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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 36

Englyst Procedure for Determination of Total, Soluble and Insoluble Dietary Fibre as Non-starch Polysaccharides: Measurement of Constituent Sugars by Gas-Liquid Chromatography and Colorimetry

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1. Scope and Field of Application

The Englyst procedure determines total, soluble and insoluble non starch polysaccharides (NSP) in plant foods and food products. The procedure is widely used in research, and it is one of the methods used for food labelling for dietary fibre within the EC.

The cell-wall material naturally present in plants is the common characteristic of the foods that comprise a high-fibre diet and approximately 90% of this material is NSP. The specific measurement of the NSP content of unfortified plant foods provides a good marker for endogenous plant cell-wail material and, therefore, for a high-fibre diet (i.e. a diet rich in plant cell-wail material, starch, minerals, vitamins and antioxidants but low in fat and sodium).

The EC Scientific Committee for Food have been considering the definition of dietary fibre for food labelling and have reported:

"There was lengthy discussion as to the material that should be defined as fibre for the purposes of nutrition labelling. In particular the inclusion or not of Resistant Starch in the definition of fibre was argued extensively. The Committee decided that the material to be considered as fibre for the purposes of nutrition labelling should be confined to non-starch polysaccharides of cell-wall origin."

NSP values will aid the consumer in choosing the high-fibre diet recommended in national dietary guidelines.

NSP values are reported as dietary fibre in food tables (e.g. **10.1-10.3**).

2. Definition

The content of dietary fibre: the content of NSP as determined by the method specified.

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3. Principle

The Englyst procedure (**10.4-10.7**) measures NSP, using enzymic-chemical methods, and has evolved from the principles laid down by McCance and Widdowson, and later by Southgate (**10.8**).

In the procedure, starch is dispersed and then hydrolysed enzymically. The non-starch polysaccharides (NSP) are precipitated with alcohol and then hydrolysed by sulfuric acid, releasing neutral sugars and uronic acids, which are then measured by colorimetry or the neutral sugars may be measured individually by gas-liquid chromatography (**10.5, 10.6**). The colorimetric end-point allows the determination of total, soluble and insoluble NSP within an 8 hr. working day and the method is suitable for routine analysis, where details of the separate components of NSP are not required (**10.6**). Alternatively, values for total, soluble and insoluble NSP as the sum of individual neutral sugars and uronic acids may be obtained within 1.5 working days using the GLC end-point. Agreement is good between the values obtained by the GLC and by the colorimetric assay, which is suitable for automation **(10.4)**. The procedure is summarised in Appendix 1

4. Reagents

High-purity reagents and distilled water, or water of an equivalent purity, should be used throughout the method.

4.1 Acetic acid. glacial

4.2 Acetic anhydride

4.3 Acetone

4.4 Ammonium hydroxide, 12 mol/l

4.5 Ammonium hydroxide/sodium borohydride solution. A solution of ammonium hydroxide, 2 mol/l, containing 200 mg of sodium borohydride, $NaBH_4$, per ml. Prepare immediately before use.

4.6 Benzoic acid, 50% saturated: prepare by diluting saturated benzoic add 1:1 v/v with water.

4.7 Bromophenol blue, 0.04% w/v.

4.8 Colorimetry stock sugar mixture. Make the stock sugar solution by weighing (all sugars dried to constant weight under reduced pressure with phosphorus pentoxide) to the nearest 1mg; 10.185 g of arabinose, 5.145 g of glucose and 2.16 g (2.36 g of the monohydrate) of galacturonic acid. Place them in a 1 litre calibrated flask and dilute to volume with 50% saturated benzoic acid (**4.6**). Store the mixture at 4°C.

4.9 3,3-Dimethylglutaric acid, 0.5mol/l. Add 98.5 g of NaOH, 5 mol/l (**4.29**), to 8.0 g (vacuum-dried; weighed to the nearest 1 mg) of dimethylglutaric acid and dilute to 100 ml with water at room temperature. Add one volume of the solution to one volume of sulfuric add, 2 mol/l (**4.32**); the pH should be between 3.6 and 3.8 at 22°C.

4.10 3,5-Dimethylphenol, dissolve 0.1 g of 3,5-dimethylphenol, $(CH3)_2C_6H_3OH$, in 100 ml of glacial acetic acid (**4.1**).

4.11 Dimethyl sulphoxide

4.12 Dinitrosalicylate. dissolve 10 g of 3,5-dinitrosalicylic acid ($C_7H_4N_20_7$; Sigma cat. no. D0550) and 300 g of sodium/potassium tartrate ($C_4H_4KNaO_6.4H_2O$; Sigma cat. no. S2377) in approx. 300 ml of high-purity water plus 400 ml of NaOH, 1 mol/l (**4.29**). Dissolve by stirring (overnight) and make to a final volume of 1 litre with high-purity water. Sparge for 10 min. with helium or nitrogen, or degas using an ultrasonic bath.

Store in well-capped opaque bottles, and keep for 2 days before use. The reagent is stable at room temperature for at least 6 months.

4.13 Enzyme solution I, take 2.5 ml of heat-stable amylase (EC. 3.2.1.1; 300 KNU/g) and make to 200 ml with the pre-equilibrated sodium acetate buffer (**4.27**), mix, and keep it in a 50°C water-bath (**5.4**). Prepare the solution immediately before use.

4.14 Enzyme solution II, take 1.2 g of pancreatin (Pancrex V powder from Paynes & Byrne Ltd, Greenford, Middlesex, UK) into a 50 ml tube, add 12 ml of water, vortex-mix initially and then mix for 10 min. with a magnetic stirrer. Vortex-mix again, then centrifuge for 10 min. Take 10 ml of the (cloudy) supernatant, add 2.5 ml of pullulanase (EC 3.2.1.41; 200 PNU/g) and vortex-mix. Prepare the solution immediately before use and keep it at room temperature.

4.15 Ethanol, absolute.

4.16 Ethanol, absolute, acidified. Add 1ml of hydrochloric acid, 5 mol/l (**4.21**), per litre of ethanol (**4.15**).

4.17 Ethanol. 85% v/v, acidified. Add 1ml of hydrochloric acid, 5 mol/l (**4.21**), per litre of ethanol (**4.15**).

4.18 Glass balls, 2.5 - 3.5 mm diameter (Merck cat. no. 33212)

4.19 GLC internal standard solution, 1 mg/ml. Weigh 500 mg of allose (dried to constant weight under reduced pressure with phosphorus pentoxide) to the nearest 0.1 mg. Make to 500 ml with 50% saturated benzoic acid (**4.6**) to give a 1 mg/ml solution. The solution is stable for several months at room temperature.

4.20 GLC stock sugar mixture, weigh (all sugars dried to constant weight under reduced pressure with phosphorus pentoxide), to the nearest 1 mg, 0.52 g of rhamnose, 0.48 g of fucose, 4.75 g of arabinose, 4.45 g of xylose, 2.3 g of mannose, 2.82 g of galactose, 9.4 g of glucose and 2.79 g (3.05 g of the monohydrate) of galacturonic acid. Place them in a 1 litre calibrated flask and dilute to volume with 50% saturated benzoic acid (**4.6**). The solution is stable at room temperature for several months.

4.21 Hydrochloric acid, 5 mol/l.

4.22 1-Methylimidazole

4.23 Octan-2-ol

4.24 Pectinase (EC 3.2.1.15), add 9 volumes of water to 1 volume of pectinase solution.

4.25 Potassium hydroxide, 7.5 mol/l.

4.26 Sand, acid-washed, 50 - 100 mesh (Merck cat. no. 33094).

4.27 Sodium acetate buffer, 0.1 mol/l, pH 5.2.

4.27.1 Sodium acetate, 0.1 mol/l: prepare by dissolve 13.6 g of sodium acetate trihydrate, $CH_3COONa.3H_2O$, and make to a final volume of 1 litre with water.

4.27.2 Acetic acid, 0.1 mol/l.

4.27.3 Calcium chloride, 1 mol/l.

4.27.4 Sodium acetate buffer, 0.1 mol/l: adjust sodium acetate, 0.1 mol/l (**4.27.1**), to pH 5.2 with acetic acid, 0.1 mol/l (**4.27.2**). To stabilise and activate enzymes, add 4 ml of calcium chloride, 1 mol/l (**4.27.3**), to 1 litre of buffer.

4.28 Sodium chloride/boric acid, dissolve 2 g of sodium chloride, NaCI, and 3 g of boric acid, H_3BO_3, in 100 ml of water.

4.29 Sodium hydroxide. 1.0 mol/l, 3.0 mol/l and 5 mol/l. Prepare from ConvoL® (Merck product no. 18023) or equivalent; do not use pellets.

4.30 Sodium phosphate buffer, 0.2 mol/l, pH 7.

4.30.1 Disodium hydrogen phosphate, 0.2 mol/l.

4.30.2 Sodium dihydrogen phosphate, 0.2 mol/l.

4.30.3 Sodium phosphate buffer, adjust $Na2HPO_4$, 0.2 mol/l (**4.30.1**), to pH 7 with $NaH2PO_4$, 0.2 mol/l (**4.30.2**).

4.31 Sulfuric acid, concentrated.

4.32 Sulfuric acid, 2 mol/l. Add 5ml of sulfuric acid, 12 mol/l (**4.33**) to 25 ml of water.

4.33 Sulfuric acid, 12 mol/l. This may be obtained as sulfuric acid, 72% w/w (Merck cat. no. 1932167).

4.34 Sulfuric acid, 2.4 mol/l. Accurately take 20 ml of sulfuric acid, 12 mol/l (**4.33**), and make to 100 ml with water.

5. Apparatus

5.1 Balance, accurate to 0.1 mg.

5.2 Centrifuge. capable of exerting 1500 g.

5.3 Centrifuge tubes, glass centrifuge tubes of 50-60 ml capacity (Kimble, cat. no. 45212-50), fitted with Teflon-lined screw tops (24 tubes are suitable for a batch), with a mark at 50ml.

5.4 Water-baths. one capable of maintaining 100°C; one capable of maintaining temperatures in the range 35°C to 70°C. Each bath should be of such a capacity that there is no significant change in temperature when a rack containing all the tubes (**5.3**) is placed in it.

5.5 Spectrophotometer, capable of measurement at 400 nm, 450 nm and 530 nm.

5.6 Vortex-mixer

5.7 GLC chromatograph, fitted with flame ionisation detector and, preferably, auto-injector and computing integrator.

5.8 GLC column. Supelco SP-2380 wide-bore capillary column (30 m × 0.53 mm: Supelco cat. no. 2-5319).

5.9 Heating block, capable of maintaining 40°C and 70°C.

6. Procedure

6.1 pH-treatment of samples

All samples should be finely divided so that representative sub-samples may be taken. Foods with a low water content (<10%) may be milled, and foods with a higher water content may be homogenised wet or milled after freeze-drying.

6.2 Test samples and sample weights

6.2.1 Test samples

Two portions, (a) and (b), of each test sample are required to obtain separate values for total, insoluble and soluble NSP. Portion (a) is used to measure total NSP; portion (b) is used to measure insoluble NSP. Soluble NSP is determined as the difference. The two portions are treated identically throughout the procedure, except for their separate treatment in steps **6.5** and **6.6**. A third portion (c) should be taken if a separate value for cellulose is required. It is carried through steps **6.1** to **6.5** of the procedure, then one proceeds to step **6.7.2**.

6.2.2 Sample weight

Weigh (**5.1**), to the nearest 0.1 mg, between 50 and 1000 mg depending on the water and NSP content of the sample (to give not more than 300 mg of dry matter; e.g. 300 mg is adequate for most dried foods but smaller amounts should be used for bran and purified fibre preparations) into 50-60 ml screw-top glass tubes (**5.3**). Add 300(±20) mg of acid-washed sand (**4.26**) and approximately 15 glass bails (**4.18**) to each tube. If the sample is dry (85 to 100% dry matter) and contains less than 10% fat, proceed to step **6.4**; otherwise, go to step **6.3**.

(*It is recommended that all analyses are carried out in duplicate.*)

6.3 Fat extraction/drying of wet samples

Add 40 ml of acetone (**4.3**), cap the tubes and mix several times over a 30 min. period. Centrifuge (**5.2**) at 1000 g for 10 min. to obtain a clear supernatant liquid and remove by aspiration as much of the supernatant liquid as possible without disturbing the residue. Vortex-mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tube.

Place the tube in a pan of water at 75°C in a fume-cupboard. Remove the tubes singly and vortex-mix vigorously at frequent intervals until the tubes and residues are dry.

6.4 Dispersion and enzymic hydrolysis

6.4.1 Treatment with dimethyl sulphoxide (DMSO)

Pre-equilibrate sufficient acetate buffer (**4.27**) at 50°C (8 ml required per sample).

Add 2 ml of DMSO (**4.11**), to the dry sample, cap the tube, and immediately mix the contents using a vortex-mixer (**5.6**). It is essential that all the sample is wetted and no material is encapsulated or adhering to the tube wall before proceeding. When DMSO has been added and mixed in all the tubes, vortex-mix a further three or four times during a 5 min. period.

Vortex-mix and immediately place two of the tubes into a boiling water-bath (**5.4**). Remove after 20 seconds, vortex-mix and replace into the boiling water-bath. Repeat this for subsequent pairs of tubes until all the tubes are in the bath; leave them there for 30 min. from that time. During this period, prepare enzyme solutions I and II (the volumes given are suitable for 24 tubes).

6.4.2 Treatment with enzymes

*Note: All portions of the sample in their associated tubes are to be treated in the same manner in the procedures given in Section **6.4.2** through to Section **6.6** of the method. Reference to "a sample" or "a tube" is to be taken to include "all samples" or "all tubes" being analysed in this part of the m*ethod.

Remove one tube at a time, vortex-mix, uncap and immediately add, by positive displacement, 8 ml of enzyme solution I (**4.13**), cap the tube, vortex-mix thoroughly, ensuring that no material adheres to the tube wall, and replace it in the boiling waterbath. Leave the tubes there for 10 min., timed from the last addition of enzyme.

Transfer the rack of tubes to the 50°C water-bath. After 3 min., add 0.5 ml of enzyme solution II (**4.14**) to each tube and mix the contents thoroughly to aid distribution of the enzyme throughout the sample. Replace the tubes in the 50°C water-bath and leave them there for 30 min. Transfer the rack of tubes to the boiling water-bath and leave them there for 10 min.

6.5 Precipitation and washing of the residue for measurement of total NSP: test sample portion (a) only

6.5.1 Cool the sample by placing in ice-water. Add 0.15ml of hydrochloric acid, 5mol/l (**4.21**), and vortex-mix thoroughly 2 or 3 times during a 5 min. period, with tubes being replaced in the ice-water. Add 40 ml of acidified ethanol, absolute (**4.16**), mix well by repeated inversion, then leave in ice-water for 30 min. Centrifuge (**5.2**) at 1500 g for 10 min. to obtain a clear supernatant liquid. Remove by decanting or by aspiration as much of the supernatant

liquid as possible, without disturbing the residue, and discard the liquid.

6.5.2 Add approximately 10 ml of acidified ethanol, 85% (**4.17**), to the residue and vortex-mix. Make to 50 ml with acidified ethanol, 85% (**4.17**), and mix thoroughly by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 ml of ethanol, absolute (**4.15**).

6.5.3 Add 30 ml of acetone (**4.3**) to the residue and vortex-mix thoroughly to form a suspension. Centrifuge and remove the supernatant liquid as in step **6.5.1**.

6.5.4 Vortex-mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tubes. Place the rack of tubes in a pan of water at 70 to 75°C in a fumecupboard. Remove the tubes singly and vortex-mix vigorously at frequent intervals, to ensure that the residue in each tube is finely divided, until the tube and residue appear dry. Place the rack of tubes in a fan oven at 80°C for 10 min. to remove any traces of acetone. It is essential that the residues and tubes are completely free of acetone.

6.6 Extraction and washing of the residue for measurement of insoluble NSP: test sample portion (b) only

6.6.1 After the treatment with enzymes in step **6.4**, add 40 ml of sodium phosphate buffer (**4.30**). Place the capped tubes in the boiling water-bath and leave them there for 30 min. Mix continuously or a minimum of 3 times during this period. Remove the tubes and equilibrate to room temperature in water. Centrifuge and remove the supernatant liquid as in step **6.5.1**.

6.6.2 Add approximately 10 ml of water and vortex-mix. Make to 50 ml with water and mix well by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 ml of ethanol, absolute.

6.6.3 Proceed as described for step **6.5.3** and step **6.5.4**.

6.7 Acid hydrolysis of the residue from enzymic digestion

6.7.1 Add 5 ml of sulfuric acid, 12 mol/l (**4.33**), to one tube and immediately vortex-mix vigorously; ensure that all the material is wetted. Repeat this for each tube in turn. Once the acid has been added to all the tubes, vortex-mix again and place the tubes into a water-bath at 35°C. Leave the tubes for 30 min. with vigorous vortex-mixing after 5, 10 and 20 min. to disperse the cellulose. To each tube in turn, add 25 ml of water rapidly and vortex-mix. Place all the tubes into a boiling water-bath (**5.4**) and leave them there for 1 hr., timed from when boiling recommences; mix after 10 min. Remove all the tubes together and cool rapidly in water.

6.7.2 This modification allows the separate measurement of cellulose and non-cellulosic polysaccharides (NCP). To portion (c), treated in steps **6.1** to **6.5**, add 30 ml of sulfuric add, 2 mol/l, and mix. Place in a boiling water-bath and leave them there for 1 hr., timed from when

boiling recommences; mix after 10 min. A value for cellulose may be obtained as the difference between glucose (measured by GLC or glucose oxidase) for sample portions (a) and (c). NCP is calculated as the difference between total NSP and cellulose.

7. Measurement of Constituent Sugars by Colorimetry

7.1 Preparation of standard sugar solutions for colorimetry

Take 0.50 ml of the stock sugar mixture (**4.8**) into a glass tube, add 2.50 ml of sulfuric add, 2.4 mol/l (**4.34**), and mix to give 3 ml of 3 mg sugars/ml standard solution in sulfuric acid, 2 mol/l. The chromogenic reaction is linear up to 3 mg sugars/mi. The absorbance of the samples should not exceed that of the standard.

7.2 Measurement of total reducing sugars

Place into separate tubes 0.5 ml of the standard solution, 0.5 ml of the hydrolysate from step 6.7, and into each of a further 2 tubes (blanks 1 and 2), 0.5 ml of sulfuric acid, 2 mol/l (**4.32**). Add 0.5 ml of dimethyl glutarate solution (**4.9**) and vortex-mix. Check the pH of 1 drop of blank 1; it should be between 3.5 and 4. [If it is different from this, check the preparation of the dimethyl glutarate solution.] Add 0.1 ml of diluted pectinase solution (**4.24**), vortex-mix and place all the tubes into a water-bath at 50°C; leave them there for 20 min. Cool the tubes to room temperature and add 0.1 ml of sodium hydroxide, 3 mol/l, vortex-mix and leave for 5 min. Check that the pH of blank 1 is between 6 and 8. Add 1 ml of dinitrosalicylate reagent (**4.12**) to each tube and vortex mix. Place all the tubes together into a briskly boiling water-bath and leave them there for 5 min. Remove the rack of tubes and cool them to room temperature in water. Add 10 ml of water and mix by inversion (do not use the vortex-mixer at this stage).

Measure the absorbance in the spectrophotometer (**5.5**) at 530 nm against blank 2.

[*Sample blanks may be prepared by diluting the hydrolysate as described above, replacing the dinitrosalicylate reagent with water and reading the absorbance against water. Alternatively, the absorbance of the undiluted hydrolysate can be measured against water and the value obtained divided by 24.4, the dilution of the hydrolysate after addition of the dinitrosalicylate reagent and water. The absorbance of the test sample is then calculated by subtracting this value. When the hydrolysate is colourless and the NSP content is more than 5%, the sample blank is not required.*]

7.3 Calculation of total, soluble and insoluble NSP as measured by colorimetry

7.3.1 Calculation for food samples

The amount of total NSP (portion (a)) and of insoluble NSP (portion (b)) in g/100 g of sample, is calculated as:

$$\frac{A_t \times V_t \times D \times F \times C \times 100}{A_s \times W_t} \times 0.89$$

where:

A_t is the absorbance of the test solution (minus the absorbance of the hydrolysate blank if measured);

V_t is the total volume of the test solution (here, 30 ml);

D is the dilution of the test solution; (D =1 if no dilution in step 7.2);

F is the factor correcting the difference between the composition of monosaccharides in the sugar mixture and that in NSP of various types of plant foods.

For the calculation of total DF: cereals, F = 0.95; fruit and non-starchy vegetables, F = 1.05; starchy vegetables, oats and unknown samples, F = 1.

For the calculation of insoluble DF: F = 1 except for cereals, where F = 0.95;

C is the concentration (in mg sugars/ml) of the standard used (here, C = 3);

A_s is the absorbance of the 3 mg/ml standard;

W_t is the weight (in mg) of sample taken for analysis;

0.89 is the factor for converting the experimentally determined monosaccharides to polysaccharides.

The amount of soluble NSP is calculated as the difference between total and insoluble NSP.

7.3.1 Calculation for food samples

When an isolated NSP preparation, e.g. pectin, has a high content of uronic acids, a more accurate value for NSP may be obtained if the uronic acids are measured separately. The standard sugar mixture for colorimetry as described here contains 12.5% uronic acids, and it has been determined (data not shown) that this leads to 17% underestimation of NSP when the sample contains only uronic acids (using F = 1). Correction for the underestimation is straightforward if a separate value for uronic acids is obtained. If the sample contains 12.5% uronic acids, the standard sugar mixture is entirely appropriate and no correction is required; otherwise, the correction required is an increment or decrement to the NSP value obtained by colorimetry equivalent to 17% of the uronic acid content that is in excess of or less than 12.5% of the sample, respectively.

The percentage of the NSP value to be corrected for is the difference (A) between the value for uronic acids *(Z;* expressed as a percentage of the NSP value, X) and 12.5, and is calculated as:

$$\Delta = \left[\frac{Z}{X} \times 100\right] - 12.5$$

the correction factor Y is calculated as:

$$Y = 0.17\left[\frac{X}{100} \times \Delta\right] = 0.17Z - 0.021X$$

and the value for total NSP after correction is X + Y.

7.4 Breaks in the procedure

The whole analysis may be completed in one day if desired. However, the procedure may be halted at either of the following stages.

After precipitation, washing and drying the starch-free residue in steps **6.5** and **6.6**. The residue may be stored for long periods.

After the hydrolysis with sulfuric acid, 2 mol/l, in step **6.7**. The hydrolysate may be kept at 4°C for 24 hr.

7.5 Trouble shooting. See Appendix 2

8. Measurement of Constituent Neutral Sugars by Gas-Liquid Chromatography and Uronic Acids by Colorimetry

8.1 Standard sugar mixture for GLC calibration

Mix 1.0 ml of the GLC stock sugar mixture (**4.20**) with 5 ml of sulfuric acid, 2.4 mol/l (**4.34**). Treat 2 × 1 ml of this standard sugar mixture for calibration of GLC in parallel with the hydrolysates from step **6.7** of the procedure.

8.2 Preparation of alditol acetate derivatives for GLC

Prepare sufficient (0.2 ml is required for each tube) of the ammonium hydroxide/sodium borohydride solution (**4.5**)

Add 0.50 ml of GLC internal standard (1 mg allose/ml (**4.19**)) to 1 ml of the cooled hydrolysates from step **6.7** and to 2 × 1 ml of the standard sugar mixture from step **11.1**; vortex-mix.

Place the tubes in ice-water, add 0.4 ml of ammonium hydroxide, 12 mol/l (**4.4**), and vortex-mix. Test that the solution is alkaline (add a little more ammonium hydroxide if necessary, but replace the ammonia solution if more than 0.1 ml extra is required), then add approximately 5 gl of the antifoam agent octan-2-ol (**4.23**) and 0.1 ml of the ammonium hydroxide/sodium borohydride solution (**4.5**), vortex-mix.

Leave the tubes in a heating block (**5.9**) or water-bath (**5.4**) at 40°C for 30 min. then remove and add 0.2 ml of glacial acetic acid (**4.1**), mix again. Remove 0.5 ml to a 30 m 1 glass tube; add 0.5 ml of 1-methylimidazole (**4.22**). Add 5 rid of acetic anhydride (**4.2**) and vortex-mix IMMEDIATELY. Leave the tubes for 10 min. for the

reaction to proceed (the reaction is exothermic and the tubes will become hot).

Add 0.9 ml of ethanol, absolute, vortex-mix and leave for 5 min. Add 10 ml of water, vortex-mix and leave for 5 min. Add 0.5 ml of bromophenol blue solution (**4.7**). Place the tubes in ice-water and add 5 ml of potassium hydroxide, 7.5 mol/l (**4.25**); a few minutes later add a further 5 ml of potassium hydroxide, 7.5 mol/l, cap the tubes and mix by inversion.

Leave the tubes until the separation into two phases is complete (10 to 15 min.) or centrifuge for a few minutes. Draw the upper phase into the tip of an automatic pipette; if any of the blue phase is included, allow it to separate then run it out of the tip before transferring a portion of the upper phase alone into a small (auto-injector) vial.

8.3 GLC measurement of neutral sugars

Carry out conventional GLC (**5.7**) measurement of the neutral sugars. At the beginning of each batch of analyses, equilibrate with the isothermal elution conditions for at least 1 hr. Carry out several calibration runs to check that the response factors are reproducible. Inject 0.5 to 1 μl of the alditol acetate derivatives prepared in step 11.2. Use a 5μl plunger-in-the-barrel type of needle (SGE; SR-SS-GT-7T) and pre-pierced septa (SGE; 7 TCSD P/N 041852) or equivalent.

8.3.1 GLC column and conditions

Injector temperature, 275°C; detector temperature, 275°C; carrier gas, helium; flowrate, 8 ml/min. Under these conditions, a GLC chromatograph fitted with flame ionization detector and, preferably, auto-injector and computing integrator, using a Supelco SP-2380 wide-bore capillary column (30 m × 0.53 mm: Supelco cat. no.2-5319) with a temperature gradient (0.5 min. at 180°C, ramp 5 deg. C/min. to upper temperature of 215°C, upper time 2 min.; helium flow-rate 16 ml/min.).

8.3.2 GLC calibration

Start at a flow-rate of 12 ml/min and inject a standard that has been reduced and derivatized according to the procedure. Increase the flow-rate by approx. 2ml/min. and inject another standard. Repeat as necessary. Increasing the flow-rate will result in a narrower solvent front, giving greater separation between the front and the sugar peaks. An excessive flow-rate will result in a broadening of the solvent front. The optimum flow-rate is just below the rate at which this broadening of the solvent front occurs, and is usually about 16ml/min. using the operating conditions described here. Accurate determination of the individual sugars in the standard sugar mixture is obtained with a run-time of 8 min.

For calibration, use the following ratio for the combination of the standard sugar mixture and internal standard (allose):

Sugar	Sugar mixture Actual (mg/dm^{-3})	(%)	Sugar mixture Apparent (mg/dm^{-3})	Calibration ratio
Rhamnose	520	52	1000	1
Fucose	480	96	500	0.5
Arabinose	4750	95	5000	5
Xylose	4450	89	5000	5
Mannose	2300	92	2500	2.5
Galactose	2820	94	3000	3
Glucose	9400	94	10,000	10
Allose (Int. Std.)				3

The Sugar mixture Actual column shows the amount of each sugar in the mixture, and the Sugar mixture Apparent column shows the values to be used for calibration taking into account the recovery of NSP constituents. The Calibration ratio column gives the ratio of sugars to the internal standard after the addition of allose to the standard sugar mixture.

8.4 Calculation of neutral sugars as measured by GLC

The amount of individual sugars (expressed as grams of polysaccharide per 100 g of sample) is calculated as:

$$\frac{A_t \times W_i \times R_f \times 100}{A_i \times W_t} \times 0.89$$

where:

A_t and A_i are the peak areas of the test sample and the internal standard, respectively;

W_i is the weight (in mg; here 15 mg; total hydrolysate 30 ml x 0.5 mg of allose) of the internal standard;

Rf is the response factor for individual sugars obtained from the calibration run with the sugar mixture treated in parallel with the samples;

W_t is the weight (in mg) of the test sample;

0.89 is the scale factor for converting the experimentally determined values for monosaccharides to polysaccharides.

There is incomplete hydrolysis of any rhamnose, which is corrected for in the sugar mixture.

8.5 Standard sugar solutions for colorimetry

The standard sugar mixture in sulfuric acid, 2 mol/l, prepared in step **7.1** (12 ml) contains, for the purposes of calculation, 0.50g of galacturonic acid/ml.

To prepare the uronic acid standard solutions, take 0.5 ml, 2.0 ml and 3.0 ml of the sugar mixture into separate tubes and dilute to 10.0 ml with sulfuric acid, 2 mol/l (**4.32**), to give standards of 0.025, 0.1 and

0.15 mg of galacturoruc acid/ml. Only the 0.10 μg/ml standard is required for routine analysis (it may be kept at 5°C for several weeks).

8.6 Measurement of uronic acids by colorimetry

Place into separate tubes (40 - 50 ml capacity) 0.3 ml of blank solution (sulfuric acid, 2 mol/l), 0.3 ml of each of the standard solutions and 0.3 ml of the test sample hydrolysates, diluted if necessary (with sulfuric acid, 2 mol/l) to contain no more than 150 μg of uronic acids per ml (e.g. no dilution for flour, 1:2 for bran, 1:5 for most fruits and vegetables). Add 0.3 ml of sodium chloride/boric acid solution (**4.28**) and mix. Add 5 ml of sulfuric acid, concentrated (**4.31**) and vortex-mix immediately. Place the tubes in a heating block or water-bath at 70°C and leave them there for 40 min. Remove the tubes and cool to room temperature in water (the tubes may be kept in the water for up to 1 hr.).

Add 0.2 ml of dimethylphenol solution (**4.10**) and vortex-mix immediately. After 15 min. measure the absorbance at 400 nm and at 450 nm in the spectrophotometer (**5.5**) against the blank solution. The timing for measurement of the absorbance of standards and samples should be identical. In practice, this is achieved by adding the chromogenic reagent at 1 min. intervals. Subtract the reading at 400 nm from that at 450 nm, to correct for interference from hexoses. A straight line should be obtained if the differences for the standards are plotted against concentration.

8.7 Calculation of uronic acids as measured by colorimetry

The amount of uronic acids (expressed as grams of polysaccharide per 100 g of sample) is calculated as:

$$\frac{At \times V_t \times D \times C \times 100}{As \times W_t} \times 0.91$$

where:

A_t is the difference in absorbance of the test solution;

V_t is the total volume of test solution (here 30 ml);

D is the dilution of the test solution;

C is the concentration of the standard (here 0.1 mg/ml);

A_s is the difference in absorbance of the 0.1 mg/ml standard;

W_t is the weight (in mg) of the test sample;

0.91 is the scale factor for converting the experimentally determined values for monosaccharides to polysaccharides.

8.8 Calculation of total. soluble and insoluble NSP

The amount of total, soluble and insoluble NSP, in g/100 g of sample, is calculated as:

Total NSP = Neutral sugars calculated for portion (a)	[step **8.4**]
+ Uronic acids calculated for portion (a)	[step **8.7**]
Insoluble NSP = Neutral sugars calculated for portion (b)	[step **8.4**]
+ Uronic acids calculated for portion (b)	[step **8.7**]
Soluble NSP = Total NSP - Insoluble NSP	

It is recommended that the results for total, insoluble and soluble NSP are expressed as grams of polysaccharide per 100 g of dry matter.

8.9 Breaks in the procedure

The procedure may be halted as described under Section **7.4**, or at any of the following stages.

After acidification of the reduced samples in step **8.2**. The samples may be stored at room temperature for two or three days.

After acetylation and transfer to small vials in step **8.2**. The samples may be kept at 0 to 5°C for two to three days before analysis by GLC.

The acid hydrolysate in step **6.7.1** may be kept at 5°C for several weeks before the measurement of uronic acids.

8.10 Trouble shooting. See Appendix 2.

9. COSHH

Analysts are reminded that appropriate hazard and risk assessments required by the Control of Substances Hazardous to Health Regulations, 1988 (see "Control of Substances Hazardous to Health - Approved Code of Practice, Control of Substances Hazardous to Health Regulations, 1988") must be made before using this method.

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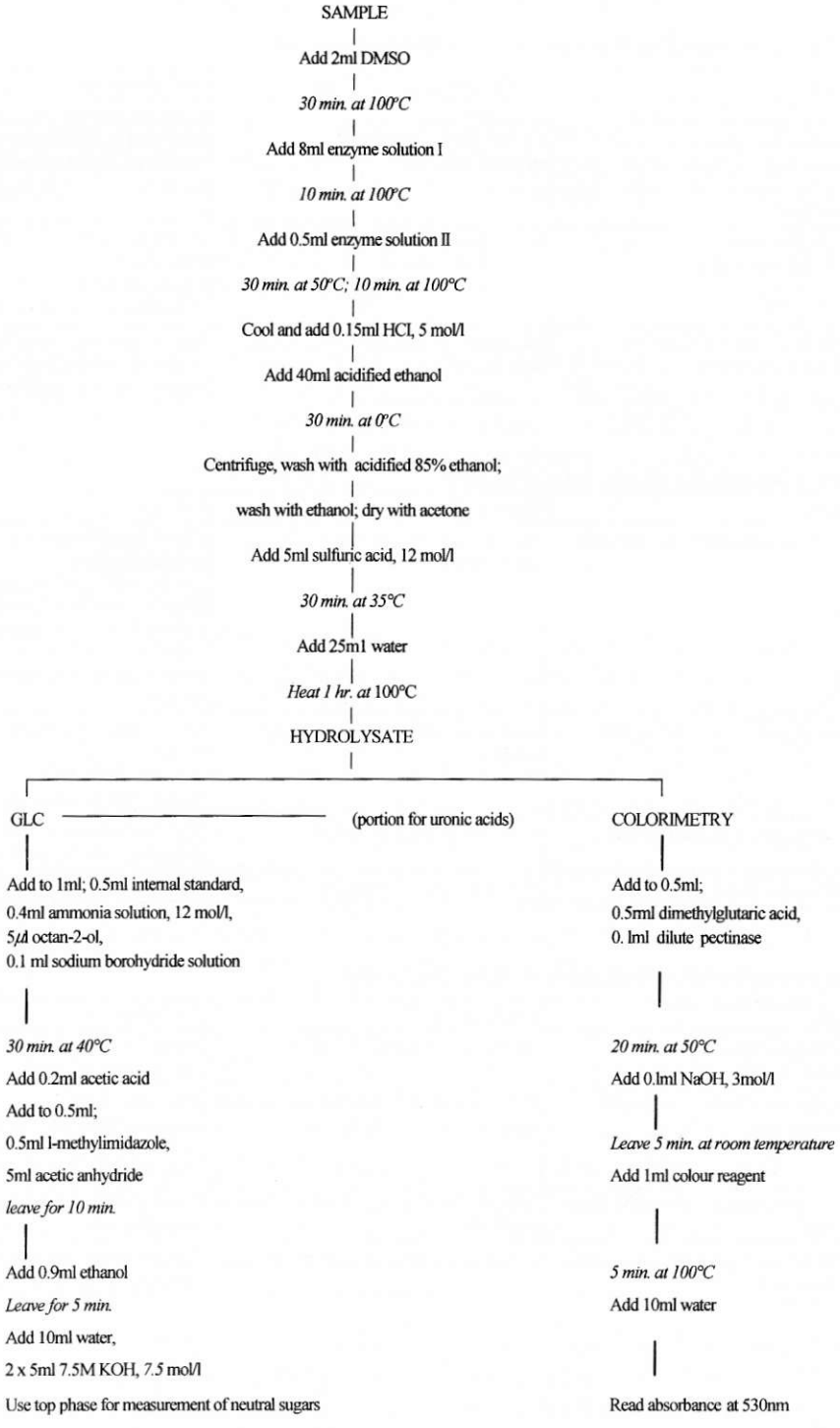
APPENDIX 1
DIAGRAM FOR ANALYSIS OF DIETARY FIBRE



Calculate dietary fibre as described for the GLC and colorimetry end-points.

APPENDIX 2

TROUBLE-SHOOTING GUIDE

Problems associated with preparation of the hydrolysate

Symptom	Likely cause	Cure/prevention
Good Duplicates (within batch) +good replicates (between batch) but values lower or higher than expected for material of known composition	Sytematic error e.g. weighing, pipetting, moisture content	Calibrate balance using 1g weight, use positive displacement pipettes ALL calibrated by weighing replicates of water
Values higher than expected and variable for duplicates.	Incomplete removal of stach leading to high glucose values	Vortex-mix vigorously at all the times indicated. Check the pH of the buffers used and temperature of water-baths
Values lower than expected and variable for duplicates.	Incomplete depolymerisation during acid hydrolysis	Prevent aggregation of the sample during the drying step by regular, vigorous vortex-mixing.

Errors associated with the GLC end-point

Symptom	Likely cause	Cure/prevention
Variation between replicate analysis	Improper calibration of the GLC instrumentation	Check the calibration of all instrumentation. For the GLC perform analysis only after the response factors for the standard sugar mixture are consistent and similar to those obtained previously. Keep a record of response factors
Extra peaks on the chromatogram	Incomplete reduction/storage of samples	Ensure alkaline pH before adding $NaBH_4$ Replace old $NaBH_4$ Use freshly prepared derivatives only
Little or no upper phase for GLC	Very high protein in sample	Use 1 ml ethanol instead of 0.9 and centrifuge the derivative
Broad solvent front	Columns set up incorrectly	Check setting-up procedure.

Errors associated with the colorimetric end-point

Symptom	Likely cause	Cure/prevention
Values higher than expected and variable	Incomplete removal of acetone	Ensure there is no trace of acetone Dry in oven longer
No colour produced for standards and/or samples	Error in preparation of sulfuric acid or sodium hydroxide solution	Use commercially prepared solutions where indicated. For sodium hydroxide (3M and 5M) solutions prepare using ConvoL® (Do not use pellets)
Values for cooked foods higher than expected	Interference from Maillard reaction products	Timing with colour reagent must not exceed 6 min.
Values for fruit and vegetables lower than expected	Incomplete hydrolysis of pectin	Ensure correct pH and temperature during enzymic hydrolysis

APPENDIX 3

CHANGES TO THE ENGLYST PROCEDURE FROM THAT USED IN COLLABORATIVE TRIALS, PART IV (**10.9**)

A3.1 Precipitation of NSP at pH 5.2 and 2

Oligosaccharides may be linked to polysaccharides by non-covalent bonds, such as calcium bridges (**10.10**), forming complexes that are insoluble in ethanol, 80%, at pH 5.2, and therefore falsely included in values for NSP. These bonds are not present, however, in ethanol, 80%, at pH 2. Lowering the pH of the 80% ethanol has a further advantage, because some free galacturoruc acid (GalA) is insoluble in ethanol, 80%, in the presence of sodium acetate buffer (0.1 mol/l, pH 5.2) but is solubilised upon addition of HCI to a final pH of 2. Values for the total NSP content of a polysaccharide mixture (duplicate analysis on 4 different occasions) were 91.2±2.8%, w/w, and 91.9±2.1%, w/w, when the precipitation in ethanol was done at pH 5.2 and pH 2, respectively, (**10.6**) showing that the recovery of NSP is the same at pH 2 as it is at pH 5.2

A3.2 Hydrolysis of NSP

We have shown (**9.5**) that cellulose and non-cellulosic NSP are completely dispersed and hydrolysed by treatment with sulfuric acid, 12 mol/l, for 1 hr. at 35°C, followed by treatment with sulfuric acid, 2 mol/l, for 1 hr. at 100°C. Independently, Hoebier *et al.* (**10.11**) showed that a wide range of samples, including microcrystalline cellulose and sunflower husks, could be dispersed completely by treatment with sulfuric acid, 12 mol/l, for 30 min. at 25°C, and we have shown (**10.6**) complete hydrolysis of purified polysaccharide preparations after only 10 min. of treatment with sulfuric acid, 12 mol/l. In order to decrease the time of treatment with sulfuric acid, 12 mol/l, for samples that have been subjected to the washing and drying procedures it is essential that the sample is finely divided before addition of the acid. The grinding action of the sand and the glass bails, which are now incorporated into the procedure, prevents aggregation and ensures that samples are finely divided after drying. Identical NSP values were obtained for a wide range of samples taken through the washing and drying procedures when either 30 min. or 1 hr. of treatment with sulfuric acid, 12 mol/l, was used (data not shown). Treatment with sulfuric acid, 12 mol/l, for 30 min. at 35°C has therefore been incorporated into the procedure.

As a test for completeness of depolymerization, high-pressure liquid chromatography with pulsed amperometric detection (HPLC-PAD) was used to detect any oligosaccharides remaining in solution after the acid hydrolysis steps. No residual oligomers were detected for cellulose or other non-cellulosic neutral sugar-containing NSP,

indicating complete hydrolysis to monosaccharides. Oligosaccharides were found, however, for uronic acid-containing polymers, such as pectin. This incomplete hydrolysis is of no importance when the uronic acid content is determined by the modified Scott procedure, which measures both free uronic acids and uronic acid polymers in solution, as used in the GLC analysis of NSP. Special treatment of the hydrolysate is required, however, when using the colorimetric procedure. Prolonged treatment (3 hr.) with sulfuric acid, 2 mol/l, is required to obtain maximal values using HPLC but such a treatment is time-consuming and results in further undesirable losses. The use of pectinases, after the treatments with sulfuric acid, to complete the hydrolysis of uronic acid-containing polymers has been described in detail for the analysis of uronic acids by HPLC **(10.7)**. Similarly, the addition of pectinase after adjustment of the pH of the hydrolysate to between 3.5 and 4 can be used to ensure complete recovery of uronic acids when using the colorimetric procedure **(10.6)**. In addition, the enzyme preparation has sufficient reducing groups that, when used at the concentration described here (0.lml of pectinase diluted 1:10, *v/v*), the glucose solution previously used to compensate for dissolved oxygen is not required.

A3.3 Interference in the reducing sugar assay

Mineral ions have been reported to increase the intensity of the colour formed upon heating dinitrosalicylic acid with reducing groups **(10.12, 10.13)**. The extent of the interference is variable and dependent upon the concentrations of both mineral ions and reducing sugars. In the analysis of NSP, the presence of mineral ions may explain the small differences obtained by GLC and colorimetry for fortified products such as cornflakes and wholemeal bread. A small proportion of minerals are insoluble in ethanol, 80%, at pH 5 and may increase the colour yield. However, all minerals tested were soluble in ethanol, 80%, at pH 2. The use of acidified ethanol in the procedure has completely removed this type of interference. In addition, the presence of mineral ions during the drying procedure results in residues that are more prone to aggregation, which hinders subsequent hydrolysis with sulfuric acid, and removal of the mineral ions as described here obviates this problem.

Advanced Maillard reaction products such as 5-hydroxymethyl furfural and 2furaldehyde have reducing aldehyde groups and their effect on colorimetry has been shown **(10.6)**. There is a time-dependent increase in absorbance for 2-furaldehyde and 5-hydroxymethyl furfural, whereas virtually maximum values are obtained for monosaccharides after 5 min. reaction. Reducing the time of reaction with the colour reagent from 15 to 5 min. therefore minimises the interference from the products formed during food processing.

A3.4 Colour yields

Previous studies have shown that different colour yields are obtained for hexoses, pentoses and uronic acids with the colour reagent used in the reducing sugar method **(10.4, 10.14, 10.15)**. In practice, a single standard may be used for the colorimetry, especially if correction is made for the colour yield from different types of NSP; e.g. cereals contain a high proportion of pentoses, whereas fruits and vegetables are high in cellulosic and uronic acid-containing polymers. The derivation of the correction factors used in the procedure has been described **(10.6)**.

A3.5 Timing in the Scott procedure

A small increase (5 to 7%) in absorbance between 10 and 15 min. of the reaction has been observed for a range of samples, and a plateau is reached after 15 min. It is important that all readings are taken after an identical reaction time.

A3.6 Ammonia solution in the GLC assay

The strength of the ammonia solution added during the reduction step needs to be sufficient to ensure alkaline conditions. The strength of the stock ammonia solution can decrease with time as ammonia evaporates. To compensate, it is now recommended that the strength of ammonia solution is greater than that used in the earlier version of the procedure.

A3.7 Aggregation of samples

It is recommended that samples are mixed after 20 seconds in the 50°C waterbath at step **6.4.1**. This prevents aggregation due to the gelatinisation of starch that occurs with some samples if mixing is delayed for several minutes.

A3.8 Conclusion

Sources of potential interference in the measurement of NSP have been identified and eliminated by the modifications described here. These changes have made the NSP procedure more accurate and robust. Virtually identical values for NSP are obtained by GLC and by the more rapid colorimetric assay of constituent sugars following the isolation and hydrolysis procedures as described.

APPENDIX 4

SUMMARY OF RESULTS FOR INDIVIDUAL SAMPLES

Sample	Code	Method	Fibre Type	Mean	r	R
Coconut	A	E-GLC	Soluble	1.21	1.46	2.54
			Insoluble	11.12	1.61	3.10
			Total	12.31	2.61	3.03
		E-COL	Soluble	1.39	1.09	2.82
			Insoluble	11.71	0.95	4.62
			Total	13.06	0.91	4.83
		PROSKY	Soluble	0.31	0.41	0.71
			Insoluble	13.38	1.86	5.64
			Total	16.41	3.37	17.00
Porridge	B	E-GLC	Soluble	3.68	1.35	1.94
			Insoluble	3.42	0.77	1.46
			Total	7.22	1.13	2.27
		E-COL	Soluble	4.46	1.56	2.38
			Insoluble	4.07	0.65	2.67
			Total	8.69	0.72	2.21
		PROSKY	Soluble	1.79	0.64	3.99
			Insoluble	5.33	1.30	1.84
			Total	7.27	1.07	4.93
Baked Beans	C	E-GLC	Soluble	7.06	1.27	1.95
			Insoluble	5.56	0.86	1.80
			Total	12.62	1.07	3.06
		E-COL	Soluble	7.29	1.88	3.00
			Insoluble	6.16	0.89	2.65
			Total	13.13	4.52	4.52
		PROSKY	Soluble	5.96	0.66	2.57
			Insoluble	11.00	0.83	1.79
			Total	16.10	2.87	5.34
Cabbage	D	E-GLC	Soluble	12.15	4.24	6.61
			Insoluble	13.04	1.80	4.93
			Total	25.25	3.17	8.54
		E-COL	Soluble	9.93	3.07	5.05
			Insoluble	13.61	1.16	4.59
			Total	23.80	2.89	5.92
		PROSKY	Soluble	6.05	2.14	4.86
			Insoluble	21.98	3.13	6.66
			Total	27.12	2.66	6.47

APPENDIX 4

SUMMARY OF RESULTS FOR INDIVIDUAL SAMPLES

(continued)

Sample	Code	Method	Fibre Type	Mean	r	R
Mashed Potato	E	E-GLC	Soluble	4.03	0.84	0.97
			Insoluble	2.83	0.84	1.29
			Total	6.87	0.67	1.44
		E-COL	Soluble	3.85	0.75	0.97
			Insoluble	2.94	0.79	1.80
			Total	7.12	0.89	2.81
		PROSKY	Soluble	2.19	0.90	1.67
			Insoluble	5.62	1.33	1.77
			Total	7.67	1.40	2.17
Banana	F	E-GLC	Soluble	2.88	0.80	1.57
			Insoluble	1.87	0.65	0.97
			Total	4.62	0.76	1.48
		E-COL	Soluble	2.73	1.60	1.66
			Insoluble	2.46	0.93	1.79
			Total	5.40	1.02	2.61
		PROSKY	Soluble	1.94	1.00	2.28
			Insoluble	5.35	1.44	2.53
			Total	7.95	2.01	6.79
Apple	G	E-GLC	Soluble	5.28	1.21	2.21
			Insoluble	6.45	1.10	2.45
			Total	11.73	1.06	3.79
		E-COL	Soluble	4.13	2.16	3.50
			Insoluble	6.93	0.89	3.83
			Total	10.78	1.55	6.23
		PROSKY	Soluble	3.78	0.99	2.07
			Insoluble	8.42	0.73	1.21
			Total	12.36	1.75	2.33
Bread	H	E-GLC	Soluble	2.82	0.61	1.61
			Insoluble	2.77	0.48	0.96
			Total	5.61	0.66	1.96
		E-COL	Soluble	3.39	1.28	1.43
			Insoluble	3.18	0.97	1.23
			Total	6.57	0.83	1.98
		PROSKY	Soluble	2.42	1.35	2.30
			Insoluble	4.29	0.90	2.26
			Total	6.83	1.25	3.15

APPENDIX 4

SUMMARY OF RESULTS FOR INDIVIDUAL SAMPLES
(continued)

Sample	Code	Method	Fibre Type	Mean	r	R
White Bread	I/P	E-GLC	Soluble	1.83	1.50	1.50
			Insoluble	1.61	0.74	0.91
			Total	3.43	0.99	1.33
		E-COL	Soluble	2.11	1.78	1.92
			Insoluble	2.00	1.05	1.35
			Total	4.16	1.62	2.42
		PROSKY	Soluble	1.60	1.25	1.71
			Insoluble	2.55	1.88	3.11
			Total	4.36	2.63	3.74
Cornflakes	J/K	E-GLC	Soluble	0.46	0.47	0.66
			Insoluble	1.04	0.69	1.69
			Total	1.48	0.73	1.54
		E-COL	Soluble	0.68	1.57	1.57
			Insoluble	1.52	0.80	2.71
			Total	2.35	1.05	3.74
		PROSKY	Soluble	0.23	0.74	0.92
			Insoluble	3.04	1.09	2.77
			Total	3.50	1.69	3.21
Wholemeal Bread	L/M	E-GLC	Soluble	2.50	1.2	1.20
			Insoluble	7.01	1.05	2.10
			Total	9.39	1.13	1.88
		E-COL	Soluble	2.88	1.45	1.64
			Insoluble	7.71	1.74	3.43
			Total	10.54	1.62	3.08
		PROSKY	Soluble	1.83	1.68	2.08
			Insoluble	10.00	2.49	4.49
			Total	11.87	2.93	4.85
1:1 Bread Mix	N/O	E-GLC	Soluble	2.16	1.33	1.39
			Insoluble	4.41	0.79	1.37
			Total	6.56	0.92	1.67
		E-COL	Soluble	2.65	1.18	1.42
			Insoluble	4.87	0.97	2.42
			Total	7.42	0.93	2.44
		PROSKY	Soluble	1.93	1.59	2.87
			Insoluble	6.53	1.01	3.29
			Total	8.45	1.72	4.12

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INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

ANALYTICAL, APPLIED, CLINICAL, INORGANIC AND PHYSICAL CHEMISTRY DIVISIONS INTERDIVISIONAL WORKING PARTY FOR HARMONIZATION OF QUALITY ASSURANCE SCHEMES FOR ANALYTICAL LABORATORIES*

HARMONISED GUIDELINES FOR INTERNAL QUALITY CONTROL IN ANALYTICAL CHEMISTRY LABORATORIES

(Technical Report)

Resulting from the Symposium on Harmonization of Internal Quality Assurance Systems for Analytical Laboratories, Washington DC, USA, 22-23 July 1993 held under the sponsorship of IUPAC, ISO & AOAC INTERNATIONAL

Prepared for publication by

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The Harmonised Guidelines for Internal Quality Control in Analytical Chemistry Laboratories (Technical Report)

Synopsis

ISO, IUPAC and AOAC INTERNATIONAL have co-operated to produce agreed protocols on the "Design, Conduct and Interpretation of Collaborative Studies"(1) and on the "Proficiency Testing of (Chemical) Analytical Laboratories"(2). The Working Group that produced these protocols was asked to prepare guidelines on the internal quality control of data produced in analytical laboratories.

Such guidelines would have to outline minimum recommendations to laboratories producing analytical data on the internal quality control procedures to be employed.

A draft of the guidelines was discussed at the Fifth International Symposium on the Harmonisation of Quality Assurance Systems in Chemical Analysis, sponsored by IUPAC/ISO/AOAC INTERNATIONAL and held in Washington D.C. in July, 1993, and finalised at a Working Group Meeting held in Delft in May 1994.

CONTENTS

1 INTRODUCTION

1.1 Basic concepts

This document sets out guidelines for the implementation of internal quality control (IQC) in analytical laboratories. IQC is one of a number of concerted measures that analytical chemists can take to ensure that the data produced in the laboratory are fit for their intended purpose. In practice, fitness for purpose is determined by a comparison of the accuracy achieved in a laboratory at a given time with a required level of accuracy. Internal quality control therefore comprises the routine practical procedures that enable the analytical chemist to accept a result or group of results as fit for purpose, or reject the results and repeat the analysis. As such, IQC is an important determinant of the quality of analytical data, and is recognised as such by accreditation agencies.
Internal quality control is undertaken by the inclusion of particular reference materials, here called "control materials", into the analytical sequence and by duplicate analysis. The control materials should, wherever possible, be representative of the test materials under consideration in respect of matrix composition, the state of physical preparation and the concentration range of the analyte. As the control materials are treated in exactly the same way as the test materials, they are regarded as surrogates that can be used to characterise the performance of the analytical system, both at a specific time and over longer intervals.

Internal quality control is a final check of the correct execution of all of the procedures (including calibration) that are prescribed in the analytical protocol and all of the other quality assurance measures that underlie good analytical practice. IQC is therefore necessarily retrospective. It is also required to be as far as possible independent of the analytical protocol, especially the calibration, that it is designed to test.

Ideally both the control materials and those used to create the calibration should be traceable to appropriate certified reference materials or a recognised empirical reference method. When this is not possible, control materials should be traceable at least to a material of guaranteed purity or other well characterised material. However, the two paths of traceability must not become coincident at too late a stage in the analytical process. For instance, if control materials and calibration standards were prepared from a single stock solution of analyte, IQC would not detect any inaccuracy stemming from the incorrect preparation of the stock solution.
In a typical analytical situation several, or perhaps many, similar test materials will be analysed together, and control materials will be included in the group.

Often determinations will be duplicated by the analysis of separate test portions of the same material. Such a group of materials is referred to in this document as an analytical "run". (The words "set", "series" and "batch" have also been used as synonyms for "run".) Runs are regarded as being analysed under effectively constant conditions. The batches of reagents, the instrument settings, the analyst, and the laboratory environment will, under ideal conditions, remain unchanged during analysis of a run. Systematic errors should therefore remain constant during a run, as should the values of the parameters that describe random errors. .As the monitoring of these errors is of concern, the run is the basic operational unit of IQC.

A run is therefore regarded as being carried out under repeatability conditions, *i.e.*, the random measurement errors are of a magnitude that would be encountered in a "short" period of time. In practice the analysis of a run may occupy sufficient time for small systematic changes to occur. For example, reagents may degrade, instruments may drift, minor adjustments to instrumental settings may be called for, or the laboratory temperature may rise. However, these systematic effects are, for the purposes of IQC, subsumed into the repeatability variations. Sorting the materials making up a run into a randomised order converts the effects of drift into random errors.

1.2 Scope of this document

This document is a harmonisation of IQC procedures that have evolved in various fields of analysis, notably clinical biochemistry, geochemistry and environmental studies, occupational hygiene and food analysis[3-9]. There is much common ground in the procedures from these various fields. However, analytical chemistry comprises an even wider range of activities and the basic principles of IQC should be able to encompass all of these. The present document provides guidelines that will be applicable in the great majority of instances. This policy necessarily excludes a number of IQC practices that are restricted to individual sectors of the analytical community. In addition in some sectors it is common to combine IQC as defined here with other aspects of quality assurance practice. There is no harm in such combination, but it must remain clear what are the essential aspects of IQC.

In order to achieve a harmonisation and provide basic guidance on IQC, some types of analytical activity have been excluded from this document. Issues specifically excluded are as follows.

(i) *Quality control of sampling.* While it is recognised that the quality of the analytical result can be no better than that of the sample, quality control

of sampling is a separate subject and in many areas is not fully developed. Moreover, in many instances analytical laboratories have no control over sampling practice and quality.

(ii) *In-line analysis and continuous monitoring.* In this style of analysis there is no possibly of repeating the measurement, so the concept of IQC as used in this document is inapplicable.

(iii) *Multivariate IQC.* Multivariate methods in IQC are still the subject of research and cannot be regarded as sufficiently established for inclusion here. The current document regards multianalyte data as requiring a series of univariate IQC tests. Caution is necessary in the interpretation of this type of data to avoid inappropriately frequent rejection of data.

(iv) *Statutory and contractual requirements.*

(v) *Quality assurance measures* such as checks on instrumental stability before and during analysis, wavelength calibration, balance calibration, tests on resolution of chromatography columns, and problem diagnostics are not included. For present purposes they are regarded as part of the analytical protocol, and IQC tests their effectiveness together with the other aspects of the methodology.

1.3 Internal quality control and uncertainty

A prerequisite of analytical chemistry is the recognition of "fitness for purpose", the standard of accuracy that is required for an effective use of the analytical data. This standard is arrived at by consideration of the intended uses of the data although it is seldom possible to foresee all of the potential future applications of analytical results. For this reason in order to prevent inappropriate interpretation, it is important that a statement of the uncertainty should accompany analytical results, or be readily available to those who wish to use the data.

Strictly speaking, an analytical result cannot be interpreted unless it is accompanied by knowledge of its associated uncertainty at a stated level of confidence. A simple example demonstrates this principle. Suppose that there is a statutory requirement that a foodstuff must not contain more than 10 $\mu g\ g^{-1}$ of a particular constituent. A manufacturer analyses a batch and obtains a result of 9 $\mu g\ g^{-1}$ for that constituent. If the uncertainty of the result expressed as a half range (assuming no sampling error) is 0.1 $\mu g\ g^{-1}$ (i.e. the true result falls, with a high probability, within the range 8.9-9.1) then it may be assumed that the legal limit is not exceeded. If, in contrast, the uncertainty is 2 $\mu g\ g^{-1}$

then there is no such assurance. The interpretation and use that may be made of the measurement thus depends on the uncertainty associated with it.

Analytical results should therefore have an associated uncertainty if any definite meaning is to be attached to them or an informed interpretation made. If this requirement cannot be fulfilled, the use to which the data can be put is limited. Moreover, the achievement of the required measurement uncertainty must be tested as a routine procedure, because the quality of data can vary, both in time within a single laboratory and between different laboratories. IQC comprises the process of checking that the required uncertainty is achieved in a run.

2 DEFINITIONS

2.1 International definitions

Quality assurance. All those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality[10].

Trueness: closeness of the agreement between the average value obtained from a large series of test results and an accepted reference value[11].

Precision: closeness of agreement between independent test results obtained under prescribed conditions[12].

Bias: difference between the expectation of the test results and an accepted reference value[11].

Accuracy: closeness of the agreement between the result of a measurement and a true value of the measurand[13].

Note 1. Accuracy is a qualitative concept.

Note 2. The term *precision* should not be used for *accuracy*.

Error: result of a measurement minus a true value of the measurand[13].

Repeatability conditions. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time[11].

Uncertainty of measurement: parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand[14].

Note 1. The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence.

Note 2. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.

Note 3. It is understood that the result of a measurement is the best estimate of the value of a measurand, and that all components of uncertainty, including those arising from systematic effects, such as components associated with corrections and reference standards, contribute to the dispersion.

Traceability: property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties[13].

Reference material: material or substance one of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials[13].

Certified reference material: reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence[13].

2.2 Definitions of terms specific to this document

Internal quality control: set of procedures undertaken by laboratory staff for the continuous monitoring of operation and the results of measurements in order to decide whether results are reliable enough to be released.

Control material: material used for the purposes of internal quality control and subjected to the same or part of the same measurement procedure as that used for test materials.

Run (analytical run): set of measurements performed under repeatability conditions.

Fitness for purpose: degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose

Analytical system: range of circumstances that contribute to the quality of analytical data, including equipment, reagents, procedures, test materials, personnel, environment and quality assurance measures.

3 QUALITY ASSURANCE PRACTICES AND INTERNAL QUALITY CONTROL

3.1 Quality assurance

Quality assurance is the essential organisational infrastructure that underlies all reliable analytical measurements. It is concerned with achieving appropriate levels in matters such as staff training and management, adequacy of the laboratory environment, safety, the storage, integrity and identity of samples, record keeping, the maintenance and calibration of instruments, and the use of technically validated and properly documented methods . Failure in any of these areas might undermine vigorous efforts elsewhere to achieve the desired quality of data. In recent years these practices have been codified and formally recognised as essential. However, the prevalence of these favourable circumstances by no means ensures the attainment of appropriate data quality unless IQC is conducted.

3.2 Choice of analytical method

It is important that laboratories restrict their choice of methods to those that have been characterised as suitable for the matrix and analyte of interest. The laboratory must possess documentation describing the performance characteristics of the method, estimated under appropriate conditions.

The use of a method does not in itself guarantee the achievement of its established performance characteristics. There is, for a given method, only the potential to achieve a certain standard of reliability when the method is applied

under a particular set of circumstances. It is this collection of circumstances, known as the "analytical system", that is therefore responsible for the accuracy of analytical data. Hence it is important to monitor the analytical system in order to achieve fitness for purpose. This is the aim of the IQC measures undertaken in a laboratory.

3.3 Internal quality control and proficiency tests

Proficiency testing is a periodic assessment of the performance of individual laboratories and groups of laboratories that is achieved by the distribution by an independent testing body of typical materials for unsupervised analysis by the participants[2]. Although important, participation in proficiency testing schemes is not a substitute for IQC measures, or *vice versa*.

Proficiency testing schemes can be regarded as a routine, but relatively infrequent, check on analytical errors. Without the support of a well-developed IQC system, the value of participation in a proficiency test is negligible. Probably the main beneficial effect of proficiency tests is that of encouraging participants to install effective quality control systems. It has been shown that laboratories with effective IQC systems performed better in a proficiency testing scheme[15].

4. INTERNAL QUALITY CONTROL PROCEDURES

4.1 Introduction

Internal quality control involves the practical steps undertaken to ensure that errors in analytical data are of a magnitude appropriate for the use to which the data will be put. The practice of IQC depends on the use of two strategies, the analysis of reference materials to monitor trueness and statistical control, and duplication to monitor precision.

The basic approach to IQC involves the analysis of control materials alongside the test materials under examination. The outcome of the control analyses forms the basis of a decision regarding the acceptability of the test data. Two key points are worth noting in this context.

(i) The interpretation of control data must be based on documented, objective criteria, and on statistical principles wherever possible.

(ii) The results of control analyses should be viewed primarily as indicators of the performance of the analytical system, and only secondarily as a guide to the errors associated with individual test results. Substantial changes in the apparent accuracy of control determinations can sometimes be taken to imply similar changes to data for contemporary test materials, but correction of analytical data on the basis of this premise is unacceptable.

4.2 General Approach - Statistical Control

The interpretation of the results of IQC analyses depends largely on the concept of statistical control, which corresponds with stability of operation. Statistical control implies that an IQC result x can be interpreted as arising independently and at random from a normal population with mean μ and variance σ^2.

Under these constraints only about 0.3% of results (x) would fall outside the bounds of $\mu \pm 3\sigma$. When such extreme results are encountered they are regarded as being "out-of- control" and interpreted to mean that the analytical system has started to behave differently. Loss of control therefore implies that the data produced by the system are of unknown accuracy and hence cannot be relied upon. The analytical system therefore requires investigation and remedial action before further analysis is undertaken. Compliance with statistical control can be monitored graphically with Shewhart control charts (see Appendix 1). An equivalent numerical approach, comparing values of $z = (x-\mu)/\sigma$ against appropriate values of the standard normal deviate, is also possible.

4.3 Internal quality control and fitness for purpose.

For the most part, the process of IQC is based on a description in terms of the statistical parameters of an ongoing analytical system in normal operation. Control limits are therefore based on the estimated values of these parameters rather than measures derived from considerations of fitness for purpose. Control limits must be narrower than the requirements of fitness for purpose or the analysis would be futile.

The concept of statistical control is inappropriate, however, when the so-called *ad hoc* analysis is being undertaken. In *ad hoc* analysis the test materials may be unfamiliar or rarely encountered, and runs are often made up of only a few such test materials. Under these circumstances there is no statistical basis for

the construction of control charts. In such an instance the analytical chemist has to use fitness for purpose criteria, historical data or consistency with the visual properties of the test material for judging the acceptability of the results obtained.

Either way, agreed methods of establishing quantitative criteria to characterise fitness for purpose would be desirable. Unfortunately, this is one of the less-developed aspects of IQC. In specific application areas guidelines may emerge by consensus. For example, in environmental studies it is usually recognised that relative uncertainties of less than ten percent in the concentration of a trace analyte are rarely of consequence. In food analysis the Horwitz curve[16] is sometimes used as a fitness for purpose criterion. Such criteria have been defined for clinical analysis[17,18]. In some areas of applied geochemistry a systematic approach has given rise to fitness for purpose criteria for sampling and analytical precisions. However, it is not practicable here to give guidelines in these areas, and at present no general principles can be advanced that would allow specific applications to be addressed.

4.4 The nature of errors

Two main categories of analytical error are recognised, namely random errors and systematic errors, which give rise to imprecision and bias respectively. The importance of categorising errors in this way lies in the fact that they have different sources, remedies and consequences for the interpretation of data.

Random errors determine the precision of measurement. They cause random positive and negative deviations of results about the underlying mean value. *Systematic errors* comprise displacement of the mean of many determinations from the true value. For the purposes of IQC two levels of systematic error are worth consideration.

(i) *Persistent bias* affects the analytical system (for a given type of test material) over a long period and affects all data. Such bias, if small in relation to random error, may be identifiable only after the analytical system has been in operation for a long time. It might be regarded as tolerable, provided it is kept within prescribed bounds.

(ii) *The run effect* is exemplified by a deviation of the analytical system during a particular run. This effect, where it is sufficiently large, will be identified by IQC at the time of occurrence as an out-of-control condition.

The conventional division of errors between the random and the systematic depends on the timescale over which the system is viewed. Run effects of unknown source can be regarded in the long-term as the manifestation of a random process. Alternatively, if a shorter-term view is taken, the same variation could be seen as a bias-like change affecting a particular run.

The statistical model used for IQC in this document is as follows[1]. The value of a measurement (x) in a particular run is given by:

x = true value + persistent bias + run effect + random error (+ gross error).

The variance of x (σ_x^2) in the absence of gross errors is given by:

$$\sigma_x^2 = \sigma_0^2 + \sigma_1^2$$

where

σ_0^2 = variance of the random error (within run) and
σ_1^2 = variance of the run effect.

The variances of the true value and the persistent bias are both zero. An analytical system in control is fully described by σ_0^2, σ_1^2 and the value of the persistent bias. Gross errors are implied when the analytical system does not comply with such a description.

5 IQC AND WITHIN-RUN PRECISION

5.1 Precision and duplication

A limited control of within-run precision is achieved by the duplication within a run of measurements made on test materials. The objective is to ensure that the differences between paired results are consistent with or better than the level implied by the value of σ_0 used by a laboratory for IQC purposes[2]. Such a test alerts the user to the possibility of poor within-run precision and provides additional information to help in interpreting control charts. The method is

[1] The model could be extended if necessary to include other features of the analytical system

[2] There is no intention here of estimating the standard deviation of repeatability σ_r from the IQC data or of comparing estimates: there would usually be too few results for a satisfactory outcome. Where such an estimate is needed the formula $s_r = \sqrt{\Sigma d^2 / 2n}$ can be used.

especially useful in *ad hoc* analysis, where attention is centred on a single run and information obtained from control materials is unlikely to be completely satisfactory.

As a general approach all of the test materials, or a random selection from them, are analysed in duplicate. The absolute differences $|d| = |x_1 - x_2|$ between duplicated analytical results x_1 and x_2 are tested against an upper control limit based on an appropriate value of σ_0 However, if the test materials in the run have a wide range of concentration of analyte, no single value of σ_0 can be assumed[19].

Duplicates for IQC must reflect as far as possible the full range of variation present in the run. They must not be analysed as adjacent members of the run, otherwise they will reveal only the smallest possible measure of analytical variability. The best placing of duplicates is at random within each run. Moreover the duplication required for IQC requires the complete and independent analysis (preferably blind) of separate test portions of the test material. A duplication of the instrumental measurement of a single test solution would be ineffective because the variations introduced by the preliminary chemical treatment of the test material would be absent.

5.2 Interpretation of duplicate data

5.2.1 *Narrow concentration range.* In the simplest situation the test materials comprising the run have a small range of analyte concentrations so that a common within-run standard deviation σ_0 can be applied. A value of this parameter must be estimated to provide a control limit. The upper 95% bound of $|d|$ is $2\sqrt{2}\,\sigma_0$ and on average only about three in a thousand results should exceed $3\sqrt{2}\,\sigma_0$.

A group of n duplicated results can be interpreted in several ways. For example, the standardised difference

$$z_d = d/\sqrt{2}\,\sigma_0$$

should have a normal distribution with zero mean and unit standard deviation. The sum of a group of n such results would have a standard deviation of $\sqrt{n}$, so only about three runs in a thousand would produce a value of $|\Sigma z_d| > 3\sqrt{n}$. Alternatively a group of n values of z_d from a run can be combined to form Σz_d^2 and the result interpreted as a sample from a chi-squared distribution with n

degrees of freedom, (χ^2_n). Some caution is needed in the use of this statistic, however, as it is sensitive to outlying results.

5.2.2 *Wide concentration range.* If the test materials comprising a run have a wide range of analyte concentrations, no common standard of precision (σ_o) can be assumed. In such an instance σ_0 must be expressed as a functional relationship with concentration. The value of concentration for a particular material is taken to be $(x_1 + x_2)/2$, and an appropriate value of σ_o obtained from the functional relationship, the parameters of which have to be estimated in advance.

6. CONTROL MATERIALS IN IQC

6.1 Introduction

Control materials are characterised substances that are inserted into the run alongside the test materials and subjected to exactly the same treatment. A control material must contain an appropriate concentration of the analyte, and a value of that concentration must be assigned to the material. Control materials act as surrogates for the test materials and must therefore be representative, *i.e.*, they should be subject to the same potential sources of error. To be fully representative, a control material must have the same matrix in terms of bulk composition, including minor constituents that may have a bearing on accuracy. It should also be in a similar physical form, *i.e.*, state of comminution, as the test materials. There are other essential characteristics of a control material. It must be adequately stable over the period of interest. It must be possible to divide the control material into effectively identical portions for analysis. It is often required in large amounts to allow its use over an extended period.

Reference materials in IQC are used in combination with control charts that allow both persistent bias and run effects to be addressed (Appendix 1). Persistent bias is evident as a significant deviation of the centre line from the assigned value. The variation in the run effect is predictable in terms of a standard deviation when the system is under statistical control, and that standard deviation is used to define action limits and warning limits at appropriate distances from the true value.

6.2 The role of certified reference materials

Certified reference materials (CRM) as defined in Section 2 (*i.e.*, with a statement of uncertainty and traceability), when available and of suitable composition, are ideal control materials in that they can be regarded for traceability purposes as ultimate standards of trueness[20]. In the past CRMs were regarded as being for reference purposes only and not for routine use. A more modern approach is to treat CRMs as consumable and therefore suitable for IQC.

The use of CRMs in this way is, however, subject to a number of constraints.

(i) Despite the constantly increasing range of CRMs available, for the majority of analyses there is no closely matching CRM available.

(ii) Although the cost of CRMs is not prohibitive in relation to the total costs of analysis, it may not be possible for a laboratory with a wide range of activities to stock every relevant kind of reference material.

(iii) The concept of the reference material is not applicable to materials where either the matrix or the analyte is unstable.

(iv) CRMs are not necessarily available in sufficient amounts to provide for IQC use over extended periods.

(v) It must be remembered that not all apparently certified reference materials are of equal quality. Caution is suggested when the information on the certificate is inadequate.

If for any of the above reasons the use of a CRM is not appropriate it falls on individual laboratories or groups of laboratories to prepare their own control materials and assign traceable[3] values of analyte concentration to them. Such a material is sometimes referred to as a "house reference material" (HRM). Suggestions for preparing HRMs are listed in Section 6.3. Not all of the methods described there are applicable to all analytical situations.

6.3 Preparation of control materials

6.3.1 *Assigning a true value by analysis.* In principle a working value can be assigned to a stable reference material simply by careful analysis. However, precautions are necessary to avoid biases in the assigned value. This requires some form of independent check such as may be provided by analysis of the materials in a number of laboratories and where possible, the use of methods based on different physico-chemical principles. Lack of attention to

[3]Where a CRM is not available traceability only to a reference method or to a batch of a reagent supplied by a manufacturer may be necessary.

independent validation of control materials has been shown to be a weakness in IQC systems[15].

One way of establishing a traceable assigned value in a control material is to analyse a run comprising the candidate material and a selection of matching CRMs, with replication and randomisation. This course of action would be appropriate if only limited amounts of CRMs were available. The CRMs must be appropriate in both matrix composition and analyte concentration. The CRMs are used directly to calibrate the analytical procedure for the analysis of the control material. An appropriate analytical method is a prerequisite for this approach. It would be a dangerous approach if, say, a minor and variable fraction of the analyte were extracted for measurement. The uncertainty introduced into the assigned value must also be considered.

6.3.2 *Materials validated in proficiency testing* comprise a valuable source of control materials. Such materials would have been analysed by many laboratories using a variety of methods. In the absence of counter-indications, such as an obvious bias or unusual frequency distribution of results, the consensus of the laboratories could be regarded as a validated assigned value to which a meaningful uncertainty could be attached. (There is a possibility that the consensus could suffer from a bias of consequence, but this potential is always present in reference values.) There would be a theoretical problem of establishing the traceability of such a value, but that does not detract from the validity of the proposed procedure. The range of such materials available would be limited, but organisers of proficiency tests could ensure a copious supply by preparing batches of material in excess of the immediate requirements of the round. The normal requirements of stability would have to be demonstrable.

6.3.3 *Assigning a true value by formulation.* In favourable instances a control material can be prepared simply by mixing constituents of known purity in predetermined amounts. For example, this approach would often be satisfactory in instances where the control material is a solution. Problems are often encountered in formulation in producing solid control materials in a satisfactory physical state or in ensuring that the speciation and physical distribution of the analyte in the matrix is realistic. Moreover an adequate mixing of the constituents must be demonstrable.

6.3.4 *Spiked control materials.* "Spiking" is a way of creating a control material in which a value is assigned by a combination of formulation and analysis. This method is feasible when a test material essentially free of the analyte is available. After exhaustive analytical checks to ensure the

background level is adequately low, the material is spiked with a known amount of analyte. The reference sample prepared in this way is thus of the same matrix as the test materials to be analysed and of known analyte level - the uncertainty in the assigned concentration is limited only by the possible error in the unspiked determination. However, it may be difficult to ensure that the speciation, binding and physical form of the added analyte is the same as that of the native analyte and that the mixing is adequate.

6.3.5 *Recovery Checks*. If the use of a reference material is not practicable then a limited check on bias is possible by a test of recovery. This is especially useful when analytes or matrices cannot be stabilised or when *ad hoc* analysis is executed. A test portion of the test material spiked with a known amount of the analyte and analysed alongside the original test material. The recovery of the added analyte (known as the "marginal recovery") is the difference between the two measurements divided by the amount that is added. The obvious advantages of recovery checks are that the matrix is representative and the approach is widely applicable - most test materials can be spiked by some means. However, the recovery check suffers from the disadvantage previously noted regarding the speciation, binding and physical distribution of the analyte. Furthermore, the assumption of an equivalent recovery of the analyte added as a spike and of the native analyte may not be valid. However, it can normally be assumed that a poor performance in a recovery check is strongly indicative of a similar or worse performance for the native analyte in the test materials.

Spiking and recovery testing as an IQC method must be distinguished from the method of standard additions, which is a measurement procedure: a single spiking addition cannot be used to fulfil the roles of both measurement and IQC.

6.4 Blank determinations

Blank determinations are nearly always an essential part of the analytical process and can conveniently be effected alongside the IQC protocol. The simplest form of blank is the "reagent blank", where the analytical procedure is executed in all respects apart from the addition of the test portion. This kind of blank, in fact, tests more than the purity of the reagents. For example it is capable of detecting contamination of the analytical system originating from any source, *e.g.*, glassware and the atmosphere, and is therefore better described as a "procedural blank". In some instances, better execution of blank determinations is achieved if a simulated test material is employed. The simulant could be an actual test material known to be virtually analyte-free or a surrogate (e.g., ashless filter paper used instead of plant material). Where it can

be contrived, the best type of blank is the "field blank", which is a typical matrix with zero concentration of analyte.

An inconsistent set of blanks in a run suggests sporadic contamination and may add weight to IQC evidence suggesting the rejection of the results. When an analytical protocol prescribes the subtraction of a blank value, the blank value must be subtracted also from the results of the control materials before they are used in IQC.

6.5 Traceability in spiking and recovery checks

Potential problems of the traceability of reagents used for spikes and recovery checks must be guarded against. Under conditions where CRMs are not available, traceability can often be established only to the batch of analyte provided by a manufacturer. In such cases, confirmation of identity and a check on purity must be made before use. A further precaution is that the calibration standards and spike should not be traceable to the same stock solution of analyte or the same analyst. If such a common traceability existed, then the corresponding sources of error would not be detected by the IQC.

7 RECOMMENDATIONS

The following recommendations represent integrated approaches to IQC that are suitable for many types of analysis and applications areas. Managers of laboratory quality systems will have to adapt the recommendations to the demands of their own particular requirements. Such adaption could be implemented, for example, by adjusting the number of duplicates and control material inserted into a run, or by the inclusion of any additional measures favoured in the particular application area. The procedure finally chosen and its accompanying decision rules must be codified in an IQC protocol that is separate from the analytical system protocol.

The practical approach to quality control is determined by the frequency with which the measurement is carried out and the size and nature of each run. The following recommendations are therefore made. The use of control charts and decision rules are covered in Appendix 1.

In each of the following the order in the run in which the various materials are analysed should be randomised if possible. A failure to randomise may result in an underestimation of various components of error.

(i) *Short (e.g., n<20) frequent runs of similar materials.* Here the concentration range of the analyte in the run is relatively small, so a common value of standard deviation can be assumed.

Insert a control material at least once per run. Plot either the individual values obtained, or the mean value, on an appropriate control chart. Analyse in duplicate at least half of the test materials, selected at random. Insert at least one blank determination.

(ii) *Longer (e.g., n>20) frequent runs of similar materials.* Again a common level of standard deviation is assumed.

Insert the control material at an approximate frequency of one per ten test materials. If the run size is likely to vary from run to run it is easier to standardise on a fixed number of insertions per run and plot the mean value on a control chart of means. Otherwise plot individual values. Analyse in duplicate a minimum of five test materials selected at random. Insert one blank determination per ten test materials.

(iii) Frequent runs containing similar materials but with a wide range of analyte concentration. Here we cannot assume that a single value of standard deviation is applicable.

Insert control materials in total numbers approximately as recommended above. However, there should be at least two levels of analyte represented, one close to the median level of typical test materials, and the other approximately at the upper or lower decile as appropriate. Enter values for the two control materials on separate control charts. Duplicate a minimum of five test materials, and insert one procedural blank per ten test materials.

(iv) *Ad hoc analysis.* Here the concept of statistical control is not applicable. It is assumed, however, that the materials in the run are of a single type, *i.e.*, sufficiently similar for general conclusions on errors to be made.

Carry out duplicate analysis on all of the test materials. Carry out spiking or recovery tests or use a formulated control material, with an appropriate number of insertions (see above), and with different concentrations of analyte if appropriate. Carry out blank determinations. As no control limits are available,

compare the bias and precision with fitness for purpose limits or other established criteria..

8 CONCLUSIONS

Internal quality control is an essential aspect of ensuring that data released from a laboratory are fit for purpose. If properly executed, quality control methods can monitor the various aspects of data quality on a run-by-run basis. In runs where performance falls outside acceptable limits, the data produced can be rejected and, after remedial action on the analytical system, the analysis can be repeated.

It must be stressed, however, that internal quality control is not foolproof even when properly executed. Obviously it is subject to "errors of both kinds", *i.e.*, runs that are in control will occasionally be rejected and runs that are out of control occasionally accepted. Of more importance, IQC cannot usually identify sporadic gross errors or short-term disturbances in the analytical system that affect the results for individual test materials. Moreover, inferences based on IQC results are applicable only to test materials that fall within the scope of the analytical method validation. Despite these limitations, which professional experience and diligence can alleviate to a degree, internal quality control is the principal recourse available for ensuring that only data of appropriate quality are released from a laboratory. When properly executed it is very successful.

Finally, it must be appreciated that a perfunctory execution of any quality system will not guarantee the production of data of adequate quality. The correct procedures for feedback, remedial action and staff motivation must also be documented and acted upon. In other words, there must be a genuine commitment to quality within a laboratory for an internal quality control programme to succeed, *i.e.,* the IQC must be part of a total quality management system.

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APPENDIX 1. SHEWHART CONTROL CHARTS

1 Introduction

The theory, construction and interpretation of the Shewhart chart[1] are detailed in numerous texts on process quality control and applied statistics, and in several ISO standards[2-5]. There is a considerable literature on the use of the control chart in clinical chemistry[6,7]. Westgard and co-workers have formulated multiple rules for the interpretation of such control charts[8], and the power of these results has been studied in detail[9-10]. In this appendix only simple Shewhart charts are considered.

In IQC a Shewhart control chart is obtained when values of concentration measured on a control material in successive runs are plotted on a vertical axis against the run number on the horizontal axis. If more than one analysis of a particular control material is made in a run, either the individual results x or the mean value $\bar{x}$ can be used to form a control chart. The chart is completed by horizontal lines derived from the normal distribution $N(\mu,\sigma^2)$ that is taken to describe the random variations in the plotted values. The selected lines for control purposes are μ, $\mu\pm2\sigma$ and $\mu\pm3\sigma$. Different values of σ are required for charts of individual values and of means. For a system in statistical control, on average about one in twenty values fall outside the $\mu\pm2\sigma$ lines, called the "warning limits", and only about three in one thousand fall outside the $\mu\pm3\sigma$ lines, the "action limits". In practice the estimates $\bar{x}$ and s of the parameters μ and σ are used to construct the chart. A persistent bias is indicated by a significant difference between $\bar{x}$ and the assigned value. A control chart showing results from a system in statistical control over 40 runs is shown in Figure 1.

2 Estimates of the parameters μ and σ

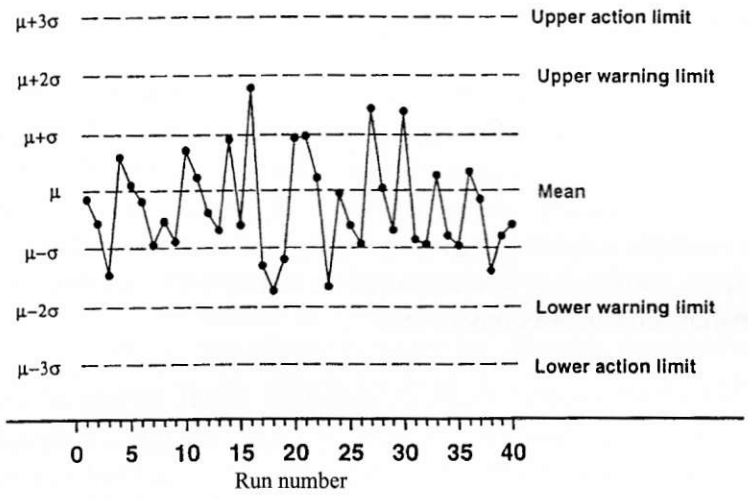
An analytical system under control exhibits two sources of random variation, the within-run, characterised by variance σ_0^2 and the between-run with variance σ_1^2. The two variances are typically comparable in magnitude. The standard deviation σ_x used in a chart of individual values is given by

$$\sigma_x = (\sigma_0^2 + \sigma_1^2)^{1/2}$$

whereas for a control chart of mean values the standard deviation is given by

$$\sigma_{\bar{x}} = (\sigma_0^2/n + \sigma_1^2)^{1/2}$$

Fig. 1 Results from a system in statistical control



where n is the number of control measurements in a run from which the mean is calculated. The value of n therefore must be constant from run to run, otherwise control limits would be impossible to define. If a fixed number of repeats of a control material per run cannot be guaranteed (*e.g.*, if the run length were variable) then charts of individual values must be used. Furthermore the equation indicates that σ_x or $\sigma_{\bar{x}}$ must be estimated with care. An attempt to base an estimate on repeat values from a single run would result in unduly narrow control limits.

Estimates must therefore include the between-run component of variance. If the use of a particular value of n can be assumed at the outset, then $\sigma_{\bar{x}}$ can be estimated directly from the m means $\bar{x}_i = \sum_{j=1}^{n} x_{ij} / n$,

($i = 1,.....,m$) of the n repeats in each of m successive runs. Thus the estimate of μ is

$$\bar{\bar{x}} = \sum_i \bar{x}_i / m ,$$

and the estimate of $\sigma_{\bar{x}}$ is

$$s_{\bar{x}} = \sqrt{\frac{\sum_i (\bar{x}_i - \bar{\bar{x}})^2}{m-1}}$$

If the value of n is not predetermined, then separate estimates of σ_0 and σ_1 could be obtained by one-way analysis of variance. If the mean squares within- and between- groups are MS_W and MS_B respectively, then

σ_0^2 is estimated by MS_W and

σ_1^2 is estimated by $(MS_b - MS_W)/n$

Often in practice it is necessary to initiate a control chart with data collected from a small number of runs, which may be to a degree unrepresentative, as estimates of standard deviation are very variable unless large numbers of observations are used. Moreover, during the initial period, the occurrence of out-of-control conditions are more than normally likely and will produce outlying values. Such values would bias $\bar{x}$ and inflate s beyond its proper value. It is therefore advisable to recalculate $\bar{x}$ and s after a further "settling down" period. One method of obviating the effects of outliers in the calculation is to reject them after the application of Dixon's Q or Grubbs'[11] test, and then use the classical statistics given above. Alternatively, the methods of robust statistics could be applied to the data[12, 13].

3 The interpretation of control charts.

The following simple rules can be applied to control charts of individual results or of means.

Single control chart. An out-of-control condition in the analytical system is signalled if any of the following occur.

(i) The current plotting value falls outside the action limits.
(ii) The current value and the previous plotting value fall outside the warning limits but within the actions limits.
(iii) Nine successive plotting values fall on the same side of the mean line.

Two control charts. When two different control materials are used in each run, the respective control charts are considered simultaneously. This increases the chance of a type 1 error (rejection of a sound run) but decreases the chance of a type 2 error (acceptance of a flawed run). An out-of-control condition is indicated if any of the following occur.

(i) At least one of the plotting values falls outside the action limits.
(ii) Both of the plotting values are outside the warning limits.
(iii) The current value and the previous plotting value on the same control chart both fall outside the warning limits.

(iv) Both control charts simultaneously show that four successive plotting values on the same side of the mean line.
(v) One of the charts shows nine successive plotting values falling on the same side of the mean line.

A more thorough treatment of the control chart can be obtained by the application of the full Westgard rules, illustrated in Figure 2.

The analytical chemist should respond to an out-of-control condition by cessation of analysis pending diagnostic tests and remedial action followed by rejection of the results of the run and reanalysis of the test materials.

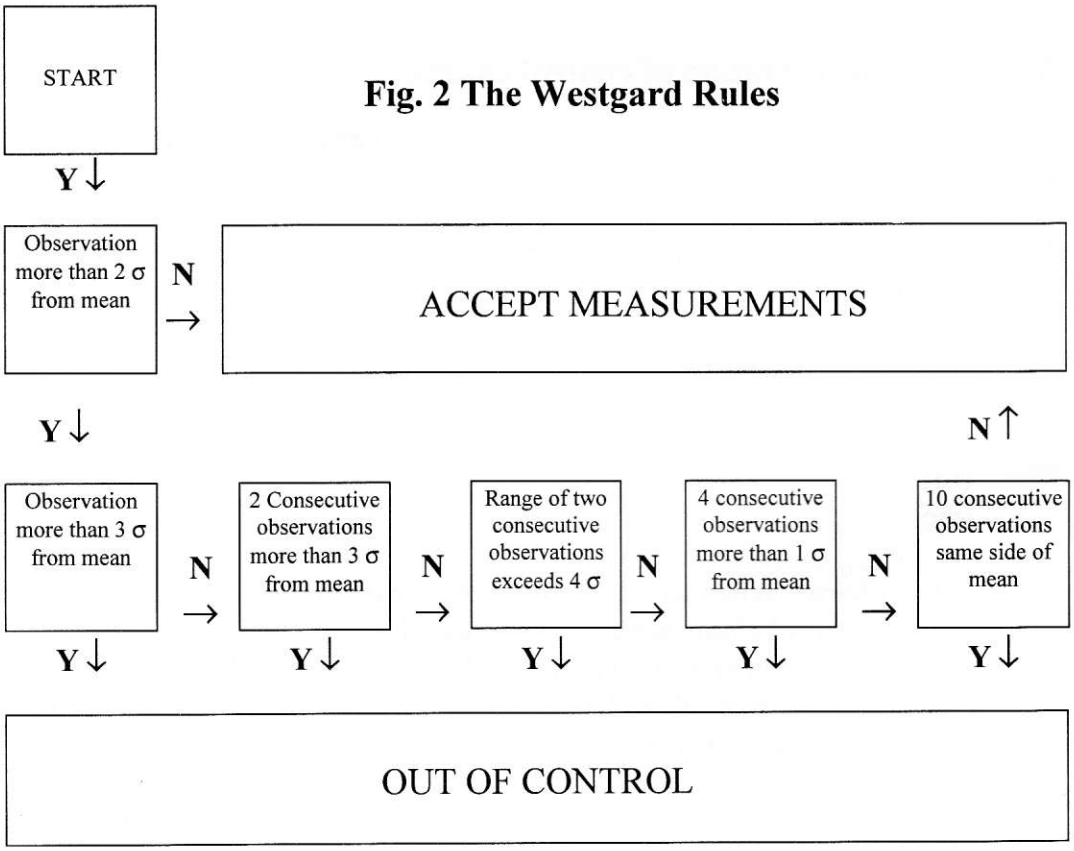


Fig. 2 The Westgard Rules

4 References

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