1976 No. 255

AGRICULTURE

The Fertilisers and Feeding Stuffs (Amendment) Regulations (Northern Ireland) 1976

Made	*23rd August* 1976
Coming into operation—	
Regulations 2(1), (3), (4), (5), (7), (8), (15) and (17) . .	*29th September* 1976
Regulation 3	*30th September* 1976
Regulations 2(2), (6), (9), (10), (11), (12), (13), (14), (16) and (18) . . .	*1st October* 1976

The Department(a) of Agriculture, after consultation with such persons or organisations as appear to it to represent the interests concerned, in exercise of the powers conferred on it by sections 66(1), 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(b)), 75(1), 76(1), 77(1), (2) and (4), 84 and 86 of the Agriculture Act 1970(c) and of all other powers enabling it in that behalf, hereby makes the following regulations:—

Citation, commencement and interpretation

1.—(1) These regulations may be cited as the Fertilisers and Feeding Stuffs (Amendment) Regulations (Northern Ireland) 1976, and shall come into operation as respects regulations 2(1), (3), (4), (5), (7), (8), (15) and (17) on 29th September 1976, as respects regulation 3 on 30th September 1976 and as respects regulations 2(2), (6), (9), (10), (11), (12), (13), (14), (16) and (18) 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 (Northern Ireland) 1973(d).

(3) The Interpretation Act (Northern Ireland) 1954(e) shall apply to these regulations as it applies to a Measure of the Northern Ireland Assembly.

(4) 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.

(a) Formerly Ministry: *see* 1973 c. 36 Sch. 5 para. 8(1)
(b) 1972 c. 68
(c) 1970 c. 40
(d) S.R. & O. (N.I.) 1973 No. 494 (II, p. 2862)
(e) 1954 c. 33

Amendment of the principal regulations

2. The principal regulations shall be amended as follows:—

(1) In regulation 3(2) for "which is packed in bags, sacks or", there shall be substituted "in".

(2) There shall be substituted for regulation 6—

"6.—(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 Northern Ireland 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 any breeder of any kind of animal specified in regulation 2, for use as a feeding stuff, any material 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 any material comprised in that feeding stuff, having regard to the 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 material comprised in a 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 substances 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.

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(f).

(f) 1968 c. 67

(3) No person shall use as a feeding stuff or import into Northern Ireland 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 paragraph 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."

(3) In paragraph (*c*) of regulation 8 for "packed in bags, sacks or" there shall be substituted "in".

(4) In paragraph (*c*) of regulation 10 for "packed in bags, sacks or" there shall be substituted "in".

(5)(*a*) In paragraph 1(*c*) of regulation 11 for "packed in bags, sacks or" there shall be substituted "in".

(*b*) Paragraph 3 of regulation 11 shall be deleted.

(6) For regulation 14 there shall be substituted:—

"14. 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."

(7) In regulation 16 for "recorded delivery service" there shall be substituted "recorded delivery service or by hand".

(8)(*a*) For paragraph 1 of Part I of Schedule 1 there shall be substituted:—

"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*) For paragraph 4 of Part I of Schedule 1 there shall be substituted:—

"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*) In paragraph 8(*a*) of Part I of Schedule 1 "bottles or" shall be deleted wherever those words appear;

(*d*) In paragraph 8(*b*) of Part I of Schedule 1 "drums, kegs or other" shall be deleted;

(*e*) In paragraph 1(*a*)(i) of Part II of Schedule 1 "on a clean dry surface" shall be deleted;

(*f*) In the last sub-paragraph of paragraph 1(*a*) of Part II of Schedule 1 after "continue the quartering and rejection" there shall be inserted "if necessary";

(*g*) In Part II of Schedule 1 for paragraph 2(*a*) there shall be substituted:—

"(*a*) *In packages*

(i) The packages, selected according to the appropriate scale in paragraph 1, 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*) In paragraph 2(*b*) of Part II of Schedule 1 for "1(*a*)" there shall be substituted "2(*a*)(ii)";

(*i*) In the first sentence of paragraph 3(*a*) of Part II of Schedule 1 for "on a clean dry surface and the matted portions torn up" there shall be substituted "and the matted portions torn up and the whole shall be thoroughly mixed.";

(*j*) In the heading to paragraph 2 of Part III of Schedule 1 "whether in bags or in bulk" shall be deleted;

(*k*) In paragraph 4(*a*) of Part III of Schedule 1 "on a clean surface" shall be deleted;

(*l*) For paragraph 1 of Part IV of Schedule 1 there shall be substituted:—

"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.".

(9) For Part II of Schedule 2 there shall be substituted Schedule 1.

(10)(*a*) In Schedule 3 for paragraph 2(*g*) there shall be substituted:—

"(*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*) In the tenth line of paragraph 3 immediately after "grammes per kilogramme" there shall be added "of the whole feeding stuff.";

(*c*) For the words "per ton" in the last line of paragraph (3) of Schedule 3 there shall be substituted "per ton or grammes per litre or grammes per tonne".

(*d*) At the end of the table in Part II of Schedule 3 "Red 6B" shall be deleted.

(11) In Schedule 3, Schedule 2 shall be added as Part VI of the table.

(12) Schedule 3 shall be inserted as Schedule 3A.

(13) In Part II of Schedule 4 there shall be added 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."

(14) For Part II of Schedule 5 there shall be substituted Schedule 4.

(15) Schedule 6 shall be amended:—

(*a*) At the end of the last item of sub-division 1.16 after "containing organic matter" there shall be added "and granular potassic basic slag";

(*b*) In the eighteenth line of sub-division 3.53 for "55" there shall be substituted "15";

(*c*) In the nineteenth line of sub-division 3.53 for "twenty" there shall be substituted "forty".

(*d*) In the first line of sub-division 4.171 immediately after the word "sample" there shall be inserted "as received";

(*e*) In the first line of sub-division 4.271 immediately after the word "sample" there shall be inserted "as received";

(*f*) In the eighth line of sub-division 15.3 for "3" there shall be substituted "1.5".

(16) For Schedule 7 there shall be substituted Schedule 5.

(17) In Part I of Schedule 8 for "Bo", which appears immediately after the word "Boron" there shall be substituted "B".

(18) For Part II of Schedule 8 there shall be substituted Schedule 6.

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.

Sealed with the Official Seal of the Department of Agriculture for Northern Ireland on 23rd day of August 1976.

(L.S.) *W. H. Parker*
Assistant Secretary

SCHEDULE 1

(Sections 68(1) and 69(1) and Regulation 2(9))

PART II

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 milligrammes per kilogramme, or magnesium (if present in excess of 0·5 per cent);

(*b*) any antioxidant or colourant, either the words "contains permitted antioxidant" 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% by weight in which case it shall be expressed in milligrammes per kilogramme);

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

(iii) in sub-paragraph (*d*) above being expressed in milligrammes per kilogramme.

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 3(*a*) and 3(*b*) 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% 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 kind of animals for which the feeding stuff is intended and as to the maximum quantity that can be used in the whole feeding stuff.
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% or greater a declaration shall be made in the following manner "protein equivalent of uric acid x% (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% and of calcium if present in excess of 2%.
Dried plain beet pulp	Amount of fibre.
Dried molassed beet pulp . . .	Amounts of sugar and fibre respectively.
Dried yeast	Amount of protein.

Description of material	*Particulars to be contained in statutory statement*
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% 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.
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% or greater a declaration shall be made in the following manner "protein equivalent of uric acid x% (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, phosphorus 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.

Description of material	*Particulars to be contained in statutory statement*
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.
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% 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% or greater and of fibre respectively. Amount of calcium if the amount present is 2% or greater.
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(2) and 2(11))

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(12))

PRESCRIBED LIMITS FOR UNDESIRABLE SUBSTANCES IN FEEDING STUFFS

(Section 74A and Regulation 6(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 except:	2
	—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 except:	150
	—feeding stuffs of animal origin	500
	—phosphates	2000
	Whole feeding stuffs except:	150
	—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
	—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

Substances	Feeding stuffs	Maximum content in mg/kg of feeding stuffs referred to a moisture content of 12%
Nitrites	Whole feeding stuffs	0.1
	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 cakes	1200
	Whole feeding stuffs except:	20
	—whole feeding stuffs for cattle, sheep and goats	500
	—whole feeding stuffs for poultry (except laying hens) and calves	100
	—whole feeding stuffs for rabbits and pigs (except piglets)	60

Substances	Feeding stuffs	Maximum content in mg/kg of the feeding stuff as found
Hydrocyanic acid	Straight feeding stuffs except:	50
	—linseed	250
	—linseed cakes	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:—	All feeding stuffs	Less than the lowest level detectable
—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.		

Substances	Feeding stuffs	Maximum content in mg/kg of the feeding stuff as found
= *Illipe latifolia* (Roxb.) F. Mueller) —Physic nut —*Jatropha curcas* L. —Sareptian Mustard —*Brassica juncea* (L.) Czern. & Coss. ssp *juncea* —Unhusked beech —*Fagus sylvatica* L.		
Theobromine	Whole feeding stuffs except:	300
	—whole feeding stuffs for adult cattle	700
Vinylthiooxazolidone	Whole feeding stuffs for poultry except:	1000
	—whole feeding stuffs for laying hens	500
Volatile mustard oil	Straight feeding stuffs except:	100
	—rape cake or meal	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(14))

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.
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.

Material	*Limits of variation (percentages are percentages of the whole bulk)*
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 grain	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 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.

Material	*Limits of variation (percentages are percentages of the whole bulk)*
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.
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.
Mixtures 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 "oat-feed" to any material containing more than 27% of fibre.

Material	*Limits of variation (percentages are percentages of the whole bulk)*
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.
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 of the 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(16))

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 14)

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
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 1 mm square (1)(3) mix thoroughly and transfer a portion of not less than 100 g 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 1 mm square(1)(3) and wholly passes through a sieve having apertures from 2 to 3 mm square(2)(3) mix thoroughly and further grind a portion of not less than 100 g to pass through a sieve having apertures of about 1 mm 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 coarse condition as, for example, pieces of broken cake, carefully grind until the whole passes through a sieve having apertures of from 2 to 3 mm square(2)(3). Mix thoroughly and further grind a portion of not less than 100 g to pass through a sieve having apertures of about 1 mm 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 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 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 1 mm 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.

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

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 airtight 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.

4. *Procedure*

Weigh to the nearest 0·001 g, approximately 5 g 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 0·010 g continue the heating and cooling procedure until a weight constant within 0·002 g is attained.

5. *Calculation of Result*

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

3a. 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 0·001 g, approximately 1 g of the prepared sample and transfer to the extraction tube (4.1).

Add 9 ml water, temperature 60-70°C stopper the tube and shake vigorously until the sample is uniformly suspended. Cool to room temperature, add 1·5 ml ammonia solution (3.1) stopper and shake thoroughly. Add 10 ml ethanol (3.3) using some to rinse the stopper and collect the washings in the extraction tube. Stopper the tube and shake thoroughly. Add 25 ml diethyl

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

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 25 ml 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 ethereal layer to a flask. Raise the siphon and, before removing it from the tube, wash it down with 15 ml of diethyl ether (3.2). Remove the siphon tube and rinse the tip with ether, collecting the rinsings in the flask. Add 1 ml ethanol (3.3) to the tube, stopper, shake vigorously for 90 seconds, cool, remove the stopper, add 15 ml light petroleum (3.4) and again shake for 90 seconds. Allow to stand for 15 minutes or until the layer separates cleanly, fit the siphon tube and remove the solvent layer to the flask as before.

Carry out a third extraction with 15 ml diethyl ether (3.2) followed by 15 ml 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. The second weight should not differ by more than 0·002 g from the first weight. Add about 20 ml 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 20 ml 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.

3b. 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 0·002 g is attained. Regard this light petroleum extract as oil.

Where a sample is presumed to have an oil content in excess of 10 per cent 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 400 g sodium hydroxide in water and dilute to 1 litre.

3.8 Sodium sulphide, cold saturated solution.

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

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 0.3 g methyl red in 100 ml of ethanol (95-96 per cent, V/V).

3.15 Screened methyl red indicator solution:

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

(*b*) dissolve 0·1 g methylene blue in 100 ml of ethanol (95-96 per cent, V/V).

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

Then add a few pieces of zinc (3.4).

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 0·001 g, approximately 1 g of the prepared sample, and transfer to a Kjeldahl Flask. Add 10 g potassium sulphate (3.2) or sodium

sulphate (3.2), 0·6 to 0·7 g mercuric oxide (3.1), 25 ml 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 crystallation still occurs, repeat the analysis using a larger quantity of sulphuric acid.

5.2 Distillation.

Add 250-350 ml water carefully mixing the contents during the addition; allow to cool.

Then add a few pieces of zinc (3.4).

Transfer 25·0 ml 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 100 ml 40% sodium hydroxide (3.7) and then add either 10 ml sodium sulphide solution (3.8) or 25 ml sodium thiosulphate solution (3.9). Mix well and connect immediately to the distillation apparatus.

Heat the flask so that approximately 150 ml 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 1 g 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. 1 ml 0·1N sulphuric acid=1·4 mg 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 deescribed 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 435 nm.

3. *Reagents*

3.1 Activated charcoal.

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

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

3.4 Hydrochloric acid 0·02N.

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

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

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

4. *Apparatus*

4.1 Tumbler mixer, or shaker.

4.2 Spectrophotometer with 10 mm cells.

5. *Procedure*

5.1 Dissolution of sample.

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

5.2 Determination.

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

5.3 Calibration Curve.

Dilute 1, 2, 4, 5 and 10 ml of the urea solution (3.7) to 100 ml with water. Transfer 10 ml of each solution to ground glass stoppered test tubes, and 10 ml 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 x 0·4665 = % urea nitrogen).

NOTE: If the sample is highly coloured the proportion of activated charcoal must be increased up to 5 g. 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 50 g sodium hydroxide in 50 ml 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 3·00 ml formaldehyde solution with 50·00 ml 1N sodium hydroxide solution and 25·00 ml 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 3·00 ml water in place of the formaldehyde.

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

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

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

3.3 Neutral ethanolic formaldehyde solution: mix an appropriate volume of formaldehyde solution (3.2) containing 17·5 g of formaldehyde with 250 ml water and 500 ml 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, 29.5 g of succinic acid in 750 ml water and 20 ml sodium hydroxide solution (3.1). Cool, add an appropriate volume of formaldehyde solution (3.2) containing 17·5 g 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: 25 g sodium thiosulphate ($Na_2S_2O_3 . 5 H_2O$) per litre.

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

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

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

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

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

4. *Apparatus*

4.1 Spectrophotometer, with 10 mm silica cells.

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

5. *Procedure*

5.1 Extraction of Uric Acid.

5.1.1 From dried poultry waste:
weigh to the nearest 0·001 g about 0·40 g dried poultry waste and place in a 150 ml round-bottomed flask. Add 60 ml 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 100 ml graduated flask. Wash out the round-bottomed flask with 3 × 10 ml portions of ethanolic formaldehyde solution (3.3) passing each portion through the crucible into the graduated flask. Dilute to 100 ml with ethanolic formaldehyde solution and mix.

5.1.2 From feeding stuffs:
weigh to the nearest 0·001 g between 4 g and 5 g feed. 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 150 ml round-bottomed flask and remove the residual solvent with a slow current of air. Continue as in 5.1.1, second sentence . . . 'Add 60 ml ethanolic formaldehyde solution (3.3) . . . . '

5.2 Determination.

Transfer by pipette 20·0 ml of the sample extract prepared as in 5.1.1 or 5.1.2 to a 50 ml centrifuge tube. Add 10 ml of Benedict and Hitchcock reagent (3.8), mix well and allow to stand in the dark for 1 hour. Centrifuge at 2000 rpm 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 20·0 ml sodium thiosulphate solution (3.5) to each tube. Dissolve the precipitate by stirring with a thin glass rod. Transfer by pipette 5·0 ml of this solution into a 200 ml graduated flask containing 40·0 ml succinate buffer solution (3.4). Dilute to 200 ml with water and mix well. Measure the absorbance of the solution at 294 nm in 10 mm silica cells against a solution prepared by mixing 5·0 ml sodium thiosulphate solution (3.5) with 40·0 ml succinate buffer solution (3.4) and diluting to 200 ml 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 50 ml centrifuge tubes, transfer by pipette 2, 4, 6, 8, 10, 12 ml standard uric acid solution (3.9) (corresponding to 2, 4, 6, 8, 10 and 12 mg of uric acid) and make up to 20·0 ml with ethanolic formaldehyde solution (3.3). Add to each tube 10·0 ml Benedict and Hitchcock reagent (3.8), mix well and stand in the dark for 1 hour. Continue as in 5.2 from . . . 'Centrifuge at 2000 rpm . . . '. Measure the absorbances of the solutions and plot the calibration curve using absorbances as the ordinates and the corresponding quantitites 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 gms.

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 feeding stuffs). An acidic solution is treated with molybdovanadate reagent and the absorbance of the yellow solution is measured at 430 nm.

3. *Reagents*

3.1 Calcium carbonate.

3.2 Nitric acid (density 1·42 g/ml).

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

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

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

3.6 Molybdovanadate reagent: dissolve separately 20 g of ammonium molybdate [$(NH_4)6Mo_7O_{24}.4H_2O$] and 0·47 g of ammonium vanadate in water, mix, acidify with 140 ml of nitric acid (3.2) and dilute to 1 litre.

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

4. *Apparatus*

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

4.2 Spectrophotometer with 10 mm 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 0·001 g, approximately 2·5 g of the prepared sample and transfer to a silica or porcelain crucible. Add 1 g 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 250 ml beaker, add 20 ml of water and sufficient hydrochloric acid (3.4) until effervescence ceases, taking suitable precautions to avoid loss. Add a further 10 ml of hydrochloric acid (3.4) and evaporate to dryness to make the silica insoluble. Cool, treat the residue with 10 ml of nitric acid (3.5), and boil for five minutes, avoid evaporating to dryness. Transfer the solution to a 500 ml 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 0·001 g approximately 1 g of the prepared sample and transfer to a Kjeldahl flask. Add 20 ml 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 2 ml 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 500 ml 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 40 μg/ml. Transfer 10 ml of this solution to a glass stoppered test tube, add 10 ml of freshly prepared molybdovanadate reagent (3.6) and mix. Allow to stand for ten minutes at 20°C, and then measure the absorbance at 430 nm against a freshly prepared reference solution prepared by adding 10 ml of molybdovanadate reagent (3.6) to 10 ml of water.

5.3 Calibration curve.
From the standard solution (3.7) prepare solutions containing respectively 5, 10, 20, 30 and 40 μg of phosphorus per ml. Transfer 10 ml of each of these solutions to glass stoppered test tubes, add 10 ml 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 per cent (V/V).

3.5 Diethyl ether.

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

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

4. *Apparatus*

4.1 Conical flask, 1000 ml.

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 0·001 g 2·7 to 3·0 g 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 1000 ml conical flask (4.1). Add 200 ml of sulphuric acid (3.1), measured at ordinary temperature and brought to boiling point, the first 30 to 40 ml 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 content 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 this and subsequent washings(1) (it is convenient 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 200 ml 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 200 ml 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.

(1) Whatman 541 or equivalent.

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 per cent 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 3·0 g.

Transfer the air-dried extracted sample to a 1000 ml 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 200 ml 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.

3. *Reagents*

3.1 Fehling's solution.

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

3.1.2 Potassium sodium tartrate solution: dissolve 346 g potassium sodium tartrate and 100 g 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 10 ml is equivalent to 0·0525 g invert sugar. It should be checked as follows:

dissolve 2·375 g sucrose (dried at 100°C) in about 100 ml water in a 300 ml beaker, add 15 ml hydrochloric acid (3.2) and sufficient water to give a volume of 150 ml. 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 500 ml graduated flask and dilute to 500 ml. Then follow the procedure described in paragraph 4.2.3: 1 ml of this solution ≡ 0·00475 g sucrose ≡ 0·005 g invert sugar, ie 10 ml Fehling's solution ≡ 10·5 ml of this standard invert sugar solution.

3.2 Hydrochloric acid solution, N.

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

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

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

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

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

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

4. *Procedure*

4.1 Extraction.

4.1.1 Sample in solid form.

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

4.1.2 Sample in liquid form.

Weigh to the nearest 0·001 g, approximately 5 g of the prepared sample and wash with water into a 250 ml graduated flask, using about 200 ml water. To clear the solution add 5 ml Carrez solution I (3.3). Mix, then add 5 ml Carrez solution II (3.4), again mix, dilute to the mark with water, mix and filter. Determine the sugar in 25 ml 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 300 ml beaker, add 15 ml hydrochloric acid (3.2), dilute to 150 ml 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 200 ml 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 10·0 ml Fehling's solution (3.1) to a 250 ml conical flask and add 20 ml of water. Add from a burette approximately 10 ml 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 1 ml 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 15-40 ml otherwise the determination should be repeated using a more appropriate volume of the filtrate.

4.2.3 Exact determination.

To 10 ml Fehling's solution (3.1) in a 250 ml 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 60 ml. 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 0·25 ml 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 10 ml Fehling's solution.

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

Not more than 1 ml of filtrate should be required for the completion of the titration. If more than 1 ml 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 1·42 g/ml).

3.5 Ammonium ferric sulphate, saturated aqueous solution.

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

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

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 0·001 g a portion of the prepared sample of not more than 10 g and containing not more than 3 g of chlorine in the form of chlorides. Place with 400 ml of water in a 500 ml graduated flask at approximately 20°C. Mix for thirty 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 0·001 g, approximately 5 g of the prepared sample and place with 1 g of activated charcoal (3.1) in a 500 ml graduated flask. Add 400 ml water at approximately 20°C, shake well and allow to stand for 30 minutes. Add 5 ml Carrez solution I (3.6) mix well and then add 5 ml 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 100 ml of the supernatant liquid to a 200 ml 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 25 ml to 100 ml 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 150 mg of chlorine (Cl). Dilute if necessary to not less than 50 ml with water, add 5 ml nitric acid (3.4), 20 ml 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 5 ml is obtained. Add 5 ml 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:

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

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: 20 g ammonium nitrate per 100 ml.

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 per cent Pt, 90 per cent Au), either rectangular (60 x 40 x 25 mm) or circular (diameter: 60 to 75 mm, height: 20 to 25 mm).

5. *Procedure*

Weigh to the nearest 0·001 g approximately 5 g 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 25 g 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.

12a. 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 0·88 g/ml).

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

3.3 Nitric acid (density 1·42 g/ml).

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

3.5 Ammonium oxalate, cold saturated solution.

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

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

3.8 Potassium permanganate, 0·1N solution.

3.9 Bromocresol green indicator solution: dissolve 0·04 g of bromocresol green in 20 ml ethanol and dilute to 100 ml 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 0·001 g, approximately 5 g 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 10 ml 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 20 ml water, 40 ml of hydrochloric acid (3.2), bring to the boil, and then filter into a 250 ml 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 40 mg of calcium into a 250 ml beaker, add 1 ml of citric acid solution (3.6) and 5 ml of ammonium chloride solution (3.4). Make the volume up to approximately 100 ml with water, bring to the boil, add 8 to 10 drops of bromocresol green indicator solution (3.9) and 30 ml 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 250 ml beaker, add 50 ml of sulphuric acid (3.7) and water to give a total volume of about 100 ml, 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*

1 ml 0·1N potassium permanganate ≡ 2·004 mg 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.

12b. 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 1·18 g/ml).

3.2 Hydrochloric acid, 50 per cent 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 117·3 g lanthanum oxide (La_2O_3), low in calcium content, with water. Slowly add 350 ml hydrochloric acid (3.1) and stir until all the lanthanum oxide is dissolved. Allow to cool and dilute to 1000 ml with water.

3·4·1 Calcium standard solution: dry calcium carbonate at 105°C for one hour. Weigh accurately 2·497 g and transfer to a 1000 ml graduated flask using approximately 100 ml water. Add slowly, with swirling, 60 ml N hydrochloric acid (3.5). When all the carbonate has dissolved, make up to the mark with water. 1 ml of this solution ≡ 1·00 mg calcium (Ca).

3·4·2 Calcium standard solution (dilute); dilute 20 ml of calcium standard solution (3.4.1) to 200 ml with water. 1 ml of this solution ≡ 100 μg 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 0·001 g, approximately 5 g 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 10 ml 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 20 ml water and 10 ml 50% (V/V) hydrochloric acid (3.2), bring to the boil and filter into a 250 ml 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 422·7 nm. 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 10 ml releasing agent (3.3) to each of six 100 ml graduated flasks. Measure 0, 3, 6, 9, 12 and 15 ml of dilute calcium solution (3.4.2) into flasks and dilute to 100 ml 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.

13a. DETERMINATION OF COPPER—DIETHYLDTHIOCARBAMATE 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 436 nm, 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 1 g sodium diethyldithiocarbamate in water and dilute to 100 ml. 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 20 g ammonium citrate and 5 g of the disodium salt of ethylenediaminetetra-acetic acid (EDTA) in water and dilute to 100 ml. To purify, add 0·1 ml 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 1·18 g per ml) with an equal volume of water.

3.7 Hydrochloric acid solution, 2N.

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

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

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

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

4. *Apparatus*

4.1 Spectrophotometer with 10 mm cells.

5. *Preparation of the test sample*

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

6. *Procedure*

6.1 Dissolution of sample.

Weigh, to the nearest 0·001 g, approximately 10 g 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°C ± 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 10 ml 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 10 ml portions of boiling 2N hydrochloric acid solution (3.7) decanting the solution each time through the same suitable filter-paper(1) into a 50 ml graduated flask. Then add 5 ml of 50% (V/V) hydrochloric acid solution (3.6) and about 5 ml 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 10 ml 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 50 μg of copper. Add 10 ml 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 1 ml of sodium diethyldithiocarbamate solution (3.2) and, from a burette, 15 ml of

(1) Whatman No. 541 or equivalent.

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 10 mm spectrophotometer cell (4.1). Avoid undue exposure of the solution to light.

Measure immediately the absorbance of the sample solution at 436 nm, 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 10 ml 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	25 ml
2N H_2SO_4	25	24	22·5	20	15	10	5	0 ml

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)

13b. 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 1·18 g per 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 30 ml nitric acid (density 1·42 g per ml) with water to 100 ml.

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

6. *Procedure*

6.1 Dissolution of sample.

Weigh, to the nearest 0·001 g, approximately 10 g 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°C ± 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 10 ml portions of boiling 2N hydrochloric acid solution (3.2), decanting the solution each time through the same suitable filter-paper(1) into a 50 ml graduated flask. Then add 5 ml 50% (V/V) hydrochloric acid solution (3.1) and about 5 ml 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 10 ml 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 10 ml 50% (V/V) hydrochloric acid solution (3.1) to a silical 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 10 μg 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 10 μg per ml copper.

6.3.3 Measurement.

Set up the instrument at a wavelength of 324·7 nm. 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

(1) Whatman No. 541 or equivalent.

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.

14a. 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.

3. *Reagents*

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

3.2 Ammonia (density 0·88 g/ml).

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

3.4 Ammonium oxalate solution—saturated aqueous solution.

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

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

3.7 Hydrochloric acid (density 1·18 g/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 20 ml hydrochloric acid (3.7), with water, to 100 ml.

3.10 Methyl red indicator solution: dissolve 0·025 g methyl red in 5 ml 95 per cent (V/V) ethanol with the aid of 0·5 ml 0·1N sodium hydroxide solution. Dilute to 250 ml with 50 per cent (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 0·001 g, a quantity of the prepared sample expected to contain between 0·05 g and 0·50 g 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 10 ml 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 10 ml of 20% (V/V) hydrochloric acid (3.9), heat on a steam bath, and then filter into a 50 ml graduated flask. Repeat the extraction with two further 10 ml 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 0·06 g of magnesium, to a 500 ml beaker, and dilute with water to 100 ml. 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 3-4 ml in excess. Heat the solution to boiling and add 0·2 ml (4-5 drops) of methyl red indicator solution (3.10). Add 25 ml of hot ammonium oxalate solution (3.4) gradually with constant stirring then add dilute ammonia solution (3.3) dropwise until the solution is netural 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 10 ml of 20% (V/V) hydrochloric acid (3.9) through the filter and collect the extract in a 250 ml beaker. Repeat with two further quantities of 20% (V/V) hydrochloric acid and finally wash the filter with water. Add 2-3 drops of methyl red indicator (3.10) to the combined extracts, heat to boiling and add 25 ml 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, 20 ml 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 10 ml of ammonia (3.2) for each 100 ml in the beaker. Allow the beaker to stand for at least 4 hours, or preferably, overnight.

Transfer the precipitate to the filter 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

(1) Whatman No. 40 or equivalent.

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:

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

where m=weight of the precipitate (g);
w=weight of sample taken (g); and
v=measured volume (ml) taken for determination (5.2).

14b. 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 per cent.

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 285·2 nm.

3. *Reagents*

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

3.2 Magnesium ribbon or wire, or magnesium sulphate heptahydrate.

3.3 Releasing agent.

3.3.1 Lanthanum oxide solution: moisten 117·3 g lanthanum oxide (La_2O_3), low in magnesium content, with water, slowly add 350 ml hydrochloric acid (3.1) and stir until the lanthanum oxide is dissolved. Allow to cool and dilute to 1,000 ml with water.

3.3.2 Strontium salt solution (chloride or nitrate) containing 2·5 g per 1000 ml of strontium (=76·08 g $SrCl_2.6H_2O$ or 60·38 g $Sr(NO_3)_2$ per 1000 ml).

3.4 Magnesium standard solution: weigh to the nearest 0·001 g, exactly 1 g of magnesium (3.2) from which the oxide coating has been carefully removed or the corresponding quantity (10·143 g) of magnesium sulphate heptahydrate (3.2) and transfer to a 1000 ml graduated flask. Dissolve the metal or magnesium sulphate in 80 ml of hydrochloric acid (3.1) and dilute to the mark with water. 1 ml of this solution ≡1·000 mg 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 0·001 g, approximately 5 g of the prepared sample and transfer to a 500 ml graduated flask with 250 to 300 ml water. Add 40 ml 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 30 ml of the filtrate. In the presence of silica, treat the sample with a sufficient quantity (15-30 ml) 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 0·001 g approximately 5 g 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 (15-30 ml) 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 10 ml hydrochloric acid (3.1) and transfer to a 500 ml graduated flask using warm water. Leave to cool and make to volume with water. Mix and filter into a dry beaker through a dry pleated filter. Discard the first 30 ml of the filtrate.

5.1.3 Feeding stuffs composed predominantly of organic substances.

Weigh to the nearest 0·001 g, approximately 5 g 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 5 ml 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 5 ml hydrochloric acid (3.1), transfer to a 250 ml 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 30 ml of the filtrate.

5.2 Determination.

Transfer a suitable volume of the solution obtained under 5.1 to a 100 ml graduated flask, add 10 ml 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 285·2 nm.

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 285·2 nm. 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 10000 IU/kg for highly pigmented feeds and 4000 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 610 nm. For Group B products, the determination is by measurement of the absorbance at 325 nm.

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 10 g aluminium oxide and 0·9 ml 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(1).

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) (25 g aluminium oxide per 250 ml diethyl ether).

3.5 Ethanol, 96 per cent (V/V).

3.6 Nitrogen.

3.7 Light petroleum, boiling range: 40-60°C. If necessary, purify as follows: stir 1000 ml light petroleum with 20 ml lots of concentrated sulphuric acid until the acid remains colourless. Remove the acid and wash the light petroleum successively with 500 ml water, twice with 250 ml of sodium hydroxide solution (approximately 2·5N), and three times with 500 ml 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 per cent (V/V) diethyl ether (3.4) in light petroleum (3.7).

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

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

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

3.12 Potassium hydroxide, N solution.

3.13 Potassium hydroxide, 0·5N solution.

(1) 1 IU = 0·3 μg of retinol.

(1) 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) (50 g aluminium oxide per 200 ml chloroform; it is advisable to chromatograph the first 50 ml of the eluate a second time).

3.16 Carr-Price reagent: stir approximately 25 g antimony trichloride (kept in a desiccator) with 100 ml 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 2 ml acetic anhydride. Keep in a refrigerator in a moistureproof brown glass bottle with ground-glass stopper. The solution keeps for 2 to 3 weeks.

3.17 Retinol — standardized spectrophotometrically.

c. used exclusively for analysing Group B products

3.18 Propan-2-ol, for chromatography.

4. *Apparatus*

4.1 Rotary vacuum evaporator.

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

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

4.4 UV lamp 365 nm.

5. *Procedure*

NB. 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:

0·1-1·0 g for contents greater than 20 000 000 IU/kg;
3·0-5·0 g for contents between 400 000 and 20 000 000 IU/kg;
10-20 g for contents between 20 000 and 400 000 IU/kg; and
30 g for Group A products.

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

5.2 Hydrolysis and extraction(1).

Add successively to the sample 40 ml ethanol (3.5), 2 ml sodium ascorbate solution (3 10)(2), 10 ml potassium hydroxide solution (3.9) and 2 ml 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 50 ml ethanol (3.5) and 100 ml 1, 2-dichloroethane (3.3). Shake vigorously and then carefully decant the supernatant liquid into a separating funnel avoiding transfer of solids. Add 150 ml 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 40 ml potassium hydroxide solution (3.13), shake for 10 seconds and allow to stand until the layers have separated. Collect the dichloroethane

(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.

layer in a separating funnel, and wash at least 6 times with 40 ml lots of water. It is essential that the dichloroethane is free of alkali and washing must be continued until 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 5 ml light petroleum (3.7).

For Group A products, chromatograph as shown in 5.3.1.

For Group B products, transfer the solution to a 50 ml 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 200 mm 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 20 ml light petroleum (3.7). Elute successively with 10 ml 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 eluating 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 2 ml benzene (3.14), using a safety pipette. Take 0·3 ml of this solution and add 3 ml of the Carr-Price reagent (3.16). A blue colour develops. Measure the absorbance at 610 nm exactly 30 seconds after the reaction has begun against a reference solution prepared from 0·3 ml of benzene and 3 ml 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 0·3 ml. Treat this volume (0·3 ml) of each solution with 3 ml Carr-Price reagent (3.16) and measure the absorbance at 610 nm.

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

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

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 treat the residue with 25 ml propan-2-ol (3.18). Measure the absorbance in the spectrophotometer at 325, 310 and 334 nm. The absorption maximum is located at 325 nm. The retinol content of the solution is calculated as follows:

Absorbance at 325 nm × 1830 = 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 310 nm;

Y = absorbance at 325 nm; and

Z = absorbance at 334 nm.

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 wth 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 5 mg/kg.

2. *Principle*

The solution 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 per cent (V/V).

3.2 2-Methylpropan-1-ol.

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

3.4 Sodium metabisulphite ($Na_2S_2O_5$).

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

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

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

(1) eg Clarase.

3.8 Sodium acetate, 2·5N solution.

3.9 Sulphuric acid 0·2N solution.

3·10 Thiamine standard solution: dissolve 127·1 mg thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$), previously dried under vacuum to constant weight, in 1000 ml of dilute sulphuric acid (3.9). 1 ml of this solution contains 100 μg 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 10 mm silica cells.

5. *Procedure*

5.1 Enzymatic hydrolysis.

Place in each of two 250 ml graduated flasks, A and B, identical amounts of the prepared sample containing approximately 100 μg thiamine base and 125 ml sulphuric acid (3.0). 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 20 ml sodium acetate solution (3.8) and 0·5 g multienzymatic preparation (3.8), then allow to stand for 20 minutes. Cool to room temperature if necessary and then add 20 ml sodium acetate solution (3.8), make up to volume with water, mix and filter. Collect filtrates A and B after having discarded the first 15 ml. Prepare the following solutions:

5.1.1 Reference solution T.

Place in a centrifuge tube 5 ml filtrate A and approximately 10 mg 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 5 ml filtrate A in a centrifuge tube and 5 ml filtrate B in another centrifuge tube.

5.2 Oxidation.

Add to solutions T, A and B 5 ml of the oxidising mixture (3.7) and, one minute later, 10 ml 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 (3.2) layer to separate 25 ml 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 365 nm. 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 DEHYDRO-ASCORBIC 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 20 mg/kg. Products are classified in two groups, according to their presumed vitamin C content:

Group A: contents lower than 10 g/kg;

Group B: contents equal to or greater than 10 g/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 509 nm.

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 0·2 mm.

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

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

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

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

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

3.14 L-ascorbic acid solution: dissolve 50 mg L-ascorbe acid in approximately 20 ml metaphosphoric acid solution (3.11) and make up to 100 ml 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: 100 mm, internal diameter 20 mm), with a sintered disc (eg Allihn tubes).

4.5 Spectrophotometer with 10 mm cells.

4.6 Apparatus for thin layer chromatography, with silica gel plates (3.6) coated to a depth of 0·5 to 0·6 mm. (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 250 ml graduated flasks A and B, identical quantities (up to 10 g, according to the presumed ascorbic acid content, weighed to the nearest 0·001 g) of the prepared sample. Add to flask B (at 4°C) 30 ml chloroform (3.2) and 25 ml metaphosphoric acid solution (3.11). Add to flask A, 30 ml 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 25 ml 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 25 ml water, stopper and shake vigorously for 10 seconds and allow to stand for 10 to 15 minutes in the water bath (4.1). Centrifuge to separate the aqueous and chloroform layers. The aqueous phases are retained for subsequent analysis.

5.2 Oxidation.

Place in a 50 ml stoppered flask, a volume of extract B, expected to contain about 160 μg 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 10 ml). Place in a second 50 ml flask an identical volume of extract A. Dilute the contents of each flask to 40 ml with a mixture of equal volumes of metaphosphoric acid solution (3.11) and water. Add 0·5 to 1·0 ml of dichlorophenolindophenol solution (3.13) and mix well. A red colour develops which should persist for at least 15 minutes. Add approximately 300 mg 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 10 ml of the filtrate obtained in (5.2) to a centrifuge tube, add 2 ml 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 3 ml water, 20 ml of the ethyl acetate/glacial acetic acid/acetone mixture (3.7) and approximately 800 mg filtration aid (3.3). Stopper the tube, shake vigorously for 30 seconds and centrifuge. Place 15 ml 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 2 ml of the ethyl acetate/glacial acetic acid/acetone mixture (3.7) by heating at 50°C, allow to cool, add 10 ml 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 30 mm with the dichloromethane/glacial acetic acid mixture (3.8). Suspend (shaking vigorously) 5 g silica gel (3.5) in 30 ml 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 5 ml portions of this solvent (3.8) until the eluate is colourless. Discard the part of the eluate which is coloured yellow.

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 in vitamin C lower than 10 g/kg), dissolve the residue in 2·0 ml 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 in vitamin C equal to or greater than 10 g/kg), treat the oily residue with 4·0 ml 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) 0·5 ml 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 2 ml and twice with 1·5 ml 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 4·0 ml dilute sulphuric acid (3.12), shake vigorously to dissolve the residue completely and measure the absorbance.

5.6 Determination.

Measure the absorbance at 509 mm 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 of 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 1 mg/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 635 nm.

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: 10 g sodium carbonate (anhydrous) per 10 ml.
3.7 Tannin solution: 10 g purified powdered tannin per 100 ml.
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 0·04 g of 2, 4-dinitrophenylhydrazine in about 40 ml of boiling absolute ethanol (3.2). Allow to cool, transfer to a 50 ml graduated flask. Add 0·4 ml of hydrochloric acid (3.4) and make up to volume with absolute ethanol. Prepare immediately before use.
3.10 Standard solutions of menadione: dissolve 20 mg menadione (vitamin K_3) in 1, 2-dichloroethane (3.1) and make up to 200 ml. 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 10 mm cells.
4.5 Phase separating paper(1).

(1) Whatman No. 1PS or equivalent.

5. *Procedure*

NB 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 0·001 g, 0·1 to 5 g of prepared feed supplement, or to the nearest 0·01 g, 20 to 30 g of all other prepared feeding stuffs and transfer, without delay, to 250 ml conical flask, with ground glass stopper.

Add to the test sample exactly 96 ml dilute ethanol (3.3) and shake mechanically for 15 minutes at room temperature. Then add 4·0 ml tannin solution (3.7), mix, transfer the extract into a centrifuge tube, centrifuge until a clear solution is obtained and decant. Place 20 to 40 ml, accurately measured, of the extract in a 250 ml separator, add 50 ml 1, 2-dichloroethane (3.1), mix and add 20 ml sodium carbonate solution (3.6). Shake vigorously for 30 seconds and then collect the dichloroethane phase in a 100 ml separator. Add 20 ml 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 10 µg menadione per ml.

5.2 Hydrazone formation.

Transfer 2·0 ml of the dichloroethane extract obtained in 5.1 to a 10 ml graduated flask and add 3·0 ml 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 3·0 ml 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 635 nm against a reference solution obtained by treating 2·0 ml 1, 2-dichloroethane (3.1) as indicated in (5.2). Determine the quantity of menadione by reference to a calibration curve established for each series of analyses.

5.4 Calibration curve.

Treat 2·0 ml 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 0·88 g/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 100 ml water at 37 to 40°C. Check that there is no hydrocyanic acid in 10 ml 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: 10 g sodium acetate (anhydrous) per 100 ml.

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

4.4 Oil bath.

4.5 Burette graduated to 0·05 ml.

5. *Procedure*

Weigh to the nearest 0·005 g, approximately 20 g of the prepared sample, place in a 1 litre flat-bottomed flask (4.3) and add 50 ml of water and 10 ml 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 80 ml of water, 10 ml of sodium acetate solution (3.6) and a drop of antifoam (3.1).

Connect the flask to the stream 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 300 ml 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 50 ml of silver nitrate solution 0·02N (3.8) and 1 ml 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 250 ml of the filtrate, add approximately 1 ml 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 10 ml 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. 1 ml of $AgNO_3$ 0·02N = 0·54 mg 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 50 ml 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 per cent (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 500 ml flasks with ground-glass stoppers.
4.2 Distilling apparatus fitted with a condenser and a splash head.

5. *Procedure*

Weigh to the nearest 0·001 g approximately 10 g of the prepared sample and place in a 500 ml flat-bottomed flask (4.1) and add 2 g of finely ground white mustard (3.4) [an enzyme source] and 200 ml water at 20°C. Stopper the flask and keep at 20°C for approximately 2 hours, shaking frequently. Add 40 ml ethanol (3.2) and one drop of antifoam (3.1). Distil approximately 150 ml and collect the distillate in a 500 ml conical flask containing 20 ml ammonia (3.5) making sure that the end of the condenser is immersed in the liquid. Add to the ammoniacal solution 50 ml silver nitrate solution 0·1 N (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 250 ml graduated flask, rinsing in with water, make up to the mark, mix and filter. To 100 ml of the clear filtrate, add 5 ml nitric acid (3.3) and approximately 5 ml 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 2 g 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 0·1 N consumed by the mustard oil in the sample. 1 ml of $AgNO_3$ 0·1N = 4·956 mg 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 20 mg/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 440 nm.

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 500 ml propan-2-ol/hexane mixture (3.1), 2 ml 3-aminopropan-1-ol, 8 ml glacial acetic acid and 50 ml 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 100 ml graduated flask 2 ml 3-aminopropan-1-ol and 10 ml 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 250 ml graduated flask 27·9 mg gossypol acetate. Dissolve and make up to volume with solvent A (3.2). Place 50 ml of this solution in a 250 ml graduated flask and make up to volume with solvent A. This solution has a gossypol concentration of 0·02 mg/ml. Allow to stand for one hour at room temperature before use.

3.6 Standard gossypol solution B: place in a 50 ml graduated flask 27·9 mg gossypol acetate. Dissolve and make up to volume with solvent B (3.3). This solution has a gossypol concentration of 0·5 mg/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 10 mm 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 1 g; for compound feeding stuffs it may be as much as 5 g. A 10 ml 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 5 g so that a 2 ml aliquuot part of the filtrate contains 40 to 200 μg 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 250 ml flask with ground glass neck, the bottom of which has been covered with a layer of glass beads of approximately 6 mm diameter. Add 50·0 ml 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 25 ml graduated flasks (A and B) identical aliquot parts of filtrate containing 50 to 100 μg gossypol. If necessary make up the volume to 10 ml 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 10 ml solvent A (3.2) to each of two other 25 ml 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 2 ml 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 440 nm 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 5 mg gossypol in a 50 ml graduated flask and add 10 ml solvent B (3.3). At the same time prepare a blank test, placing 10 ml solvent B (3.3) in another 50 ml 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 2 ml of the sample filtrate to each of two 25 ml graduated flasks, and 2 ml of the blank test filtrate to two other 25 ml flasks. Take one flask from each pair and make up the contents of each to 25 ml with the propan-2-ol/hexane mixture (3.1). These solutions shall be used for reference.

Add 2 ml 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 25 ml with the propan-2-ol/hexane mixture (3.1), mix and allow to stand for one 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:

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

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

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

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

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 25 ml 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 10 ml using solvent A (3.2). Complete each series with a blank test consisting of a 25 ml graduated flask containing only 10 ml solvent A (3.2).

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

Add 2 ml 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 50 ml graduated flasks. In the first flask place 10 ml solvent B (3.3) and in the others respectively 2·0, 4·0, 6·0, 8·0 and 10·0 ml standard gossypol solution B (3.6). Make up the contents of each flask to 10 ml 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 2·0 ml of these solutions respectively in two series of six 25 ml graduated flasks. Make up the contents of the flasks in the first series to 25 ml using the propan-2-ol/hexane mixture (3.1) (reference series).

Add 2 ml 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).

22a. 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 10 μg/kg. Other products should be analysed by the method described under method 22b.

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 Offic Analyt Chemists, 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 0·20 mm.

3.9 Absorbant 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.

3.16 Standard solution with about 0·1 μg 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 0·1 μg 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.

(1) Hyflosupercel or equivalent.

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: 22 mm, length 300 mm), with a PTFE/stopcock and a 250 ml reservoir.

4.2 Rotary evaporator, with inlet for inert gas and a 500 ml roundbottom flask.

4.3 TLC apparatus.

4.4 Glass plates for TLC, 200 × 200 mm, prepared as follows (the quantities indicated are sufficient to cover five plates): put 30 g of silica gel G-HR (3.15) into a conical flask. Add 60 ml water, stopper and shake for a minute. Spread the suspension on the plates so as to obtain a uniform layer 0·25 mm 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 the 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 365 nm. The intensity of irradiation must make it possible for a spot of 1·0 nanogram of aflatoxin B_1 to be still clearly distinguished on a TLC plate at a distance of 10 cm from the lamp.

4.6 10·0 ml graduated tubes with polyethylene stoppers.

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

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 5 g of sodium sulphate (3.10). Check that the upper surface of the sodium sulphate (3.10) is flat, then add 10 g of silica gel (3.8) in small portions. Stir carefully after each addition to eliminate

(1) Whatman No. 1 or equivalent.

air-bubbles. Allow to stand for 15 minutes and then carefully add 15 g of sodium sulphate (3.10). Let the liquid fall until it is just above the upper surface of the sodium sulphate layer. Mix the 50 ml of extract collected in 5.2 with 100 ml 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 100 ml 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 8-12 ml per minute and ensure that the column does not run dry. Discard the eluate. Finally elute with 150 ml 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 10·0 ml 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 2·0 ml with chloroform (3.2) or benzene/acetonitrile mixture (3.6).

5.4 Thin-layer chromatography.

Spot on a TLC plate (4.4), 2 cm from the lower edge and at intervals of 2 cm, the volumes indicated below of the standard solution and the extract:—10, 15, 20, 30 and 40 µl of the standard aflatoxin B_1 solution (3.16);
—10 µl of the extract obtained in 5.3 and, *superimposed on the same point,* 20 µl 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 5 mm. 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 10 cm 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 superimposition of the extract on the standard solution must be more intense than that of the 10 µl of extract and there must not be more than one visible spot. If the flourescence intensity given by the 10 µl 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 365 nm and an emission wavelength of 443 nm. 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).

NB: 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 (6 cm 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*: 25 µl 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) (1 cm 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 2·5 cm 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) (1 cm 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 formuula

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

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 extracts 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 20 µl 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 10 µg per ml. Determine the absorption spectrum between 330 and 370 nm. Measure the absorbance (A) at 363 nm in the case of the chloroform solution: or at 348 nm 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 0·1 µg 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) 5 µl of the standard solution of aflatoxin B_1 containing 8 to 10 µg/ml (sect 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 between 10 and 20 µg/kg.

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

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

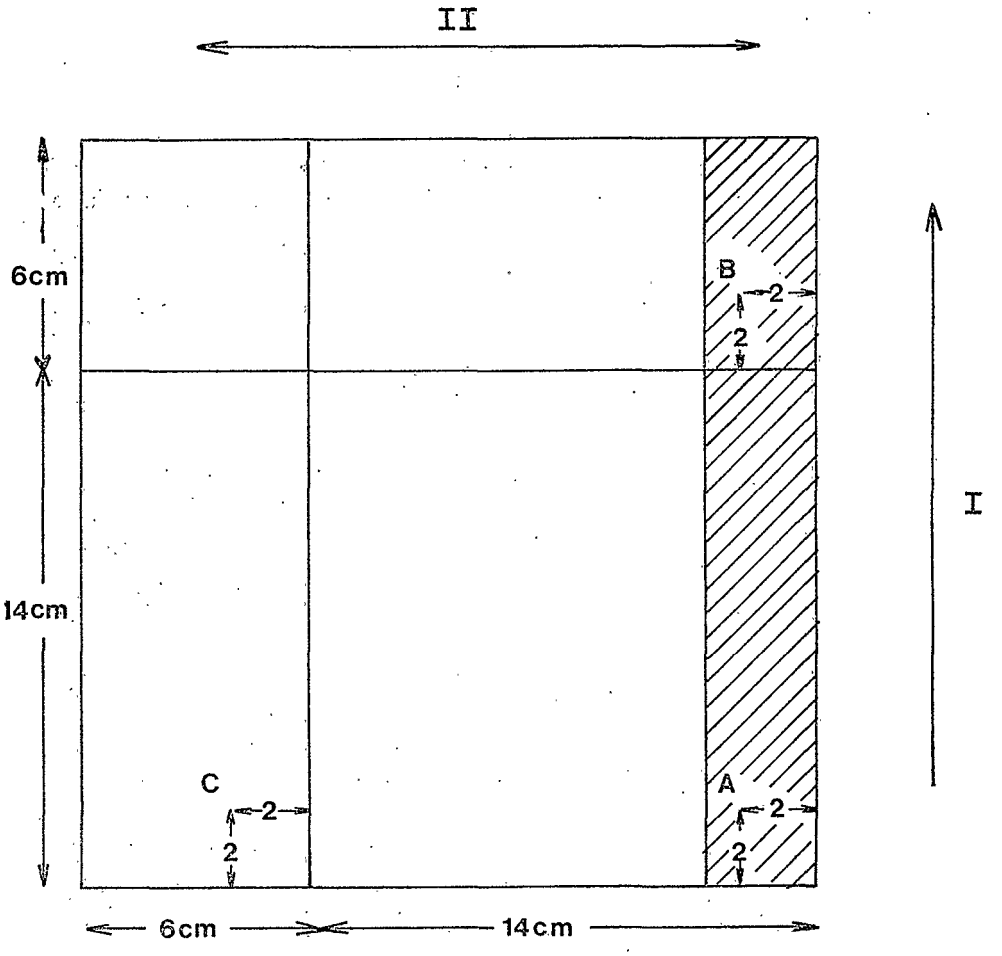

Fig. 1

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

22b. 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 22a. The lower limit of determination is 10 μg/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 0·20 nm.

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

3.10 Sodium sulphate, anhydrous, granular.

3.11 Inert gas, e.g. nitrogen.

3.12 Hydrochloric acid solution, IN.

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.

3.16 Standard solution with about 0·1 μg 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 22a.

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 proportion by volume 9 + 1, unsaturated tank.

4. *Apparatus*

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

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

4.3 TLC apparatus.

4.4 Glass plates for TLC, 200 × 200 mm, prepared as follows (the quantities indicated are sufficient to cover five plates): put 30 g of silica gel G-HR (3.15) into a conical flask. Add 60 ml water, stopper and shake for a minute. Spread the suspension on the plates so as to obtain a uniform layer 0·25 mm 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 the 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 365 nm. 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 10 cm from the lamp.

4.6 10·0 ml graduated tubes with polyethylene stoppers.

4.7 UV Spectrophotometer with 10 mm cells.

4.8 Fluorodensitometer (optional).

(1) Hyflosupercel or equivalent.

5. *Procedure*

5.1 Defatting.

Samples containing more than 5% oil or fat must be defatted with a 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 50·0 g of the prepared sample into a 500 ml conical flask. Add 25 g of diatomaceous earth (3.14), 25 ml of water and 250 ml of chloroform (3.2). Stopper the flask, shake or stir for 30 minutes and filter through a fluted filter paper(1). Discard the first 10 ml of the filtrate and then collect 50 ml.

5.3 Column clean-up.

Insert into the lower end of a chromatography tube (4.1) with top closed a cotton or glass wool plug (3.9), fill two-thirds of the tube with chloroform (3.2) and add 5 g of sodium sulphate (3.10). Check that the upper surface of the sodium sulphate (3.10) is flat, then add 10 g 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 15 g of sodium sulphate (3.10). Let the liquid fall until it is just above the upper surface of the sodium sulphate layer.

Mix the 50 ml of extract collected in 5.2 with 100 ml 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 100 ml 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 8-12 ml per minute and ensure that the column does not run dry. Discard the eluate. Finally elute with 150 ml 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 10·0 ml 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 2.0 ml 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 (5 cm and 6 cm from each side respectively), to limit migration of the solvent fronts. Spot the following solutions on the plate:

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

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

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

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

—*on point E*, 40 µl 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 5 mm.

(1) Whatman No. 1 or equivalent.

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) (1 cm 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) (1 cm 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 10 cm 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) 20 µl of the purified sample extract obtained in 5.3 and, superimposed on it, 20 µl 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 20 µl of extract is greater than that of the 40 µl 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 365 nm and an emission wavelength of 443 nm. 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).

NB: 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 (6 cm 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*: 25 µl 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) (1 cm 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 2·5 cm 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) (1 cm 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 20 µl 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:

$\pm$ 50% of the mean value for mean values of aflatoxin B_1 between 10 and 20 µg/kg

$\mp$ 10µg/g on the mean value for mean values greater than 20 and up to 50 µg/kg

$\pm$ 20% of the mean value for mean values above 50 µg/kg

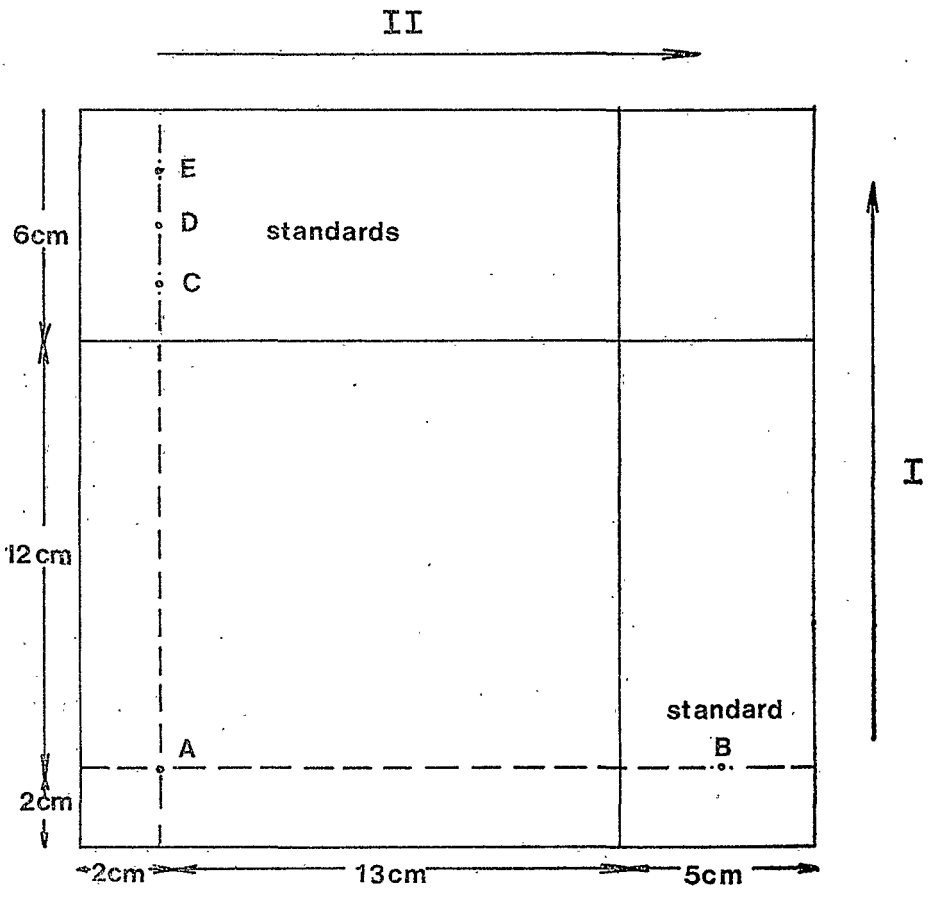

Fig. 2

II

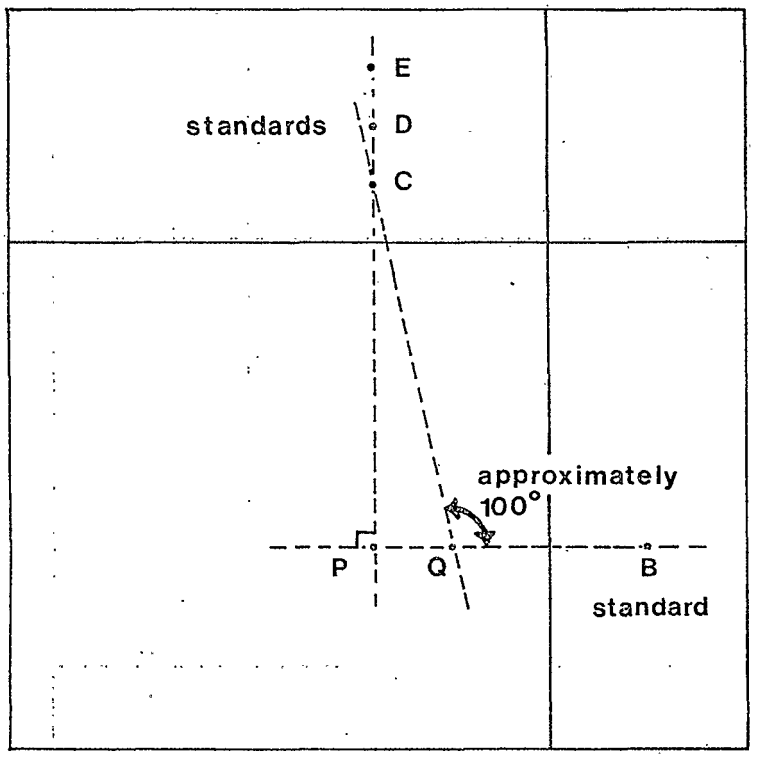

Fig 3

SCHEDULE 6

(Sections 77(4), 78(3), and 79(5) to (8) and Regulation 2(18))

CERTIFICATE OF ANALYSIS OF FEEDING STUFF (1)

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

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:— (7)

	% of Units/kg		Units/kg or IU/kg
Oil		Vitamin A	
Protein: Total, including protein equivalent of biuret, isobutylidene diurea, urea or urea phosphate and protein equivalent of uric acid		Vitamin D_2	
		Vitamin D_3	
		Vitamin E	
Protein equivalent of biuret, isobutylidene diurea, urea or urea phosphate		Other vitamins or pro vitamins	
Protein equivalent of uric acid			
Fibre		Permitted antioxidant (8)	
Sugar		Permitted colourant (8)	
Salt (NaCl)		(9)	
Phosphorus (P)		(10)	
Calcium (Ca)		(11)	
Copper (Cu)		(14)	
Magnesium (Mg)		(15)	
Molybdenum (Mo)			
Selenium (Se)			
Iron (Fe)			
Iodine (I)			
Cobalt (Co)			
Manganese (Mn)			
Zinc (Zn)			

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

The analysis was made in accordance with the Fertilisers and Feeding Stuffs Regulations (Northern Ireland) 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 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.

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

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

(5) 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.

(6) 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.

(7) Insert relevant results under the appropriate headings, ie percentage, units/kg or IU/kg.

(8) 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.

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

(10) Here indicate the presence of any preservative.

(11) 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 6 and Schedule 3 apply the name and estimated percentage of any such substance which is deleterious to human beings.

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

(13) 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.

(14) 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.

(15) 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*
5000 litres (l)	1,000 gallons
25 kilogrammes (kg)	56 lbs
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
	5000 litres (l)	1000 gallons
	25000 litres (l)	5000 gallons
	50000 litres (l)	10000 gallons
	75000 litres (l)	15000 gallons
	100000 litres (l)	20000 gallons
	250000 litres (l)	50000 gallons
	500000 litres (l)	100000 gallons
2. Weight		
	1-2 kilogrammes (kg)	2-4 lb
	2-3 kilogrammes (kg)	4-6 lb
	2·5 kilogrammes (kg)	6 lb
	6·0 kilogrammes (kg)	14 lb
	7·0 kilogrammes (kg)	15 lb
	25 kilogrammes (kg)	56 lb (½ cwt)
	100 kilogrammes (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)	$\frac{3}{16}$ 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 kilogramme (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 Northern Ireland, are made after consultation with persons and organisations representing the interests concerned and amend the Fertiliser and Feeding Stuffs Regulations (Northern Ireland) 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(2) 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(6) and Schedule 5);

(*c*) implement the provisions of Commmission Directive 75/696/EEC (OJ No. L. 229, 19.11.75, p. 19.) on the use of added non protein nitrogenous compounds in feeding stuffs (regulation 2(2) 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(9) 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(9) 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(14) 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(14) 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).