Appendix II Methods for chemical analysis & sensory evaluation

222

Determination of Moisture (ISI, 1981)

Procedure

Weigh 5-10 of the sample accurately in a predried and preweighed petri dish. Place the dish in the oven maintained at about 100^0C overnight. Cool in the dessicator and weigh.

Calculation

$$\text{Moisture (\% by weight)} = \frac{100 \times (M_1 - M_2)}{M_1 - M}$$

where :

M = weight in g of empty petridish

M_1 = weight in g of the dish with material before drying

M_2 = weight in g of the dish with the material after drying

Determination of fat acidity (AACC, 1983)

Reagents

1 *Petroleum ether ($35-60^0$)*

2. *Toluene alcohol phenolphthalein solution (TAP). Mix equal parts by volume of CP toluene and 95 % ethyl alcohal. Add 0.2 g phenolphthalein per liter to form 0.02 % solution.*

3. *KOH. Prepare 0.0178 N KOH (1 ml = 1 mg KOH).*

Colour standard

The intensity of yellow color in grain varies, depending upon the type of grain, therefore a colour standard is helpful in making titration end points uniform. Prepare as follows :

To 50 ml water in flask of type used for titrating add dropwise 0.05 % potassium dichromate until water solution matches in colour the grain extracted solution to be titrated. Add 2.5 ml freshly prepared 0.01 % potassium permanganate solution and mix. Colour of titration end point should match this standard. Prepare colour standard for titration blank by adding 2.5 ml of 0.01 % potassium permanganate to 50 ml water.

Procedure

1 *Grind at least 40 g of representative sample of small grains such as wheat, or 200 g of larger grains such as corn. (In the present study, biscuits were powdered on pastle and mortar). Once ground, sample must be carried to extraction step within 1 hr to forestall changes caused by lipolytic enzymes.*

2. *Extract 10 g ground sample with pet ether in extractor at a rate of one siphoning every 3 min. (In the present experiment the fat was extracted by keeping the sample overnight in ether).*

3. *Evaporate pet ether from extract and redissolve in extraction flask with 50 ml TAP solution. (After evaporation the flask which was preweighed was kept in the oven at 60^{0} and weighed again to get the weight of the fat extracted.)*

4. *Titrate extracted solution with 0.0178 N KOH to end point matching colour of standard.*

5. *Determine blank by titrating 50 ml TAP solution to end point matching colour of standard for titration blank.*

Note : *In the case of grains having high fat acidity values, emulsions are sometimes formed during titration, partially marking the end point. When emulsion appears, 50 ml additional TAP solution may be added to ensure clear solution for titration. Blank titration value in this case may be doubled that determined on single 50 ml portion of solvent.*

Calculation

Fat acidity is reported as mg KOH required to neutralize free fatty acids from 100 g grain on dry matter basis by formula :

$$\text{Fat Acidity (as oleic acid percent by mass)} = \frac{28 \times \text{titre value} \times \text{Normality of KOH}}{\text{weight of fat taken for estimation}}$$

Determination of Peroxide Value (AOAC, 1980)

REAGENTS

1 **Solvents :** *Mix 3 volumes of glacial acetic acid and 2 volumes of chloroform.*

2 **Saturated KI solution :** *4 parts of pure potassium iodide are dissolved in 3 parts of distilled water. The solution is stored in brown bottle. It is discarded when the blank titration becomes greater than 0.2 ml of 0.002 N sodium thiosulphate solution.*

3. **Sodium thiosulfate (0.1 N) :** *Weigh out 25 g ordinary CP sodium thiosulfate or 24.83 g of pure dry recrystallized salt. Dissolve in water and dilute to a litter. Boiled distilled water must be used.*

Standardization using potassium dichromate : *Accurately weigh 0.20 - 0.23 g* $K_2Cr_2O_7$ *(dried for 2 hr at* 100^oC*) and place in glass-stoppered inodine flask. Dissolve in 80 ml* H_2O *containing 2 g KI. Add with swirling 20 ml of 1N HCl and immediately place in dark for 10 min Tirate with* $Na_2S_2O_3$ *solution adding starch solution after most of iodine has been consumed.*

$$\text{Normality} = \frac{g\ K_2Cr_2O_7\ X\ 1000}{ml\ Na_2S_2O_2\ X\ 49.032}$$

4 ***Starch solution :*** *Mix about 1 g soluble starch with enough cold water to make thin paste. Add 100 ml boiling* H_2O *and boil for 1 min while stirring.*

PROCEDURE

Weigh 5 g of sample of oil or fat into 250 ml glass-stoppered flask. Add 30 ml saturated KI solution from Mohr pipet. Let stand with occasonal shaking for 1 min and add 30 ml H_2O*. Slowly titrate with 0.1 N* $Na_2S_2O_3$ *with vigorous shaking until yellow is almost gone. Add 0.5 ml of 1 % starch solution and continue titration shaking vigorously to release all iodine from* $CHCl_3$ *layer until blue just disappears. If 0.5 ml 0.1 N* $Na_2S_2O_3$ *is used, repeat determination with 0.01 N* $Na_2S_2O_3$*.*

Calculations

$$\text{Peroxide value (meq peroxide per Kg sample)} = \frac{S\ X\ N\ X\ 100}{\text{Weight (g) of sample}}$$

Where S = ml $Na_2S_2O_3$ *N = Normality of* $Na_2S_2O_3$*.*

225

Sensory Evaluation Proforma for shelf life studies

Product _____ *day of testing* _____

Date _____

Name of the Panel member _____

Instruction *The given biscuit samples are to be evaluated for their shelf life i.e. the duration for which they can be kept without spoilage. Keeping that in mind please evaluate the samples for the follwing :*

1 ***Off Flavour***

Undetectable	*4*	*Acceptable*	*1*
Detectable	*3*	*Unacceptable*	*0*
Slightly rancid	*2*		
Rancid	*1*		
Very Rancid	*0*		

2 Texture and Mouthfeel

Excellen t	*5*	*Acceptable*	*1*
Very Good	*4*	*Unacceptable*	*0*
Good	*3*		
Fair	*2*		
Bad	*1*		
Very Bad	*0*		

Sample	*Off Flavour*		*Texture and Score*	
	*4 - 0**	*1 - 0**	*5 - 0**	*1 - 0**
A				
B				

Comments:

* *Example:*

Give a score of 4 if off flavour is undetectable and 0 if the sample is very rancid.

Give a score of 1 if the flavour of the sample is acceptable and 0 if not.

Give a score of 5 if the texture and mouth feel are excellent and 0 if very bad.

Give a score of 1 if the texture and mouth-feel are acceptable and 0 if not.

226

Method of estimation of protein (*Micro-Kjeldahl method*)
(Ranganna 1977)

Principle *Nitrogen content is estimated by the Kjeldahl method which is based on the determination of the amount of reduced nitrogen (NH_2 and NH) present in the sample. The various nitrogenous compounds are converted into ammonium sulfate by boiling with conc H_2SO_4. The ammonium sulfate formed is decomposed with an alkali (NaOH), and the ammonia liberated is absorbed in excess of neutral boric acid solution and then titrated with standard acid.*

Reagents

1 *Mixed indicator : Prepare 0.1 % bromocresol green and 0.1% methyl red indicators in 95 % alcohol separately. Mix 10 ml of the bromocresol green with 2 ml of the methyl red solution.*

2 2 *Boric Acid - 2% : Dissolve 10 g of boric acid (crystals) in 500 ml of boiling distilled water. After cooling, transfer the solution into a glass-stoppered bottle.*

3 *HCl - 0.01 N : Check the normality against pure sodium carbonate.*

4 *NaOH - 30 % : Dissolve 150 g of sodium hydroxide pellets in 350 ml of distilled water. Store the solution in a bottle closed with a rubber stopper.*

5 *Catalysts for digestion : Mix 2.5 g of powdered selenium dioxide (SeO_2), 100 g of potassium sulfate (K_2SO_4) and 20 g copper sulfate ($CuSO_4, 5H_2O$).*

Procedure

Digestion

1 *Weigh 1-2 g of the powdered sample and transfer to a 250 ml Kjeldahl flask taking care to see that no portion of the sample clings to the neck of the flask. Add 1-2 g of catalyst mixture and 25 ml of conc H_2SO_4. Place the flask in an inclined position on the stand in the digestion chamber and digest. Heat the flask gently over a low flame until the initial frothing ceases and the mixture boils*

briskly at a moderate rate. During heating, rotate the flask several times. Continue heating for about an hour or more until the colour of the digest is pale blue. Cool the digest, and add slowly, 30-40 ml water in 5 ml portions with mixing. Cool and transfer the digest to a 100 ml volumetric flask. Rinse the digestion flask 2-3 times with water, transfer to the volumetric flask, cool and make to volume with water.

Distillation and titration

Set up the distillation apparatus as shown in Fig A . Place a flask under the condenser F. Boil the distilled water in the steam generator A using bunsen burner. Close stopcock E and pinch clamp D. Run cold water through the condensor from which about 5 ml of distillate should collect per min. Remove the burner, whereupon, the condensate in the distilling flask G is sucked back into the steam trap C. Fill funnel E with distilled water and open stopcock momentarily to drain the water into G. Replace the burner under the steam generator for about 20 sec and remove it again. Pipette 5 ml of 2 % boric acid and add 4 drops of mixed indicator into a clean conical flask. Fill the micro burette with 0.01 N HCL to the zero mark. By this time, the distilling flask G would have become empty. Replace the burner under the steam generator, and open pinch clamp D to remove liquid from the steam trap C. Leave the pinch clamp on the glass tubing through which the steam escapes. Replace the beaker under condensor with the conical flask containing boric acid, and support the flask in the oblique position, so that the tip of the condensor is completely immersed in the liquid. Open the stopcock E with one hand and with the other hand, pipette 5.0 ml of the digest into G. Rinse the funnel twice with about 2 to 3 ml portions of distilled water. Then introduce 10 ml of 30 % NaOH and close stopcock E. Replace the pinch cock D on the rubber tubing, whereupon steam enters G, stirs up the digestion mixture and sodium hydroxide, and liberates ammonia which escapes with steam through the condensor in to the boric acid solution.

The colour of boric acid changes from bluish purple to bluish green as soon as it comes in contact with ammonia. The change which is very sharp, takes place between 20 to 30 sec after the pinch clamp is closed. Three to five min after the boric acid has changed colour, lower the conical flask so that the condensor tip is 1 cm above the liquid. Wash the end of the condensor with a little distill water. Continue distillation for another min and then remove the burner. Titrate with standard HCl unil the blue colour disappears. Wash E with distilled water as described earlier and continue with the distillation of the next step.

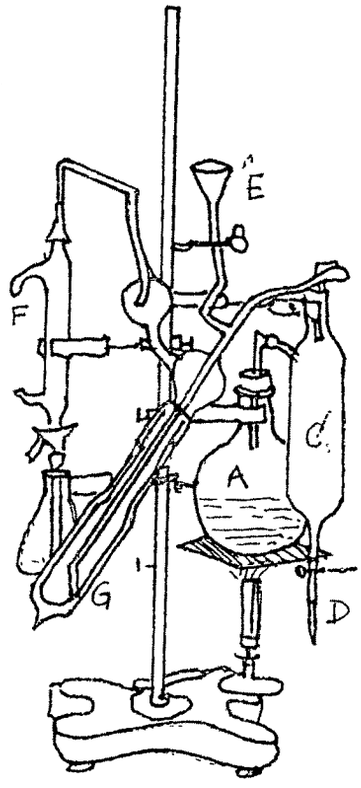

Fig A : steam distillation apparatus

Calculations

$$\text{Nitrogen (\%)} = \frac{\text{Sample titre} \times \text{Normality} \times 14 \times \text{Vol. of digest} \times 100}{\text{Aliquot of the digest} \times \text{Weight of sample} \times 1000}$$

Protein (%) = Nitrogen (%) X 6.25*

Method for estimation of crude fibre (NIN 1983)

Reagents

1. H_2SO_4 - 0.255 N (1.25% W/V)
2. NaOH - 0.313 N (1.25% W/V)

Procedure

About 2-5 g of moisture and fat-free sample are weighed into a 500 ml beaker and 200 ml of boiling 0.255 N sulfuric acid added. The mixture is boiled for 30 min keeping the volume constant by the addition of water at frequent intervals (a glass rod inserted in the beaker helps smooth boiling). At the end of this period, the mixture is filtered through a muslin cloth and the residue washed with hot water till free from acid. The material is then transferred to the same beaker and 200 ml of boiling 0.313 N (1.25%) NaOH added. After boiling for 30 min (keeping the volume constant as before) the mixture is filtered through muslin cloth. The residue is washed with hot water till free from alkali followed by washing with some alcohol and ether. It is then transferred to a crucible, dried overnight at 80-100°C and weighed (W_1). The crucible is heated in a muffle furnace at 600°C for 2-3 h, cooled and weighed again (W_2). The difference in the weights (W_1-W_2) represents the weight of crude fibre.

Crude fibre (g/100 g) sample

$$= \frac{100 - (\text{Moisture}^{**} + \text{Fat}^{**}) \times \text{weight of fibre}}{\text{Weight of sample taken (moisture and fat-free)}}$$

*Based on the assumption that plant protein contains 16% nitrogen.
** (g/100 g sample)

Method for estimation of calcium *(Pearson 1962 , Oser 1976)*

Principle *Calcium is precipitated as oxalate and the oxalate is titrated with potassium permanganate.*

Reagents

1 *HCl - 1 N*

2 *Ammonium oxalate solution 5%*

3 *Methyl red indicator - 0.1 % in 95% alcohol*

4 *Dilute ammonia - 2 ml NH_3 + 98 ml distilled water*

5 *Glacial acetic acid*

6 *Potassium permanganate - 0.02 N (Standardised against 0.02 N sodium oxalate)*

Procedure

1 *Ash 5-10 g of sample in a muffle furnace at 550°C*

2 *Add 2 ml conc HCl to the ash and evaporate to dryness*

3 *Boil with 10 ml 1 N HCl. Filter into a 25 ml vol flask, wash in and make up the mark with more of 1 N HCl*

4 *Pipet 10 ml in a centrifuge tube.*

5 *Add 1 ml of 5 % ammonium oxalate solution and a few drops of methyl red indicator.*

6 *Make alkaline with ammonia.*

7 *Add glacial acid until just pink (pH - 5.0).*

8 *Allow to stand at least 4 h and centrifuge (3000 rpm for 10 min).*

9 *Cautiously decant off the supernatant liquor and wash twice with dil NH_3 (2%) thoroughly mixing the ppt with the fluid and centrifuging as before after each eddition.*

10 *After the final decantation, add 2 ml dilute H_2SO_4 (1 : 4 H_2O). Dispense the ppt and heat to 85°C and titrate with 0.02 N $KMnO_4$.*
(1 ml = 0.4 g of Ca)

Calculation

$$\text{mg Ca per 100 g sample} = \frac{0.4 \times \text{titre value} \times \text{Vol of digest for estimation}}{0.2 \times \text{Total vol of digest}} \times \frac{100}{\text{wt of sample}}$$

Method for estimation of riboflavin (AACC 1983)

Principle : *The flourimetric procedure for the determination of riboflavin depends upon the extraction of the vitamin with dilute acid, filtration, treatment of the filtrate with permanganate and hydrogen peroxide to destroy interferring pigments, and measurement of the flourescence. The vitamin content of the extract is evaluated by means of an internal standard.*

Reagents

1 H_2SO_4 - 0.1 N
2 Sodium acetate 2.5 M. 340 g sodium acetate trihydrate is dissolved in water and diluted to 1 L.
3 $KMnO_4$: 4%. Prepare fresh daily.
4 H_2O_2 3% : Dilute 30% H_2O_2 1:10 with water.
5 Riboflavin standard.

(a) Dry USP riboflavin reference standard over phosphorus pentoxide in dessicator for 24 h. Dissolve 50 mg in 1500 ml water and 2.4 ml glacial acetic acid in 2 L flask. Warm to aid solution. Cool and make up the volume. Store under toluene in amber bottle in refrigerator.

(b) Dilute 20 ml B_2 std (a) to 50 ml with water.

(c) Working soln. Dilute 10 ml B_2 std (b) with H_2O to 100 ml. Prepare fresh daily and protect from light. (1 ml = 1 ug B_2).

6 Sodium hydrosulfite.

Procedure

Accurately weigh well mixed sample into 100 ml vol. flask according to follwing schedule :

For samples containing (mg/lb)	Weigh (g)
0.0 - 0.8	5
0.8 - 2.0	4
2.0 - 4.0	2

Size of sample is not critical. If large samples are taken, proportionately larger amounts of reagents must be used.

Extraction

Add 75 ml 0.1 N H_2SO_4 , mix and either autoclave at 15 lb for 30 min or immerse flask in boiling water bath for 30 min shaking flask every 5 min. Let cool to room temperature.

232

Adjustment of pH

1 *Add 5 ml 2.5 M sodium acetate solution. Mix and let stand at least 1 hr. solution is now at pH 4.5 .*

2 *Make to volume and filter through medium-fast paper. Filter paper may be tested for B_2 adsorption by comparing galvanometer readings of filtered and unfiltered standard riboflavin solution.*

Oxidation of impurities

1 *In two test tubes of 1 inch diameter with stirring rods, marked A and B conduct oxidation as follows :*

	Tube A	**Tube B**
Sample solution (ml)	*10*	*10*
Standard solution (ml)	*1*	-
Water (ml)	*1*	*2*
$KMnO_4$ (4%) ml	*0.5*	*0.5*
Time lapse (min)	*2*	*2*
H_2O_2 3% (ml)	*0.5*	*0.5*

2 *Stir samples after each addition of permanganate. Shake after adding peroxide until foaming is negligible. This prevents formation of gas bubbles in cuvettes*

Measurement

1 *Adjust flourimeter so that sodium flourescence solution gives suitable galvanometer deflection. Determine flourescence of solutions A and B. Make readings with no more than 10 sec of exposure in flourimeter.*

2 *To solution B in cuvette add 20 mg sodium hydrosulfite, stir and determine blank flourescence C. Do not use reading C after colloidal sulfur begins to form.*

Avoid excess of hydrosulfite. It is necessary to take readings rapidly before colloidal sulfur begins to form. Colloidal sulfur will raise apparent flourescence. Hydrosulfite may be added while cuvette is in the instrument.

Calculations

$$Riboflavin = \frac{B - C}{A - B} \; X \; \frac{R}{S} \; X \; \frac{V}{V_1} \; X \; 100$$

where :

A = Flourescence reading of sample + B_2 std

B = Flourescence reading of sample + water

C = Flourescence reading after addition of sodium hydrosulfite

233

R = *Std riboflavin added, ug/W_1 of sample solution*

V = *Original volume of sample solution, ml*

V_1 = *Volume of sample solution taken for measurement, ml*

S = *Sample wt (g).*

If dilutions recommended above are used;

$R = 1$, $V = 100$, $V_1 = 10$

Method for estimation of thiamin

Thiochrome method - AACC 1983

Principle *See page*

Reagents

1 *NaoH - 15%. Dissolve 15 g NaOH in H_2O, coll and dilute to 100 ml.*

2 *Potassium ferricyanide solution - 1 % : Dissolve 1 g potassium ferricyanide H_2O and dilute to 100 ml. If stored in stoppered brown bottle in cool, dark place, this reagent is stable.*

3 ***H_2SO_4 solution - 0.1 N :*** *Dilute 2.8 ml conc. H_2SO_4 to 1 L with water.*

4 *Sodium Acetate solution 2 5 M : Dissolve 205 g anhydrous sodium acetate or 340 g sodium acetate trihydrate in water and dil to 1 L.*

5 *Enzyme preparation :*

Dissolve 150 mg of taka-diastase and 75 mg of papain in 5 ml of acetate buffer.

6 *Isobutanol : This should give blank reading of 1.5 galvanometer scale division or less.*

7 *Stock thiamine solution : Dry thiamine hydrochloride (USP ref std) over phosphorus pentoxide in dessicator at least 24 h. Dissolve 100 mg in 25% etha nol and dilute to 1 L with 25 % ethanol. This solution is stable for several months if kept in refrigerator.*

8 *Working standard : Dilute 5 ml stock thiamin solution (warmed to room temperture) to 100 ml with water. Dilute 4 ml of this intermediate conc to 100 ml with 0.1 N H_2SO_4*

9 *Quinine sulfate solution : Dissolve 100 mg USP quinine sulfate in 0.1 N H_2SO_4 and dilute to 1 L with H_2SO_4. Dilute 3 ml of this solution to 1 L with 0.1 N H_2SO_4 . Store in brown bottle.*

Procedure

1 *Grind material to pass through 20 mesh.*

Extraction

1 *Take weight of sample containing 20 ug B_1 or such that final portion (5ml) to be oxidized will contain approximately 1 ug B_1. (In the present estimation 6-7 g of biscuit were taken for estimation.)*

2 *Place sample in 100 ml vol flask; add 50 ml 0.1 N H_2SO_4 and heat in boiling water for 10 min*

3 *Cool flask to 40^o or lower, add 5 ml enzyme suspension. Incubate at least 4 hr at 37-40^o. Cool and dilute to 100 ml.*

4 *Mix digested sample extract thoroughly and filter through Whatman no.41.*

5 *If purification is not required proceed directly to oxidation using appropriate aliquot.*

Purification : *This was not done because the extract obtained was clear.*

Oxidation

1 *Mix eluate well by inverting 3-4 times and pipet 5 ml into 25 ml glass-stoppered (g-s) separating funnel. Pipet similar 5 ml aliquots into 2nd g-s sep. funnel to be used for blank. Number these funnels 1 and 2.*

2 *To first funnel, add 3 ml alkaline ferricynide (prepare alkaline potassium ferricyanide fresh daily by diluting 3 ml 1 % potassium ferricyanide to 100 ml with 15% NaOH solution. If preferred add 3 ml alkaly and 1 drop potassium ferricyanide separately, obviating daily preparation of mixture). To second funnel add 3 ml 15% NaOH. Mix these gently for 30 sec and add 15 ml isobutanol to each. Shake vigorously for 60 sec.*

3 *Let the funnels stand for sometime till the layers (aqueous alcohol) are separated.*

4 *Decant approx. 10 ml isobutanol into cuvette for reading flourescence of thiochrome.*

Measurement

1 *Make the measurements as quickly as possible to minimize destruction of thiochrome by the inciting light. It is advisable to conduct measurements in a room with subdued light.*

2 *Standardize flourimeter with quinine sulfate. (Adjust it to 50 or 100 depending upon the range of readings for the samples.) Calibrate galvanometer in*

terms of deflection with standard thiamine solution with each set of samples treat one or two samples of standard B_1 solution exactly as the unknown. Use std B_1 solution at a conc of 1 ug/5 ml final aliquot. Difference between total and blank readings of galvanometer corresponds to 1 ug of B_1.

3. Determine flourescence on approx 10 ml isobutanol in terms of galvanometer deflections operating flourimeter as per manufacturer's instructions.

Calculations

$$\text{Thiamine} = \frac{Rx - Rxb}{Rs - Rsb} \times \frac{V}{Z} \times \frac{E}{5} \times \frac{1}{S}$$

where

Rx = Flourimeter reading with unknown
Rxb = Blank reading with unknown
Rs = Flourimeter reading with B_1 std
Rsb = Blank reading with B_1 std
Z = Volume of extract taken for oxidation
E = volume in ml of extract collected
S = Sample weight in g
V = Volume of extract.

Principle : The thiochrome method for the determination of thiamine involves extraction of the vitamin, enzyme hydrolysis, adsorption, elution, and oxidation to the flourescent thiochrome which is extracted with isobutanol and determined flourimetrically.

Method for estimation of Bioavailability of Lysine (Carpenter 1960; NIN 1983) -

Principle : Lysine residues with reactive ϵ-NH_2 groups in the food proteins are converted into the yellow ϵ-DNP lysine by treatment of the material with FDNB followed by acid hydrolysis. Other soluble interfering compounds are removed by extraction and the extinction of the residual acqueous layer is measured. A blank value is obtained by treatment with methoxy carbonyl chloride and extraction of the other soluble lysine compound which results.

Reagents

1. 1.Flouro - 2-4 denitrobenzene (FDNB) - 2.5% (VN) solution in ethanol (made up freshly for each determination - because of the danger of its vesicant effects if spilt on skin. It may be solid at room temperature but can be measured out

by holding the bottle in warm water and warming the pipet before use. Accuracy is not essential as the reagent is used in excess.

2. *Methoxy carbonyl chloride*

3. *Diethyl ether*

4. *$NaHCO_3$ W/V - 8% (buffer- pH 8.5)*

5. *Na_2CO_3 - 8%. 19.1% W/V with a final adjustment with NaOH or HCl as required.*

6. ***Standard** : ϵ-Dinitrophenyl (DNP) lysine hydrochloride. Prepare a stock solution having 1 mg DNP lysine per ml in 1 N HCl. Working standard is made daily by diluting 1 ml of the stock standard solution to 100 ml in 1 N HCl.*

Procedure

Stage I

1. *Grind 50 g material fine (to pass through 1/50 inch sieve).*

2. *Two portions, each containing an estimated 30-50 mg of nitrogen are taken into round bottomed flasks and to each is added 8 ml of 8% W/V $NaHCO_3$.*

3. *They ae shaken gently to disperse the material and then left for 10 min.*

4. *FDNB (0.3 ml) previously dissolved in 12 ml of ethanol is added to each flask, stoppered and shaken gently on a mechanical shaker for 2 h.*

5. *Stoppers are removed and the flasks stood in boiling water until there is no more effervascence - even on shaking. (It may be checked that this point corresponds to a loss of weight of 10 g i.e. the weight of ethanol added).*

6. *8.1 N HCl (24 ml) is added immediately and the flasks are refluxed gently for 16 h with condensers adequate to prevent loss of HCl.*

7. *The flasks are then disconnected after washing the condensers with water. (The condensers still give a yellow colour on being placed in alkaline washing water, owing to the presence of dinitrophenol, a decomposition product of FDNB which is colouress in acid solution.*

8. *Place the flasks in ice-water for 1-2 h.*

9. *Filter the contents through Whatman 41 with water washings. Make the volume of the filtrate to 250 ml.*

Stage 2

1. *2 ml portions from each diluted filtrate is pipetted into each of two glass-stopered tubes A and B, graduated at 10 ml and a small conical flask C.*

2. *The contents of the tube are extracted twice with approx 5 ml portions of ether. The ether layers are discarded and the tubes are held in boiling water until effervascence from the residual ether ceases and then they are cooled.*

3. *Tube A is made upto 10 ml with HCl and kept for the final readings.*

Stage 3

1. *The contents of flask C are titrated with 10% (W/V) NaOH with phenolphthalein (1%) as indicator and then discarded.*

2. *The same volume of NaOH is then added to tube B, followed by 2 ml of buffer solution (pH 8.5).*

3. *0.045 to 0.055 ml methoxycarbonyl chloride is then added and the tubes shaken vigorously to disperse and dissolve the compound. After 5 to 10 min 0.75 ml of conc HCl is added, cautiously at first and with agitation to prevent the contents frothing over.*

4. *The contents are again extracted twice with 5 ml ether (the ether washings are discarded). The residual ether in the acqueous layer is evaporated by standing the tube in boiling water and the volume is made to 10 ml with water.*

Stage 4

The extinction coefficient of the contents of tubes A and B are measured in 1 cm cell at 435 mu (or a filter having maximum transmission between 430 and 450 mu if necessary).

Reading A minus B is taken as the extinction due to epsilon DNP lysine, the concentration of which is extrapolated from the standard graph obtained by using concentrations of standard DNP-lysine solution.

$$\text{Available lysine (g/100 g protein)} = \frac{0.851 \times 0.4682 \times \text{dil factor} \times 100 \times 100 \times \text{conc of DNP-HCl}}{\text{Weight of sample} \times \% \text{ protein}}$$

238

Molecular weight of epsilon DNP lysine = 312,3

Molecular weight of epsilon DNP Lysine HCl, H_2O = 366.8

∴ Conversion of epsilon DNP lysine HCl, H_2O to

$$\text{epsilon DNP lysine} = \frac{312.3}{366.8} = 0.851.$$

$$\text{Conversion of epsilon DNP lysine to lysine} = \frac{146.19}{312.3} = 0.4682.$$

239

Method of estimation of bioavailability of iron (Narsinga Rao and Prabhavathi, 1978).

Reagents

1. *Papain HCℓ : 5 g of papain (Sigma Chemicals 1: 10,000) were dissolved in 1000 ml of 0.1 N HCℓ.*
2. *Hydroxylamine hydrochloride : 10 g of hydroxylamine hydrochloride were dissolved in 100 ml of double distilled water.*
3. *Acetate buffer : To 8.3 g anhydrous sodium acetate were added 12 ml glacial acetic acid and the volume made upto 100 ml with double distilled water. (pH - 4.2).*
4. *α - α' dipyridyl solution : 100 mg of α-α'dipyridyl were dissolved in 100 ml of 3% acetic acid.*
5. *Acidified potassium permangnate 3 g of potassium permanganate were dissolved in 100 ml of double distilled water. To 8 ml of permanganate solution were added 5 ml of 0.5 N HCℓ and the volume made upto 20 ml.*
6. *Ascorbic acid : 20 g ascorbic acid were dissolved in 100 ml double distilled water. This reagent was stored in refrigerator.*
7. *Standard iron solution: 99.57 mg of Ferrous sulfate ($FeSO_4$, $7H_2O$)were dissolved in 100 ml double distilled water. This solution contained 200 ug per ml of iron (to be prepared fresh).*
8. *Standard for use : 1 ml of the stock solution was diluted to 100 ml with double distilled water. This solution contained 2 ug per ml. (to be prepared fresh).*
9. *6 N HCℓ*
10. *NaOH : 0.5 N, 0.1 N.*

Procedure

Bioavailability of iron was estimated using the 'in vitro' method. The biscuits were powdered and homogenized to a creamy consistency with double distilled water. 25 ml of the homogenate were taken in a 150 ml conical flask and the pH of this was recorded. To this 25 ml of papain HCℓ mix were added. The pH of the solution was adjusted to 1.35 by dropwise addition of distilled 6N HCℓ. The mixture was incubated for exactly 90 min 37^{o} C in a mechanical shaker (100-200 oscillation per min). The contents of the flask were then centrifuged at 2000 rpm for 30 min and the supernanant was transferred to a clean 150 ml conical flask.

The volume of the supernatant and the weight of the flask with the supernatant was recorded. The supernatant was heated in a water bath for 15 min, cooled and reweighed. The loss in weight due to evaporation was made up with double distilled water. The volume of the solution was then recorded after which it was filtered through whatman no 44 filter paper. The pH of this solution was then adjusted to 7.5 using NaOH solutions of varying strengths (0.5 to 0.1 N) taking care to record the total amount of NaOH added. The mixture was then incubated in shaker water bath at 37^{o} C for 45 min, after which it was filtered again through Whatman no 44. This filtrate was used for the estimation of soluble iron and ionizable iron.

Ionizable iron

For estimating iron, 7 ml of the filtrate were taken in test tubes in duplicates to which 1 ml of 10% hydroxylamine hydrochloride, 5 ml of acetate buffer (pH 4.2) and 2 ml of α-α' dipyridyl solution were added in that order. The color intesity was read in a spectro-photometer after 30 min at a wave length of 540 nm. A reagent blank consisting of 7 ml of distilled water, 1 ml hydroxylamine hydro-chloride, 5 ml acetate buffer and 2 ml α-α' dipyridyl was also prepared. As the filtrated obtained tended to be colored, sample blank solutions were prepared for each sample using 7 ml of the final filtrate, 1 ml of hydroxylamine hydrochloride, 5 ml of acetate buffer and 2 ml of double distilled water. The instrument was set to zero with the reagent blank. The sample blank and the sample were then read in that order. The reading, if any, of the sample blank was subtracted from that of the sample so that the effect of color due to extraneous material in the filtrate was cancelled out. The corrected readings of the unknown solutions were compared with those of the standard solutions containg 2 ug to 10 ug per ml of iron ($Feso_4$, 7 H_2O), which were reached with the above reagents in the same manner as the unknown filtrate. The ionizable iron in the sample was calculated as follows :

$$\text{Ionizable iron} = \frac{\text{Corrected read. of the sample} \times \text{Conc. of std.(ug)} \times \text{Vol} \times 50 \times \text{Vol. of homogenate} \times 1}{\text{Read. of std} \times 7 \times \text{Vol of super nat.} \times 25 \times 1000}$$

The ionizable iron was then expressed as a percentage of the total iron. This figure represented the percentage bioavailable iron.

Soluble iron

Soluble iron was estimated by the method of Tannat and Greenman (1969). Six ml of the final filtrate were taken in a test tube to which 4 ml of acidified

$KMNO_4$ were added. The mixture was shaken and kept at room temperature for 15 min after which 2 ml of 20 % ascorbic acid were added. After shaking, this mixture was incubated for 120 min at 56^{o} C in an ordinary water bath. The solution was filtered through Whatman no 44 filte r paper and the iron in the supernatant was estimated as described earlier for ionizable iron. The soluble iron in the filtrate was calculated by comparing the readings with those of standards run simulataneously. Sample blanks were also run in order to cancel the effect of color due to extraneous material. Soluble iron in the sample was calculated as follows:

$$\text{Soluble iron (mg per meal)} = \frac{\text{Corrected read. of sample} \times \text{Conc of std ug} \times 12 \times \text{Vol} \times 50 \times \text{Vol. of homogenate} \times 1}{\text{Read of std} \times 7 \times 6 \times \text{Vol of supernat} \times 25 \times 1000}$$

Total iron (Wong's method- Oser , 1976).

Principle : Iron is determined colorimetrically making use of the fact that ferric iron gives a blood-red color with potassium thiocyanate.

Reagents

1. 30% sulphuric acid (30% ml conc H_2SO_4 diluted to 100 ml).
2. Saturated potassium persulfate solution : 7 g of potassium persulfate were dissolved in glass distilled water and the solution made upto 100 ml.
3. Potassium thiocyanate : 40% solution. 40 g KCNS were dissovled in 90 ml glass distilled water, 4 ml acetone added and the volume made upto 100 ml.
4. Standard iron solution: 0.7022 g ferrous ammonium sulfate was dissolved in 100 ml glass distlled water and after addition of 5 ml of conc H_2SO_4, the solution was made upto 1 litre and mixed throughly. (1 ml = 0.1 mg Fe). The standard solution was prepared fresh.
5. Working standard solution: 0.01 mg Fe per ml was prepared by diluting the above solution ten-fold.

Procedure

1,2,3,4 and 5 ml aliquots of the standard solution were taken and volume made to 6.5 ml with distilled water. 1 ml of 30 % H_2SO_4, 1 ml of potassium persulfate and 1.5 ml 40% KCNS solutions were then added. The color was measured at 540 nm within 20 min.

Sample treatment

About 1 to 2 g of the sample were weighed accurately and digested with a mixture

of conc H_2SO_4 and HNO_3 (1:3), till a clear solution was obtained. The volume was made to 100 ml with distilled water. Suitable aliquots of the digest were treated like the standards and the intensity of the color measured at 540 nm.

Calculations

$$\text{mg iron per} = \frac{\text{Read. of sample}}{\text{Read of std}} \times \text{Conc of std} \times \frac{\text{Total vol of digest}}{\text{Vol. of alliquot}} \times \frac{100}{\text{Wt. of the sample}}$$