, AOAC Method 936.14 45.3.01 (1).