STATUTORY INSTRUMENTS

1976 No. 840

AGRICULTURE

The Fertilisers and Feeding Stuffs (Amendment) Regulations 1976

Made - - - - - - -	*27th May* 1976
Laid before Parliament - - -	*10th June* 1976
Coming into Operation—	
Regulations 2(1), (2), (4), (5), (6), (8), (9), (16) and (18) - - -	*1st July* 1976
Regulation 3 - - - - - -	*1st August* 1976
Regulations 2(3), (7), (10), (11), (12), (13), (14), (15), (17) and (19) -	*1st October* 1976

The Minister of Agriculture, Fisheries and Food and the Secretary of State, acting jointly, in exercise of the powers conferred on them by sections 66(1), 67(5), 68, 69(1), 69(3), 70(1), 74, 74A (inserted by section 4(1) of, and paragraph 6 of Schedule 4 to, the European Communities Act 1972(**a**)), 75(1), 76(1), 77(1), (2) and (4) and 84 of the Agriculture Act 1970(**b**) and of all other powers enabling them in that behalf, hereby make the following regulations after consultation with such persons or organisations as appear to them to represent the interests concerned:—

Citation, commencement and interpretation

1.—(1) These regulations may be cited as the Fertilisers and Feeding Stuffs (Amendment) Regulations 1976, and shall come into operation as respects regulations 2(1), (2), (4), (5), (6), (8), (9), (16) and (18) on 1st July 1976, as respects regulation 3 on 1st August 1976 and as respects regulations 2(3), (7), (10), (11), (12), (13), (14), (15), (17) and (19) on 1st October 1976.

(2) In these regulations, unless the context otherwise requires—

"the Act" means the Agriculture Act 1970, as amended by section 4(1) of, and paragraph 6 of Schedule 4 to, the European Communities Act 1972;

"the principal regulations" means the Fertilisers and Feeding Stuffs Regulations 1973(**c**).

(3) Any reference in these regulations to a numbered section shall, unless the reference is to a section of a specified Act, be construed as a reference to the section bearing that number in the Act.

(4) The Interpretation Act 1889(**d**) shall apply to the interpretation of these regulations as it applies to the interpretation of an Act of Parliament.

Amendment of the principal regulations

2.—(1) Regulation 3(2) shall be amended by substituting the word "in" for the words "which is packed in bags, sacks or".

(**a**) 1972 c. 68.
(**b**) 1970 c. 40.
(**c**) S.I. 1973/1521 (1973 II, p. 4604).
(**d**) 1889 c. 63.

(2) The following regulation shall be substituted for regulation 5:—

"5. The prescribed qualifications for an agricultural analyst or a deputy agricultural analyst for the purposes of section 67(5) are that he shall be a Chartered Chemist, being a Fellow or a Member of the Royal Institute of Chemistry, and that his practical experience of chemical analysis and microscopy, as applied to fertilisers and feeding stuffs, shall be attested by another agricultural analyst or deputy agricultural analyst appointed under section 67(3) of the Act or in accordance with section 11 of the Fertilisers and Feeding Stuffs Act 1926(a)."

(3) The following regulation shall be substituted for regulation 7:—

"7.—(1) No person shall sell or have in possession with a view to sale—

(*a*) for use as a feeding stuff or use as a feeding stuff or import into Great Britain for such use any material containing any added antioxidant, colourant, emulsifier, stabiliser, binder, vitamin D_2 or D_3, copper, any added substance of a description specified in the first column of Part V of the table in Schedule 3 or, any added substance of a description specified in the first column of Part VI of the table in Schedule 3, unless, in each case, the material complies with the provisions of that Schedule as respects content and, where appropriate, marking and it shall be an offence if a sampled portion of any such material does not comply with the provisions of the said Schedule 3 as respects content;

(*b*) to any keeper or breeder of any kind of animal specified in regulation 2, for use as a feeding stuff, any substance specified in the first column of the table in Schedule 3A comprised in a feeding stuff corresponding thereto in the second column thereof in excess of the quantity specified in relation thereto in the third column of the said Schedule:

Provided that where a feeding stuff is not specified in the second column in relation to a substance specified in the first column that feeding stuff, having regard to the quantity and nature of other feeding stuffs commonly used in compiling the daily feed intake of the animal for which the feed is intended, shall not contain more than the levels specified for whole feeding stuffs and it shall be an offence if a sampled portion of any such feeding stuff does not comply with the provisions of Schedule 3A as regards content.

(2) The provisions of paragraph (1) of this regulation shall not apply to any substance referred to in sub-paragraph (*a*) thereof or to any substance of a description referred to in sub-paragraph (*b*) thereof, which is—

(*a*) for use only in accordance with a prescription given by a veterinary surgeon or veterinary practitioner for the treatment of a particular animal or animals under his care;

(*b*) a medicinal product or for use for a medicinal purpose in a feeding stuff;

(*c*) for use only for the purpose of scientific research or experiment and is not generally available for sale, purchase or use in a feeding stuff ;

(*d*) intended for exportation to any place outside the United Kingdom and is clearly marked or labelled to that effect.

(a) 1926 c. 45.

In this regulation and in Part II of Schedule 2 the expressions "a medicinal product" and "a medicinal purpose" have the meanings assigned to them by section 130(1) and (2) respectively of the Medicines Act 1968(a).

(3) No person shall use as a feeding stuff or import into Great Britain for such use any material containing any added substance, not being a substance of a name or description specified in the table in Schedule 3 or in sub-paragraphs 2(*f*) or (*g*) of that Schedule, which is deleterious either to animals of any description specified in regulation 2 or to human beings, and it shall be an offence if a sampled portion of any such material is shown by an analysis of the sample taken from it to contain an added substance which is deleterious as aforesaid.

(4) In relation to any material to which this regulation, Schedule 3 or Schedule 3A applies the operation of the provisions of sections 66(2), 73(1), 80(2) and 82 shall be modified as follows:—

(*a*) section 66(2) shall have effect as if—

(i) the words "imported or" were inserted immediately before the word "sold" in both places where that word appears, and

(ii) the words "or as so used" were inserted immediately after the words "feeding stuff", and

(iii) the words "or is so used" were inserted immediately after the words "to be so used".

(*b*) section 73(1) shall have effect as if there were added at the end of that subsection the words "or to human beings".

(*c*) sections 80(2) and 82 shall apply in relation to proceedings for an offence under this regulation and section 74A(3) as they apply respectively to proceedings for an offence under any of the provisions mentioned in them."

(4) Regulation 9 shall be amended by substituting the word "in" for the words "packed in bags, sacks or" in paragraph (*c*) thereof.

(5) Regulation 11 shall be amended by substituting the word "in" for the words "packed in bags, sacks or" in paragraph (*c*) thereof.

(6) Regulation 12 shall be amended:—

(*a*) by substituting the word "in" for the words "packed in bags, sacks or" in paragraph 1(*c*) thereof;

(*b*) by deleting paragraph (3) thereof.

(7) The following regulation shall be substituted for regulation 15:—

"15. In respect of substances for which methods of analysis are specified in Schedules 6 and 7, the methods by which analyses of fertilisers and feeding stuffs respectively shall be made for the purpose of the Act shall be those set out in the said Schedules."

(8) Regulation 17 shall be amended by substituting for the words "recorded delivery service" the words "recorded delivery service or by hand".

(a) 1968 c. 67.

(9) Schedule 1 to the principal regulations shall be amended:—

(*a*) by substituting for paragraph 1 of Part I thereof the following paragraph:—

"1. In the case of material in packages or containers, only unopened packages or containers which appear to the inspector proposing to take the sample to be the original packages or containers of the material shall be selected for the purpose of the sample.";

(*b*) by substituting for paragraph 4 of Part I thereof the following paragraph:—

"4. In every case the sampling shall be carried out in a manner which will protect the sample from contamination and shall be done as quickly as is possible, consistent with due care, and the material shall not be exposed any longer than is necessary.";

(*c*) by deleting the words "bottles or" in paragraph 8(*a*) of Part I thereof, wherever those words appear;

(*d*) by deleting the words "drums, kegs or other" in paragraph 8(*b*) of Part I thereof;

(*e*) by deleting the words "on a clean dry surface" in paragraph 1(*a*)(i) of Part II thereof;

(*f*) by inserting after the words "continue the quartering and rejection" in the last sub-paragraph of paragraph 1(*a*) of Part II thereof, the words "if necessary";

(*g*) by substituting for paragraph 2(*a*) of Part II thereof the following sub-paragraph:—

"(*a*) *In packages*

(i) The packages, selected according to the appropriate scale in paragraph 1(*a*), shall be emptied separately and worked up with a shovel and one shovelful taken from each; and

(ii) The shovelfuls so taken shall be crushed immediately and the whole passed through a sieve with meshes one and a quarter inch square. It shall be mixed thoroughly and rapidly and a sample of about 4lb. to 6lb. in weight drawn in the manner described in paragraph 1(*a*).";

(*h*) by substituting for the number and letter "1(*a*)" in paragraph 2(*b*) of Part II thereof the numbers and letter "2(*a*)(ii)";

(*i*) by substituting for the words "on a clean dry surface and the matted portions torn up" in the first sentence of paragraph 3(*a*) of Part II thereof, the words "and the matted portions torn up and the whole shall be thoroughly mixed.";

(*j*) by deleting the words "whether in bags or in bulk" in the heading to paragraph 2 of Part III thereof;

(*k*) by deleting the words "on a clean surface" in paragraph 4(*a*) of Part III thereof;

(*l*) by substituting for paragraph 1 of Part IV thereof the following paragraph:—

"1. Where the sample has been taken in the prescribed manner the person taking the sample shall divide it into three parts, or, in the

circumstances set out in section 77(2), four parts, as nearly as possible equal, in the following manner:—

(*a*) *In the case of dry or powdered substances*

The sample, drawn as described in the foregoing paragraphs, shall be thoroughly mixed and divided into three or, as the case may be, four similar and approximately equal parts. Each of these parts shall be placed in an appropriate container such that the composition at the time of sampling of the fertiliser or feeding stuff is preserved.

(*b*) *In the case of substances in a liquid or semi-liquid condition*

The sample, drawn as described in the foregoing paragraphs, shall be thoroughly mixed and at once divided into similar and approximately equal parts by pouring successive portions into each of three or, as the case may be, four appropriate containers. The containers used shall be such that the composition at the time of sampling of the fertiliser or feeding stuff is preserved and shall be so fastened that spillage or evaporation of the contents is prevented.

Each of the containers referred to in sub-paragraphs (*a*) and (*b*) above shall be so secured and sealed that it cannot be opened without breaking the seal; or alternatively the container may be placed in a stout envelope or in a linen, cotton or plastic bag and the envelope or bag then secured and sealed in such a manner that the part of the sample cannot be removed without breaking the seal or the envelope or the bag.".

(10) Schedule 1 to these regulations shall be substituted for Part II of Schedule 2 to the principal regulations.

(11) Schedule 3 to the principal regulations shall be amended:—

(*a*) by substituting for paragraph 2(*g*) thereof the following paragraph:—

"(*g*) any added non-protein nitrogenous compound save that material intended for use as a feeding stuff for the following kinds of ruminant animals namely, bulls, cows, steers, heifers, calves, sheep or goats may contain the non-protein nitrogenous compounds specified in Part VI of the table in Schedule 3.";

(*b*) by substituting for the words "grammes per kilogramme" in the ninth line of paragraph 3 the words "grams per kilogram of the whole feeding stuff";

(*c*) by substituting for the final sentence of paragraph 3 thereof the following sentence:—

"In this statement there may be substituted for the words "grams per kilogram", the symbol "%", "lb. per cwt.", "lb. per ton", "grams per litre" or "grams per tonne".";

(*d*) by deleting the colourant "Red 6B" at the end of the table in Part II thereof.

(12) Schedule 2 to these regulations shall be added as Part VI of the table in Schedule 3 to the principal regulations.

(13) Schedule 3 to these regulations shall be inserted as Schedule 3A to the principal regulations.

(14) Part II of Schedule 4 to the principal regulations shall be amended by adding immediately below the entry for Pea Meal:—

"Poultry waste	The waste from intensive poultry units which consists principally of excreta with or without litter and which has been suitably treated for use as a feeding stuff.".

(15) Schedule 4 to these regulations shall be substituted for Part II of Schedule 5 to the principal regulations.

(16) Schedule 6 to the principal regulations shall be amended:—

(*a*) by adding at the end of the last item of sub-division 1.16 thereof, after the words "containing organic matter" the words "and granular basic slag and granular potassic basic slag";

(*b*) by substituting in the eighteenth line of sub-division 3.53 thereof the figure "15" in place of the figure "55";

(*c*) by substituting in the nineteenth line of sub-division 3.53 thereof the word "forty" in place of the word "twenty";

(*d*) by inserting in the first line of sub-division 4.171 thereof the words "as received" immediately after the word "sample";

(*e*) by inserting in the first line of sub-division 4.271 thereof the words "as received" immediately after the word "sample";

(*f*) by substituting in the eighth line of sub-division 15.3 thereof the figure "1.5" in place of the figure "3".

(17) Schedule 5 to these regulations shall be substituted for Schedule 7 to the principal regulations.

(18) Part I of Schedule 8 to the principal regulations shall be amended by deleting the chemical symbol "Bo", which appears immediately after the word "Boron" and substituting therefor the chemical symbol "B".

(19) Schedule 6 to these regulations shall be substituted for Part II of Schedule 8 to the principal regulations.

Amendment as respects metrication

3.—(1) The metric units of measurement specified in the first column of Schedule 7 to these regulations shall be substituted in the Act for the corresponding imperial units specified in the second column of the said Schedule.

(2) The metric units of measurement specified in the first column of Schedule 8 to these regulations shall be substituted in the principal regulations for the corresponding imperial units specified in the second column of the said Schedule.

In Witness whereof the Official Seal of the Minister of Agriculture, Fisheries and Food is hereunto affixed on 25th May 1976.

(L.S.)

Frederick Peart,
Minister of Agriculture, Fisheries and Food.

Bruce Millan,
Secretary of State for Scotland.

27th May 1976.

SCHEDULE 1

(Sections 68(1) and 69(1) and Regulation 2(10))

FEEDING STUFFS

1. In the case of material of any description specified in the first column of the table in this Part of this Schedule, the statutory statement shall contain the particulars specified in relation to that material in the second column thereof, save that where there has been added in the course of manufacture or preparation for sale any of the undermentioned substances (other than any addition of a medicinal product or any addition for a medicinal purpose) the statutory statement shall also contain the details specified in relation to each substance—

(*a*) any copper or magnesium, a statement of the total amount present (whether naturally present or added) of any copper (if present in excess of 50 milligrams per kilogram or magnesium (if present in excess of 0·5 per cent.);

(*b*) any antioxidant or colourant, either the words "contains permitted anti-oxidant" or "contains permitted colourant" as appropriate, or the name of the antioxidant or colourant;

(*c*) any vitamin A, D or E, the name of the vitamin and a statement of the total amount present (whether naturally present or added) and an indication of the period during which that amount will remain present;

(*d*) any molybdenum or selenium, a statement of the total amount of molybdenum or selenium present (whether naturally present or added);

(*e*) any preservative, the name of the preservative;

any amount referred to—

(i) in sub-paragraph (*a*) above being expressed as a percentage by weight (unless the amount present is less than 0·1 per cent. by weight in which case it shall be expressed in milligrams per kilogram);

(ii) in sub-paragraph (*c*) above being expressed in international units per kilogram or units per kilogram;

(iii) in sub-paragraph (*d*) above being expressed in milligrams per kilogram.

2. The provisions of this Part of this Schedule shall apply to material of any description specified therein under whatever name it may be sold or offered for sale and notwithstanding that it contains a substance not mentioned in this Part of this Schedule.

3. In the said particulars—

(*a*) the amount shall in each case be expressed as a definite percentage of the weight of the material, and not as a range of percentages;

(*b*) phosphorus shall be expressed as phosphorus (P).

4. In this Part of this Schedule, subject to the provisions of paragraph 2(*g*) of Schedule 3, and, as respects the definitions of "compound feeding stuff" and "feed supplement", in these regulations—

"amount of protein" means—

(*a*) except in the case of compound feeding stuffs or feed supplements for the following kinds of ruminant animals, namely bulls, cows, steers, heifers, calves, sheep or goats, the amount of nitrogen, other than ammoniacal, nitrate, urea, biuret, isobutylidene diurea, urea phosphate or uric acid nitrogen, multiplied by 6·25;

(*b*) in the case of compound feeding stuffs or feed supplements for the following kinds of ruminant animals, namely bulls, cows, steers, heifers,

calves, sheep or goats, the amount of nitrogen, including urea, biuret, isobutylidene diurea, urea phosphate and uric acid nitrogen, but not including ammoniacal or nitrate nitrogen, multiplied by 6·25;

"amount of protein equivalent of urea, biuret, isobutylidene diurea, or urea phosphate" means the amount of urea, biuret, isobutylidene diurea or urea phosphate nitrogen multiplied by 6·25;

"amount of protein equivalent of uric acid" means the amount of uric acid nitrogen and its salts multiplied by 6·25;

"compound feeding stuff" means a product, other than a feed supplement, obtained by mixing two or more materials, including at least one of the materials mentioned in the first column of the table in this Part of this Schedule save that for the purposes of this definition the presence of any added substance of a kind referred to in regulation 7(1)(*a*) (except any added substance of a description specified in the first column of Part VI of the table in Schedule 3) shall be disregarded;

"feed supplement" means a product obtained by mixing two or more materials, being a product of a kind commonly sold or used to supplement other feeding stuffs to an extent of not more than one-twentieth of the total quantity;

"fibre" means the organic matter calculated as the result of treatment of the feeding stuff according to the procedure described in method 8 of Schedule 7;

"oil" means the extract obtained as a result of treatment of a feeding stuff according to the procedure described in methods 3a and 3b of Schedule 7;

"preservative" means any substance which delays, retards or prevents the development in a feeding stuff of rancidity or other deterioration arising from microbial activity but excludes any antioxidant, colourant, emulsifier, stabiliser or binder permitted under these regulations;

"sugar" means total reducing sugars after inversion expressed as sucrose, determined according to the procedure described in method 9 of Schedule 7.

TABLE

Description of material	Particulars to be contained in statutory statement
Compound feeding stuff for the following kinds of ruminant animals, namely bulls, cows, steers, heifers, calves, sheep or goats.	Amount of protein (stating as being included therein the amount, if any, of protein equivalent of urea, biuret, isobutylidene diurea, or urea phosphate and, if 1 per cent. or greater, the amount of protein equivalent of uric acid) and amounts, if any, of oil and fibre respectively. Where the feeding stuff contains any of the substances listed in the first column of Part VI of the table in Schedule 3, instructions for use and information as to the kinds of animals for which the feeding stuff is intended and as to the maximum quantity that can be used in the whole feeding stuff.

Description of material	Particulars to be contained in statutory statement
Compound feeding stuff for animals other than the following kinds of ruminant animals, namely bulls, cows, steers, heifers, calves, sheep or goats.	Amounts, if any, of protein, oil and fibre respectively. If protein equivalent of uric acid is present in amounts of 1 per cent. or greater a declaration shall be made in the following manner "protein equivalent of uric acid *x* per cent. (not available to non ruminants)" where *x* equals the amount declared.
Alfalfa (lucerne) meal.	Amounts of protein and fibre respectively.
Artificially dried grass, clover, lucerne, sainfoin, green cereals or any other artificially dried green crops or a mixture of any of them.	Amount of protein.
Barley meal, barley meal Grade II, bean meal, dari or durra meal, ground oats, Indian or maize meal, locust bean meal, pea meal, wheat meal.	None.
Clover meal.	Amounts of protein and fibre respectively.
Coconut or copra cake or meal.	Amounts of oil and protein respectively.
Cotton cakes or meals, not decorticated.	Amounts of oil and protein respectively.
Cotton cakes or meals from decorticated or partly decorticated cotton seed.	Amounts of oil, protein and fibre respectively.
Dried brewery grains.	Amounts of oil and protein respectively.
Dried distillery by-products (other than malt culms and dried yeast).	Amounts of oil and protein, of fibre if present in excess of 2 per cent. and of calcium if present in excess of 2 per cent.
Dried plain beet pulp.	Amount of fibre.
Dried molassed beet pulp.	Amounts of sugar and fibre respectively.
Dried yeast.	Amount of protein.
Feed supplement for the following kinds of ruminant animals, namely bulls, cows, steers, heifers, calves, sheep or goats.	Protein equivalent of urea, biuret, isobutylidene diurea, or urea phosphate, if any. Protein equivalent of uric acid if 1 per cent. or greater. Instructions for mixing with other feeding stuffs, or information as to use where the supplement is fed direct to animals, and where the feeding stuff includes any added urea, biuret, isobutylidene diurea or urea phosphate, information as to the kinds of animals for which the feeding stuff is intended and as to the maximum quantity that can be used in the whole feeding stuff.

Description of material	Particulars to be contained in statutory statement
Feed supplement for animals other than the following kinds of ruminant animals, namely bulls, cows, steers, heifers, calves, sheep or goats.	Instructions for mixing with other feeding stuffs, or information as to use where the supplement is fed direct to animals. If protein equivalent of uric acid is present in amounts of 1 per cent. or greater a declaration shall be made in the following manner "protein equivalent of uric acid *x* per cent. (not available to non ruminants)" where *x* equals the amount declared.
Feeding bone flour.	Amounts of phosphorus and protein respectively.
Feeding bone meal, ground bone or any other bone product for feeding purposes.	Amounts of phosphorus and protein respectively.
Feeding dried blood.	Amount of protein.
Feeding meat and bone meal, or any other product of meat and bone for feeding purposes.	Amounts of oil, protein and phosphorus respectively.
Feeding meat meal, or any other product of meat for feeding purposes.	Amounts of oil, protein and phosphorus respectively.
Fish meal, white fish meal, or other product obtained by drying and grinding or otherwise treating fish or fish waste.	Amounts of oil, protein, phosphorus and salt respectively.
Linseed cakes and the meals of such cakes; extracted linseed meal.	Amounts of oil and protein respectively.
Linseed meal.	Amount of oil.
Maize by-products not otherwise mentioned in this table.	Amounts of oil, protein and fibre respectively.
Maize, flaked.	Amounts of oil and protein respectively.
Maize germ cake or meal.	Amounts of oil and protein respectively.
Maize gluten feed.	Amounts of oil and protein respectively.
Malt culms.	Amounts of protein and fibre respectively.
Milk powders, including oil or fat fortified milk powders.	Amounts of oil and protein respectively.
Mixtures of molasses and urea, biuret, isobutylidene diurea or urea phosphate.	Amounts of sugar and protein equivalent of urea, biuret, isobutylidene diurea or urea phosphate. Instructions for use and information as to the kinds of animals for which the material is intended and as to the maximum quantity that can be used in the whole feeding stuff.

Description of material	Particulars to be contained in statutory statement
Molasses feeds (other than dried molassed beet pulp and mixtures of molasses and urea, biuret, isobutylidene diurea or urea phosphate) including any feeding stuffs, composed of treacle or molasses with an absorbent, containing not less than 10 per cent. of sugar.	Amounts of sugar and fibre respectively.
Oatmeal by-products.	Amount of fibre.
Oil cakes or meals, not otherwise mentioned in this table, which are the product of any one undecorticated substance or seed from which oil has been removed.	Amounts of oil and protein respectively.
Oil cakes or meals, not otherwise mentioned in this table, which are the product of any one decorticated or partly decorticated substance or seed from which oil has been removed.	Amounts of oil, protein and fibre respectively.
Palm kernel cake or meal.	Amounts of oil and protein respectively.
Poultry waste.	Amounts of protein, of protein equivalent of uric acid if 1 per cent. or greater and of fibre respectively. Amount of calcium if the amount present is in excess of 2 per cent.
Rape cake or meal.	Amounts of oil and protein respectively.
Rice bran or rice meal, or the by-product produced in milling shelled rice.	Amounts of oil, protein and fibre respectively.
Soya cake or meal.	Amounts of oil and protein respectively.
Treacle or molasses.	Amount of sugar.
Wheat offals or millers' offals.	Amount of fibre.

SCHEDULE 2

(Section 74A and Regulations 2(3) and 2(12))

PART VI

NON-PROTEIN NITROGENOUS COMPOUNDS

Substance	Empirical Formulae
Biuret	$C_2H_5O_2N_3$
Isobutylidene diurea	$C_6H_{14}O_2N_4$
Urea	CH_4ON_2
Urea phosphate	$CH_7O_5N_2P$

SCHEDULE 3

(Section 74A and Regulation 2(13))

PRESCRIBED LIMITS FOR UNDESIRABLE SUBSTANCES IN FEEDING STUFFS

(Section 74A and Regulation 7(1)(*b*))

In this Schedule—

"complementary feeding stuff" means a product obtained by mixing two or more materials being a product of a kind commonly sold or used to supplement other feeding stuffs for dairy cattle to an extent greater than one-fifth of the total daily intake of dry matter;

"mineral mixture" means a product obtained by mixing two or more minerals, being a product of a kind commonly sold or used to supplement other feeding stuffs;

"straight feeding stuff" means a single feeding stuff of animal or vegetable origin whether or not processed which is intended for sale, or sold, to keepers or breeders for the feeding of any animal specified in regulation 2 and which does not include material sold to manufacturers for further processing;

"whole feeding stuff" has the meaning assigned to it by paragraph 1 of Schedule 3.

TABLE

Substances	Feeding stuffs	Maximum content in mg/kg of feeding stuffs referred to a moisture content of 12%
CHAPTER A		
Arsenic	Straight feeding stuffs	2
	except:	
	—meal made from grass, from dried lucerne, or from dried clover	4
	—dried sugar beet pulp or dried molassed sugar beet pulp	4
	—phosphates and feeding stuffs obtained from the processing of fish or other marine animals	10
	Whole feeding stuffs	2
Fluorine	Straight feeding stuffs	150
	except:	
	—feeding stuffs of animal origin	500
	—phosphates	2000
	Whole feeding stuffs	150
	except:	
	—whole feeding stuffs for cattle, sheep and goats	
	—in milk	30
	—other	50
	—whole feeding stuffs for pigs	100
	—whole feeding stuffs for poultry	350

Substances	Feeding stuffs	Maximum content in mg/kg of feeding stuffs referred to a moisture content of 12%
Fluorine (cont.)	—whole feeding stuffs for chicks	250
	Mineral mixtures for cattle, sheep and goats	2000
Lead	Straight feeding stuffs except:	10
	—phosphates	30
	—yeasts	5
	Whole feeding stuffs	5
Mercury	Straight feeding stuffs except:	0·1
	—feeding stuffs produced by the processing of fish or other marine animals	0·5
	Whole feeding stuffs	0·1
Nitrites	Fish meal	60 (expressed as sodium nitrite)
	Whole feeding stuffs	15 (expressed as sodium nitrite)

Substances	Feeding stuffs	Maximum content in mg/kg of the feeding stuff as found
CHAPTER B		
Aflatoxin B_1	Straight feeding stuffs	0·05
	Whole feeding stuffs for cattle, sheep and goats (except dairy cattle, calves, lambs and kids)	0·05
	Whole feeding stuffs for pigs and poultry (except piglets and chicks)	0·02
	Other whole feeding stuffs	0·01
	Complementary feeding stuffs	0·02
Castor oil plant —*Ricinus communis* L	All feeding stuffs	10 (expressed in terms of castor oil plant husks)
Crotalaria L. spp	All unmilled materials	100
Free Gossypol	Straight feeding stuffs except:	20
	—cotton cake or meal	1200
	Whole feeding stuffs except:	20
	—whole feeding stuffs for cattle, sheep and goats	500

Substances	Feeding stuffs	Maximum content in mg/kg of the feeding stuff as found
Free Gossypol (cont.)	—whole feeding stuffs for poultry (except laying hens) and calves	100
	—whole feeding stuffs for rabbits and pigs (except piglets)	60
Hydrocyanic acid	Straight feeding stuffs except:	50
	—linseed	250
	—linseed cake or meal	350
	—manioc products and almond cakes	100
	Whole feeding stuffs except:	50
	—whole feeding stuffs for chicks	10
Rye Ergot *Claviceps purpurea* (Fr.) Tul	All feeding stuffs containing unground cereals	1000
The following seeds and their processing derivatives:— —Apricot—*Prunus armeniaca* L. —Bitter almond—*Prunus dulcis* (Mill.) D. A. Webb (=*Prunus amygdalus* Batsch var. *amara* (DC.) Focke) —Black mustard—*Brassica nigra* (L.) Koch —Camelina—*Camelina sativa* (L.) Crantz —Croton—*Croton tiglium* L. —Chinese Yellow Mustard—*Brassica juncea* (L.) Czern & Coss. ssp. *juncea* var. *lutea* Batalin —Ethiopian Mustard —*Brassica carinata* A. Braun —Mowrah, bassia, madhuca—*Madhuca longifolia* (L.) Macbr. (=*Bassia longifolia* L. = *Illipe malabarorum* Engl.) Madhuca indica Gmelin (= *Bassia latifolia* Roxb. = *Illipe latifolia* (Roxb.) F. Mueller)	All feeding stuffs	Less than the lowest level detectable

Substances	Feeding stuffs	Maximum content in mg/kg of the feeding stuff as found
—Physic nut —*Jatropha curcas* L. —Sareptian Mustard —*Brassica juncea* (L.) Czern. & Coss. ssp *juncea* —Unhusked beech —*Fagus sylvatica* L.		
Theobromine	Whole feeding stuffs except: —whole feeding stuffs for adult cattle	300 700
Vinylthiooxazolidone	Whole feeding stuffs for poultry except: —whole feeding stuffs for laying hens	1000 500
Volatile mustard oil	Straight feeding stuffs except: —rape cake or meal	100 4000 (expressed as allyl isothiocyanate)
	Whole feeding stuffs except:	150 (expressed as allyl isothiocyanate)
	—whole feeding stuffs for cattle, sheep and goats, (except calves, lambs and kids)	1000 (expressed as allyl isothiocyanate)
	—whole feeding stuffs for pigs (except piglets) and poultry	500 (expressed as allyl isothiocyanate)
Weed seeds and unground and uncrushed fruit containing alkaloids, glucoside or other toxic substances separately or in combination including:—	All feeding stuffs	3000
(*a*) *Lolium temulentum* L.		1000
(*b*) *Lolium remotum* Schrank		1000
(*c*) *Datura stramonium* L.		1000

SCHEDULE 4

(Section 74 and Regulation 2(15))

FEEDING STUFFS

Material	Limits of variation (percentages are percentages of the whole bulk)
Compound feeding stuff	Oil, 0·75% or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated; protein equivalent of biuret, isobutylidene diurea, urea, or urea phosphate, 1·25% or one-fifth of the amount stated, whichever is the greater; protein equivalent of uric acid if present in quantities of 1% or greater, 1·25% or one-quarter of the amount stated, whichever is the greater; fibre, if the actual amount exceeds that stated, 0·5% or one-eighth of the amount stated, whichever is the greater, if the actual amount is less than that stated, 0·5% or one-half of the amount stated, whichever is the greater.
Any material, not being a feed supplement, mentioned in this Part of this Schedule, containing cobalt, iodine, manganese, molybdenum, selenium or zinc	One-half of the amount of cobalt, iodine, manganese, molybdenum, selenium or zinc stated.
Any material, not being a feed supplement, mentioned in this Part of this Schedule containing copper	Where the amount of copper stated is between 50 mg/kg and 200 mg/kg, one-half of the amount stated. Where the amount stated exceeds 200 mg/kg, 30% of the amount stated.
Any material, not being a feed supplement, mentioned in this Part of this Schedule containing iron	Where the amount of iron stated is less than 250 mg/kg, one-half of the amount stated. Where the amount of iron stated is 250 mg/kg or greater, 30% of the amount stated.
Any material, not being a feed supplement, mentioned in this Part of this Schedule containing vitamins other than vitamin D_2 or vitamin D_3	Where the actual amount is less than that stated, 30% of the amount stated; in the case of an excess, no limit.
Any material, not being a feed supplement, mentioned in this Part of this Schedule containing vitamin D_2 or vitamin D_3	Where the amount of vitamin D_2 or vitamin D_3 stated does not exceed 4000 IU/kg, 50% of the amount stated. Where the amount of vitamin D_2 or vitamin D_3 stated exceeds 4000 IU/kg, 30% of the amount stated.
Any material mentioned in this Part of this Schedule containing magnesium	In the case of a deficiency of magnesium, 30% of the amount stated; in the case of an excess, no limit.
Alfalfa meal; lucerne meal	Protein, one-tenth of the amount stated; fibre, one-eighth of the amount stated.

Material	Limits of variation (percentages are percentages of the whole bulk)
Clover meal	Protein, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Coconut or copra cake or meal	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated.
Cotton cakes or meals not decorticated	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated.
Cotton cakes or meals from decorticated or partly decorticated cotton seed	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Dried brewery grains	Oil, 0·75%, or one-fifth of the amount stated, whichever is the greater; protein, one-fifth of the amount stated.
Dried distillery by-products (other than malt culms and dried yeast)	Oil, 0·75%, or one-fifth of the amount stated, whichever is the greater; protein, one-fifth of the amount stated; fibre (where present in excess of 2%) if the actual amount exceeds that stated, one-eighth of the amount stated, if the actual amount is less than that stated, one-half of the amount stated; lime expressed as calcium (Ca), if present in excess of 2%, one-fifth of the amount stated.
Dried grass Dried grass (maintenance quality) Dried green fodder crops Dried green roughage	Protein, one-tenth of the amount stated, provided that this limit of variation shall not operate so as to permit the application of the name "dried grass" to any article containing less than 13% protein or the names "dried grass (maintenance quality)" or "dried green fodder crops" to any material containing less than 10% protein.
Dried plain beet pulp	Fibre, one-eighth of the amount stated.
Dried yeast	Protein, one-twentieth of the amount stated.
Dried molassed beet pulp	Sugar, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Feed supplement	Protein equivalent of biuret, isobutylidene diurea, urea or urea phosphate, 1·25% or one-fifth of the amount stated, whichever is the greater; protein equivalent of uric acid if present in quantities of 1% or greater, 1·25% or one-quarter

Material	Limits of variation (percentages are percentages of the whole bulk)
	of the amount stated, whichever is the greater; copper, where the amount stated is 200 mg/kg or less, 50%, where the amount stated exceeds 200 mg/kg, 30%; iodine, iron, manganese and zinc, where the amount stated is 250 mg/kg or less, 50%, where the amount stated exceeds 250 mg/kg, 30%; cobalt, molybdenum and selenium, 50% of the amount stated; vitamin D_2 and vitamin D_3, 30% of the amount stated; vitamins other than vitamin D_2 or vitamin D_3, if the actual amount is less than that stated, 30% of the amount stated, in the case of an excess, no limit.
Feeding bone flour	Phosphorus, one-twentieth of the amount stated; protein, one-fifth of the amount stated.
Feeding bone meal, ground bone or any other bone product for feeding purposes	Phosphorus and protein, one-tenth of the respective amounts stated.
Feeding dried blood	Protein, one-twentieth of the amount stated.
Feeding meat meal or any other product of meat for feeding purposes Feeding meat and bone meal or any other product of meat and bone for feeding purposes	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein and phosphorus, one-tenth of the respective amounts stated; provided that these limits of variation shall not operate so as to permit the application of the names "feeding meat meal" and "feeding meat and bone meal" to materials containing less than 55% and less than 40% of protein respectively.
Fish meal, white fish meal, or any other product obtained by drying or grinding or otherwise treating fish or fish waste	Oil, 0·75% or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated; phosphorus, one-sixth of the amount stated; salt, 0·75%; provided that these limits of variation shall not operate so as to permit the application of the name "white fish meal" to material containing more than 6% of oil or 4% of salt.
Linseed cakes and the meals of such cakes; extracted linseed meal	Oil, 0·75%, or one-eighth of the amount stated, whichever is the greater; protein, one-eighth of the amount stated.
Linseed meal	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater.

Material	Limits of variation (percentages are percentages of the whole bulk)
Maize by-products, not otherwise mentioned in this Part of this Schedule	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Maize, flaked	Oil, 0·75%, or one-eighth of the amount stated, whichever is the greater; protein, one-eighth of the amount stated.
Maize germ cake or meal	Oil, 0·75%, or one-eighth of the amount stated, whichever is the greater; protein, one-eighth of the amount stated.
Maize gluten feed	Oil, 0·75%, or one-eighth of the amount stated, whichever is the greater; protein, one-eighth of the amount stated.
Malt culms	Protein, one-fifth of the amount stated; fibre, one-eighth of the amount stated.
Milk powders including oil or fat fortified milk powders	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated.
Mixture of molasses and biuret, isobutylidene diurea, urea, or urea phosphate	Sugar, one-tenth of the amount stated; protein equivalent of biuret, isobutylidene diurea, urea or urea phosphate, one-fifth of the amount stated.
Molasses feeds, as described in the table in Part II of Schedule 2	Sugar, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Oatmeal by-products	Fibre, one-eighth of the amount stated; provided that this limit of variation shall not operate so as to permit the application of the name "oatfeed" to any material containing more than 27% of fibre.
Oil cakes or meals not otherwise mentioned in this Part of this Schedule which are the product of any one decorticated or partly decorticated substance or seed from which oil has been removed	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Oil cakes or meals not otherwise mentioned in this Part of this Schedule which are the product of any one undecorticated substance or seed from which oil has been removed	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated.

Material	Limits of variation (percentages are percentages of the whole bulk)
Palm kernel cake or meal	Oil, 0·75%, or one-tenth of the amount stated, whichever is the greater; protein, one-tenth of the amount stated.
Poultry waste	Protein, one-fifth of the amount stated; protein equivalent of uric acid if present in quantities of 1% or greater, 1·25% or one-quarter of the amount stated, whichever is the greater; fibre, if the actual amount exceeds that stated, 0·5% or one-eighth of the amount stated, whichever is the greater, if the actual amount is less than stated, 0.5% or one-half of the amount stated, whichever is the greater; calcium, if present in excess of 2%, one-fifth of the amount stated.
Rape cake or meal	Oil, 0·75%, or one-eighth of the amount stated, whichever is the greater; protein, one-eighth of the amount stated.
Rice bran or rice meal, or the by-product produced in milling shelled rice	Oil, 0·75%, or one-tenth the of amount stated, whichever is the greater; protein, one-tenth of the amount stated; fibre, one-eighth of the amount stated.
Soya cake or meal	Oil, 0·75%, or one-eighth of the amount stated, whichever is the greater; protein, one-eighth of the amount stated.
Treacle or molasses	Sugar, one-twentieth of the amount stated.
Wheat offals or millers' offals	Fibre, if the actual amount exceeds that stated, one-eighth of the amount stated; if the actual amount is less than that stated, one-half of the amount stated.

SCHEDULE 5

(Sections 68(5), 69(5), 70(4), 71(3), 73(1), 74A, 75(1), 77(4), 78(6) and 79(3) and Regulation 2(17))

METHODS OF ANALYSIS OF FEEDING STUFFS

(Sections 68(5), 69(5), 70(4), 71(3), 73(1), 74A, 75(1), 77(4), 78(6) and 79(3) and Regulation 15)

1. *General*

When two or more methods are prescribed in this Schedule to determine a component of a feeding stuff the choice of method shall, except where otherwise indicated, be left to the agricultural analyst concerned; the method used must however be indicated in the certificate of analysis.

2. *Reagents and apparatus*

(*a*) All reagents used should be of analytical quality.

(*b*) Where water is mentioned this means purified water as defined in the European Pharmacopoeia.

(*c*) Solutions for which solvents are not prescribed must be aqueous.

(*d*) Only special instruments or apparatus requiring special standards are mentioned in the descriptions of the methods of analysis.

3. *Methods of Analysis*

1. Preparation of sample for analysis
2. Determination of moisture
3.*a* Determination of oil—in the presence of milk powder
b Determination of oil—in the absence of milk powder
4. Determination of protein
5. Determination of urea
6. Determination of uric acid
7. Determination of phosphorus
8. Determination of fibre
9. Determination of sugar
10. Determination of water-soluble chlorides
11. Determination of ash
12.*a* Determination of calcium—volumetric method
b Determination of calcium—atomic absorption method
13.*a* Determination of copper—diethyldithiocarbamate spectrophotometric method
b Determination of copper—atomic absorption spectrophotometric method
14.*a* Determination of magnesium—gravimetric method
b Determination of magnesium—atomic absorption spectrophotometric method
15. Determination of vitamin A (retinol)
16. Determination of thiamine hydrochloride (vitamin B_1, aneurine)
17. Determination of ascorbic acid and dehydroascorbic acid (vitamin C)
18. Determination of menadione (vitamin K_3)
19. Determination of hydrocyanic acid
20. Determination of volatile mustard oil
21. Determination of free and total gossypol
22.*a* Determination of aflatoxin B_1
b Determination of aflatoxin B_1

1. PREPARATION OF SAMPLE FOR ANALYSIS

1. With some materials, fine grinding may lead to loss or gain of moisture, and allowance for this must be made. Grinding should be as rapid as possible and unnecessary exposure to the atmosphere avoided. Grinding in a laboratory mill is usually quicker than grinding in a mortar although the latter is permissible.

2. If the sample is in a fine condition and passes through a sieve having apertures of about 1mm square(1)(3) mix thoroughly and transfer a portion of not less than 100g to a non-corrodible container provided with an air-tight closure.

3. If the sample does not wholly pass through a sieve having apertures of about 1mm square(1)(3) and wholly passes through a sieve having apertures from 2 to 3mm square(2)(3) mix thoroughly and further grind a portion of not less than 100g to pass through a sieve having apertures of about 1mm square (1)(3). Transfer the portion so prepared to a non-corrodible container provided with an air-tight closure.

4. If the sample is in a coarse condition as, for example, pieces of broken cake, carefully grind until the whole passes through a sieve having apertures of from 2 to 3mm square(2)(3). Mix thoroughly and further grind a portion of not less than 100g to pass through a sieve having apertures of about 1mm square(1)(3). Transfer the portion so prepared to a non-corrodible container provided with an air-tight closure.

5. If the sample is appreciably moist or if for any reason the processes of grinding and mixing are likely to result in loss or gain of moisture, take a sample immediately after the preliminary mixing procedure described in paragraph 3 or after the preliminary grinding and mixing procedure described in paragraph 4 for the determination of moisture by the method described in method 2. Determine also the moisture content in the finally prepared sample so that the results of the analysis may be corrected to correspond with the sample in its original condition as regards moisture.

6. If, because of its physical condition, grinding is difficult, take a portion immediately after the preliminary mixing procedure described in paragraph 3 or after the preliminary grinding and mixing procedure described in paragraph 4 for the determination of moisture by the method described in method 2. Dry the sample until grinding with an iron mortar and pestle, or by other means, enables the sample to be passed completely through a sieve having apertures of about 1mm square(1)(3). Determine also the moisture content in the finally prepared sample so that the results of the analysis may be corrected to correspond with the sample in its original condition as regards moisture.

7. Treat by any other suitable means materials which cannot conveniently be ground or passed through a sieve.

2. DETERMINATION OF MOISTURE

1. PURPOSE AND SCOPE

This method is for the determination of moisture in feeding stuffs.

2. PRINCIPLE

The sample is dried to constant weight in an oven at 100°C. The loss in weight corresponds to the moisture content of the sample.

3. APPARATUS

3.1 Suitable containers with lids ensuring air-tight closure; the dimensions should allow the sample to be spread at about 0·3g per cm^2.

3.2 Electrically heated oven, suitably ventilated and capable of being maintained at 100 ± 1°C.

(1) Test sieves conforming to British Standard 410: 1969 are suitable.

(2) Test sieves of nominal aperture sizes 2·00, 2·36 or 2·80mm conforming to British Standard 410: 1969 are suitable.

(3) Where an analysis for copper has to be carried out, a stainless steel sieve should be used.

4. PROCEDURE

Weigh to the nearest 0·001g, approximately 5g of the prepared sample and transfer to a previously weighed container (3.1). Place the uncovered container and the lid in the oven (3.2) for 2 to 3 hours. Replace the lid on the container, remove from the oven and allow to cool in a desiccator and weigh. Reheat for another hour, cool and reweigh. If the difference in weight exceeds 0·010g continue the heating and cooling procedure until a weight constant within 0·002g is attained.

5. CALCULATION OF RESULT

Calculate the total loss of weight and express it as a percentage of the original weight.

3*a*. DETERMINATION OF OIL—IN THE PRESENCE OF MILK POWDER

1. PURPOSE AND SCOPE

This method is for the determination of oil in milk powders, including oil or fat fortified milk powders, and also for feeding stuffs containing milk powder and/or oil or fat fortified milk powder.

2. PRINCIPLE

The sample is suspended in water, alcohol and ammonia added and the oil is extracted with a mixture of diethyl ether and light petroleum. The mixed solvent is distilled off and the residue dried and weighed.

3. REAGENTS

3.1 Ammonia solution (density 0·88g/ml).

3.2 Diethyl ether, peroxide free.

3.3 Ethanol 95% (V/V).

3.4 Light petroleum, boiling range 40–60°C.

4. APPARATUS

4.1 Fat extraction tube(1) provided with a glass stopper and siphon tube.

5. PROCEDURE

Weigh to the nearest 0·001g, approximately 1g of the prepared sample and transfer to the extraction tube (4.1).

Add 9ml water, temperature 60–70°C, stopper the tube and shake vigorously until the sample is uniformly suspended. Cool to room temperature, add 1·5ml ammonia solution (3.1) stopper and shake thoroughly. Add 10ml ethanol (3.3) using some to rinse the stopper and collect the washings in the extraction tube. Stopper the tube and shake thoroughly. Add 25ml diethyl ether (3.2) using some to wash the stopper as before, stopper the tube and shake vigorously for 90 seconds. Cool the tube and remove the stopper cautiously so as to avoid loss of contents. Add 25ml light petroleum (3.4), washing the stopper as before, stopper the tube and shake vigorously for 90 seconds. Centrifuge or allow to stand so that the solvent layer separates cleanly. Remove the stopper, insert a siphon tube and transfer the ether layer to a flask. Raise the siphon and, before removing it from the tube, wash it down with 15ml of diethyl ether (3.2). Remove the siphon tube and rinse the tip with ether, collecting the rinsings in the flask. Add 1ml ethanol (3.3) to the tube, stopper, shake vigorously for 90 seconds, cool, remove the stopper, add 15ml light petroleum (3.4) and again shake for 90 seconds. Allow to stand for 15 minutes or until the solvent layer separates cleanly, fit the siphon tube and remove the solvent layer to the flask as before.

Carry out a third extraction with 15ml diethyl ether (3.2) followed by 15ml light petroleum (3.4) in the same way, collecting the solvent in the flask. Remove the solvent from the flask by evaporation and dry the flask lying on its side at 100°C for 2 hours; cool in a desiccator and weigh. Reheat at 100°C for 30 minutes, cool and weigh.

(1) British Standard 1743: 1968, fig. 1 is suitable.

The second weight should not differ by more than 0·002g from the first weight. Add about 20ml light petroleum (3.4) to the flask and swirl gently to dissolve the oil, warming if necessary. Allow any residue to settle, then decant the supernatant solution taking care to retain any insoluble residue. Add another 20ml light petroleum (3.4) swirl cautiously and decant as before. Repeat with further small quantities of light petroleum until all the oil has been removed from the flask. Reheat the flask, lying on its side, at 100°C for 1 hour, allow to cool and weigh.

6. CALCULATION OF RESULTS

The oil content of the sample is calculated from the weight of extract soluble in light petroleum expressed as a percentage of the weight of the test portion.

3*b*. DETERMINATION OF OIL—IN THE ABSENCE OF MILK POWDER

1. PURPOSE AND SCOPE

This method is for the determination of oil in feeding stuffs *not* containing milk powder, or oil or fat fortified milk powder.

2. PRINCIPLE

The sample is extracted with light petroleum, the solvent is distilled off and the extract dried and weighed.

3. REAGENTS

3.1 Light petroleum, boiling range 40–60°C.

4. APPARATUS

4.1 Extraction apparatus.

5. PROCEDURE

Weigh to the nearest 0·001g, 3 to 5g of the prepared sample, transfer it to an extraction thimble, place in the apparatus (4.1) and extract with light petroleum (3.1) for a period of at least 4 hours; collect the extract in a suitable flask. Remove the thimble from the apparatus, allow to dry in the air, transfer the feeding stuff to a small mortar and grind lightly. Return the feeding stuff to the thimble, wash the mortar with a small quantity of light petroleum and add the washings to the contents of the extraction flask. Continue the extraction for at least another hour. Filter the extract quantitatively through suitable paper or a cotton wool plug into a weighed flask rinsing the extraction flask and filter, with light petroleum (3.1). Remove the bulk of the solvent by evaporation, dry at 100°C for 2 hours, cool and weigh. Reheat at 100°C for 30 minutes, cool and weigh. Continue, if necessary, the heating and cooling procedure until a weight constant within 0·002g is attained. Regard this light petroleum extract as oil.

Where a sample is presumed to have an oil content in excess of 10% or where there is reason to believe that the whole of the oil will not be removed from the feeding stuff in a 5 hours extraction, place a fresh flask on the extraction apparatus and continue the extraction with a fresh quantity of light petroleum for at least a further hour. Filter and wash into a second weighed flask; dry and weigh as described in the preceding paragraph.

6. CALCULATION OF RESULTS

Calculate the weight of material extracted as a percentage of original weight and regard as oil.

4. DETERMINATION OF PROTEIN

1. PURPOSE AND SCOPE

This method is for the determination of protein in feeding stuffs by the Kjeldahl method for nitrogen.

2. PRINCIPLE

The sample is digested by mineral acid. The acid solution is made alkaline by a sodium hydroxide solution. The ammonia released is removed by distillation and collected in a measured quantity of sulphuric acid, the excess of which is titrated with a solution of sodium hydroxide.

3. REAGENTS

3.1 Catalyst: mercuric oxide.

3.2 Potassium sulphate or anhydrous sodium sulphate.

3.3 Sucrose.

3.4 Zinc, granulated.

3.5 Pumice stone, granulated, washed in hydrochloric acid and ignited.

3.6 Sulphuric acid (density 1·84 g/ml).

3.7 Sodium hydroxide solution, carbonate free: dissolve 400g sodium hydroxide in water and dilute to 1 litre.

3.8 Sodium sulphide, cold saturated solution.

3.9 Sodium thiosulphate solution: 8g sodium thiosulphate ($Na_2S_2O_3$. $5H_2O$) per 100ml.

3.10 Sodium hydroxide, 0·1N solution.

3.11 Sodium hydroxide, 0·25N solution.

3.12 Sulphuric acid, 0·1N solution.

3.13 Sulphuric acid, 0·5N solution.

3.14 Methyl red indicator solution: dissolve 0·3g methyl red in 100ml of ethanol (95 96% V/V).

3.15 Screened methyl red indicator solution:

(*a*) dissolve 0·2g methyl red in 100ml of ethanol (95–96% V/V).

(*b*) dissolve 0·1g methylene blue in 100ml of ethanol (95–96% V/V).

Mix 1 volume of (*a*) with 1 volume of (*b*).

4. APPARATUS

Apparatus for mineral acid digestion and distillation according to Kjeldahl's method.

5. PROCEDURE

5.1 *Mineral acid digestion*

Weigh to the nearest 0·001g, approximately 1g of the prepared sample, and transfer to a Kjeldahl Flask. Add 10g potassium sulphate (3.2) or sodium sulphate (3.2), 0·6 to 0·7g mercuric oxide (3.1), 25ml sulphuric acid (3.6) and a few grains of pumice stone (3.5). Mix. Heat the flask moderately at first, shaking from time to time, until the mass is carbonised and the froth has disappeared; then increase the heat and bring the liquid to a steady boil. Avoid overheating the walls which may cause organic particles to stick to the sides of the flask. When the solution appears clear and colourless continue boiling for a further two hours. Then allow to cool.

Note: If after the digestion and cooling crystallisation occurs repeat the analysis. If crystallisation still occurs, repeat the analysis using a larger quantity of sulphuric acid.

5.2 *Distillation*

Add 250–350ml water carefully mixing the contents during the addition; allow to cool. Then add a few pieces of zinc (3.4).

Transfer 25·0ml 0·1N or 0·5N sulphuric acid (3.12) or (3.13) to the collecting flask of the distillation apparatus, according to the presumed level of nitrogen and a few drops of methyl red indicator (3.14) or (3.15).

Taking precautions against loss of ammonia, carefully add 100ml sodium hydroxide solution (3.7) and then add either 10ml sodium sulphide solution (3.8) or 25ml sodium thiosulphate solution (3.9). Mix well and connect immediately to the distillation apparatus.

Heat the flask so that approximately 150ml of the liquid is distilled in 30 minutes. At the end of this time, check the pH of the resulting distillate with indicator paper. If the reaction is alkaline, continue the distillation. Discontinue distillation when the distillate appears neutral to indicator paper. During the distilling process, swirl the contents of the collecting flask from time to time.

Note: If the contents of the collecting flask become alkaline, the determination should be abandoned and the experiment repeated making appropriate adjustments.

5.3 *Titration*

In the collecting flask titrate the excess sulphuric acid with sodium hydroxide solution 0·1N (3.10) or 0·25N (3.11), according to the normality of the sulphuric acid employed, to the end point of the indicator, (3.14) or (3.15).

5.4 *Blank test*

Carry out a blank test on the reagents using 1g sucrose in place of the sample, and allow for this in the calculation of the results.

6. CALCULATION OF RESULTS

Determine the volume of sulphuric acid consumed. 1ml 0·1N sulphuric acid = 1·4mg nitrogen.

Calculate the percentage of nitrogen in the sample and convert to percentage protein by multiplying the result by 6·25.

Note: Where it is believed that the sample contains nitrogen in the form of ammoniacal or nitrate nitrogen, the appropriate determination should be made as described in paragraph 3.52, 3.53, 3.6 or 3.7 of Schedule 6 of the 1973 Fertilisers and Feeding Stuffs Regulations and the amount so obtained deducted from the total nitrogen content. Except in the case of compound feeding stuffs and feed supplements for ruminants:—

(*a*) where it is believed also that the sample contains nitrogen in the form of urea nitrogen or urea phosphate, the appropriate determination should be made as described in method 5 of this Schedule and the amount so obtained deducted from the total nitrogen content;

(*b*) where it is believed also that the sample contains nitrogen in the form of uric acid nitrogen, the appropriate determination should be made as described in method 6 of this Schedule and the amount so obtained deducted from the total nitrogen content; and

(*c*) where it is believed also that the sample contains nitrogen in the form of biuret nitrogen or in the form of 1,1′—isobutylidenediurea nitrogen, the amount of nitrogen contributed by these ingredients should be determined and deducted from the total nitrogen content.

5. DETERMINATION OF UREA

1. PURPOSE AND SCOPE

This method is for the determination of urea in feeding stuffs.

2. PRINCIPLE

The sample is suspended in water with a clarifying agent and filtered. The urea content of the filtrate is determined after the addition of 4-dimethylaminobenzaldehyde (4-DMAB) by measuring the absorbance at 435nm.

3. REAGENTS

3.1 Activated charcoal.

3.2 Carrez solution I: dissolve 21·9g of zinc acetate dihydrate in water, add 3ml of glacial acetic acid and dilute to 100ml with water.

3.3 Carrez solution II: 10·6g potassium ferrocyanide per 100ml.

3.4 Hydrochloric acid 0·02N.

3.5 Sodium acetate solution: 136g sodium acetate trihydrate per litre.

3.6 4-Dimethylaminobenzaldehyde solution: dissolve 1·6g of 4-dimethylaminobenzaldehyde (4-DMAB) in 100ml of 96% ethanol and add 10ml of hydrochloric acid (density 1·18 g/ml).

3.7 Urea standard solution: 1·0g urea per 100ml.

4. APPARATUS

4.1 Tumbler mixer, or shaker.

4.2 Spectrophotometer with 10mm cells.

5. PROCEDURE

5.1 *Dissolution of sample*

Weigh to the nearest 0·001g, approximately 2g of the prepared sample or a suitable amount expected to contain between 50 and 500mg of urea, and transfer to a 500ml graduated flask. Add 150ml 0·02N hydrochloric acid (3.4) shake for 30 minutes then add 10ml sodium acetate solution (3.5) and mix well. Add 1g activated charcoal (3.1) to the flask and shake well and stand for a further 15 minutes. Add 5ml Carrez solution I (3.2), followed by 5ml Carrez solution II (3.3), mixing well between additions. Dilute to volume with water and mix well. Filter a portion through a dry filter paper into a clean dry 250ml beaker.

5.2 *Determination*

Transfer 10ml of the filtrate to a ground glass stoppered test tube, add 10ml of 4-DMAB solution (3.6) mix and allow to stand for 15 minutes. Measure the absorbance of the solution at 435nm, in a 10mm cell, against a reference solution prepared from the reagents.

5.3 *Calibration curve*

Dilute 1, 2, 4, 5 and 10ml of the urea solution (3.7) to 100ml with water. Transfer 10ml of each solution to ground glass stoppered test tubes, and add 10ml of 4-DMAB solution (3.6) to each; mix and proceed as described above (5.2). Construct a graph relating the absorbances to the amounts of urea present.

6. CALCULATION OF RESULTS

Determine the amount of urea in the sample by reference to the calibration curve. Express the result as a percentage of the sample. (% urea × 0·4665 = % urea nitrogen).

Note: If the sample is highly coloured the proportion of activated charcoal must be increased up to 5g. The final solution after filtering should be colourless.

6. DETERMINATION OF URIC ACID

1. PURPOSE AND SCOPE

This method is for the determination of uric acid and its salts in dried poultry waste and in feeding stuffs containing dried poultry waste.

2. PRINCIPLE

Uric acid is extracted with neutral ethanolic formaldehyde solution, precipitated as silver magnesium urate, redissolved in sodium thiosulphate solution and determined spectrophotometrically.

3. REAGENTS

3.1 Sodium hydroxide solution: dissolve 50g sodium hydroxide in 50ml water, mix well and store in a suitable plastic container.

3.2 Formaldehyde solution: the strength of the commercially available solution should be checked as follows:

mix 3·00ml formaldehyde solution with 50·00ml 1N sodium hydroxide solution and 25·00ml hydrogen peroxide solution (20 volumes). Heat on a steam bath until effervescence stops. Cool, and titrate with 1N hydrochloric acid using phenolphthalein indicator. Carry out a blank titration using 3·00ml water in place of the formaldehyde.

1ml of 1N sodium hydroxide≡0·0300g formaldehyde

strength of formaldehyde solution $= (B-T) \times \frac{0{\cdot}0300 \times 100}{3}$ g per 100ml

where B = blank titre; and
T = sample titre.

3.3 Neutral ethanolic formaldehyde solution: mix an appropriate volume of formaldehyde solution (3.2) containing 17·5g of formaldehyde with 250ml water and 500ml ethanol. Adjust the pH of the solution to 7·0 with 0·1N sodium hydroxide solution. Dilute to 1 litre with water, mix and again adjust the pH to 7·0 if necessary.

3.4 Succinate buffer solution: dissolve by heating, 29·5g of succinic acid in 750ml water and 20ml sodium hydroxide solution (3.1). Cool, add an appropriate volume of formaldehyde solution (3.2) containing 17·5g of formaldehyde, mix well and adjust the pH to 6·0 with sodium hydroxide solution (3.1). Dilute to 1 litre with water, mix and again adjust the pH to 6·0 if necessary.

3.5 Sodium thiosulphate solution: 25g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) per litre.

3.6 Silver lactate solution: dissolve, by heating, 3g silver lactate in 50ml water and 1ml lactic acid. Dilute to 100ml with water, filter, and store in dark glassware. Do not expose to strong light.

3.7 Ammoniacal magnesium solution: dissolve 8·75g magnesium sulphate ($MgSO_4.7H_2O$) and 17·5g ammonium chloride in 50ml water. Add 30ml ammonia solution (density 0·88g per ml) mix well and dilute to 100ml with water.

3.8 Benedict and Hitchcock reagent: mix 35ml silver lactate solution (3.6) with 15ml ammoniacal magnesium solution (3.7). Add 50ml ammonia solution (density 0·88g per ml). Mix well. Prepare *immediately* before use.

3.9 Standard uric acid solution: weigh to the nearest 0·1mg, 250mg of uric acid and transfer to a 150ml round-bottom flask fitted with a reflux condenser. Add 100ml ethanolic formaldehyde solution (3.3) and boil under reflux on a steam bath for 30 minutes, shaking frequently. Cool, transfer the solution to a 250ml graduated flask, wash the round-bottomed flask with ethanolic formaldehyde solution (3.3), combine the washings with the uric acid solution. Dilute to the mark with ethanolic formaldehyde solution (3.3) and mix. 1ml contains 1mg of uric acid.

3.10 Light petroleum, boiling range 40–60°C.

4. APPARATUS

4.1 Spectrophotometer, with 10mm silica cells.

4.2 Percolation tubes, glass. Upper part: approximately 24cm long, 18mm internal diameter; lower part approximately 12cm long 8mm internal diameter.

5. PROCEDURE

5.1 Extraction of Uric Acid.

5.1.1 From dried poultry waste:

weigh to the nearest 0·001g about 0·40g dried poultry waste and place in a 150ml round-bottomed flask. Add 60ml ethanolic formaldehyde

solution (3.3), fit a reflux condenser onto the flask and heat on a steam bath for 1 hour. Cool and filter by suction through a sintered glass crucible (porosity 4) into a 100ml graduated flask. Wash out the round-bottomed flask with 3 × 10ml portions of ethanolic formaldehyde solution (3.3) passing each portion through the crucible into the graduated flask. Dilute to 100ml with ethanolic formaldehyde solution and mix.

5.1.2 From feeding stuffs:
weigh to the nearest 0·001g between 4g and 5g of prepared sample. Transfer to a glass percolation tube (4.2) fitted with a small paper cup to retain the feed. Remove the fat from the feed by extraction with light petroleum (3.10). Transfer quantitatively the defatted sample to a 150ml round-bottomed flask and remove the residual solvent with a slow current of air. Continue as in 5.1.1, second sentence . . . 'Add 60ml ethanolic formaldehyde solution (3.3) . . .'

5.2 DETERMINATION

Transfer by pipette 20·0ml of the sample extract prepared as in 5.1.1 or 5.1.2 to a 50ml centrifuge tube. Add 10ml of Benedict and Hitchcock reagent (3.8), mix well and allow to stand in the dark for 1 hour. Centrifuge at 2000rpm for 15 minutes, pour off the supernatant liquid and allow to drain for 10 minutes. Carefully wipe off any remaining liquid without disturbing the precipitate, and add 20·0ml sodium thiosulphate solution (3.5) to each tube. Dissolve the precipitate by stirring with a thin glass rod. Transfer by pipette 5·0ml of this solution into a 200ml graduated flask containing 40·0ml succinate buffer solution (3.4). Dilute to 200ml with water and mix well. Measure the absorbance of the solution at 294nm in 10mm silica cells against a solution prepared by mixing 5·0ml sodium thiosulphate solution (3.5) with 40·0ml succinate buffer solution (3.4) and diluting to 200ml with water. Determine the quantity of uric acid present by reference to the calibration curve (5.3).

5.3 CALIBRATION CURVE

Into a series of 50ml centrifuge tubes, transfer by pipette 2, 4, 6, 8, 10, 12ml standard uric acid solution (3.9) (corresponding to 2, 4, 6, 8, 10 and 12mg of uric acid) and make up to 20·0ml with ethanolic formaldehyde solution (3.3). Add to each tube 10·0ml Benedict and Hitchcock reagent (3.8), mix well and stand in the dark for 1 hour. Continue as in 5.2 from . . . . 'Centrifuge at 2000rpm . . .'. Measure the absorbances of the solutions and plot the calibration curve using absorbances as the ordinates and the corresponding quantities of uric acid, in mg (as shown above) as the abscissae.

6. CALCULATION

The uric acid nitrogen content per cent of the sample is given by the formula:

$$\frac{A}{6 \times W}$$

where A = mg uric acid (in the aliquot volume of the sample extract) as determined by photometric measurement; and

W = weight of sample in grams.

7. DETERMINATION OF PHOSPHORUS

1. PURPOSE AND SCOPE

This method is for the determination of total phosphorus in feeding stuffs.

2. PRINCIPLE

The sample is either ashed (in the case of organic feeding stuffs) or digested with acid (in the case of mineral compounds and liquid feedingstuffs). An acidic solution is treated with molybdovanadate reagent and the absorbance of the yellow solution is measured at 430nm.

3. REAGENTS

3.1 Calcium carbonate.

3.2 Nitric acid (density 1·42g/ml).

3.3 Sulphuric acid (density 1·84 g/ml).

3.4 Hydrochloric acid, 50% (V/V): dilute an appropriate volume of hydrochloric acid (density 1·18g/ml) with an equal volume of water.

3.5 Nitric acid, 10% (V/V): dilute 10ml of nitric acid (3.2) to 100ml with water.

3.6 Molybdovanadate reagent: dissolve separately 20g of ammonium molybdate $[(NH_4)_6\ Mo_7O_{24}.4H_2O]$ and 0·47g of ammonium vanadate in water, mix, acidify with 140ml of nitric acid (3.2) and dilute to 1 litre.

3.7 Phosphorus standard solution: dissolve 4·387g of potassium dihydrogen phosphate, previously dried at 105°C for 1 hour, in water and dilute to 1 litre. 1ml = 1mg phosphorus (P).

4. APPARATUS

4.1 Muffle furnace capable of being maintained at 550 ± 5°C.

4.2 Spectrophotometer with 10mm cells.

5. PROCEDURE

5.1 *Dissolution of the sample*

According to the nature of the sample, prepare a solution as indicated in either 5.1.1 or 5.1.2.

5.1.1 *For samples containing organic feeding stuffs free from calcium and magnesium phosphates*

Weigh to the nearest 0·001g, approximately 2·5g of the prepared sample and transfer to a silica or porcelain crucible. Add 1g of calcium carbonate (3.1), mix well and incinerate at 550°C ± 5°C until a white or grey ash is obtained. Allow to cool, transfer the ash quantitatively to a 250ml beaker, add 20ml of water and sufficient hydrochloric acid (3.4) until effervescence ceases, taking suitable precautions to avoid loss. Add a further 10ml of hydrochloric acid (3.4) and evaporate to dryness to make the silica insoluble. Cool, treat the residue with 10ml of nitric acid (3.5), and boil for five minutes, avoid evaporating to dryness. Transfer the solution to a 500ml graduated flask, rinsing the beaker several times with hot water. Cool to room temperature, make up to volume with water, mix and filter.

5.1.2 *Alternative procedure, especially suitable for mineral compounds and liquid feeding stuffs*

Weigh to the nearest 0·001g approximately 1g of the prepared sample and transfer to a Kjeldahl flask. Add 20ml of sulphuric acid (3.3), shake to break up the sample and to prevent it sticking to the sides of the flask. Boil for ten minutes, allow to cool, add 2ml of nitric acid (3.2) and bring back to boiling point. Repeat the procedure of addition of nitric acid until a colourless solution is obtained. Cool, cautiously add a small volume of water and transfer the solution to a 500ml graduated flask, rinsing the Kjeldahl flask with hot water. Cool to room temperature, make up to volume with water, mix and filter.

5.2 *Determination*

Dilute if necessary, the filtrate as obtained under 5.1.1 or 5.1.2 in order to obtain a phosphorus concentration of not more than 40μg/ml. Transfer 10ml of this solution to a glass stoppered test tube, add 10ml of freshly prepared molybdovanadate reagent (3.6) and mix. Allow to stand for ten minutes at 20°C, and then measure the absorbance at 430nm against a freshly prepared reference solution prepared by adding 10ml of molybdovanadate reagent (3.6) to 10ml of water.

5.3 *Calibration curve*

From the standard solution (3.7) prepare solutions containing respectively 5, 10, 20, 30 and 40μg of phosphorus per ml. Transfer 10ml of each of these solutions to glass stoppered test tubes, add 10ml of molybdovanadate reagent, mix and proceed as under 5.2. Construct a graph relating the absorbances to the amount of phosphorus present. The calibration curve should be newly constructed, if possible at the same time as the determination is carried out.

6. CALCULATION OF RESULTS

Determine the amount of phosphorus in the test sample by reference to the calibration curve. Express the result as a percentage of the sample.

8. DETERMINATION OF FIBRE

1. PURPOSE AND SCOPE

This method is for the determination of the fibre content of feeding stuffs.

2. PRINCIPLE

The sample is defatted and treated successively with boiling solutions of sulphuric acid and sodium hydroxide of specified concentrations. The residue is separated by filtration, washed, dried, weighed and ashed. The loss of weight resulting from ashing corresponds to the fibre present in the test sample.

3. REAGENTS

3.1 Sulphuric acid, 0·255N solution.

3.2 Sodium hydroxide, 0·313N solution: the solution must be free or nearly free from sodium carbonate.

3.3 Antifoam agent (eg silicone).

3.4 Ethanol, 95% (V/V).

3.5 Diethyl ether.

3.6 Light petroleum, boiling range 40–60°C.

3.7 Hydrochloric acid: dilute 10ml of hydrochloric acid (density 1·18g/ml) with water to 1 litre.

4. APPARATUS

4.1 Conical flask, 1000ml.

4.2 Buchner flask.

4.3 Buchner funnel.

4.4 Platinum or silica crucibles.

4.5 Electric muffle furnace.

4.6 Extraction apparatus, for removal of fatty material.

5. PROCEDURE

Weigh to the nearest 0·001g 2·7 to 3·0g of the prepared sample, transfer to the extraction apparatus (4.6) and extract with light petroleum (3.6). Alternatively extract with light petroleum by stirring, settling and decanting three times. Air dry the extracted sample and transfer to a dry 1000ml conical flask (4.1). Add 200ml of sulphuric acid (3.1), measured at ordinary temperature and brought to boiling point, the first 30 to 40ml being used to disperse the sample; heat to boiling point within 1 minute. An appropriate amount of antifoam agent (3.3) may be added if necessary. Boil gently for exactly 30 minutes, maintaining a constant volume and rotating the flask every few minutes in order to mix the contents, and remove particles from the sides.

Meantime fit a Buchner funnel with suitable filter paper. This should be of such quality that it does not release any paper fibre during washings(1) (it is convenient

(1) Whatman No. 541 or equivalent.

to use two filter papers, thereby facilitating the transfer of the insoluble matter at a later stage). Pour boiling water into the funnel, allow to remain until the funnel is hot and then drain by applying suction.

At the end of the 30 minutes boiling period, allow the acid mixture to stand for 1 minute and then pour immediately into a shallow layer of hot water under gentle suction in the prepared funnel. Adjust the suction so that the filtration of the bulk of the 200ml is completed within 10 minutes. Repeat the determination if this time is exceeded.

Wash the insoluble matter with boiling water until the washings are free from acid; then wash back into the original flask by means of a wash bottle containing 200ml 0·313N sodium hydroxide solution (3.2) measured at ordinary temperature and brought to boiling point. Boil for 30 minutes with the same precautions as those used in the earlier boiling and treatment. Allow to stand for 1 minute and then filter immediately through a suitable filter paper. Transfer the whole of the insoluble material to the filter paper by means of boiling water, wash first with boiling water then with dilute hydrochloric acid (3.7) and finally with boiling water until free from acid. Then wash twice with ethanol (3.4) and three times with diethyl ether (3.5). Transfer the insoluble matter to a dried weighed ashless filter paper(1) and dry at 100°C to a constant weight. Allow to cool in a desiccator and weigh. Transfer the paper and insoluble matter to a crucible (4.4) previously ignited to constant weight. Incinerate the paper and contents to an ash at a dull red heat, to a constant weight. Allow to cool in a desiccator and weigh.

6. CALCULATION OF RESULTS

The fibre content, as a percentage of the sample, is given by the formula:

$$\frac{d-(p+a)\times 100}{w}$$

in which: d = weight of the paper + insoluble matter after drying (g);
p = weight of the paper (g);
a = weight of the ash (g); and
w = weight of sample (g).

Note: In the event of the sample containing 3% or more of calcium carbonate (chalk or limestone flour), it will be necessary to remove the calcium carbonate before digesting the sample with acid. This can be done at the stage in the procedure when the portion taken for analysis has been extracted with light petroleum. The original weight taken for the determination should be such that the actual amount of feeding stuff free from calcium carbonate is between 2·7 and 3·0g.

Transfer the air-dried extracted sample to a 1000ml conical flask, add a quantity of hydrochloric acid (3.7) more than sufficient to neutralise the calcium carbonate present and stir well. Allow to settle, decant off the supernatant liquid through a filter and wash the residue twice by decantation with water, passing the washings through the filter. Allow the residue and the filter to drain thoroughly. Bring 200ml 0·255N sulphuric acid (3.1) (measured at ordinary temperature) to boiling point and use a portion of this to wash any particles on the filter back into the flask. Add the remainder of the acid to the flask and heat to boiling point within 1 minute. Add an appropriate amount of antifoam agent (3.3) if necessary and continue the determination as described in section 5, commencing—'boil gently for exactly 30 minutes'.

9. DETERMINATION OF SUGAR

1. PURPOSE AND SCOPE

This method is for the determination of sugar in feeding stuffs.

2. PRINCIPLE

The sugar is extracted from the sample with water, the solution clarified and the total reducing sugar content is determined after inversion of the sucrose.

(1) Whatman No. 541 or equivalent.

3. REAGENTS

3.1 *Fehling's solution*

3.1.1 Copper sulphate solution: dissolve 69·28g copper sulphate ($CuSO_4.5H_2O$) in water and dilute to 1 litre.

3.1.2 Potassium sodium tartrate solution: dissolve 346g potassium sodium tartrate and 100g sodium hydroxide in water and dilute to 1 litre. For use, mix equal volumes of solutions 3.1.1 and 3.1.2.

Note: The strength of the Fehling's solution should be such that 10ml is equivalent to 0·0525g invert sugar. It should be checked as follows: dissolve 2·375g sucrose (dried at 100°C) in about 100ml water in a 300ml beaker, add 15ml hydrochloric acid (3.2) and sufficient water to give a volume of 150ml. Heat to boiling point, boil for 2 minutes; cool, add 2 or 3 drops of phenolphthalein indicator solution (3.8), just neutralise with sodium hydroxide solution (3.6) transfer to a 500ml graduated flask and dilute to 500ml. Then follow the procedure described in paragraph 4.2.3: 1ml of this solution≡ 0·00475g sucrose≡0·005g invert sugar, ie 10ml Fehling's solution≡ 10·5ml of this standard invert sugar solution.

3.2 Hydrochloric acid solution, N.

3.3 Carrez solution I: dissolve 21·9g zinc acetate dihydrate in water, add 3ml glacial acetic acid and dilute to 100ml with water.

3.4 Carrez solution II: dissolve 10·6g potassium ferrocyanide in water and dilute to 100ml.

3.5 Potassium oxalate solution: dissolve 5g potassium oxalate in water and dilute to 100ml.

3.6 Sodium hydroxide solution: dissolve 10g sodium hydroxide in water and dilute to 100ml.

3.7 Methylene blue solution: dissolve 2·5g methylene blue in water and dilute to 250ml.

3.8 Phenolphthalein indicator solution: dissolve 0·25g phenolphthalein in 150ml industrial methylated spirit and dilute with water to 250ml.

4. PROCEDURE

4.1 *Extraction*

4.1.1 Sample in solid form

Weigh to the nearest 0·01g, approximately 10g of the prepared sample, or a sufficient quantity to obtain about 2g sugar. Grind in a mortar with warm water at a temperature not exceeding 60°C and transfer to a 500ml graduated flask using about 400ml water. Shake the flask at intervals during 30 minutes. Add 5ml potassium oxalate solution (3.5) to the contents of the flask, followed by 5ml Carrez solution I (3.3); mix well and then add 5ml Carrez solution II (3.4) make up to the mark with water at room temperature, mix well and filter. Determine the sugar in 100ml of the filtrate by the procedure described in paragraph 4.2.

4.1.2 Sample in liquid form

Weigh to the nearest 0·001g, approximately 5g of the prepared sample and wash with water into a 250ml graduated flask, using about 200ml water. To clear the solution add 5ml Carrez solution I (3.3). Mix, then add 5ml Carrez solution II (3.4), again mix, dilute to the mark with water, mix and filter. Determine the sugar in 25ml of the filtrate by the method described in paragraph 4.2.

4.2 *Determination*

4.2.1 Inversion

Transfer the measured volume of filtrate obtained as described in paragraph 4.1.1 or 4.1.2 to a 300ml beaker, add 15ml hydrochloric acid (3.2),

dilute to 150ml with water, cover with a watch glass and heat to boiling point. Continue to boil for 2 minutes, cool, add 2 or 3 drops of phenolphthalein indicator solution (3.8), just neutralise with sodium hydroxide solution (3.6), transfer to a 200ml graduated flask and dilute to the mark. Filter if necessary.

4.2.2 Preliminary estimation

(This estimation is usually necessary where the percentage of sugar is unknown)

Transfer 10·0ml Fehling's solution (3.1) to a 250ml conical flask and add 20ml of water. Add from a burette approximately 10ml of the filtrate prepared as described in paragraph 4.2.1, heat to boiling point and boil briskly for 1 minute. Add 3 drops of methylene blue solution (3.7) and titrate from the burette at the rate of 1ml per 15 seconds until the blue colour is discharged, the contents of the flask being kept boiling throughout the titration. Note the total number of ml required, and call this x ml. This titration should not be outside the range of 15–40ml otherwise the determination should be repeated using a more appropriate volume of the filtrate.

4.2.3 Exact determination

To 10ml Fehling's solution (3.1) in a 250ml conical flask add from a burette (x–1)ml of the filtrate prepared as described in paragraph 4.2.1, together with sufficient water to make a total volume 60ml. Heat to boiling point, boil briskly for 1½ minutes and add 3 drops of methylene blue solution (3.7). Titrate from the burette at the rate of approximately 0·25ml per 15 seconds until the blue colour is discharged, the contents of the flask being kept boiling briskly throughout the titration which must not take more than 1½ minutes. The total number of ml used in the determination equals the sugar equivalent of 10ml Fehling's solution

10ml Fehling's solution ≡ 0·0525g invert sugar

Not more than 1ml of filtrate should be required for the completion of the titration. If more than 1ml is required, then the determination should be repeated, using a more closely calculated volume of filtrate for the original addition. The time taken from the initial boiling point until the end of the titration should be about 3 minutes. If this time is exceeded by more than 20 seconds, the titration should be repeated.

5. EXPRESSION OF RESULT

The total copper reducing power should be calculated as invert sugar and diminished by 1/20th to give the sugar content.

10. DETERMINATION OF WATER-SOLUBLE CHLORIDES

1. PURPOSE AND SCOPE

This method is for the determination of the amount of water-soluble chloride expressed as sodium chloride. It is applicable to all feeding stuffs.

2. PRINCIPLE

The sample is extracted with water and the solution clarified if necessary. The chloride is precipitated in acid solution with a known amount of standard silver nitrate solution and the excess of silver is titrated with standard thiocyanate solution, using ferric alum as indicator.

3. REAGENTS

3.1 Activated charcoal.

3.2 Acetone.

3.3 Diethyl ether.

3.4 Nitric acid (density 1·42g/ml).

3.5 Ammonium ferric sulphate, saturated aqueous solution.

3.6 Carrez solution I: dissolve 21·9g zinc acetate dihydrate in water, add 3ml glacial acetic acid. Make up to 100ml with water.

3.7 Carrez solution II: 10·6g potassium ferrocyanide per 100ml.

3.8 Ammonium thiocyanate, 0·1N solution, or potassium thiocyanate, 0·1N solution.

3.9 Silver nitrate, 0·1N solution.

4. APPARATUS

Mixer (tumbler): approximately 35 to 40 rpm.

5. PROCEDURE

5.1 *Preparation of the solution*

According to the nature of the sample, prepare a solution as shown under 5.1.1, 5.1.2 or 5.1.3.

At the same time carry out a blank test omitting the sample to be analysed.

5.1.1 Samples free from organic matter

Weigh to the nearest 0·001g a portion of the prepared sample of not more than 10g and containing not more than 3g of chlorine in the form of chlorides. Place with 400ml of water in a 500ml graduated flask at approximately 20 C. Mix for 30 minutes in the tumbler, bring up to volume, mix and filter.

5.1.2 Samples containing organic matter, excluding the products listed under 5.1.3

Weigh to the nearest 0·001g, approximately 5g of the prepared sample and place with 1g of activated charcoal (3.1) in a 500ml graduated flask. Add 400ml water at approximately 20°C, shake well and allow to stand for 30 minutes. Add 5ml Carrez solution I (3.6) mix well and then add 5ml Carrez solution II (3.7). Mix for 30 minutes in the tumbler, make up to the mark, mix, and filter.

5.1.3 Cooked feeding stuffs, linseed cakes and flour, products rich in linseed flour and other products rich in mucilage or in colloidal substances (for example, dextrinated starch).

Prepare the solution as described under 5.1.2 but do not filter. Decant (if necessary centrifuge), transfer 100ml of the supernatant liquid to a 200ml graduated flask. Mix with acetone (3.2) and bring up to the mark with this solvent, mix and filter.

5.2 *Titration*

Transfer to an Erlenmeyer flask from 25ml to 100ml of the filtrate (according to the presumed chlorine content) obtained as described under 5.1.1, 5.1.2 or 5.1.3. The aliquot portion must not contain more than 150mg of chlorine (Cl). Dilute if necessary to not less than 50ml with water, add 5ml nitric acid (3.4), 20ml saturated solution of ammonium ferric sulphate (3.5) and two drops of the thiocyanate solution (3.6) transferred by means of a burette filled up to the zero mark. Add from a burette the silver nitrate solution (3.9) in such a way that an excess of 5ml is obtained. Add 5ml of diethyl ether (3.3) and shake hard to coagulate the precipitate.

Titrate the excess silver nitrate with the thiocyanate solution (3.8), until the reddish-brown tint persists for one minute.

6. CALCULATION OF RESULTS

The weight of chlorine in mg (W), expressed as sodium chloride, present in the volume of filtrate taken for titration is calculated by using the following formula:

$$W \quad 5{\cdot}845 \times (V_1 \quad V_2)$$

where:

V_1 ml of silver nitrate solution 0·1N added; and

V_2 ml of ammonium thiocyanate solution 0·1N or potassium thiocyanate solution 0·1N, used for titration.

If the blank test indicates that silver nitrate solution 0·1N has been consumed deduct this value from the volume $(V_1 - V_2)$.

Express the result as a percentage of the sample.

11. DETERMINATION OF ASH

1. PURPOSE AND SCOPE

This method is for the determination of the ash content of feeding stuffs.

2. PRINCIPLE

The sample is ashed at 550°C; the residue is weighed.

3. REAGENTS

3.1 Ammonium nitrate solution: 20g ammonium nitrate per 100ml.

4. APPARATUS

4.1 Hot-plate.

4.2 Muffle furnace capable of being maintained at 550°C ± 5°C.

4.3 Crucibles for ashing made of platinum or an alloy of platinum and gold (10% Pt, 90% Au), either rectangular (60 × 40 × 25mm) or circular (diameter: 60 to 75mm, height: 20 to 25mm).

5. PROCEDURE

Weigh to the nearest 0·001g approximately 5g of the prepared sample (2·5 in the case of products which have a tendency to swell) and place in a crucible for ashing which has first been heated at 550°C, cooled in a desiccator and weighed. Either place the crucible in a cold muffle and bring up to a temperature of 550°C overnight, or carbonise the contents of the crucible before placing in the hot muffle. Keep at this temperature until white, light grey or reddish ash is obtained which appears to be free from carbonaceous particles. Place the crucible in a desiccator, allow to cool and weigh.

6. CALCULATION OF RESULTS

Calculate the weight of the residue and express the result as a percentage of the sample.

7. OBSERVATIONS

7.1 Substances which are difficult to ash must be subjected to an initial ashing of at least three hours, cooled and then a few drops of ammonium nitrate solution (3.1) added to it (carefully, to avoid dispersal of the ash or the formation of lumps). Dry in an oven at 100°C and then incinerate at 550°C. Repeat the operation as necessary until ashing is complete.

7.2 In the case of substances resistant to the treatment described under 7.1, proceed as follows: after ashing for three hours, place the ash in warm water and filter through a small, ash-free filter. Ash the filter and its contents in the original crucible. Place the filtrate in the cooled crucible, evaporate until dry, ash and weigh.

7.3 In the case of oils and fats, weigh accurately a sample of approximately 25g in a suitably sized crucible. Carbonise by setting light to the substance with a strip of ash-free filter paper. After combustion, moisten with as little water as possible. Dry and ash as described under 5.

12*a*. DETERMINATION OF CALCIUM—VOLUMETRIC METHOD

1. PURPOSE AND SCOPE

This method is for the determination of the total calcium content of feeding stuffs.

2. PRINCIPLE

The sample is ashed, the ash treated with hydrochloric acid and the calcium precipitated as calcium oxalate. The precipitate after filtering and washing is dissolved in acid and the liberated oxalic acid is titrated with standard potassium permanganate solution.

3. REAGENTS

3.1 Ammonia (density 0·88g/ml).

3.2 Hydrochloric acid, 50% solution (V/V): dilute an appropriate volume of concentrated hydrochloric acid (density 1·18g/ml) with an equal volume of water.

3.3 Nitric acid (density 1·42g/ml).

3.4 Ammonium chloride solution: 5g ammonium chloride per 100ml.

3.5 Ammonium oxalate, cold saturated solution.

3.6 Citric acid monohydrate solution: 30g citric acid monohydrate per 100ml.

3.7 Sulphuric acid, 20% solution (V/V): 20ml sulphuric acid (density 1·84g/ml) per 100ml.

3.8 Potassium permanganate, 0·1N solution.

3.9 Bromocresol green indicator solution: dissolve 0·04g of bromocresol green in 20ml ethanol and dilute to 100ml with water.

4. APPARATUS

4.1 Muffle furnace capable of being maintained at 550°C.

4.2 Platinum, silica or porcelain crucibles for ashing.

4.3 Glass filter crucibles, No. 4 porosity.

5. PROCEDURE

5.1 *Dissolution of sample*

Weigh to the nearest 0·001g, approximately 5g of the prepared sample into the crucible (4.2) and incinerate at a temperature not exceeding 550°C until all the organic matter has been destroyed. Allow to cool, moisten the ash with water and cautiously add 10ml of hydrochloric acid (3.2), avoiding loss by use of a cover glass. Wash the cover glass with water adding the washings to the crucible and evaporate to dryness. Continue the heating for at least one hour in order to dehydrate any silica that might be present. Cool, add 20ml water, 40ml of hydrochloric acid (3.2), bring to the boil, and then filter into a 250ml graduated flask. Wash the crucible and filter with hot water collecting the washings in the flask. Cool, make up to the mark and mix.

5.2 *Determination*

Transfer an aliquot of the solution from 5.1 containing 10 to 40mg of calcium into a 250ml beaker, add 1ml of citric acid solution (3.6) and 5ml of ammonium chloride solution (3.4). Make the volume up to approximately 100ml with water, bring to the boil, add 8 to 10 drops of bromocresol green indicator solution (3.9) and 30ml of a warm solution of ammonium oxalate (3.5). If any precipitate forms dissolve it by adding a few drops of hydrochloric acid (3.2). Neutralise very slowly with ammonia (3.1), stirring continuously until a pH of 4·4–4·6 is obtained (ie when the indicator changes colour). Place the beaker on a steam bath and keep it there for 30 minutes to allow the precipitate which has formed to settle. Remove the beaker from the steam bath and allow it to stand for one hour. Transfer the precipitate to the glass filter crucible (4.3) with water and wash the beaker and the precipitate with water until the excess ammonium oxalate is removed. (The absence of chloride in the washing water indicates that they have been sufficiently washed). Rinse the outside of the glass filter crucible with water and discard the rinsings. Place the crucible containing the precipitate in the original 250ml beaker, add 50ml of sulphuric acid (3.7) and water to give a total volume of about 100ml, and heat the contents to 70–80°C in order to dissolve the precipitate. Titrate the hot solution with potassium permanganate (3.8) until a pink colour persists for one minute.

6. CALCULATION OF RESULTS

1ml 0·1N potassium permanganate≡2·004mg calcium. Express the result obtained as a percentage of the sample.

Note: Where the magnesium content of the sample exceeds that of the calcium, the calcium oxalate should be re-dissolved and re-precipitated before titration with potassium permanganate.

12*b*. DETERMINATION OF CALCIUM—ATOMIC ABSORPTION METHOD

1. PURPOSE AND SCOPE

This method is for the determination of the calcium content of feeding stuffs.

2. PRINCIPLE

The sample is ashed, the ash treated with hydrochloric acid, and dissolved in hydrochloric acid. The calcium content of the solution is determined by atomic absorption spectrophotometry in the presence of lanthanum salt. The lanthanum salt is added to eliminate the interference of other elements (phosphorus, alkali metals).

3. REAGENTS

3.1 Hydrochloric acid (density 1·18g/ml).

3.2 Hydrochloric acid, 50% solution (V/V): dilute an appropriate volume of hydrochloric acid (3.1) with an equal volume of water.

3.3 Lanthanum oxide solution (releasing agent): moisten 117·3g lanthanum oxide (La_2O_3), low in calcium content, with water. Slowly add 350ml hydrochloric acid (3.1) and stir until all the lanthanum oxide is dissolved. Allow to cool and dilute to 1000ml with water.

3.4.1 Calcium standard solution: dry calcium carbonate at 105°C for one hour. Weigh accurately 2·497g and transfer to a 1000ml graduated flask using approximately 100ml water. Add slowly, with swirling, 60ml N hydrochloric acid (3.5). When all the carbonate has dissolved, make up to the mark with water. 1ml of this solution ≡ 1·00mg calcium (Ca).

3.4.2 Calcium standard solution (dilute): dilute 20ml of calcium standard solution (3.4.1) to 200ml with water. 1ml of this solution ≡ 100μg calcium (Ca).

3.5 Hydrochloric acid, 1N solution.

4. APPARATUS

4.1 Muffle furnace capable of being maintained at 550°C.

4.2 Platinum or silica crucibles for ashing.

4.3 Atomic absorption spectrophotometer with a calcium hollow cathode lamp.

5. PROCEDURE

5.1 *Dissolution of sample*

Weigh to the nearest 0·001g, approximately 5g of the prepared sample into a platinum or silica crucible (4.2), and ash at a temperature not exceeding 550°C until all the organic matter has been destroyed. Allow to cool, moisten the ash with water and cautiously add 10ml 50% (V/V) hydrochloric acid (3.2) avoiding loss by use of a cover glass. Wash the cover glass with water, adding the washings to the crucible and evaporate to dryness. Continue the heating for at least one hour to dehydrate any silica which may be present. Cool, add 20ml water and 10ml 50% (V/V) hydrochloric acid (3.2), bring to the boil and filter into a 250ml graduated flask. Wash the crucible and filter with hot water, collecting the washings in the flask. Cool, make up to volume and mix.

5.2 *Determination*

Set up the instrument using the line at 422·7nm. Use a fuel rich flame. Add releasing agent (3.3) and water to a suitable aliquot of the sample solution to produce a standard volume of solution to contain between 5 and 10 micrograms of calcium per ml and 10% (V/V) releasing agent. Prepare a blank solution from which only the sample has been omitted. Spray water into the flame and zero the instrument. Spray successively in triplicate, the standard solution (5.3), sample and blank, washing the instrument through with water between each spraying. Determine the calcium content of the sample and blank solution by reference to the calibration curve (5.3) and from the difference between them calculate the calcium content of the sample.

5.3 *Calibration curve*

Add 10ml releasing agent (3.3) to each of six 100ml graduated flasks. Measure 0, 3, 6, 9, 12 and 15ml of dilute calcium solution (3.4.2) into flasks and dilute to 100ml with water. The flasks contain 0, 3, 6, 9, 12 and 15 micrograms calcium per ml respectively. Carry out the measurements as indicated under 5.2. Plot the mean reading obtained for each standard solution against its calcium content.

6. CALCULATION OF RESULTS

Express the result as a percentage of the sample.

7. OBSERVATIONS

If no organic matter is present, the sample should be dissolved directly in hydrochloric acid. For products, such as calcium aluminium phosphates which are not readily soluble in acid, an alkaline fusion should be adopted as follows:

Mix the sample to be analysed in a platinum crucible with a mixture five times its weight, consisting of equal parts of potassium carbonate and sodium carbonate. Heat carefully until the mixture is completely fused. Cool and dissolve carefully in hydrochloric acid and proceed as in 5.1.

13*a*. DETERMINATION OF COPPER—DIETHYLDITHIOCARBAMATE SPECTROPHOTOMETRIC METHOD

1. PURPOSE AND SCOPE

This method is for the determination of the quantity of copper in feeding stuffs.

2. PRINCIPLE

The sample is ashed and the residue treated with hydrochloric acid. Copper is extracted from the resulting solution as its diethyldithiocarbamate complex into carbon tetrachloride. The copper content is measured at 436nm, by reference to a calibration curve.

3. REAGENTS

The water used should be free from copper.

3.1 Carbon tetrachloride, redistilled.

3.2 Sodium diethyldithiocarbamate solution: dissolve 1g sodium diethyldithiocarbamate in water and dilute to 100ml. Filter the solution if it is not clear. The solution may be stored, protected from light, in a refrigerator but should not be used after seven days.

3.3 EDTA-citrate solution: dissolve 20g ammonium citrate and 5g of the disodium salt of ethylenediaminetetra-acetic acid (EDTA) in water and dilute to 100ml. To purify, add 0·1ml sodium diethyldithiocarbamate solution (3.2) and extract with carbon tetrachloride (3.1). Add a further quantity of sodium diethyldithiocarbamate solution (3.2) to ensure that it is in excess.

3.4 Ammonium hydroxide solution approximately 6N: this may be prepared by passing gaseous ammonia into water, or by purifying ammonia solution as described for the EDTA-citrate solution (3.3).

3.5 Sulphuric acid solution, 2N.

3.6 Hydrochloric acid solution, 50% (V/V): dilute an appropriate volume of hydrochloric acid (density 1·18g/ml) with an equal volume of water.

3.7 Hydrochloric acid solution, 2N.

3.8 Nitric acid solution, 30% (V/V): dilute 30ml of nitric acid (density 1·42g/ml) with water to 100ml.

3.9 Copper standard solution: weigh to the nearest 0·1mg, 393mg of copper sulphate ($CuSO_4 . 5H_2O$), dissolve in 100ml 2N sulphuric acid (3.5) and dilute to one litre with water.

3.10 Copper standard working solution: dilute 5ml of the copper sulphate standard solution (3.9) to 250ml with 2N sulphuric acid (3.5) immediately before use. 1ml of this solution ≡ 2μg copper (Cu).

3.11 Thymol blue indicator solution: dissolve 0·1g thymol blue in 2·15ml of 0·1N sodium hydroxide and dilute to 100ml with water.

4. APPARATUS

4.1 Spectrophotometer with 10mm cells.

5. PREPARATION OF THE TEST SAMPLE

Grind the sample to pass through a stainless steel sieve having apertures about 1mm square.

6. PROCEDURE

6.1 *Dissolution of sample*

Weigh, to the nearest 0·001g, approximately 10g of the sample prepared under 5 into a silica dish or basin, and place a silica cover on top. Transfer to a cool muffle furnace. Raise the temperature to 450°±10°C and allow to ash until all the carbonaceous matter has disappeared; a slow current of air through the furnace during the initial stages of ashing is desirable. In the case of high-fat content materials, care must be taken to avoid ignition of the sample. When all the organic matter has been destroyed, cool, add 10ml 50% (V/V) hydrochloric acid solution (3.6) and evaporate to dryness on a water-bath. Extract the soluble salts from the residue with two successive 10ml portions of boiling 2N hydrochloric acid solution (3.7) decanting the solution each time through the same suitable filter-paper(1) into a 50ml graduated flask. Then add 5ml of 50% (V/V) hydrochloric acid solution (3.6) and about 5ml of 30% (V/V) nitric acid solution (3.8) to the residue in the basin, and evaporate the mixture to dryness on a hot-plate at low heat. Finally, add a further 10ml of boiling 2N hydrochloric acid solution (3.7) to the residue and filter the solution through the same filter-paper into the flask. Wash the basin and the filter with water, and collect the washings in the flask, make up to the mark with water and mix.

6.2 *Determination*

Transfer to a separating funnel a suitable aliquot of the solution prepared in accordance with 6.1, (or a dilution of this solution in N hydrochloric acid), containing not more than 50μg of copper. Add 10ml EDTA-citrate solution (3.3), two drops of thymol blue indicator solution (3.11) and ammonium hydroxide solution (3.4) until the mixture is coloured green or bluish-green. Cool the mixture, add 1ml of sodium diethyldithiocarbamate solution (3.2) and, from a burette, 15ml of carbon tetrachloride (3.1). Stopper the funnel, shake vigorously for two minutes and allow the layers to separate. Place a piece of cotton-wool in the stem of the funnel and run off the carbon tetrachloride layer into a dry 10mm spectrophotometer cell (4.1). Avoid undue exposure of the solution to light.

Measure immediately the absorbance of the sample solution at 436nm, against carbon tetrachloride as reference. Determine the quantity of copper by reference to the calibration curve (6.4).

6.3 *Blank test*

Carry out a blank test omitting only the sample and following the procedure described under 6.2.

6.4 *Calibration curve*

To a series of separating funnels transfer 10ml EDTA-citrate solution (3.3) and the following amounts of copper standard working solution (3.10) and 2N sulphuric acid (3.5):

Copper solution ...	0	1	2·5	5	10	15	20	25ml
2N H_2SO_4	25	24	22·5	20	15	10	5	0ml

(1) Whatman No. 541 or equivalent.

Proceed as for the test solution, as described in 6.2 commencing '... two drops thymol blue indicator (3.11)...'. Measure the absorbances of the solutions and plot the calibration curve using absorbances as the ordinates and the corresponding quantities of copper in μg as the abscissae.

7. CALCULATION OF RESULTS

The copper content in mg/kg of sample is given by the formula:

$$\frac{A \times 50 \times F}{V \times W}$$

in which:

A = weight of copper in aliquot taken for colour development as read from the calibration curve after allowing for blank reading (μg);

V = volume of aliquot taken for colour development (ml);

W = weight of test portion in g; and

F = dilution factor (from 6.2).

13*b*. DETERMINATION OF COPPER—ATOMIC ABSORPTION SPECTROPHOTOMETRIC METHOD

1. PURPOSE AND SCOPE

This method is for the determination of the quantity of copper in feeding stuffs.

2. PRINCIPLE

The sample is ashed and the residue treated with hydrochloric acid. The copper content of the sample is determined by atomic absorption spectrophotometry.

3. REAGENTS

The water used should be free from copper.

3.1 Hydrochloric acid solution, 50% (V/V): dilute an appropriate volume of hydrochloric acid (density 1·18g/ml) with an equal volume of water.

3.2 Hydrochloric acid solution, 2N.

3.3 Hydrochloric acid solution, 0·5N.

3.4 Nitric acid solution, 30% (V/V): dilute 30ml nitric acid (density 1·42g/ml) with water to 100ml.

3.5 Copper standard solution: weigh to the nearest 0·1mg, 393mg of copper sulphate ($CuSO_4.5H_2O$), dissolve in 0·5N hydrochloric acid solution (3.3) and dilute to 100ml with 0·5N hydrochloric acid solution (3.3). 1ml of this solution ≡ 1mg of copper (Cu).

4. APPARATUS

4.1 Atomic absorption spectrophotometer with a copper hollow cathode lamp.

5. PREPARATION OF THE TEST SAMPLE

Grind the sample to pass through a stainless steel sieve having apertures about 1mm square.

6. PROCEDURE

6.1 *Dissolution of sample*

Weigh, to the nearest 0·001g, approximately 10g of the sample as prepared under 5 into a silica dish or basin, and place the silica cover on top. Transfer to a cool muffle furnace. Raise the temperature to 450° ± 10°C and heat until no carbonaceous material remains. A slow current of air through the furnace during the initial stages of the ashing is advantageous. Care must be taken with high-fat content material to avoid ignition of the sample. When all the organic matter has been destroyed, cool, add 10 ml 50% (V/V) hydrochloric acid solution (3.1) and evaporate to dryness on a water-bath. Extract the soluble salts from the residue with two successive 10ml portions of boiling 2N hydrochloric acid solution (3.2),

decanting the solution each time through the same suitable filter paper(1) into a 50ml graduated flask. Then add 5ml 50% (V/V) hydrochloric acid solution (3.1) and about 5ml 30% (V/V) nitric acid solution (3.4) to the residue in the basin, and take the mixture to dryness on a hot-plate at low heat. Finally, add a further 10ml of boiling 2N hydrochloric acid solution (3.2) to the residue and filter the solution through the same paper into the flask. Wash the basin and the filter with water, and collect the washings in the graduated flask. Make up to the mark with water and mix.

6.2 *Blank test*

Simultaneously with the test determination prepare a blank of all the reagents which have been used in the preparation of the sample, and, starting at 6.1, 'add 10ml 50% (V/V) hydrochloric acid solution (3.1) to a silica dish'.

6.3 *Determination*

6.3.1 Preparation of sample and blank test solutions

Take an aliquot of the extract prepared as in 6.1 and dilute with 0·5N hydrochloric acid solution (3.3) to a known volume containing between 0 and 10μg per ml of copper.

Treat the blank test solution (6.2) identically.

6.3.2 Preparation of standard solutions for calibration

Prepare from the copper standard solution (3.5) a series of solutions in 0·5N hydrochloric acid solution (3.3) containing between 0 and 10μg per ml copper.

6.3.3 Measurement

Set up the instrument at a wavelength of 324·7nm. Spray distilled water into the flame and zero the instrument. Spray successively in triplicate the standard solutions (6.3.2), sample and blank (6.3.1) rinsing the liquid channels with water between each spraying. Plot the calibration curve using the mean absorbances as the ordinates and the corresponding concentrations of copper in μg per ml as the abscissae. Determine the concentration of copper in the blank and test solutions by reference to the calibration curve.

7. CALCULATION OF RESULTS

The copper content in mg/kg of sample is given by the formula:

$$\frac{C \times V_2 \times 50}{W \times V_1}$$

in which:

C = concentration of copper in final solution after subtracting the blank value (μg per ml);

V_2 volume of final solution;

V_1 volume of aliquot taken in para 6.3.1 (ml); and

W = weight of test portion in g.

14*a*. DETERMINATION OF MAGNESIUM—GRAVIMETRIC METHOD

1. PURPOSE AND SCOPE

This method is for the determination of magnesium in feeding stuffs. It is particularly appropriate for contents of magnesium of 1% and above, but it should not be used when substantial quantities of mineral phosphates are present.

2. PRINCIPLE

The sample is ashed and taken up in acid solution. Calcium is separated, by the addition of ammonium oxalate, and the magnesium precipitated with ammonium phosphate. The magnesium ammonium phosphate is ignited to magnesium pyrophosphate and weighed.

(1) Whatman No. 541 or equivalent.

3. REAGENTS

3.1 Citric acid solution: 30g citric acid monohydrate per 100ml.

3.2 Ammonia (density 0·88g/ml).

3.3 Ammonia solution: dilute 5ml ammonia (3.2) with water to 100ml.

3.4 Ammonium oxalate solution: saturated aqueous solution.

3.5 Ammonium phosphate solution: 20g diammonium hydrogen phosphate per 100ml.

3.6 Calcium wash solution: dissolve 1g oxalic acid, $[(COOH)_2.2H_2O]$ and 2g ammonium oxalate in water and dilute to 100ml.

3.7 Hydrochloric acid, (density 1·18g/ml).

3.8 Hydrochloric acid 50% (V/V): dilute an appropriate volume of hydrochloric acid (3.7), with an equal volume of water.

3.9 Hydrochloric acid 20% (V/V): dilute 20ml hydrochloric acid (3.7) with water to 100ml.

3.10 Methyl red indicator solution: dissolve 0·025g methyl red in 5ml 95% (V/V) ethanol with the aid of 0·5ml 0·1N sodium hydroxide solution. Dilute to 250ml with 50% (V/V) ethanol.

4. APPARATUS

4.1 Platinum, silica or porcelain crucible, suitable for ashing.

4.2 Electric muffle furnace, capable of being maintained at temperatures up to 950°C.

4.3 Filter crucible of 5 to 15 microns porosity, suitable for ignition at temperatures up to 1000°C.

5. PROCEDURE

5.1 *Dissolution of sample*

Weigh to the nearest 0·001g, a quantity of the prepared sample expected to contain between 0·05g and 0·50g of magnesium and incinerate at a temperature not exceeding 550°C until all the organic matter has been destroyed. Allow to cool, moisten the ash with water and cautiously add 10ml of 50% (V/V) hydrochloric acid (3.8), avoiding loss by use of a cover glass. Wash the cover glass with water and add the washings to the basin, and evaporate to dryness. Continue the heating for at least one hour in order to dehydrate any silica that might be present. Cool, add 10ml of 20% (V/V) hydrochloric acid (3.9), heat on a steam bath, and then filter into a 50ml graduated flask. Repeat the extraction with two further 10ml portions of 20% (V/V) hydrochloric acid (3.9). Wash the basin and filter with hot water and collect the washings in the flask. Cool, make up to the mark and mix.

5.2 *Precipitation and determination*

Transfer a measured volume (v) of the solution prepared under 5.1, containing between 0·04 and 0·06g of magnesium, to a 500ml beaker, and dilute with water to 100ml. Add ammonia solution (3.3) until a slight precipitate is formed then add citric acid solution (3.1) until the precipitate just dissolves and add 3–4ml in excess. Heat the solution to boiling and add 0·2ml (4–5 drops) of methyl red indicator solution (3.10). Add 25ml of hot ammonium oxalate solution (3.4) gradually with constant stirring then add dilute ammonia solution (3.3) dropwise until the solution is neutral or faintly alkaline (colour changes from red to yellow). Heat the mixture on a steam bath for one hour. Decant the clear supernatant liquid through a suitable filter paper(1), transfer the precipitate quantitatively to the filter using the calcium wash solution (3.6), rinse the beaker with the same solution, and pass the rinsings through the precipitate and collect them in the filtrate. [Test the filtrate for absence of calcium by adding a few drops of ammonium oxalate solution (3.4)]. Reserve the filtrate.

Dissolve the precipitate by passing 10ml of 20% (V/V) hydrochloric acid (3.9) through the filter and collect the extract in a 250ml beaker. Repeat with two further quantities of 20% (V/V) hydrochloric acid and finally wash the filter with water.

(1) Whatman No. 40 or equivalent.

Add 2–3 drops of methyl red indicator (3.10) to the combined extracts, heat to boiling and add 25ml of ammonium oxalate solution (3.4). Neutralise the solution by adding dilute ammonia (3.3) with constant stirring until the colour changes from red to yellow. Heat on a steam bath for one hour and then filter through a suitable filter paper(1), finally transferring the precipitate with the aid of calcium wash solution. Retain the filtrate and add it to the filtrate reserved from the first precipitation. Measure the approximate volume of the combined filtrates and add 20% (V/V) hydrochloric acid (3.9) until just acid to methyl red. Add, while stirring with a glass rod, 20ml of ammonium phosphate solution (3.5) taking care not to touch the sides of the beaker with the rod. Continue to stir and add ammonia (3.2) dropwise until the mixture is neutralised, then add a further 10ml of ammonia (3.2) for each 100ml in the beaker. Allow the beaker to stand for at least 4 hours, or preferably, overnight.

Transfer the precipitate to a tared crucible (4.3) and wash the residue with cold ammonia solution (3.3), ensuring that any precipitate adhering to the beaker and glass rod is transferred to the crucible. Discard the filtrate and washings. Dry the crucible and precipitate at 120°C, transfer to a muffle furnace and ensure that a temperature of at least 950°C is maintained for one hour. Allow the crucible to cool in a desiccator and weigh. Repeat the heating and cooling until constant weight is achieved.

6. CALCULATION OF RESULTS

Calculate the percentage of magnesium in the sample using the formula:

$$\text{Magnesium}\ (\%) = m \times \frac{21{\cdot}85}{w} \times \frac{50}{v}$$

where m = weight of the precipitate (g);

w = weight of sample taken (g); and

v = measured volume (ml) taken for determination (5.2).

14*b*. DETERMINATION OF MAGNESIUM—ATOMIC ABSORPTION METHOD

1. PURPOSE AND SCOPE

This method is for the determination of magnesium in feeding stuffs. It is particularly appropriate for determining magnesium contents lower than 5%.

2. PRINCIPLE

The sample is ashed and dissolved in dilute hydrochloric acid, or if it contains no organic substances, it is dissolved directly in dilute hydrochloric acid. The solution is diluted and the magnesium content determined by atomic absorption spectrophotometry at 285·2nm.

3. REAGENTS

3.1 Hydrochloric acid (density 1·18g/ml).

3.2 Magnesium ribbon or wire, or magnesium sulphate heptahydrate.

3.3 Releasing agent.

3.3.1 Lanthanum oxide solution: moisten 117·3g lanthanum oxide (La_2O_3), low in magnesium content, with water, slowly add 350ml hydrochloric acid (3.1) and stir until the lanthanum oxide is dissolved. Allow to cool and dilute to 1000ml with water.

3.3.2 Strontium salt solution (chloride or nitrate) containing 25g per 1000ml of strontium (= 76·08g $SrCl_2.6H_2O$ or 60·38g $Sr(NO_3)_2$ per 1000ml).

3.4 Magnesium standard solution: weigh to the nearest 0·001g, exactly 1g of magnesium (3.2) from which the oxide coating has been carefully removed or the corresponding quantity (10·143g) of magnesium sulphate heptahydrate (3.2) and

(1) Whatman No. 40 or equivalent.

transfer to a 1000ml graduated flask. Dissolve the metal or magnesium sulphate in 80ml of hydrochloric acid (3.1) and dilute to the mark with water. 1ml of this solution ≡ 1·000mg of magnesium (Mg).

4. APPARATUS

4.1 Electric muffle furnace capable of being maintained at 550°C.

4.2 Atomic absorption spectrophotometer, with magnesium hollow cathode lamp.

5. PROCEDURE

5.1 *Dissolution of sample*

5.1.1 *Feeding stuffs composed exclusively of mineral substances*

Weigh to the nearest 0·001g, approximately 5g of the prepared sample and transfer to a 500ml graduated flask with 250 to 300ml water. Add 40ml hydrochloric acid (3.1), bring to the boil and keep the liquid gently boiling for 30 minutes. Allow to cool, make up to volume with water, mix and filter into a dry beaker through a dry pleated filter. Discard the first 30ml of the filtrate. In the presence of silica, treat the sample with a sufficient quantity (15–30ml) of hydrochloric acid (3.1), evaporate to dryness on a steam bath and transfer to an oven at 105°C for one hour. Proceed as from the third sentence of 5.1.2.

5.1.2 *Feeding stuffs composed predominantly of mineral substances*

Weigh to the nearest 0·001g approximately 5g of the prepared sample into a crucible and ash at 550°C in the muffle furnace until an ash which is free from carbonaceous particles is obtained, and leave to cool. In order to eliminate silica, add to the ash a sufficient quantity (15–30ml) of hydrochloric acid (3.1) evaporate to dryness on a steam bath and transfer to an oven at 105°C for one hour. Treat the residue with 10ml hydrochloric acid (3.1) and transfer to a 500ml graduated flask using warm water. Leave to cool and make up to volume with water. Mix and filter into a dry beaker through a dry pleated filter. Discard the first 30ml of the filtrate.

5.1.3 *Feeding stuffs composed predominantly of organic substances*

Weigh to the nearest 0·001g, approximately 5g of the prepared sample into a crucible and ash at 550°C in the muffle furnace until an ash which is free from carbonaceous particles is obtained. Treat the ash with 5ml hydrochloric acid (3.1), evaporate to dryness on a steam bath and then dry for one hour in an oven at 105°C in order to render any silica insoluble. Treat the ash with 5ml hydrochloric acid (3.1), transfer to a 250ml graduated flask using warm water, bring to the boil, leave to cool and make up to volume with water. Mix and filter into a dry beaker through a dry pleated filter. Discard the first 30ml of the filtrate.

5.2 *Determination*

Transfer a suitable volume of the solution obtained under 5.1 to a 100ml graduated flask, add 10ml of the releasing agent solution (3.3.1) or (3.3.2) make up to the mark with water and mix. The magnesium content of this solution must be within the optimal measuring range of the spectrophotometer, and the hydrochloric concentration must not exceed 0·4N. Measure the absorption of the solution at 285·2nm.

5.3 *Calibration curve*

Prepare at least five standard solutions of increasing concentration of magnesium, corresponding to the optimal measuring range of the instrument, by diluting suitable volumes of the magnesium standard solution (3.4). Measure the absorption of these solutions at 285·2nm. Construct a graph relating absorbances to the amounts of magnesium present.

6. CALCULATION OF RESULTS

Calculate the quantity of magnesium in the sample by relation to the reference solutions. Express the result as a percentage of the sample.

15. DETERMINATION OF VITAMIN A (RETINOL)

1. PURPOSE AND SCOPE

This method is for the determination of Vitamin A in feeding stuffs. The lower limit of the determination is 10 000 IU/kg for highly pigmented feeds and 4 000 IU/kg for others(1). Products are classified in two groups, according to their presumed retinol content:

Group A: contents lower than 200 000 IU/kg;

Group B: contents equal to or greater than 200 000 IU/kg.

2. PRINCIPLE

The sample is hydrolysed in hot ethanolic potassium hydroxide solution, either in the presence of an antioxidant or in a nitrogen atmosphere. The mixture is extracted with 1,2-dichloroethane. The extract is evaporated to dryness and treated with light petroleum. The solution is chromatographed on a column of aluminium oxide (for Group B products, chromatography is only required in certain cases). For Group A products, the retinol is determined by development of a coloured complex according to the Carr-Price reaction and measurement of its absorbance at 610nm. For Group B products, the determination is by measurement of the absorbance at 325nm.

3. REAGENTS

(*a*) *used for analysing products of Groups A and B*

3.1 Aluminium oxide, neutral; ignite for 8 hours at 750° C, cool in a desiccator and keep in a brown glass bottle fitted with a ground-glass stopper. Before use in chromatography moisten as follows: place in a brown glass bottle 10g aluminium oxide and 0·9ml water, seal with a stopper, reheat for 5 minutes in a boiling water bath while shaking. Allow to cool. Verify the activity of the aluminium oxide thus prepared by subjecting a known quantity of retinol (3.17) (ca. 500 IU) to the procedure of 5.3 and 5.4 and checking recovery.

3.2 Aluminium oxide, basic, degree of activity 1(2).

3.3 1,2-Dichloroethane.

3.4 Diethyl ether: remove peroxides and traces of water by chromatography on a column of basic aluminium oxide (3.2) (25g aluminium oxide per 250ml diethyl ether).

3.5 Ethanol, 96% (V/V).

3.6 Nitrogen.

3.7 Light petroleum, boiling range, 40–60° C. If necessary, purify as follows: stir 1000ml light petroleum with 20ml lots of concentrated sulphuric acid until the acid remains colourless. Remove the acid and wash the light petroleum successively with 500ml water, twice with 250ml of sodium hydroxide solution (approximately 2·5N), and three times with 500ml water. Remove the aqueous layer, dry the light petroleum for 1 hour over active carbon and anhydrous sodium sulphate, filter and distil.

3.8 Diethyl ether solutions: prepare a series of solutions containing 4, 8, 12, 16 and 20% (V/V) diethyl ether (3.4) in light petroleum (3.7).

3.9 Potassium hydroxide solution: dissolve 500g potassium hydroxide in water and dilute to 1 litre.

3.10 Sodium ascorbate solution: 10g sodium ascorbate per 100ml.

3.11 Sodium sulphide, 0·5 molar solution in 70% (V/V) glycerine.

3.12 Potassium hydroxide, N solution.

3.13 Potassium hydroxide, 0·5N solution.

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(1) 1 IU = 0·3 μg of retinol.

(2) Woelm, Merck or equivalent.

(*b*) *used exclusively for analysing Group A products*

3.14 Benzene, crystallizable.

3.15 Chloroform: remove the ethanol, phosgene and traces of water by chromatography on a column of basic aluminium oxide (3.2) (50g aluminium oxide per 200ml chloroform; it is advisable to chromatograph the first 50ml of the eluate a second time).

3.16 Carr-Price reagent: stir approximately 25g antimony trichloride (kept in a desiccator) with 100ml chloroform (3.15) until the solution is saturated. (If necessary warm to 50°C and allow to cool). A slight deposit of antimony trichloride causes no problem. Add 2ml acetic anhydride. Keep in a refrigerator in a moisture-proof brown glass bottle with ground-glass stopper. The solution keeps for 2 to 3 weeks.

3.17 Retinol—standardised spectrophotometrically.

(*c*) *used exclusively for analysing Group B products*

3.18 Propan-2-ol.

4. APPARATUS

4.1 Rotary vacuum evaporator.

4.2 Glass chromatography tubes (length: 300mm; internal diameter: about 13mm).

4.3 Spectrophotometer with 10mm cells. Measurements in the UV require silica cells.

4.4 UV lamp 365nm.

5. PROCEDURE

Note: All operations must be carried out away from direct sunlight using amber glass where necessary.

5.1 *Test portion*

From the prepared sample, take a quantity proportional to the presumed retinol content, thus:

0·1–1·0g for contents greater than 20 000 000 IU/kg;

3·0–5·0g for contents between 400 000 and 20 000 000 IU/kg;

10–20g for contents between 20 000 and 400 000 IU/kg; and

30g for Group A products.

Immediately place the test sample in a 500ml flask with a ground-glass stopper.

5.2 *Hydrolysis and extraction*[1]

Add successively to the sample 40ml ethanol (3.5), 2ml sodium ascorbate solution (3.10)[2], 10ml potassium hydroxide solution (3.9) and 2ml sodium sulphide solution (3.11).

Heat for 30 minutes at 70–80°C under a reflux condenser and then cool under a stream of water. Add 50ml ethanol (3.5) and 100ml 1,2-dichloroethane (3.3). Shake vigorously and then carefully decant the supernatant liquid into a separating funnel avoiding transfer of solids. Add 150ml potassium hydroxide solution (3.12), shake for 30 seconds and allow to stand until the layers have separated. Collect the lower dichloroethane layer in a separating funnel, add 40ml potassium hydroxide solution (3.13), shake for 10 seconds and allow to stand until the layers have separated. Collect the dichloroethane layer in a separating funnel, and wash at least 6 times with 40ml lots of water. It is essential that the dichloroethane is free of alkali and washing must be continued until

[1] For milk feeds and products with a tendency to agglomerate or swell, double the quantity of the reagents shown in the first and second paragraphs of 5.2.

[2] Sodium ascorbate need not be added when hydrolysis is carried out in a nitrogen atmosphere.

the wash water gives no positive reaction to phenolphthalein. Collect the dichloroethane layer and remove the last traces of water using strips of filter paper.

Evaporate to dryness an aliquot part of the solution under vacuum on a water bath at 40°C. Rapidly treat the residue with 5ml light petroleum (3.7).

For Group A products, chromatograph as shown in 5.3.1.

For Group B products, transfer the solution to a 50ml graduated flask, make up to volume with light petroleum (3.7), mix and measure the absorbance as shown in 5.4.1.

5.3 *Chromatography*

5.3.1 Group A products

Fill a chromatography tube (4.2) to a height of 200mm with aluminium oxide (3.1) previously slurried with light petroleum (3.7). Place in the tube the solution obtained in 5.2 and immediately add 20ml light petroleum (3.7). Elute successively with 10ml lots of the light petroleum solutions at 4, 8, 12, 16 and 20% diethyl ether (3.8) under pressure or partial vacuum, the rate of flow being 2 to 3 drops per second.

The carotene is eluted first(1). The retinol is generally eluted with the light petroleum solution at 20% diethyl ether (3.8). The elution is followed under UV light (brief irradiation of the column with the mercury lamp). The fluorescent zone of the retinol is clearly separated from the yellow xanthophyll zones following it. If the zones have not separated the chromatography should be repeated, using increased concentrations of diethyl ether in the eluting solvent. Collect the eluate fraction containing the retinol in an Erlenmeyer flask.

5.3.2 Group B products

Chromatography must only be carried out if the absorbance measurements obtained in 5.4.3 do not conform to the requirements given in 5.4.3.

If chromatography proves necessary, place in the chromatography column an aliquot part of the solution in the light petroleum obtained in 5.2, containing approximately 500 IU of retinol, and chromatograph as shown in 5.3.1.

5.4 *Determination*

5.4.1 Group A products

Evaporate to dryness under vacuum the eluate containing the retinol obtained in 5.3.1. Treat the residue with 2ml benzene (3.14), using a safety pipette. Take 0·3ml of this solution and add 3ml of the Carr-Price reagent (3.16). A blue colour develops. Measure the absorbance at 610nm exactly 30 seconds after the reaction has begun against a reference solution prepared from 0·3ml of benzene and 3ml of Carr-Price reagent. Determine the retinol content by reference to the standard curve (5.4.2.).

5.4.2 Calibration curve

Prepare in benzene (3.14) a series of solutions of retinol (3.17) containing 2 to 16 IU per 0·3ml. Treat this volume (0·3ml) of each solution with 3ml Carr-Price reagent (3.16) and measure the absorbance at 610nm.

5.4.3 Group B products

Take an aliquot part of the solution in light petroleum obtained in 5.2 containing approximately 200 IU retinol. Evaporate to dryness under vacuum and dissolve the residue in 25ml propan-2-ol (3.18). Measure the

(1) Carotene content may be determined by absorbance measurement at 450nm.

$E^{1\%}_{1cm}$ 2600

absorbance in the spectrophotometer at 325, 310 and 334nm. The absorption maximum is located at 325nm. The retinol content of the solution is calculated as follows:

Absorbance at 325nm × 18·30 = IU of retinol/ml

However, the ratios of the absorbances $\frac{X}{Y}$ and $\frac{Z}{Y}$ must be 0·857 where:

X = absorbance at 310nm;

Y = absorbance at 325nm; and

Z = absorbance at 334nm.

If one of these ratios differs appreciably from this value (<0·830 or >0·880), the measurement of the absorbances must be preceded by chromatography in accordance with the method given in 5.3.2. If the measurement of the absorbances carried out after chromatography shows that the above-mentioned ratios still differ appreciably from the value of 0·857 (<0·830 or >0·880), the determination must be carried out in accordance with the method given for Group A products.

6. CALCULATION OF RESULTS

Calculate the retinol content of the sample taking into account the weight of the test sample and the dilutions carried out in the course of analysis. Express the results in IU of retinol per kg of feeding stuff.

16. DETERMINATION OF THIAMINE HYDROCHLORIDE (VITAMIN B_1, ANEURINE)

1. PURPOSE AND SCOPE

This method is for the determination of thiamine hydrochloride (aneurine, Vitamin B_1) in feeding stuffs. The lower limit of the determination is 5mg/kg.

2. PRINCIPLE

The sample is treated with hot dilute sulphuric acid, hydrolysed enzymatically and then subjected to alkaline oxidation. The thiochrome formed is extracted with 2-methylpropan-1-ol, and determined spectrofluorimetrically.

3. REAGENTS

3.1 Ethanol, 96% (V/V).

3.2 2-Methylpropan-1-ol.

3.3 Multienzymatic preparation containing protease, phosphatase and amylase (1).

3.4 Sodium metabisulphite ($Na_2S_2O_5$).

3.5 Potassium ferricyanide solution: 20g potassium ferricyanide per 100ml.

3.6 Potassium hydroxide solution: 25g potassium hydroxide per 100ml.

3.7 Oxidising mixture: mix 2ml of potassium ferricyanide solution (3.5) with 48ml of potassium hydroxide solution (3.6). This mixture does not keep for more than 4 hours.

3.8 Sodium acetate, 2·5N solution.

3.9 Sulphuric acid, 0·2N solution.

3.10 Thiamine standard solution: dissolve 127·1mg thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$), previously dried under vacuum to constant weight, in 1000ml of dilute sulphuric acid (3.9). 1ml of this solution contains 100μg of thiamine base ($C_{12}H_{17}N_4OS$). It will keep for one month if stored in a cool, dark place.

4. APPARATUS

4.1 Centrifuge.

4.2 Spectrofluorimeter with 10mm silica cells.

(1) eg Clarase.

5. PROCEDURE

5.1 *Enzymatic hydrolysis*

Place in each of two 250ml graduated flasks, A and B, identical amounts of the prepared sample containing approximately 100μg thiamine base and 125ml sulphuric acid (3.9). Also add, to flask A only, 1.0 ml standard solution (3.10) (internal standard).

Shake the flasks vigorously, place on a steam bath and keep there for 15 minutes, shaking occasionally. Allow to cool to approximately 45°C. Add to each flask 20ml sodium acetate solution (3.8) and 0·5g multienzymatic preparation (3.3), then allow to stand for 20 minutes. Cool to room temperature if necessary and then add 20ml sodium acetate solution (3.8), make up to volume with water, mix and filter. Collect filtrates A and B after having discarded the first 15ml. Prepare the following solutions:

5.1.1 Reference solution T
Place in a centrifuge tube 5ml filtrate A and approximately 10mg sodium metabisulphite (3.4). Immerse the tube in a boiling water bath for 15 minutes and then allow to cool to room temperature.

5.1.2 Solutions A (internal standard) and B (sample)
Place 5ml filtrate A in a centrifuge tube and 5ml filtrate B in another centrifuge tube.

5.2 *Oxidation*

Add to solutions T, A and B, 5ml of the oxidising mixture (3.7) and, one minute later, 10ml 2-methylpropan-1-ol (3.2). Stopper the tubes and shake vigorously for 5 seconds. Allow to stand for one minute and centrifuge (4.1) so as to separate the layers. From each tube transfer 5 ml of the supernatant 2-methylpropan-1-ol layer to separate 25ml graduated flasks, make up to volume with ethanol (3.1) and mix (- extracts T, A and B).

5.3 *Measurement of fluorescence*

Carry out the measurements at the wavelength for which the spectrofluorimeter (4.2) gives an optimal response to the fluorescence of the thiochrome. Irradiate at approximately 365nm. Adjust the instrument to zero using extract T. Measure the intensity of fluorescence of extracts A and B.

6. CALCULATION OF RESULTS

The thiamine hydrochloride content in mg/kg of the sample is calculated from the formula:

$$\frac{b \times d}{c \times (a - b)}$$

where:

a - intensity of fluorescence of extract A (internal standard);

b - intensity of fluorescence of extract B (sample);

c - weight of the test sample in grams; and

d - amount of thiamine hydrochloride in μg added to the test sample (internal standard).

17. DETERMINATION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID (VITAMIN C)

1. PURPOSE AND SCOPE

This method is for the determination of the total quantity of ascorbic and dehydroascorbic acids (vitamin C) in feeding stuffs. The lower limit of the determination is 20mg/kg. Products are classified in two groups, according to their presumed vitamin C content:

Group A: contents lower than 10g/kg;

Group B: contents equal to or greater than 10g/kg.

2. PRINCIPLE

The sample is suspended in a dilute solution of metaphosphoric acid and extracted with chloroform. The aqueous phase is treated with a solution of 2,6-dichlorophenolindophenol in order to transform the ascorbic acid into dehydroascorbic acid, and then with a solution of 2,4-dinitrophenylhydrazine. The hydrazone formed is extracted with a mixture of ethyl acetate, glacial acetic acid and acetone. The solution is chromatographed on a column of silica gel, the eluate evaporated to dryness and the residue dissolved in dilute sulphuric acid. The absorbance of the solution is measured at 509nm.

For Group A products the eluate resulting from chromatography on the column is further subjected to thin layer chromatography to isolate the hydrazone.

3. REAGENTS

3.1 Carbon dioxide (gas).

3.2 Chloroform.

3.3 Filtration aid.

3.4 Nitrogen.

3.5 Silica gel, particle size 0·05 to 0·2mm.

3.6 Silica gel, Stahl grade H, for thin layer chromatography.

3.7 Mixture of ethyl acetate (96 parts by volume), glacial acetic acid (2 parts by volume) and acetone (2 parts by volume).

3.8 Mixture of dichloromethane (97 parts by volume) and glacial acetic acid (3 parts by volume).

3.9 2,4-Dinitrophenylhydrazine: dissolve 2g 2,4-dinitrophenylhydrazine in 100ml dilute sulphuric acid (25ml sulphuric acid (density 1·84 g/ml) diluted by making up to 100ml with water). Stored at a cool temperature this solution keeps for one week.

3.10 Eluting solvent for thin layer chromatography: mix 75ml diethyl ether, 25ml ethyl acetate and 4ml acetic acid (96g per 100ml). Renew after two to three chromatographic runs.

3.11 Metaphosphoric acid solution: dissolve 200g of ground metaphosphoric acid in water and make up to 2000ml with water. Keep at 4°C. This solution is stable for one week.

3.12 Sulphuric acid, dilute solution: place 105ml water in a 200ml graduated flask and cautiously make up to volume with sulphuric acid (density 1·84g/ml).

3.13 2,6-Dichlorophenolindophenol solution: 0·5g per 100ml. Prepare immediately before use.

3.14 L-Ascorbic acid solution: dissolve 50mg L-ascorbic acid in approximately 20ml metaphosphoric acid solution (3.11) and make up to 100ml with water. Prepare immediately before use.

4. APPARATUS

4.1 Water bath controlled at 20°C.

4.2 Centrifuge.

4.3 Rotary vacuum evaporator.

4.4 Glass chromatography tubes (length: 100mm; internal diameter: 20mm), with a sintered disc (eg Allihn tubes).

4.5 Spectrophotometer with 10mm cells.

4.6 Apparatus for thin layer chromatography, with silica gel plates (3.6) coated to a depth of 0·5 to 0·6mm. (Ready-made plates are suitable). Dry the plates for 2½ to 3 hours in a drying oven at 120 to 130°C. Allow to cool and then keep in a desiccator for at least 24 hours before use.

5. PROCEDURE

5.1 *Extraction*

Place in each of two 250ml graduated flasks A and B, identical quantities (up to 10g, according to the presumed ascorbic acid content, weighed to the nearest 0·001g) of the prepared sample. Add to flask B (at 4°C) 30ml chloroform (3.2) and 25ml metaphosphoric acid solution (3.11). Add to flask A, 30ml chloroform (3.2) and an aliquot portion of the standard solution (3.14) corresponding to the amount of ascorbic acid presumed to be present in the sample; make up the volume of the aqueous phase to 25ml with metaphosphoric acid solution (3.11). Stopper the flasks, shake briefly, and then allow to stand for 10 to 15 minutes.

To each flask add 25ml water, stopper and shake vigorously for 10 seconds and allow to stand for 10 to 15 minutes in the water bath (4.1). Centifruge to separate the aqueous and chloroform layers. The aqueous phases are retained for subsequent analysis.

5.2 *Oxidation*

Place in a 50ml stoppered flask, a volume of extract B, expected to contain about 160μg ascorbic acid (if necessary, dilute the extract with a mixture of equal volumes of metaphosphoric acid solution (3.11) and water, such that this weight of ascorbic acid is contained in about 10ml) Place in a second 50ml flask an identical volume of extract A. Dilute the contents of each flask to 40ml with a mixture of equal volumes of metaphosphoric acid solution (3.11) and water. Add 0·5 to 1·0ml of dichlorophenolindophenol solution (3.13) and mix well. A red colour develops which should persist for at least 15 minutes. Add approximately 300mg filtration aid (3.3), shake and filter through a dry pleated filter. The filtrate need not necessarily be clear.

5.3 *Reaction with 2,4-dinitrophenylhydrazine and hydrazone extraction.*

Pipette 10ml of the filtrate obtained in 5.2 to a centrifuge tube, add 2ml 2,4-dinitrophenylhydrazine solution (3.9) and mix. Pass a stream of nitrogen (3.4) or carbon dioxide (3.1) rapidly into the tube, stopper the tube and immerse it for approximately 15 hours (overnight) in the water bath (4.1).

Then add 3ml water, 20ml of the ethyl acetate/glacial acetic acid/acetone mixture (3.7) and approximately 800mg filtration aid (3.3). Stopper the tube, shake vigorously for 30 seconds and centrifuge. Place 15ml of the supernatant phase in an evaporation flask and evaporate under reduced pressure in the rotary evaporator (4.3) until an oily residue is obtained. Dissolve the residue in 2ml of the ethyl acetate/glacial acetic acid/acetone mixture (3.7) by heating at 50°C, allow to cool, add 10ml of the dichloromethane/glacial acetic acid mixture (3.8) and mix.

5.4 *Chromatography on a column*

Fill a chromatography tube (4.4) up to a level of 30mm with the dichloromethane/glacial acetic acid mixture (3.8). Suspend (shaking vigorously) 5g silica gel (3.5) in 30ml of the dichloromethane/glacial acetic acid mixture (3.8); pour the suspension into the tube, allow to stand and then compress under nitrogen (3.4) at low pressure. Decant into the tube the solution obtained in 5.3, rinse the flask with a small quantity of the dichloromethane/glacial acetic acid mixture (3.8) and decant into the tube, then fill the latter with this solvent (3.8). Wash the column with 5ml portions of this solvent (3.8) until the eluate is colourless; discard this eluate.

Elute the reddish zone at the top of the column with the ethyl acetate/glacial acetic acid/acetone mixture (3.7), collect the eluate and evaporate to dryness.

5.4.1 For Group A products (contents of vitamin C lower than 10g/kg), dissolve the residue in 2·0ml of the ethyl acetate/glacial acetic acid /acetone mixture (3.7) and chromatograph immediately on a thin layer plate as shown in 5.5.

5.4.2 For Group B products (contents of vitamin C equal to or greater than 10g/kg), treat the oily residue with 4.0ml dilute sulphuric acid (3.12), shake vigorously to dissolve the residue completely and measure the absorbance as shown in 5.6.

5.5 *Thin layer chromatography*

Carry out in duplicate the operations described as follows. Place in a thin line on the plate (4.6) 0·5ml of the solution obtained in 5.4.1. Using the eluting solvent (3.10) develop for at least 20 minutes in a tank saturated with solvent vapour, until the pink-coloured hydrazone zone is clearly separated. Allow to dry in the open. Remove the pink zone quantitatively from the plate and transfer it into a chromatography tube (4.4).

Elute successively once with 2ml and twice with 1·5ml of the ethyl acetate/glacial acetic acid/acetone mixture (3.7). Collect the eluate in a small flask (the last part must be colourless). Evaporate to dryness, treat the oily residue with 4·0ml dilute sulphuric acid (3.12), shake vigorously to dissolve the residue completely and measure the absorbance.

5.6 *Determination*

Measure the absorbance at 509nm, 20 to 30 minutes after dissolving the residue in sulphuric acid, against a reference solution of dilute sulphuric acid (3.12).

5.7 *Blank test*

Carry out a blank test applying the same procedure but without the sample.

6. CALCULATION OF RESULTS

The vitamin C content of the sample in g per kg is calculated from the formula:

$$\frac{e \times (c - a) \times F}{1000 \times d \times (b - c)}$$

where

a absorbance of the blank;

b absorbance of the sample plus internal standard solution;

c absorbance of the sample solution;

d weight, in grams, of the test sample;

e weight, in µg, of ascorbic acid added as internal standard; and

F dilution factor (section 5.2).

18. DETERMINATION OF MENADIONE (VITAMIN K_3)

1. PURPOSE AND SCOPE

This method is for the determination of menadione (vitamin K_3) in feeding stuffs. The lower limit of the determination is 1mg/kg.

2. PRINCIPLE

The sample is extracted with diluted ethanol, clarified with tannin solution and centrifuged. The extract is treated with a solution of sodium carbonate; the menadione is extracted with 1, 2-dichloroethane. The dichloroethane extract is treated, according to its menadione content, either directly or after evaporation, with 2, 4-dinitrophenylhydrazine in solution in ethanol acidified with hydrochloric acid. The resulting hydrazone is treated with ammonia in excess to form a blue-green complex the absorbance of which is measured at 635nm.

3. REAGENTS

3.1 1, 2-Dichloroethane.

3.2 Ethanol, absolute.

3.3 Ethanol, 40 per cent (V/V).

3.4 Hydrochloric acid (density 1·18 g/ml).

3.5 Ammonia, 25 per cent solution (V/V): dilute one volume of ammonia (density 0·88 g/ml) with 3 volumes of water.

3.6 Sodium carbonate solution: 10g sodium carbonate (anhydrous) per 100ml.

3.7 Tannin solution: 10g purified powdered tannin per 100ml.

3.8 Ammonia-ethanol mixture: mix 1 volume of ethanol (3.2) with 1 volume of ammonia solution (3.5).

3.9 2,4-Dinitrophenylhydrazine reagent: dissolve 0·04g of 2,4-dinitrophenylhydrazine in about 40ml of boiling absolute ethanol (3.2). Allow to cool, transfer to a 50ml graduated flask. Add 0·4ml of hydrochloric acid (3.4) and make up to volume with absolute ethanol. Prepare immediately before use.

3.10 Standard solutions of menadione: dissolve 20mg menadione (vitamin K_3) in 1, 2-dichloroethane (3.1) and make up to 200ml. Dilute aliquots of this stock solution with 1, 2-dichloroethane (3.1) to obtain a series of solutions with menadione concentrations between 2 and 10 μg per ml. These solutions must be freshly prepared.

4. APPARATUS

4.1 Mechanical shaker.

4.2 Centrifuge.

4.3 Rotary vacuum evaporator.

4.4 Spectrophotometer, with 10mm cells.

4.5 Phase separating paper(1).

5. PROCEDURE

Note: 1. All operations must be carried out away from direct sunlight, using amber glass where necessary.

2. All glassware must be free from detergent and washed first with 50 per cent hydrochloric acid (V/V) then with acetone and dried.

5.1 *Extraction*

Weigh to the nearest 0·001g, 0·1 to 5g of prepared feed supplement, or to the nearest 0·01g, 20 to 30g of all other prepared feeding stuffs and transfer to a 250ml conical flask with ground glass stopper.

Add to the test sample exactly 96ml dilute ethanol (3.3) and shake mechanically for 15 minutes at room temperature. Then add 4·0ml tannin solution (3.7), mix, transfer the extract into a centrifuge tube, centrifuge until a clear solution is obtained and decant. Place 20 to 40ml, accurately measured, of the extract in a 250ml separator, add 50ml 1,2-dichloroethane (3.1), mix and add 20ml sodium carbonate solution (3.6). Shake vigorously for 30 seconds and then collect the dichloroethane phase in a 100ml separator. Add 20ml water, shake again for 15 seconds, allow the phases to separate and collect the dichloroethane phase through a phase separating paper in order to remove traces of water.

For feed supplements, take an aliquot part of the extract and dilute with 1,2-dichloroethane (3.1) to obtain a menadione concentration of 2 to 10 μg per ml. For all other feeding stuffs, evaporate to dryness an aliquot part of the extract under reduced pressure in an atmosphere of nitrogen on a water bath at 40°C. Rapidly treat the residue with 1,2-dichloroethane (3.1) to obtain a solution containing 2 to 10μg menadione per ml.

5.2 *Hydrazone formation*

Transfer 2·0ml of the dichloroethane extract obtained in 5.1 to a 10ml graduated flask and add 3·0ml 2,4-dinitrophenylhydrazine reagent (3.9), securely stopper the flask with a cork or teflon stopper so as to prevent evaporation and heat for two hours at 70°C on a water bath. Allow to cool, add 3·0ml ammoniacal ethanol (3.8), mix, make up to volume with absolute ethanol (3.2) and mix again.

5.3 *Determination*

Measure the absorbance of the blue-green complex at 635nm against a reference solution obtained by treating 2·0ml 1,2-dichloroethane (3.1) as indicated in (5.2).

(1) Whatman No. 1PS or equivalent.

Determine the quantity of menadione by reference to a calibration curve established for each series of analyses.

5.4 *Calibration curve*

Treat 2·0ml of the menadione standard solutions (3.10) as described in 5.2. Measure the absorbance as indicated in 5.3. Construct a graph relating the absorbances to the amount of menadione present.

6. CALCULATION OF RESULTS

Calculate the menadione content of the sample by taking account of the weight of the test sample and of the dilutions carried out in the course of analysis. Express the result in mg menadione per kg.

19. DETERMINATION OF HYDROCYANIC ACID

1. PURPOSE AND SCOPE

This method is for the determination of hydrocyanic acid, free and combined in the form of glycosides, in feeding stuffs and in particular in products derived from linseed, manioc flour and certain species of beans.

2. PRINCIPLE

The sample is suspended in water. The hydrocyanic acid is released by the action of enzymes, separated by steam distillation and collected in a specific volume of acidified silver nitrate solution. The silver cyanide is separated by filtration and the excess silver nitrate is titrated with a solution of ammonium thiocyanate.

3. REAGENTS

3.1 Antifoam (eg silicone).

3.2 Nitric acid (density 1·42 g/ml).

3.3 Ammonia solution dilute. Prepare by diluting 1 volume of ammonia (density 0·88g/ml) with two volumes of water.

3.4 Ammonium ferric sulphate, saturated aqueous solution.

3.5 Sweet almonds suspension; crush twenty blanched sweet almonds in 100ml water at 37 to 40°C. Check that there is no hydrocyanic acid in 10ml of the suspension using sodium picrate paper or by carrying out a blank test as described in the last paragraph of 5.

3.6 Sodium acetate solution neutral to phenolphthalein: 10g sodium acetate (anhydrous) per 100ml.

3.7 Ammonium thiocyanate solution, 0·02N.

3.8 Silver nitrate solution, 0·02N.

4. APPARATUS

4.1 Oven regulated at 37–38°C.

4.2 Apparatus for steam distillation fitted with a condenser with a curved extension piece.

4.3 1000ml flat-bottomed flasks with ground-glass stoppers.

4.4 Oil bath.

4.5 Burette graduated to 0·05ml.

5. PROCEDURE

Weigh to the nearest 0·005g, approximately 20g of the prepared sample, place in a one litre flat-bottomed flask (4.3) and add 50ml of water and 10ml of sweet almond suspension (3.5). Stopper the flask and transfer to the oven (4.1) for sixteen hours at 37–38°C. Cool to room temperature and add 80ml of water, 10ml of sodium acetate solution (3.6) and a drop of anti-foam (3.1).

Connect the flask to the steam distillation apparatus (4.2) and place in the oil bath (4.4) which has first been brought to a temperature slightly above 100°C. Distil 200

to 300ml of liquid by passing a current of steam through the flask and gently heating the oil bath. Collect the distillate in an Erlenmeyer flask protected from the light and containing exactly 50ml of silver nitrate solution 0·02N (3.8) and 1ml of nitric acid (3.2). Make sure that the condenser's extension piece is immersed in the silver nitrate solution.

Transfer the contents of the Erlenmeyer flask to a 500 ml graduated flask, make up to volume with water, mix and filter. Remove 250ml of the filtrate, add approximately 1ml ammonium ferric sulphate (3.4) and titrate the excess silver nitrate with the solution of ammonium thiocyanate 0·02N (3.7). A blank test may, if required, be carried out by applying the same procedure to 10ml of sweet almond suspension (3.5), omitting the sample to be analysed.

6. CALCULATION OF RESULTS

If the blank test indicates that silver nitrate solution 0·02N has been consumed, subtract the value of this from the volume consumed by the distillate of the sample. 1ml of $AgNO_3$ 0·02N = 0·54mg of HCN. Express the result as a percentage of the sample.

Note: If the sample contains a large quantity of sulphides (eg beans) a black precipitate of silver sulphide is formed which is filtered together with the silver cyanide deposit. The formation of this precipitate causes a loss of silver nitrate solution 0·02N, the volume of which must be subtracted from the volume used to calculate the HCN content. To do this, proceed as follows:

Treat the deposit left on the filter with 50ml of ammonia (3.3) in order to dissolve the silver cyanide. Wash the residue in dilute ammonia and then determine its silver content. Convert the value obtained into ml of silver nitrate solution 0·02N.

20. DETERMINATION OF VOLATILE MUSTARD OIL

1. PURPOSE AND SCOPE

This method is for the determination of volatile mustard oil contained in cakes made from the *Brassica* and *Sinapis* species, and in feeding stuffs which contain cakes made from those species. The steam separated component is expressed as allyl isothiocyanate.

2. PRINCIPLE

The sample is suspended in water. The volatile mustard oil is released by the action of enzymes, entrained by distillation with ethanol and collected in dilute ammonia. The solution is treated while warm with a given volume of silver nitrate solution, then cooled and filtered. The excess silver nitrate is titrated with a solution of ammonium thiocyanate.

3. REAGENTS

3.1 Antifoam (eg silicone).

3.2 Ethanol, 96% (V/V).

3.3 Nitric acid (density 1·42 g/ml).

3.4 White mustard (*Sinapis alba*).

3.5 Ammonia, dilute solution: prepare by diluting 1 volume of ammonia (density 0·88 g/ml) with 2 volumes of water.

3.6 Ammonium ferric sulphate, saturated solution.

3.7 Ammonium thiocyanate solution, 0·1N.

3.8 Silver nitrate solution, 0·1N.

4. APPARATUS

4.1 Flat-bottomed 500ml flasks with ground-glass stoppers.

4.2 Distilling apparatus fitted with a condenser and a splash head.

5. PROCEDURE

Weigh to the nearest 0·001g approximately 10g of the prepared sample and place in a 500ml flat-bottomed flask (4.1) and add 2g of finely ground white mustard (3.4) [an enzyme source] and 200ml water at 20°C. Stopper the flask and keep at 20°C for approximately 2 hours, shaking frequently. Add 40ml ethanol (3.2) and one drop of antifoam (3.1). Distil approximately 150ml and collect the distillate in a 500ml conical flask containing 20ml ammonia (3.5) making sure that the end of the condenser is immersed in the liquid. Add to the ammoniacal solution 50ml silver nitrate solution 0·1N, (3.8) (or more if necessary), place a small funnel over the neck of the flask, and heat the mixture on a steam bath for one hour. Allow to cool, transfer to a 250ml graduated flask, rinsing in with water, make up to the mark, mix and filter. To 100ml of the clear filtrate, add 5ml nitric acid (3.3) and approximately 5ml ammonium ferric sulphate solution (3.6). Titrate the excess silver nitrate with the 0·1N ammonium thiocyanate solution (3.7).

Carry out a blank test by applying the same procedure to 2g finely ground white mustard, omitting the sample for analysis.

6. CALCULATION OF RESULTS

Subtract the volume of silver nitrate solution 0·1N consumed in the blank test from that consumed by the sample in solution. The value obtained gives the number of ml of silver nitrate solution 0·1N consumed by the mustard oil in the sample. 1ml of $AgNO_3$ 0·1N 4·956mg of allyl isothiocyanate. Express the result as allyl isothiocyanate as a percentage of the sample.

21. DETERMINATION OF FREE AND TOTAL GOSSYPOL

1. PURPOSE AND SCOPE

This method is for the determination of free gossypol, total gossypol and chemically related substances in seed, flour and cotton seed cake, and feeding stuffs containing these substances. The lowest limit of determination is 20mg/kg.

2. PRINCIPLE

The gossypol is extracted in the presence of 3-aminopropan-1-ol either by a mixture of propan-2-ol and hexane for the determination of free gossypol, or by dimethylformamide for the determination of total gossypol. The gossypol is converted by aniline to gossypol-dianiline, the absorbance of which is measured at 440nm.

3. REAGENTS

3.1 Propan-2-ol/hexane mixture: mix 60 parts by volume propan-2-ol with 40 parts by volume hexane.

3.2 Solvent A: place in a 1 litre graduated flask about 500ml propan-2-ol/hexane mixture (3.1), 2ml 3-aminopropan-1-ol, 8ml glacial acetic acid and 50ml water. Make up to volume with the propan-2-ol/hexane mixture (3.1.) This reagent will remain stable for one week.

3.3 Solvent B: place in a 100ml graduated flask 2ml 3-aminopropan-1-ol and 10ml glacial acetic acid. Cool to room temperature and make up to volume with dimethylformamide. This reagent will remain stable for one week.

3.4 Aniline: if the absorbance of the blank test exceeds 0·022, distil the aniline over zinc dust rejecting the first and last 10% fractions of the distillate. This reagent will keep for several months refrigerated in a stoppered dark glass flask.

3.5 Standard gossypol solution A: place in a 250ml graduated flask 27·9mg gossypol acetate. Dissolve and make up to volume with solvent A (3.2). Place 50ml of this solution in a 250ml graduated flask and make up to volume with solvent A. This solution has a gossypol concentration of 0·02mg/ml. Allow to stand for one hour at room temperature before use.

3.6 Standard gossypol solution B: place in a 50ml graduated flask 27·9mg gossypol acetate. Dissolve and make up to volume with solvent B (3.3). This solution has a gossypol concentration of 0·5mg/ml.

Standard gossypol solutions A and B will remain stable for 24 hours if kept away from light.

4. APPARATUS

4.1 Mixer (tumbler); approximately 35 revolutions per minute.

4.2 Spectrophotometer with 10mm cells.

5. PROCEDURE

5.1 *Sample for analysis*

The sample taken for analysis depends on the supposed level of gossypol in the sample. It is preferable to work on a small sample for analysis together with a relatively large aliquot part of the filtrate, so as to obtain a sufficient quantity of gossypol to be able to carry out a precise photometric measurement. *For the determination of free gossypol* in seeds, flour and cotton seed cake, the sample for analysis must not exceed 1g; for compound feeding stuffs it may be as much as 5g. A 10ml aliquot part of the filtrate is suitable in most cases; it should contain from 50 to 100 μg gossypol. *For the determination of total gossypol*, the sample for analysis may vary from 0·5 to 5g so that a 2ml aliquot part of the filtrate contains 40 to 200μg gossypol. *The analysis must be carried out at a room temperature close to 20°C.*

5.2 *Determination of free gossypol*

Place the prepared sample in a 250ml flask with ground glass neck, the bottom of which has been covered with a layer of glass beads of approximately 6mm diameter. Add 50·0ml solvent A (3.2) stopper the flask and mix for one hour in the mixer (4.1). Filter through a dry filter and collect the filtrate in a small flask with ground glass neck. During filtration, cover the funnel with a watch glass. Transfer to two 25ml graduated flasks (A and B) identical aliquot parts of filtrate containing 50 to 100μg gossypol. If necessary make up the volume to 10ml using solvent A (3.2). Then make up to volume the contents of flask (A) with the propan-2-ol/hexane mixture (3.1). This solution is used as a reference solution against which the sample is measured.

Transfer 10ml solvent A (3.2) to each of two other 25ml graduated flasks (C and D). Make up to volume the contents of flask (C) with the propan-2-ol/hexane mixture (3.1). This solution is used as a reference solution against which to measure the blank.

Add 2ml aniline (3.4) to flasks (D) and (B). Heat for 30 minutes on a steam bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol/hexane mixture (3.1), mix and allow to stand for one hour. Measure the absorbance of the blank test solution (D) compared with the solution for reference (C), and the absorbance of the sample solution (B) compared with the solution for reference (A), at 440nm in the spectrophotometer (4.2).

Subtract the absorbance of the blank test solution from that of the sample solution (=corrected absorbance). From this value calculate the amount of free gossypol as indicated in 6.

5.3 *Determination of total gossypol*

Place a prepared sample for analysis containing 1 to 5mg gossypol in a 50ml graduated flask and add 10ml solvent B (3.3). At the same time prepare a blank test, placing 10ml solvent B (3.3) in another 50ml graduated flask. Heat the two flasks for 30 minutes on a steam bath. Cool to room temperature and make up the volume of the contents of each flask with the propan-2-ol/hexane mixture (3.1). Mix and allow to settle for 10 to 15 minutes, then filter.

Transfer 2ml of the sample filtrate to each of two 25ml graduated flasks, and 2ml of the blank test filtrate to two other 25ml flasks. Take one flask from each pair and make up the contents of each to 25ml with the propan-2-ol/hexane mixture (3.1). These solutions shall be used for reference.

Add 2ml aniline (3.4) to each of the other two flasks. Heat for 30 minutes on a steam bath to develop the colour. Cool to room temperature, make up to 25ml with the propan-2-ol/hexane mixture (3.1), mix and allow to stand for 1 hour.

Measure the absorbance as indicated in 5.2 for free gossypol. From this value calculate the amount of total gossypol as indicated in 6.

6. CALCULATION OF RESULTS

Results may be calculated either from the specific absorbance (6.1) or by reference to a calibration curve (6.2).

6.1 *From the specific absorbance*

In the conditions described, the specific absorbances are as follows:

free gossypol: $E^{1\%}_{1cm}$ - 625

total gossypol: $E^{1\%}_{1cm}$ 600

The amount of free or total gossypol in the sample is given by the following formula:

$$\text{gossypol } \% = \frac{E \times 1250}{E^{1\%}_{1cm} \times p \times a}$$

in which:

E = corrected absorbance, determined as indicated in 5.2;

p = sample taken for analysis in grams; and

a = aliquot part of the filtrate in ml.

6.2 *From a calibration curve*

6.2.1 *Free gossypol*

Prepare 2 series of five 25ml graduated flasks. Transfer to each series of flasks respectively 2·0, 4·0, 6·0, 8·0 and 10·0 ml aliquots of standard gossypol solution A (3.5). Make up the volumes to 10ml using solvent A (3.2). Complete each series with a blank test consisting of a 25ml graduated flask containing only 10ml solvent A (3.2).

Make up the volumes of the first series to 25ml (including the blank test) with the propan- 2-ol/hexane mixture (3.1) (reference series).

Add 2ml aniline (3·4) to each flask in the second series (including the blank test). Heat for 30 minutes on a steam bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol/hexane mixture (3.1) mix and allow to stand for 1 hour (standard series).

Measure, under the conditions indicated in 5.2 the absorbances of the solutions in the standard series compared with the corresponding solutions in the reference series. Plot a calibration curve of absorbances against quantities of gossypol (in μg).

6.2.2 *Total gossypol*

Prepare six 50ml graduated flasks. In the first flask place 10ml solvent B (3.3) and in the others respectively 2·0, 4·0, 6·0, 8·0 and 10·0ml standard gossypol solution B (3.6). Make up the contents of each flask to 10ml using solvent B (3.3). Heat for 30 minutes on a steam bath. Cool to room temperature, make up to volume with the propan-2-ol/hexane mixture (3.1) and mix.

Place 2·0ml of these solutions respectively in two series of six 25ml graduated flasks. Make up the contents of the flasks in the first series to 25ml using the propan-2-ol/hexane mixture (3.1) (reference series).

Add 2ml aniline (3.4) to each flask in the second series. Heat for 30 minutes on a steam bath. Cool to room temperature, make up to volume with the propan-2-ol/hexane mixture (3.1) mix and allow to stand for 1 hour (standard series).

Under the conditions indicated in 5.2 measure the absorbances of the solutions in the standard series compared with the corresponding solutions in the reference series. Plot the calibration curve of absorbances against quantities of gossypol (in μg).

22*a*. DETERMINATION OF AFLATOXIN B_1—METHOD I

1. PURPOSE AND SCOPE

This method is for the determination of aflatoxin B_1 in the following feeding stuffs only: groundnut, copra, linseed, soya, sesame, babassu palm and maize germ oilcakes, cereals and cereal products, pea meal, potato pulp and starch. The lower limit of determination is 10μg/kg. Other products should be analysed by the method described under method 22*b*.

2. PRINCIPLE

The sample is extracted with chloroform, the extract filtered, and an aliquot portion of the filtrate purified by column chromatography on silica gel. The eluate is evaporated and the residue redissolved in a specific volume of chloroform or of a mixture of benzene and acetonitrile. An aliquot portion of this solution is subjected to thin-layer chromatography. The quantity of aflatoxin B_1 is determined under UV irradiation of the chromatogram, either visually or by fluorodensitometry, by comparison with known quantities of standard aflatoxin B_1. The identity of the aflatoxin B_1 must be confirmed by the procedure indicated.

3. REAGENTS

Note: Aflatoxin is a very hazardous material in view of its carcinogenic nature. It is suggested that particular attention be paid to the decontamination recommendations given in J. Assoc. Off. Anal. Chem., 1965, **48**, 681.

3.1 Acetone.

3.2 Chloroform stabilised with 0·5 to 1·0% of 96% ethanol (V/V).

3.3 n-Hexane.

3.4 Methanol.

3.5 Diethyl ether, anhydrous, free from peroxides.

3.6 Mixture of benzene and acetonitrile in the proportions by volume 98+2.

3.7 Mixture of chloroform (3.2) and methanol (3.4) in the proportions by volume 97+3.

3.8 Silica gel, for column chromatography, particle size 0·05 to 0·20mm.

3.9 Absorbent cotton wool, previously degreased with chloroform, or glass wool.

3.10 Sodium sulphate, anhydrous, granular.

3.11 Inert gas, eg nitrogen.

3.12 Hydrochloric acid solution, 1N.

3.13 Sulphuric acid solution: mix one volume of sulphuric acid (density 1·84 g/ml) with one volume of water.

3.14 Diatomaceous earth(1), acid washed.

3.15 Silica gel G-HR or equivalent, for TLC.

(1) Hyflosupercel or equivalent.

3.16 Standard solution with about 0·1μg of aflatoxin B_1 per ml in chloroform (3.2) or the benzene/acetonitrile mixture (3.6), prepared and checked as indicated in Section 7.

3.17 Standard solution for qualitative testing purposes containing about 0·1μg of aflatoxin B_1 and B_2 per ml in chloroform (3.2), or benzene/acetonitrile mixture (3.6). These concentrations are given as a guide. They must be adjusted so as to obtain the same intensity of fluorescence for both aflatoxins.

3.18 Developing solvents:

3.18.1 Mixture of chloroform (3.2) and acetone (3.1) in the proportions by volume 9+1, unsaturated tank;

3.18.2 mixture of diethyl ether (3.5) and methanol (3.4) and water in the proportions by volume 96+3+1, unsaturated tank;

3.18.3 mixture of diethyl ether (3.5) and methanol (3.4) and water in the proportions by volume 94+4·5+1·5, saturated tank;

3.18.4 mixture of chloroform (3.2) and methanol (3.4) in the proportions by volume 94+6, saturated tank;

3.18.5 mixture of chloroform (3.2) and methanol (3.4) in the proportions by volume 97+3, saturated tank.

4. APPARATUS

4.1 Glass tube for chromatography (internal diameter: 22mm; length: 300mm), with a PTFE stopcock and a 250ml reservoir.

4.2 Rotary evaporator, with inlet for inert gas and a 500ml round-bottom flask.

4.3 TLC apparatus.

4.4 Glass plates for TLC, 200×200mm, prepared as follows (the quantities indicated are sufficient to cover five plates):

put 30g of silica gel G-HR (3.15) into a conical flask. Add 60ml water, stopper and shake for a minute. Spread the suspension on the plates so as to obtain a uniform layer 0·25mm thick. Leave to dry in the air and then store in a desiccator containing silica gel. At the time of use, activate the plates by keeping them in an oven at 110°C for one hour. Ready-to-use plates are suitable if they give results similar to those obtained with the plates prepared as indicated above.

4.5 UV lamp, wavelength 365nm. The intensity of irradiation must make it possible for a spot of 1·0 nanogram of aflatoxin B_1 to be clearly distinguished on a TLC plate at a distance of 10cm from the lamp.

4.6 10·0ml graduated tubes with polyethylene stoppers.

4.7 UV Spectrophotometer with 10mm silica cells.

4.8 Fluorodensitometer (optional).

5. PROCEDURE

5.1 *Defatting*

Samples containing more than 5% oil or fat must be defatted with light petroleum (boiling range 40–60°C) before the material is ground and sieved as described under Method 1 (Preparation of sample for analysis). In such cases the analytical results must be expressed in terms of the weight of the non-defatted sample.

5.2 *Extraction*

Put 50·0g of the prepared sample into a 500ml conical flask. Add 25g of diatomaceous earth (3.14), 25ml of water and 250ml of chloroform (3.2). Stopper the flask, shake or stir for 30 minutes and filter through a fluted filter paper(1). Discard the first 10ml of the filtrate and then collect 50ml.

(1) Whatman No. 1 or equivalent.

5.3 *Column clean-up*

Insert into the lower end of a chromatography tube (4.1) with tap closed a cotton or glass wool plug (3.9), fill two-thirds of the tube with chloroform (3.2) and add 5g of sodium sulphate (3.10). Check that the upper surface of the sodium sulphate (3.10) is flat, then add 10g of silica gel (3.8) in small portions. Stir carefully after each addition to eliminate air-bubbles. Allow to stand for 15 minutes and then carefully add 15g of sodium sulphate (3.10). Let the liquid fall until it is just above the upper surface of the sodium sulphate layer. Mix the 50ml of extract collected in 5.2 with 100ml of hexane (3.3) and quantitatively transfer the mixture to the column. Allow the liquid to fall until it is just above the upper surface of the sodium sulphate layer. Discard this eluate. Then add 100ml of diethyl ether (3.5) and again allow it to fall to the upper surface of the sodium sulphate layer. During these operations adjust the rate of flow to 8–12ml per minute and ensure that the column does not run dry. Discard the eluate. Finally elute with 150ml of the chloroform/methanol mixture (3.7) and collect the whole of this eluate. Evaporate the latter *almost* to dryness in the rotary evaporator (4.2) at a temperature not exceeding 50°C, under a stream of inert gas (3.11). Quantitatively transfer the residue, using chloroform (3.2) to a 10·0ml graduated tube (4.6). [Note: chloroform may be unsuitable for some residues in which case benzene/acetonitrile (3.6) should be used.] Concentrate the solution under a stream of inert gas (3.11) and then adjust the volume to 2·0ml with chloroform (3.2) or benzene/acetonitrile mixture (3.6).

5.4 *Thin-layer chromatography*

Spot on a TLC plate (4.4) 2cm from the lower edge and at intervals of 2cm, the volumes indicated below of the standard solution and the extract:

– 10, 15, 20, 30 and 40μl of the standard aflatoxin B_1 solution (3.16);

– 10μl of the extract obtained in 5.3 and, *superimposed on the same point*, 20μl of the standard solution (3.16); and

– 10 and 20 μl of the extract obtained in 5.3.

The spots obtained must have a diameter of about 5mm. Dry in a slow stream of air. Develop the chromatogram in the dark with one of the developing solvents (3.18). The choice of the solvent must be made beforehand, by depositing 25 μl of the qualitative standard solution (3.17) on a plate and checking that, when developed, aflatoxin B_1 and B_2 are completely separated. Remove the plate from the tank and allow the solvents to evaporate in the dark and then irradiate with UV light, placing the plate 10cm from the lamp (4.5). The spots of aflatoxin B_1 give a blue fluorescence.

5.5 *Quantitative determination*

Determine either visually or by fluorodensitometry as indicated below.

5.5.1 *Visual measurements*

Determine the quantity of aflatoxin B_1 in the extract by matching the fluorescence intensity of the extract spots with that of one of the standard solution spots; interpolate if necessary. The fluorescence obtained by the super-imposition of the extract on the standard solution must be more intense than that of the 10μl of extract and there must not be more than one visible spot. If the fluorescence intensity given by the 10μl of extract is greater than that of the 40μl of standard solution, dilute the extract 10 or 100 times with chloroform (3.2) or benzene/acetonitrile mixture (3.6) before subjecting it again to thin-layer chromatography.

5.5.2 *Measurements by fluorodensitometry*

Measure the fluorescence intensity of the aflatoxin B_1 spots with the fluorodensitometer (4.8) at an excitation wavelength of 365nm and an emission wavelength of 443nm. Determine the quantity of aflatoxin B_1 in the extract spots by comparison of their fluorescence intensities with that of the standard aflatoxin B_1 spots.

5.6 *Confirmation of the identity of aflatoxin B_1*

Confirm the identity of the aflatoxin B_1 in the extract by the procedures indicated below.

5.6.1 *Treatment with sulphuric acid*

Spray the chromatogram obtained in 5.4 with sulphuric acid (3.13). The fluorescence of the aflatoxin B_1 spots after spraying with sulphuric acid must be yellow under UV irradiation.

5.6.2 *Two dimensional chromatography involving the formation of aflatoxin B_1—hemiacetal (aflatoxin B_{2a})*

Note: The operations described below must be carried out following carefully the diagram in fig 1.

5.6.2.1 *Application of the solutions*

Score two straight lines on a TLC plate (4.4) parallel to two contiguous sides (6cm in from each side) to limit migration of the solvent fronts. Spot the following solutions on the plate using capillary pipettes or microsyringes:

—*on point A:* a volume of purified extract of the sample, obtained in 5.3 containing about 2.5 nanograms of aflatoxin B_1;

—*on points B and C:* 25µl of the standard solution (3.16).

5.6.2.2 *Development*

Develop the chromatogram in direction I, in the dark, using the developing solvent (3.18.1) (1cm depth of solvent in an unsaturated tank) until the solvent front reaches the solvent limit line. Remove the plate from the tank and allow to dry in the dark at ambient temperature for five minutes. Cover the plate with a glass sheet so that a band 2·5cm wide, containing points A and B, is left exposed (indicated by the hatched area in fig 1). Spray the exposed band with hydrochloric acid (3.12) until it darkens, the cover sheet is overlaid with a sheet of filter paper to absorb excess hydrochloric acid. Allow to react for 10 minutes in the dark and dry with a stream of air at ambient temperature. Develop the chromatogram in direction II, in the dark, using the developing solvent (3.18.1) (1cm layer in an unsaturated tank) until the solvent front reaches the solvent limit line. Remove the plate from the tank and allow to dry at ambient temperature.

5.6.2.3 *Interpretation of the chromatogram*

Examine the chromatogram under UV light (4.5) and check for the following features:

(*a*) appearance of a blue fluorescent spot of aflatoxin B_1 originating from the standard solution applied at C (migration in the direction I);

(*b*) appearance of a blue fluorescent spot of unreacted (with the hydrochloric acid) aflatoxin B_1 and a more intense blue fluorescent spot of aflatoxin B_1-hemiacetal, both originating from the standard solution applied at B (migration in direction II); and

(*c*) appearance of spots matching those described in *b*, originating from the sample extract applied at A. The position of these spots is defined first by the migration distance of the aflatoxin B_1 from point A in direction I (same as that travelled by the standard applied at C), and then by the migration distances from there in direction II of the aflatoxin B_1-hemiacetal (same as those travelled by the standard applied at B). The fluorescence intensities of hemiacetal spots originating from the extract and from the standard applied at B should match.

6. CALCULATION OF THE RESULTS

6.1 *From the visual measurements*

The content in micrograms of aflatoxin B_1 per kg of sample is given by the formula $\frac{R \times Y \times V}{W \times Z}$ in which:

Y and Z are respectively the volumes in microlitres of the standard solution of aflatoxin B_1 (3.16) and of the extract having a similar intensity of fluorescence;

R = concentration in micrograms of aflatoxin B_1 per ml in the standard solution (3.16);

V = final volume of the extract in microlitres, allowing for any dilution that was necessary; and

W = weight in grams of the sample corresponding to the volume of extract subjected to column clean-up.

6.2 *From the fluorodensitometric measurements*

The content in micrograms of aflatoxin B_1 per kg of sample is given by the formula $\frac{S \times V}{W \times Z}$ in which:

Z = volume in microlitres of the extract spotted on the plate;

S = quantity in nanograms of aflatoxin B_1 in the extract spot, 10 or 20µl related to Y deduced from the measurements;

V = final volume of the extract in microlitres, allowing for any dilution that was necessary; and

W = weight in grams of the sample corresponding to the volume of extract subjected to column clean-up.

7. PREPARATION AND TESTING OF THE STANDARD SOLUTION (3.16)

7.1 *Determination of the concentration of aflatoxin B_1*

Caution: Aflatoxin solid is a most hazardous material and analysts are urged to minimise the handling of it in this form. The use of stock solutions, wherever possible, is recommended. See also note under 'Reagents' paragraph 3.

Prepare a standard solution of aflatoxin B_1 in chloroform (3.2) or benzene/acetonitrile mixture (3.6) with a concentration of 8 to 10µg per ml. Determine the absorption spectrum between 330 and 370nm. Measure the absorbance (A) at 363 nm in the case of the chloroform solution: or at 348nm in the case of the solution in benzene/acetonitrile mixture. Calculate the concentration in micrograms of aflatoxin B_1 per ml of solution from the formulae below:

$\frac{312 \times A \times 1000}{20\,600}$ for the chloroform solution;

$\frac{312 \times A \times 1000}{19\,800}$ for the solution in the benzene/acetonitrile mixture.

Dilute as appropriate, away from daylight, to obtain a working standard solution with a concentration of aflatoxin B_1 of about 0·1µg per ml. If kept in a refrigerator at 4°C, this solution is stable for two weeks.

7.2 *Testing of chromatographic purity*

Spot on a plate (4.4) 5µl of the standard solution of aflatoxin B_1 containing 8 to 10µg/ml (7.1). Develop the chromatogram as indicated in 5.4. In UV light the chromatogram should show only one spot and no fluorescence must be perceptible in the original deposit zone.

8. OBSERVATIONS ON REPRODUCIBILITY OF RESULTS

The variation between the results obtained by two or more laboratories on the same sample has been estimated at:

±50% of the mean value for mean values of aflatoxin B_1 greater than 10 and up to and including 20μg/kg.

±10μg/kg on the mean value for mean values greater than 20 and up to and including 50μg/kg.

±20% of the mean value for mean values above 50μg/kg.

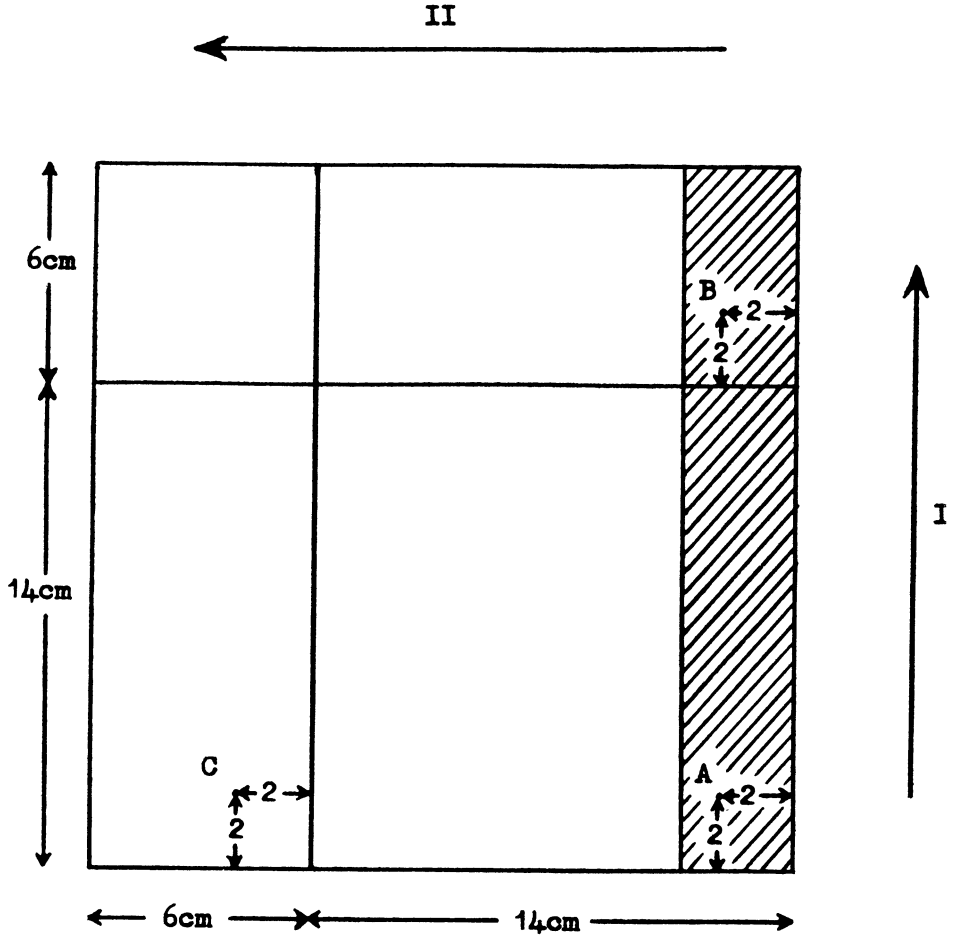

Fig. 1

Secure plate with B in top right hand corner when spraying with hydrochloric acid.

22*b*. DETERMINATION OF AFLATOXIN B_1—METHOD II

1. PURPOSE AND SCOPE

This method is for the determination of aflatoxin B_1 in feeding stuffs not falling within the scope of method 22*a*. The lower limit of determination is 10μg/kg.

2. PRINCIPLE

The sample is subjected to extraction with chloroform, the extract filtered, and an aliquot portion of the filtrate purified by column chromatography on silica gel. The eluate is evaporated and the residue redissolved in a specific volume of chloroform or of a mixture of benzene and acetonitrile. An aliquot portion of this solution is subjected to two-dimensional thin-layer chromatography. The quantity of aflatoxin B_1 is determined under UV irradiation of the chromatogram, either visually or by fluorodensitometry, by comparison with known quantities of standard aflatoxin B_1. The identity of the aflatoxin B_1 must be confirmed by the procedure indicated.

3. REAGENTS

3.1 Acetone.

3.2 Chloroform, stabilised with 0·5 to 1·0% of 96% ethanol (V/V).

3.3 n-Hexane.

3.4 Methanol.

3.5 Diethyl ether, anhydrous, free from peroxides.

3.6 Mixture of benzene and acetonitrile in the proportions by volume 98+2.

3.7 Mixture of chloroform (3.2) and methanol (3.4) in the proportions by volume 97+3.

3.8 Silica gel, for column chromatography, particle size 0·05 to 0·20mm.

3.9 Absorbent cotton wool, previously degreased with chloroform, or glass wool.

3.10 Sodium sulphate, anhydrous, granular.

3.11 Inert gas, eg nitrogen.

3.12 Hydrochloric acid solution, 1N.

3.13 Sulphuric acid solution: mix one volume of sulphuric acid (density 1·84g/ml) with one volume of water.

3.14 Diatomaceous earth(1), acid washed.

3.15 Silica gel G-HR or equivalent, for TLC.

3.16 Standard solution with about 0·1μg aflatoxin B_1 per ml in chloroform (3.2) or benzene/acetonitrile mixture (3.6), prepared and checked as described in Section 7 of method 22*a*.

3.17 Developing solvents

3.17.1 Mixture of diethyl ether (3.5) and methanol (3.4) and water in the proportions by volume 94+4·5+1·5, saturated tank.

3.17.2 Mixture of chloroform (3.2) and acetone (3.1) in the proportions by volume 9+1, unsaturated tank.

4. APPARATUS

4.1 Glass tube for chromatography (internal diameter: 22mm; length: 300mm), with a PTFE stopcock and a 250ml reservoir.

4.2 Rotary evaporator, with inlet for inert gas and a 500ml round-bottomed flask.

4.3 TLC apparatus.

4.4 Glass plates for TLC, 200×200mm, prepared as follows (the quantities indicated are sufficient to cover five plates): put 30g of silica gel G-HR (3.15) into a conical flask. Add 60ml water, stopper and shake for a minute. Spread the suspension on the plates so as to obtain a uniform layer 0·25mm thick. Leave to dry in the

(1) Hyflosupercel or equivalent.

air and then store in a desiccator containing silica gel. At the time of use, activate the plates by keeping them in an oven at 110°C for one hour. Ready-to-use plates are suitable if they give results similar to those obtained with the plates prepared as indicated above.

4.5 UV lamp, wavelength 365nm. The intensity of irradiation must make it possible for a spot of 1·0 nanogram of aflatoxin B_1 to be clearly distinguished on a TLC plate at a distance of 10cm from the lamp.

4.6 10·0ml graduated tubes with polyethylene stoppers.

4.7 UV Spectrophotometer with 10mm silica cells.

4.8 Fluorodensitometer (optional).

5. PROCEDURE

5.1 *Defatting*

Samples containing more than 5% oil or fat must be defatted with light petroleum (boiling range 40–60°C) before the material is ground and sieved as described under Method 1 (Preparation of sample for analysis). In such cases the analytical results must be expressed in terms of the weight of the non-defatted sample.

5.2 *Extraction*

Put 50·0g of the prepared sample into a 500ml conical flask. Add 25g of diatomaceous earth (3.14), 25ml of water and 250ml of chloroform (3.2). Stopper the flask, shake or stir for 30 minutes and filter through a fluted filter paper (1). Discard the first 10ml of the filtrate and then collect 50ml.

5.3 *Column clean-up*

Insert into the lower end of a chromatography tube (4.1) with tap closed a cotton or glass wool plug (3.9), fill two-thirds of the tube with chloroform (3.2) and add 5g of sodium sulphate (3.10). Check that the upper surface of the sodium sulphate (3.10) is flat, then add 10g of silica gel (3.8) in small portions. Stir carefully after each addition to eliminate air-bubbles. Allow to stand for 15 minutes and then carefully add 15g of sodium sulphate (3.10). Let the liquid fall until it is just above the upper surface of the sodium sulphate layer.

Mix the 50ml of extract collected in 5.2 with 100ml of hexane (3.3) and quantitatively transfer the mixture to the column.

Allow the liquid to fall until it is just above the upper surface of the sodium sulphate layer. Discard this eluate. Then add 100ml of diethyl ether (3.5) and again allow it to fall to the upper surface of the sodium sulphate layer. During these operations adjust the rate of flow to 8–12ml per minute and ensure that the column does not run dry. Discard the eluate. Finally elute with 150ml of the chloroform/methanol mixture (3.7) and collect the whole of this eluate.

Evaporate the latter *almost* to dryness in the rotary evaporator (4.2) at a temperature not exceeding 50°C, under a stream of inert gas (3.11).

Quantitatively transfer the residue, using chloroform (3.2), to a 10·0ml graduated tube (4.6). [Note: chloroform may be unsuitable for some residues in which case benzene/acetonitrile (3.6) should be used].

Concentrate the solution under a stream of inert gas (3.11) and then adjust the volume to 2·0ml with chloroform (3.2) or benzene/acetonitrile mixture (3.6).

5.4 *Two dimensional thin layer chromatography*

5.4.1 *Application of the solutions (follow the diagram in fig* 2)

Score two straight lines on a plate (4.4) parallel to two contiguous sides (5cm and 6cm from each side respectively), to limit migration of the solvent fronts. Spot the following solutions on the plate:

—*on point A*, 20μl of the purified sample extract obtained in 5.3;

—*on point B*, 20μl of the standard solution (3.16);

(1) Whatman No. 1 or equivalent.

—*on point C*, 10μl of the standard solution (3.16);

—*on point D*, 20μl of the standard solution (3.16); and

—*on point E*, 40μl of the standard solution (3.16).

Dry in a slow stream of air or inert gas (3.11). The spots obtained must have a diameter of about 5mm.

5.4.2 *Development (following the diagram in fig 2)*

Develop the chromatogram in direction I, in the dark, using the developing solvent (3.17.1) (1cm layer in a saturated tank) until the solvent front reaches the limit line.

Remove the plate from the tank and allow to dry, in the dark, at ambient temperature for 15 minutes.

Develop the chromatogram in direction II, in the dark, using the developing solvent (3.17.2) (1cm layer in an unsaturated tank) until the solvent front reaches the limit line.

Remove the plate from the tank and allow to dry, in the dark, at ambient temperature.

5.4.3 *Interpretation of the chromatogram (follow the diagram in fig 3)*

Irradiate the chromatogram with UV light by placing the plate 10cm from the lamp (4.5). Locate the position of the blue fluorescent spots B, C, D and E of the aflatoxin B_1 from the standard solution. Project two imaginary lines passing through these spots and at right angles to the development directions. The intersection P of these lines is the location in which to expect to find the aflatoxin B_1 spot originating from the sample extract applied at A (fig 2). However, the actual location of the aflatoxin B_1 spot may be at a point Q at the intersection of two imaginary straight lines forming an angle of about 100° between them and passing through spots B and C respectively. Determine the quantity of aflatoxin B_1 in the sample extract as indicated in 5.5.

5.4.4 *Supplementary chromatography*

Score two straight lines on a new plate (4.4) parallel to two contiguous sides, as indicated on the diagram in fig 2, and apply on point A (see fig 2) 20μl of the purified sample extract obtained in 5.3 and, superimposed on it, 20μl of the standard solution (3.16). Develop as indicated in 5.4.2. Irradiate the chromatogram with UV light (4.5) and check for the following features:

(*a*) the aflatoxin B_1 spots from the extract and the standard solution are superimposed; and

(*b*) the fluorescence of this spot is more intense than that of the aflatoxin B_1 spot developed at Q on the first plate.

5.5 *Quantitative determination*

Determine either visually or by fluorodensitometry as indicated below.

5.5.1 *Visual measurements*

Determine the quantity of aflatoxin B_1 in the extract by matching the fluorescence intensity of the extract spot with one of the standard solution spots (C, D or E); interpolate if necessary. If the fluorescence intensity given by the 20μl of extract is greater than that of the 40μl of standard solution, dilute the extract 10 or 100 times with chloroform (3.2) or benzene/acetonitrile mixture (3.6) before subjecting it again to thin-layer chromatography.

5.5.2 *Measurements by fluorodensitometry*

Measure the fluorescence intensity of the aflatoxin B_1 spots with the fluorodensitometer (4.8), using an excitation wavelength of 365nm and

an emission wavelength of 443nm. Determine the quantity of aflatoxin B_1 in the extract spot by comparison of its fluorescence intensity with that of the standard aflatoxin B_1 spots.

5.6 *Confirmation of the identity of aflatoxin B_1*

Confirm the identity of the aflatoxin B_1 in the extract by the procedures indicated below.

5.6.1 *Treatment with sulphuric acid*

Spray the chromatogram obtained in 5.4 with sulphuric acid (3.13). The fluorescence of the aflatoxin B_1 spots after spraying with sulphuric acid must be yellow under UV irradiation.

5.6.2 *Two dimensional chromatography involving the formation of aflatoxin B_1—hemiacetal (aflatoxin B_{2a})*

Note: The operations described below must be carried out following carefully the diagram in fig 1.

5.6.2.1 *Application of the solutions*

Score two straight lines on a TLC plate (4.4) parallel to two contiguous sides (6cm in from each side) to limit migration of the solvent fronts. Spot the following solutions on the plate using capillary pipettes or microsyringes:

on point A: a volume of purified extract of the sample, obtained in 5.3 containing about 2·5 nanograms of aflatoxin B_1; and

on points B and C: 25µl of the standard solution (3.16).

5.6.2.2 *Development*

Develop the chromatogram in direction I, in the dark, using the developing solvent (3.17.1) (1cm depth of solvent in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry in the dark at ambient temperature for five minutes. Cover the plate with a glass sheet so that a band 2·5cm wide, containing points A and B, is left exposed (indicated by the hatched area in fig 1). Spray the exposed band with hydrochloric acid (3.12) until it darkens, the cover sheet is overlaid with a sheet of filter paper to absorb excess hydrochloric acid. Allow to react for 10 minutes in the dark and dry with a stream of air at ambient temperature.

Develop the chromatogram in direction II, in the dark, using the developing solvent (3.17.1) (1cm layer in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry at ambient temperature.

5.6.2.3 *Interpretation of the chromatogram*

Examine the chromatogram under UV light (4.5) and check for the following features:

(*a*) appearance of a blue fluorescent spot of aflatoxin B_1 originating from the standard solution applied at C (migration in the direction I);

(*b*) appearance of a blue fluorescent spot of unreacted (with the hydrochloric acid) aflatoxin B_1 and a more intense blue fluorescent spot of aflatoxin B_1-hemiacetal, both originating from the standard solution applied at B (migration in direction II); and

(*c*) appearance of spots matching those described in (*b*), originating from the sample extract applied at A. The position of these spots is defined first by the migration distance of the aflatoxin B_1 from point A in direction I (same as that travelled by the standard applied at C), and then by the migration distances from there in direction II of the aflatoxin B_1-hemiacetal (same as those travelled by the standard applied at B). The fluorescence intensities of hemiacetal spots originating from the extract and from the standard applied at B should match.

6. CALCULATION OF THE RESULTS

6.1 *From the visual measurements*

The content in micrograms of aflatoxin B_1 per kg of sample is given by the formula:

$$\frac{R \times Y \times V}{W \times Z}$$

in which:

Y and Z are respectively the volumes in microlitres of the standard solution of aflatoxin B_1 (3.16) and of the extract having a similar intensity of fluorescence;

R = concentration in micrograms of aflatoxin B_1 per ml in the standard solution (3.16);

V = final volume of the extract in microlitres, allowing for any dilution that was necessary; and

W = weight in grams of the sample corresponding to the volume of extract subjected to column clean-up.

6.2 *From the fluorodensitometric measurements*

The content in micrograms of aflatoxin B_1 per kg of sample is given by the formula:

$$\frac{S \times V}{W \times Z}$$

in which:

Z = volume in microlitres of the extract spotted on the plate;

S = quantity in nanograms of aflatoxin B_1 in the extract spot, 10 or 20µl related to Y deduced from the measurements;

V = final volume of the extract in microlitres, allowing for any dilution that was necessary; and

W = weight in grams of the sample corresponding to the volume of extract subjected to column clean-up.

7. OBSERVATIONS ON REPRODUCIBILITY OF RESULTS

The variation between the results obtained by two or more laboratories on the same sample has been estimated at:

±50% of the mean value for mean values of aflatoxin B_1 greater than 10 and up to and including 20µg/kg.

±10µg/kg on the mean value for mean values greater than 20 and up to and including 50µg/kg.

±20% of the mean value for mean values above 50µg/kg.

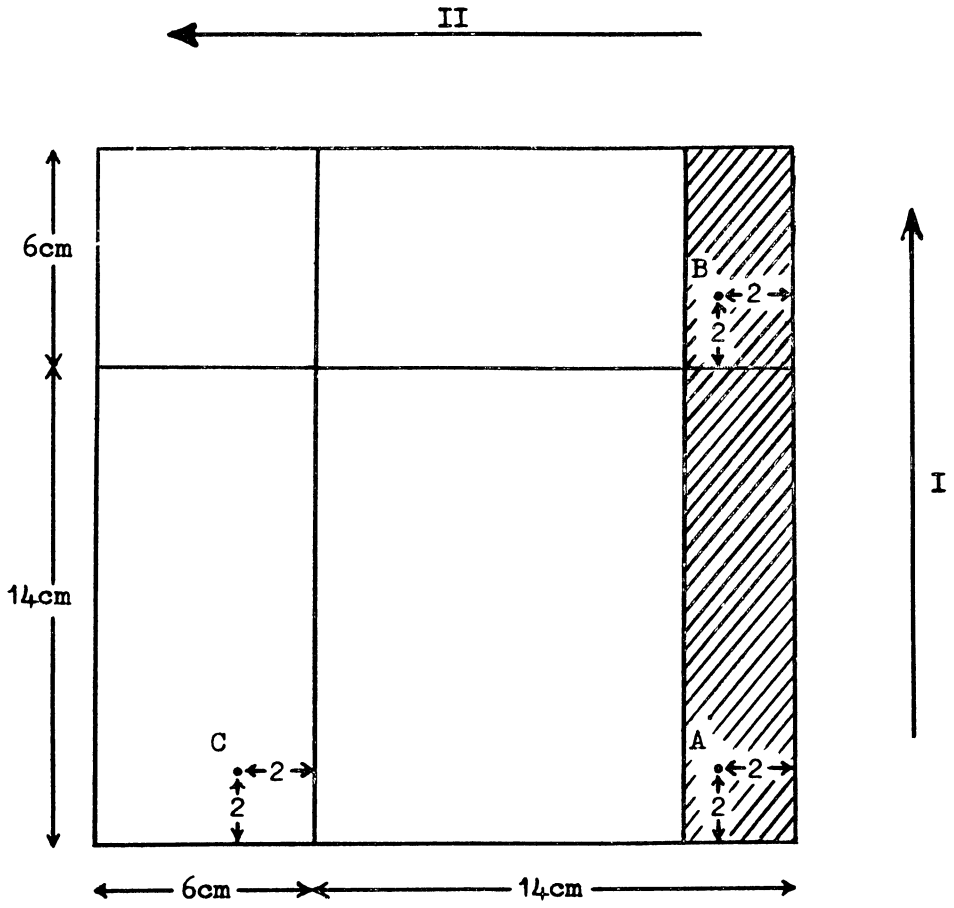

Fig.1

Secure plate with B in top right hand corner when spraying with hydrochloric acid.

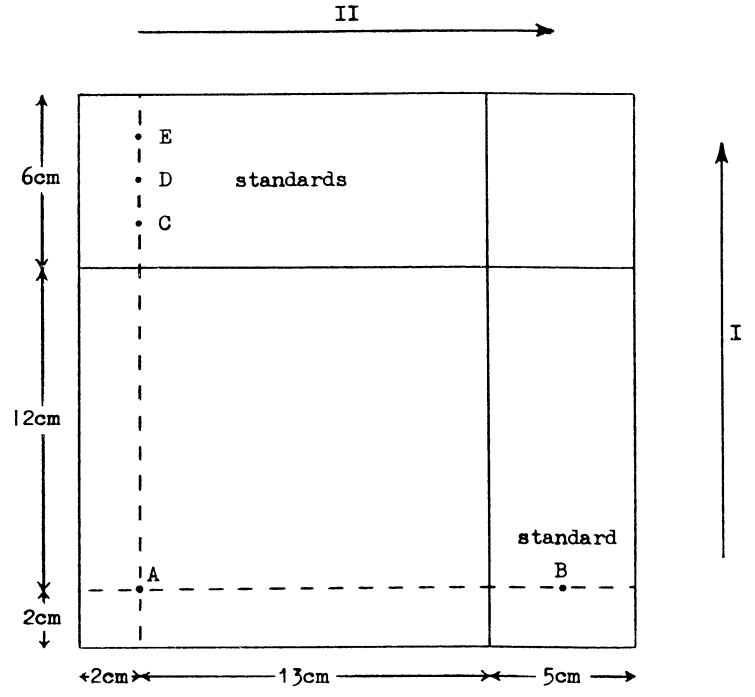
II
E
D
C
standards
6cm
12cm
2cm
A
standard
B
I
2cm
13cm
5cm

Fig.2

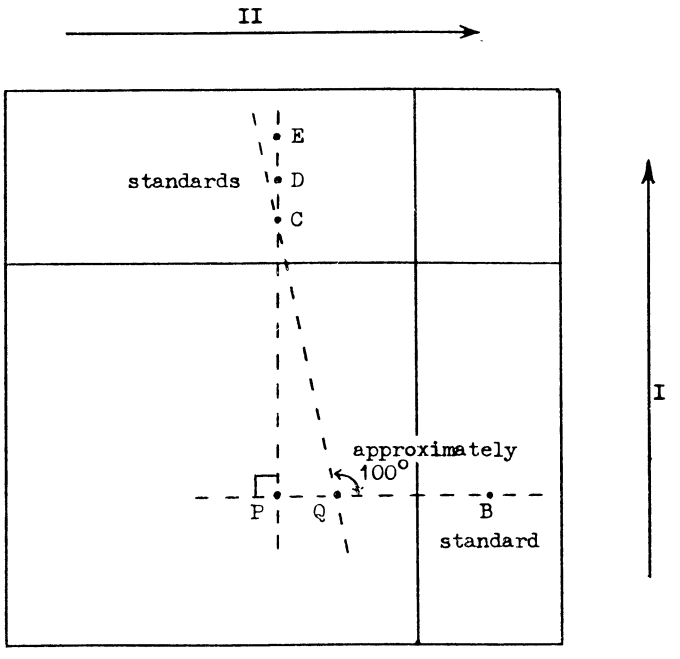
II
E
D
C
standards
I
approximately
100°
P
Q
B
standard

Fig.3

SCHEDULE 6

(Sections 77(4), 78(3) and 79(5) to (8) and Regulation 2(19))

CERTIFICATE OF ANALYSIS OF FEEDING STUFF (1)

I, the undersigned, agricultural analyst for the (2) , in pursuance of the provisions of the Agriculture Act 1970, Part IV, hereby certify that I received on the day of 19 , from (3) one part of a sample of (4) for analysis; which was duly sealed and fastened up and marked (5) and was accompanied by a (6) , as follows:— (7)

and also by a signed statement that the sample was taken in the prescribed manner; and that the said part has been analysed by me or under my direction, and I declare the results of analysis to be as follows:— (8)

	% or Units/kg		Units/kg or IU/kg
Oil			
Protein: Total, including protein equivalent of biuret, isobutylidene diurea, urea or urea phosphate and protein equivalent of uric acid		Vitamin A	
		Vitamin D_2	
		Vitamin D_3	
Protein equivalent of biuret, isobutylidene diurea, urea or urea phosphate		Vitamin E	
		Other vitamins or pro vitamins	
Protein equivalent of uric acid ...			
Fibre		Permitted antioxidant (9)	
Sugar		Permitted colourant (9)	
Salt (NaCl)		(10)	
Phosphorus (P)		(11)	
Calcium (Ca)		(12)	
Copper (Cu)		(15)	
Magnesium (Mg)		(16)	
Molybdenum (Mo)			
Selenium (Se)			
Iron (Fe)			
Iodine (I)			
Cobalt (Co)			
Manganese (Mn)			
Zinc (Zn)			

(13) Analysis for oil was completed on and I am of the opinion that (14)

The analysis was made in accordance with the Fertilisers and Feeding Stuff Regulations 1973.

As witness my hand this day of 19 .

(Signature and address of analyst)

(1) Statements made in certificates are to be confined to matters which are necessary to verify compliance with the Act.

(2) Here insert the name of the local authority.

(3) Here insert the name of the inspector who submitted the sample for analysis; and also the mode of transit, i.e. "by hand", "by registered post", "by rail", or as the case may be.

(4) Here insert the name or description applied to the material.

(5) Here insert the distinguishing mark on the sample and the date of sampling shown thereon.

(6) Here insert either "statutory statement", "copy of statutory statement", "copy of particulars marked on the material" or "copy of particulars indicated by a mark applied to the material", or as the case may be.

(7) Here insert the particulars contained in the statutory statement, or particulars marked on or indicated by a mark applied to the material, or as the case may be.

(8) Insert relevant results under the appropriate headings, i.e. percentage, units/kg or IU/kg.

(9) Here indicate whether the antioxidant or colourant is an antioxidant listed in Part I of the table to Schedule 3 or a colourant listed in Part II of the table to Schedule 3.

(10) Here indicate the presence of any emulsifier, stabiliser or binder not listed in Part III of the Table to Schedule 3.

(11) Here indicate the presence of any preservative.

(12) Here insert the name and estimated percentage of any ingredient found in the sample, being an ingredient deleterious to animals of any description prescribed for the purpose of the definition of feeding stuff in section 66(1) of the Agriculture Act 1970, having regard to section 73 of that Act or in the case of substances to which regulation 7 and Schedule 3 apply the name and estimated percentage of any such substance which is deleterious to human beings.

(13) In the case of a sample of any feeding stuff containing oil insert the date of completion of the oil analysis.

(14) Here enter information as follows:—

(*a*) If the material was sold under a name mentioned in the first column of Schedule 4, state whether it accords with the meaning given in the second column; and if not, in what respect.

(*b*) If the composition of the material agrees with or does not differ by more than the limits of variation from the statement of particulars contained in the statutory statement, or the particulars marked on or indicated by a mark associated with the material, state that the particulars are correct within the limits of variation.

(*c*) If the composition of the material differs by more than the limits of variation from the statement of particulars contained in the statutory statement, or the particulars marked on or indicated by a mark associated with the material, or as the case may be, state the difference between the amount found and the amount stated, and that the difference is outside the limits of variation; and that the difference is to the prejudice of the purchaser, if such is believed to be the case.

(*d*) If the material is not suitable for use as a feeding stuff having regard to Section 72, state in what respect.

(15) In the case of analysis of substances for which no analytical method is prescribed in Schedule 7 here indicate the method used. If analysis cannot be carried out because no suitable method exists then the certificate should be noted accordingly.

(16) Here indicate the amount of any of the substances listed in the first column of the table in Schedule 3 and Schedule 3A for which an analysis has been requested.

(These notes and the numbers referring to them are for guidance only and do not form part of and need not appear on the certificate.)

SCHEDULE 7

METRIC SUBSTITUTIONS FOR IMPERIAL UNITS

(Section 66(1) and Regulation 3(1))

Units of Measurement

Metric	*Imperial*
5,000 litres (l)	1,000 gallons
25 kilograms (kg)	56 lb.
5 tonnes (t)	5 tons

SCHEDULE 8

METRIC SUBSTITUTIONS FOR IMPERIAL UNITS

(Regulation 3(2))

Unit of Measurement

	Metric	*Imperial*
1. Capacity		
	0·5 litre (l)	1 pint
	1·0 litre (l)	2 pints (1 quart)
	1·5 litres (l)	3 pints
	2·0 litres (l)	4 pints (½ gallon)
	2·5 litres (l)	5 pints
	3·0 litres (l)	6 pints
	3·5 litres (l)	7 pints
	5·0 litres (l)	10 pints
	10·0 litres (l)	20 pints
	200 litres (l)	40 gallons
	5,000 litres (l)	1,000 gallons
	25,000 litres (l)	5,000 gallons
	50,000 litres (l)	10,000 gallons
	75,000 litres (l)	15,000 gallons
	100,000 litres (l)	20,000 gallons
	250,000 litres (l)	50,000 gallons
	500,000 litres (l)	100,000 gallons
2. Weight		
	1–2 kilograms (kg)	2–4 lb.
	2–3 kilograms (kg)	4–6 lb.
	2·5 kilograms (kg)	6 lb.
	6·0 kilograms (kg)	14 lb.
	7·0 kilograms (kg)	15 lb.
	25 kilograms (kg)	56 lb. (½ cwt.)
	100 kilograms (kg)	2 cwt.
	1 tonne (t)	1 ton
	3 tonnes (t)	3 tons
	5 tonnes (t)	5 tons
	25 tonnes (t)	25 tons
3. Length		
	12 millimetres (mm)	½ inch
4. Sieve aperture size		
	3·35 millimetres (mm)	⅛ inch square
	4·75 millimetres (mm)	3/16 inch square
	6·7 millimetres (mm)	¼ inch square
	31·8 millimetres (mm)	1¼ inch square
	75 millimetres (mm)	3 inch square
5. Ratios		
	milligram per kilogram (mg/kg)	parts per million (ppm)

EXPLANATORY NOTE

(This Note is not part of the Regulations.)

These Regulations, made under Part IV of the Agriculture Act 1970 (as amended by Schedule 4E to the European Communities Act 1972) apply throughout Great Britain, are made after consultation with persons and organisations representing the interests concerned and amend the Fertiliser and Feeding Stuffs Regulations 1973 (the principal regulations) in order to:—

(*a*) implement the provisions of Council Directive 74/63/EEC (OJ No. L.38, 11.2.74, p.31.) and Commission Directive 76/14/EEC (OJ No. L.4, 9.1.76, p.24.) on the fixing of maximum permitted levels for undesirable substances and products in feeding stuffs (regulation 2(3) and Schedule 3);

(*b*) implement certain methods of analysis agreed by the Community and set out in Commission Directives 71/250/EEC (OJ No. L.155, 12.7.71, p.13; OJ/SE 1971 (II), p.480.), 71/393/EEC (OJ No. L.279, 20.12.71, p.7; OJ/SE 1971 (III) p.987.), 72/199/EEC (OJ No. 123, 29.5.72, p.6.) and 73/46/EEC (OJ No. L.83, 30.7.73, p.21.) (regulation 2(7) and Schedule 5);

(*c*) implement the provisions of Commission Directive 75/696/EEC (OJ No. L.299, 19.11.75, p.19.) on the use of added non protein nitrogenous compounds in feeding stuffs (regulation 2(3) and Schedule 2);

(*d*) implement the provisions of paragraph 4 of article 7 of Commission Directive 75/296/EEC (OJ No. L. 124, 15.5.75, p. 29.) so as to provide that the presence of a preservative shall be declared (regulation 2(10) and Schedule 1);

(*e*) extend the labelling provisions of Part II of Schedule 2 to the principal regulations to cover the use of poultry waste and non protein nitrogenous compounds in feeding stuffs (regulation 2(10) and Schedule 1) and expand Part II of Schedule 5 to the principal regulations to set limits of variation for these new declarations (regulation 2(15) and Schedule 4);

(*f*) revise the tolerances set out in Part II of Schedule 5 to the principal regulations for copper and trace elements in feed supplements (regulation 2(15) and Schedule 4);

(*g*) provide metric substitutions for the imperial units specified in Part IV of the Agriculture Act 1970 (regulation 3(1) and Schedule 7) and in the principal regulations (regulation 3(2) and Schedule 8).

SI 1976/840
ISBN 0-11-060840-2