STATUTORY INSTRUMENTS

1983 No. 62

MEDICINES

The Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983

Made - - - -	*24th January* 1983
Laid before Parliament	*10th February* 1983
Coming into Operation	*3rd March* 1983

The Minister of Agriculture, Fisheries and Food, the Secretaries of State respectively concerned with agriculture in Scotland and in Wales and the Department of Agriculture for Northern Ireland, acting jointly, in exercise of powers conferred by sections 117(1), (2) and (3) of the Medicines Act 1968**(a)** and now vested in them**(b)** and of all other powers enabling them in that behalf, after consulting such organisations as appear to them to be representative of interests likely to be substantially affected by these regulations in accordance with Section 129(6) of the said Act, hereby make the following regulations:—

Title, commencement and interpretation

1.—(1) These regulations may be cited as the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983, shall come into operation on 3rd March 1983.

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

"the Act" means the Medicines Act 1968;

"agricultural analyst" means an agricultural analyst appointed under section 67 of the Agriculture Act 1970**(c)** and includes a deputy agricultural analyst so appointed for the same area;

"animal feeding stuff" includes a complete feeding stuff, a feed supplement and a protein concentrate;

"complete feeding stuff" means a substance or a mixture of substances

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(a) 1968 c. 67.

(b) In the case of the Secretary of State concerned with agriculture in Wales by virtue of Article 2(3) of, and Schedule 1 to, the Transfer of Functions (Wales) (No. 1) Order 1978 (S.I. 1978/272) and in the case of the Department of Agriculture for Northern Ireland by virtue of section 40 of, and Schedule 5 to, the Northern Ireland Constitution Act 1973 (c. 36) and section 1(3) of, and paragraph 2(1)(*b*) of Schedule 1 to, the Northern Ireland Act 1974 (c. 28).

(c) 1970 c. 40.

designed for feeding to animals without further mixing with other feeding stuffs;

"feed supplement" means a substance or a mixture of substances designed for further mixing before feeding to animals at an inclusion rate of less than 5 per cent with other animal feeding stuffs;

"inspector" means a person authorised by an enforcement authority for the purposes of the enforcement of the Act;

"protein concentrate" means a substance or a mixture of substances designed for further mixing before feeding to animals at an inclusion rate of 5 per cent or more with other animal feeding stuffs;

"sampled portion", in relation to any animal feeding stuff means an amount of that feeding stuff (as prescribed under the provisions of Part I of Schedule 2 to these regulations) constituting a unit and having characteristics presumed to be uniform from which a sample has been taken or set aside by an inspector in the manner prescribed in Parts II and III of the said Schedule, being an amount—

(a) consisting either—

(i) entirely of feeding stuffs packed in one or more containers; or

(ii) entirely of feeding stuffs not so packed; and

(b) not exceeding, in the case of an amount consisting of feeding stuffs so packed, the requisite quantity, that is to say, five tonnes or 5,000 litres, except where—

(i) it consists of feeding stuffs packed in a single container; or

(ii) it consists of feeding stuffs packed in two or more containers each of which holds less than the requisite quantity, in which case the prescribed amount may be the contents of the lowest number of those containers which together hold the requisite quantity;

"unit" means an animal feeding stuff identifiable as such by reason of its manufacturer, packer, uniform manner of packaging or labelling:

Modification of certain provisions of the Act

2. For the purposes of the application of the provisions of sections 112, 113 and 115 of the Act in relation to animal feeding stuffs, those provisions shall have effect subject to the modifications specified in Part 1 of Schedule 1 to these regulations, such provisions as so modified being set out in Part II of the said Schedule.

Sampling and analysis

3.—(1) A sample which is taken in accordance with the provisions of sections 112(2) or 115(1) of the Act, shall be taken, submitted for analysis and dealt with in the manner provided in Schedule 2 to these regulations.

(2) Where pursuant to section 113(2) of the Act a sample is set aside from a substance or article seized in pursuance of section 112(4) of the Act or a substance or article so seized is itself treated as a sample, such a sample or such a substance or article shall be set aside, submitted for analysis and dealt with in

the manner provided in Schedule 2 to these regulations, but subject to the modifications set out in Part VII to that Schedule.

(3) Without prejudice to his powers and duties under section 112 of the Act an inspector may for the purposes of the Act take a sample otherwise than in accordance with these regulations of any substance or article appearing to him to be an animal feeding stuff but in all other respects these regulations shall not apply and no proceedings under the Act shall be taken in relation to such a sample.

Methods of analysis

4.—(1) Paragraphs (2) and (3) of this regulation shall have effect for the purposes of proceedings for offences under any of the following provisions of the Act, that is to say subsections (1), (2), (4) and (5) of section 45, subsection (2) of section 67, subsections (1) and (2) of section 91 and subsections (1) and (2) of section 123 and for the purposes of proceedings for offences under the Medicines (Labelling of Medicated Animal Feeding Stuffs) Regulations 1973**(a)**.

(2) For the purpose of determining what quantity or proportion (if any) of a substance or article of a description or class specified in Schedule 3 to these regulations has been incorporated in a sample of an animal feeding stuff to be analysed in pursuance of section 112, section 113 or section 115 of the Act the methods of analysis set out in the said Schedule 3 shall, whenever they are applicable, be used.

(3) On the production in the proceedings of a certificate of the results of analysis of a sample of the animal feeding stuff performed by a method prescribed by paragraph (2) of this regulation evidence of the results of any analysis of a part of the sample performed by any other method shall not be admissible in the proceedings.

Forms of certificate of analysis or examination

5. A certificate of analysis issued by an agricultural analyst in accordance with Schedule 2 to these regulations shall be in the form set out in Schedule 4 Part I to these regulations, and a certificate of analysis or examination made by any laboratory for the purposes of the said Schedule 2 shall be in the form set out in Schedule 4 Part II to these regulations.

Revocation

6. The Medicines (Animal Feeding Stuffs)(Enforcement) Regulations 1976**(b)** and the Medicines (Animal Feeding Stuffs)(Enforcement)(Amendment) Regulations 1977**(c)** are hereby revoked.

In Witness whereof the Official Seal of the Minister of Agriculture, Fisheries and Food is hereunto affixed on 12th January 1983.

(a) S.I. 1973/1530.
(b) S.I. 1976/30.
(c) S.I. 1977/1584.

L.S.

Peter Walker,
Minister of Agriculture, Fisheries and Food.

George Younger,
Secretary of State for Scotland.

14th January 1983.

Nicholas Edwards,
Secretary of State for Wales.

18th January 1983.

Sealed with the Official Seal of the Department of Agriculture for Northern Ireland this 24th January 1983.

L.S.

J. A. Young,
Permanent Secretary.

Regulation 2

SCHEDULE 1

PART I

MODIFICATIONS OF SECTIONS 112, 113 AND 115 OF THE MEDICINES ACT 1968

Section 112

1. In subsection (1), immediately after the words "that authority" there shall be inserted the words "(in this section referred to as an "inspector")", in paragraph (*a*), for the words "a medicinal product" there shall be substituted the words "an animal feeding stuff", in paragraph (*b*), for the words "any medicinal product" there shall be substituted the words "any animal feeding stuff" and for the words "a medicinal product" there shall be substituted the words "an animal feeding stuff", and paragraph (*c*) shall be omitted.

2. In subsection (2), for the words "a person authorised as mentioned in that subsection" there shall be substituted the words "an inspector", for paragraphs (*a*) and (*b*) there shall be substituted the words "an animal feeding stuff" and the words "if he does not obtain the sample by purchase" shall be omitted.

3. In subsection (3), for the words "person authorised as mentioned in that subsection" there shall be substituted the word "inspector", for the words "medicinal products" there shall be substituted the words "animal feeding stuffs" and immediately after the words "medicinal products" there shall be inserted the words "or a business which includes the mixing of animal feeding stuffs for use in the course of that business".

4. In subsection (4), for the words "person so authorised" there shall be substituted the word "inspector" and immediately before the word "document" wherever it appears, there shall be inserted the words "book or".

5. In subsection (5), for the words "subsection (4)" there shall be substituted the words "subsections (1), (2) or (4)", for the words "person having that right" there shall be substituted the word "inspector" and the words "or open any vending machine" shall be omitted.

6. In subsection (6), for the words "a person" there shall be substituted the words "an inspector", immediately before the word "document" there shall be inserted the words "book or" and the words from "and, in the case of" to the end of the subsection shall be omitted.

7. In subsection (7), for the words "person duly authorised in writing by the licensing authority" there shall be substituted the word "inspector" and for the words "a person" there shall be substituted the words "an inspector".

8. In subsection (9), for the words "The provisions of Schedule 3 to" there shall be substituted the words "Regulations made under section 117(2) of".

Section 113

9. In subsections (1) to (5), for the words "authorised officer", wherever they appear, there shall be substituted the word "inspector".

10. In subsection (1), immediately before the word "document" there shall be inserted the words "book or".

11. In subsection (2), immediately after the word "nature" there shall be inserted the words "or quantity".

12. In subsection (3), immediately after the word "nature" there shall be inserted the words "or quantity".

13. In subsection (4), for the words from "into three parts" to the end of the subsection there shall be substituted the words "into such number of parts as shall be required by regulations made under section 117 of this Act".

14. In subsection (5), for the words "Paragraphs 10, 11 and 12 and paragraphs 15 to 27 of Schedule 3 to" there shall be substituted the words "Regulations made under section 117(2) of", and for the words from "as they have effect" to the end of the subsection there shall be substituted the words "and as if any reference to a sample included a reference to a substance or article treated as a sample".

Section 115

15. In subsection (1), for the words from "a medicinal product" to the end of the subsection there shall be substituted the words "an animal feeding stuff shall be entitled to have a sample taken of it by a person authorised in that behalf by an enforcement authority (in this section referred to as an "inspector") and analysed by the agricultural analyst for the inspector's area".

16. At the end of subsection (1) there shall be added the following subsections:—

"(1A) Any request for a sample to be taken and analysed under this section shall be accompanied by such fee as may be fixed by the enforcement authority of the inspector's area; and different fees may be fixed for different animal feeding stuffs and for different analyses of the same animal feeding stuff.

(1B) Regulations made under section 117(2) of this Act shall have effect in relation to a sample taken in accordance with subsection (1) of this section.

(1C) Where a sample of any animal feeding stuff has been taken pursuant to

the request of a purchaser under subsection (1) of this section any of the following persons, that is to say, the purchaser, the person who sold the animal feeding stuff to him and any other person against whom a cause of action may lie in respect of the sale of the animal feeding stuff, shall be entitled to require the inspector—

(a) to send one of the parts retained by the inspector in accordance with regulations made under section 117 of the Act (hereafter in this section referred to as "the first remaining part") for analysis or other appropriate examination to the Government Chemist;

(b) to supply the person making the request with a copy of the Government Chemist's certificate of analysis or other appropriate examination of that first remaining part, whether that part was sent to the Government Chemist for analysis or other appropriate examination in pursuance of the request of that person or otherwise.

(1D) Where a sample of any animal feeding stuff has been taken by an inspector in the prescribed manner and it is intended to institute proceedings against any person for an offence under this Act and to adduce on behalf of the prosecution evidence of the result of an analysis or examination of the sample—

(a) the prosecutor, if a person other than the inspector, shall be entitled to require the inspector—

(i) to send the first remaining part of the sample for analysis or other appropriate examination to the Government Chemist;

(ii) to supply the prosecutor with a copy of the Government Chemist's certificate of analysis or other appropriate examination of that first remaining part, whether that part was sent to the Government Chemist for analysis or other appropriate examination in pursuance of the request of the prosecutor or otherwise;

(b) the inspector, if he is the prosecutor, shall be entitled himself so to send that first remaining part.

(1E) Where a prosecutor avails himself of his rights under subsection (1D) of this section he shall cause to be served with the summons a copy of the agricultural analyst's certificate of analysis, and of any certificate of analysis or examination of any laboratory to which the sample was referred pursuant to paragraph 7(3) of Part V of Schedule 2 to these regulations, and a copy of the Government Chemist's certificate of analysis or other appropriate examination; and where a prosecutor does not avail himself of his rights under that subsection he shall, not less than fourteen days before the service of the summons, cause to be served on the person charged a copy of the agricultural analyst's certificate of analysis (and of any laboratory certificate above referred to) and a notice of intended prosecution, and if, within the period of fourteen days beginning with the service of the notice, that person sends the prosecutor a written request to that effect accompanied by the amount of the fee payable by the prosecutor for the purpose under subsection (1K) of this section (which shall be refunded to that person by the prosecutor if the prosecution is not brought) the prosecutor shall exercise his rights under subsection (1D) of this section and the proceedings shall not be instituted until he has sent that person a copy

of the Government Chemist's certificate of analysis or other appropriate examination.

(1F) Where proceedings are brought against any person for an offence under this Act and evidence is given or sought to be given of the result of an analysis or examination of a sample of any animal feeding stuff taken by an inspector in the prescribed manner but it appears that the sample has not been analysed or examined by the Government Chemist, the court may, of its own motion or on the application of either party, order the remaining part of the sample to be sent for analysis or other appropriate examination to the Government Chemist.

(1G) Where under this section a part of a sample is sent for analysis or other appropriate examination to the Government Chemist there shall be sent with it—

(a) a copy of any document which was sent with the part of the sample sent to the agricultural analyst; and

(b) if the part is sent to the Government Chemist under subsection (1D) or (1F) of this section, a statement of the particulars on which the proceedings or intended proceedings are based.

(1H) The Government Chemist shall analyse or examine or cause to be analysed or examined any part of a sample sent to him under this section but, where the part is accompanied by a statement such as is mentioned in subsection (1G) of this section, the analysis or other appropriate examination shall be made only with respect to the particulars in the statement unless the person or court requesting or ordering the analysis or other appropriate examination requires it to extend also to other matters.

(1J) A certificate of any analysis or other appropriate examination under this paragraph shall be sent by the Government Chemist—

(a) if the material analysed or examined was sent to him in pursuance of subsection (1C) or (1D) of this section, to the inspector;

(b) if it was sent to him in pursuance of an order of the court under subsection (1F) of this section, to the court.

(1K) A request for an analysis or other appropriate examination under subsection (1C) or (1D) of this section shall be of no effect unless accompanied by the appropriate fee; and the appropriate fee for any analysis or other appropriate examination ordered by the court under subsection (1F) of this section shall be paid by such party to the proceedings as the court may direct.

(1L) In the application of this section to Scotland—

(a) for any reference to the court there shall be substituted a reference to the sheriff;

(b) in subsection (1D), in paragraph *(a)* the words "if a person other than the inspector", and paragraph *(b)* shall be omitted;

(c) in subsection (1E), for any reference to the summons there shall be substituted a reference to the complaint;

(d) in subsection (1F), the words "of its own motion" there shall be substituted the words "of his own accord";

(e) for subsection (1K), there shall be substituted the following sub-paragraph—

"(1K) A request for an analysis or other appropriate examination—

(a) under subsection (1C) of this section; or

(b) under subsection (1D) thereof where the request is made at the instance of a person charged with an offence who has received a notice of intended prosecution,

shall be of no effect unless accompanied by the appropriate fee; and the appropriate fee for any analysis or other appropriate examination ordered by the sheriff under subsection (1F) of this section shall be paid by such party to the proceedings as the sheriff may direct.".

(1M) In the application of this section to Northern Ireland—

(a) in subsection (1), for the words "the agricultural analyst for the inspector's area", there shall be substituted the words "an agricultural analyst in Northern Ireland";

(b) in subsection (1A), for the words "the enforcement authority of the inspector's area" there shall be substituted the words "the Department of Agriculture for Northern Ireland";

(c) for any reference to the Government Chemist there shall be substituted a reference to the Chief Agricultural Analyst for Northern Ireland, and the expression "agricultural analyst" shall not include the Chief Agricultural Analyst for Northern Ireland.

(1N) In subsection (1K) of this section "the appropriate fee" means such fee as may be fixed by the Secretary of State for Industry with the approval of the Treasury, and different fees may be fixed for different animal feeding stuffs and for different analyses of the same animal feeding stuff.

(1P) For the purposes of this section, the appropriation of any animal feeding stuff by one person for use under arrangements with another person not constituting a sale of the feeding stuff to that other person, being arrangements which are intended to benefit both the person appropriating the feeding stuff and that other person but under which the probability or extent of any benefit to that other person may be affected by the quality of the feeding stuff, shall be treated as a sale of that feeding stuff to that other person by the person so appropriating it, and references to sale or purchase and cognate expressions shall be construed accordingly."

17. Subsections (2) to (10) shall be omitted.

PART II

SECTIONS 112, 113 AND 115 OF THE MEDICINES ACT 1968 AS MODIFIED IN ACCORDANCE WITH PART I OF THIS SCHEDULE

112.—(1) For the purpose of ascertaining whether there is or has been a contravention of this Act or of any regulations or order made thereunder

which, by or under any provisions of sections 108 to 110 of this Act an enforcement authority is required or empowered to enforce, any person duly authorised in writing by that authority (in this section referred to as an "inspector") shall have a right to inspect—

(a) any substance or article appearing to him to be an animal feeding stuff;

(b) any article appearing to him to be a container or package used or intended to be used to contain any animal feeding stuff or to be a label or leaflet used or intended to be used in connection with an animal feeding stuff.

(2) Where for the purpose specified in the preceding subsection an inspector requires a sample of any substance or article appearing to him to be an animal feeding stuff he shall have a right to take a sample of that substance or article.

(3) For the purpose specified in subsection (1) of this section, any inspector shall have a right—

(a) to require any person carrying on a business which consists of or includes the manufacture, assembly, sale or supply of animal feeding stuffs or a business which includes the mixing of animal feeding stuffs for use in the course of that business, and any person employed in connection with such a business, to produce any books or documents relating to the business which are in his possession or under his control;

(b) to take copies of, or of any entry in, any book or document produced in pursuance of the preceding paragraph.

(4) Any inspector shall have a right to seize and detain any substance or article which he has reasonable cause to believe to be a substance or article in relation to which, or by means of which, an offence under this Act is being or has been committed, and any book or document which he has reasonable cause to believe to be a book or document which may be required as evidence in proceedings under this Act.

(5) For the purpose of exercising any such right as is specified in subsections (1), (2) or (4) of this section the inspector may, so far as is reasonably necessary in order to secure that the provisions of this Act and any regulations or order made thereunder are duly observed, require any person having authority to do so to break open any container or package, or to permit him to do so.

(6) Where an inspector seizes any substance or article (including any book or document) in the exercise of such a right as is specified in subsection (4) of this section, he shall inform the person from whom it is seized.

(7) Without prejudice to the preceding provisions of this section, any inspector shall have the rights conferred by those provisions in relation to things belonging to, or any business carried on by, an applicant for a licence or certificate under Part II of this Act, and may exercise those rights for the purpose of verifying any statement contained in the application for the licence or certificate; and, where by virtue of this subsection an inspector exercises any such right as is specified in subsection (4) of this section, he shall be subject to the duty imposed by subsection (6) of this section.

(8) Notwithstanding anything in the preceding provisions of this section, where a person claiming to exercise a right by virtue of this section is required to produce his credentials, the right shall not be exercisable by him except on production of those credentials.

(9) Regulations made under section 117(2) of this Act shall have effect with

respect to samples obtained on behalf of enforcement authorities for the purposes of this Act.

113.—(1) The provisions of this section shall have effect where a person (in this section referred to as an "inspector") seizes a substance or article (other than a book or document) in the exercise of such a right as is specified in subsection (4) of section 112 of this Act (including that subsection as applied by subsection (7) of that section).

(2) If any person who in accordance with subsection (6) of that section is entitled to be informed of the seizure so requests, either at the time of the seizure or at any subsequent time, not being later than twenty-one days after he is informed of the seizure, then, subject to the next following subsection, the inspector shall either—

(a) set aside a sample of the substance or article seized, or

(b) treat that substance or article as a sample,

whichever he considers more appropriate having regard to the nature or quantity of that substance or article.

(3) An inspector shall not be required by virtue of subsection (2) of this section to set aside a sample, or to treat a substance or article as a sample, if the nature or quantity of the substance or article is such that it is not reasonably practicable to do either of those things.

(4) Where in accordance with subsection (2) of this section an inspector sets aside a sample, or treats a substance or article as a sample, he shall divide it into such number of parts as shall be required by regulations made under section 117 of this Act.

(5) Regulations made under section 117(2) of this Act shall have effect in relation to a sample set aside, or a substance or article treated as a sample, in accordance with subsection (2) of this section and as if any reference to a sample included a reference to a substance or article treated as a sample.

115.—(1) A person who, not being a person authorised in that behalf by an enforcement authority, has purchased an animal feeding stuff shall be entitled to have a sample taken of it by a person authorised in that behalf by an enforcement authority (in this section referred to as an "inspector") and analysed by the agricultural analyst for the inspector's area.

(1A) Any request for a sample to be taken and analysed under this section shall be accompanied by such fee as may be fixed by the enforcement authority of the inspector's area; and different fees may be fixed for different animal feeding stuffs and for different analyses of the same animal feeding stuff.

(1B) Regulations made under section 117(2) of this Act shall have effect in relation to a sample taken in accordance with subsection (1) of this section.

(1C) Where a sample of any animal feeding stuff has been taken pursuant to the request of a purchaser under subsection (1) of this section any of the following persons, that is to say, the purchaser, the person who sold the animal feeding stuff to him and any other person against whom a cause of action may lie in respect of the sale of the animal feeding stuff, shall be entitled to require the inspector:—

(a) to send one of the parts retained by the inspector in accordance with

regulations made under section 117 of the Act (hereafter in this section referred to as "the first remaining part") for analysis or other appropriate examination to the Government Chemist;

(b) to supply the person making the request with a copy of the Government Chemist's certificate of analysis or other appropriate examination of that first remaining part, whether that part was sent to the Government Chemist for analysis or other appropriate examination in pursuance of the request of that person or otherwise.

(1D) Where a sample of any animal feeding stuff has been taken by an inspector in the prescribed manner and it is intended to institute proceedings against any person for an offence under this Act and to adduce on behalf of the prosecution evidence of the result of an analysis or examination of the sample:—

(a) the prosecutor, if a person other than the inspector, shall be entitled to require the inspector—

(i) to send the first remaining part of the sample for analysis or other appropriate examination to the Government Chemist;

(ii) to supply the prosecutor with a copy of the Government Chemist's certificate of analysis or other appropriate examination of that first remaining part, whether that part was sent to the Government Chemist for analysis or other appropriate examination in pursuance of the request of the prosecutor or otherwise;

(b) the inspector, if he is the prosecutor, shall be entitled himself so to send that first remaining part.

(1E) Where a prosecutor avails himself of his rights under subsection (1D) of this section he shall cause to be served with the summons a copy of the agricultural analyst's certificate of analysis, and of any certificate of analysis or examination of any laboratory to which the sample was referred pursuant to paragraph 7(3) of Part VI of Schedule 2 to these regulations, and a copy of the Government Chemist's certificate of analysis or other appropriate examination; and where a prosecutor does not avail himself of his rights under that subsection he shall, not less than fourteen days before instituting proceedings against any person for an offence under this Act, cause to be served on the person to be charged a copy of the agricultural analyst's certificate of analysis (and of any laboratory certificate above referred to) and a notice of intended prosecution, and if, within the period of fourteen days beginning with the service of the notice, that person sends the prosecutor a written request to that effect accompanied by the amount of the fee payable by the prosecutor for the purpose under subsection (1K) of this section (which shall be refunded to that person by the prosecutor if the prosecution is not brought) the prosecutor shall exercise his rights under subsection (1D) of this section and the proceedings shall not be instituted until he has sent that person a copy of the Government Chemist's certificate of analysis or other appropriate examination.

(1F) Where proceedings are brought against any person for an offence under this Act and evidence is given or sought to be given of the result of an analysis or examination of a sample of any animal feeding stuff taken by an inspector in the prescribed manner but it appears that the sample has not been analysed or examined by the Government Chemist, the court may, of its own motion or on the application of either party, order the remaining part of the sample to be sent for analysis or other appropriate examination to the Government Chemist.

(1G) Where under this section a part of a sample is sent for analysis or other

appropriate examination to the Government Chemist there shall be sent with it:—

(a) a copy of any document which was sent with the part of the sample sent to the agricultural analyst; and

(b) if the part is sent to the Government Chemist under subsection (1D) or (1F) of this section, a statement of the particulars on which the proceedings or intended proceedings are based.

(1H) The Government Chemist shall analyse or examine or cause to be analysed or examined any part of a sample sent to him under this section but, where the part is accompanied by a statement such as is mentioned in subsection (1G) of this section, the analysis or other appropriate examination shall be made only with respect to the particulars in the statement unless the person or court requesting or ordering the analysis or other appropriate examination requires it to extend also to other matters.

(1J) A certificate of any analysis or other appropriate examination under this paragraph shall be sent by the Government Chemist—

(a) if the material analysed or examined was sent to him in pursuance of subsection (1C) or (1D) of this section, to the inspector;

(b) if it was sent to him in pursuance of an order of the court under subsection (1F) of this section, to the court.

(1K) A request for an analysis or other appropriate examination under subsection (1C) or (1D) of this section shall be of no effect unless accompanied by the appropriate fee; and the appropriate fee for any analysis or other appropriate examination ordered by the court under subsection (1F) of this section shall be paid by such party to the proceedings as the court may direct.

(1L) In the application of this section to Scotland—

(a) for any reference to the court there shall be substituted a reference to the sheriff;

(b) in subsection (1D), in paragraph *(a)* the words, "if a person other than the inspector" and paragraph *(b)* shall be omitted;

(c) in subsection (1E), for any reference to the summons there shall be substituted a reference to the complaint;

(d) in subsection (1F), for the words "of its own motion" there shall be substituted the words "of his own accord";

(e) for subsection (1K), there shall be substituted the following sub-paragraph—

"(1K) A request for an analysis or other appropriate examination—

(a) under subsection (1C) of this section; or

(b) under subsection (1D) thereof where the request is made at the instance of a person charged with an offence who has received a notice of intended prosecution,

shall be of no effect unless accompanied by the appropriate fee; and the appropriate fee for any analysis or other appropriate examination ordered by the sheriff under subsection (1F) of this section shall be paid by such party to the proceedings as the sheriff may direct".

(1M) In the application of this section to Northern Ireland—

(a) in subsection (1), for the words "the agricultural analyst for the inspector's area" there shall be substituted the words "an agricultural analyst in Northern Ireland";

(b) in subsection (1A), for the words "the enforcement authority of the inspector's area" there shall be substituted the words "the Department of Agriculture for Northern Ireland";

(c) for any reference to the Government Chemist there shall be substituted a reference to the Chief Agricultural Analyst for Northern Ireland, and the expression "agricultural analyst" shall not include the Chief Agricultural Analyst for Northern Ireland.

(1N) In subsection (1K) of this section "the appropriate fee" means such fee as may be fixed by the Secretary of State for Industry with the approval of the Treasury, and different fees may be fixed for different animal feeding stuffs and for different analyses of the same animal feeding stuff.

(1P) For the purposes of this section, the appropriation of any animal feeding stuff by one person for use under arrangements with another person not constituting a sale of the feeding stuff to that other person, being arrangements which are intended to benefit both the person appropriating the feeding stuff and that other person but under which the probability or extent of any benfit to that other person may be affected by the quality of the feeding stuff, shall be treated as a sale of that feeding stuff to that other person by the person so appropriating it, and references to sale or purchase and cognate expressions shall be construed accordingly.

SCHEDULE 2 Regulation 3

MANNER OF TAKING DIVIDING, MARKING, SEALING AND FASTENING OF SAMPLES

PART I

GENERAL PROVISIONS AND DEFINITIONS

1. The prescribed amount of animal feeding stuff for the purposes of the definition of sampled portion in regulation 1(2) of these regulations shall be determined in accordance with the following provisions:—

(i) In relation to solid feeding stuff in packages, the prescribed amount shall be the quantity of feeding stuff present or 5 tonnes, whichever is the less.

(ii) In relation to solid feeding stuff which is packed in bulk containers—

(a) if any of those containers holds not less than 5 tonnes of feeding stuff, the prescribed amount shall be the contents of any such container;

(b) if all the containers together hold not less than 5 tonnes of feeding stuff and every container holds less than 5 tonnes, the prescribed amount shall be the contents of the lowest number of containers which together holds not less than 5 tonnes;

(c) if all the containers together hold less than 5 tonnes of feeding stuff or if all the feeding stuff is in one container, the prescribed amount shall be the quantity of feeding stuff present.

(iii) In relation to solid feeding stuff which is loose in heaps or bays—

(a) if the feeding stuff is in more than one heap or bay, any of which contains not less than 5 tonnes of feeding stuff, the prescribed amount shall be the contents of any heap or bay containing not less than 5 tonnes;

(b) if all the heaps or bays together contain not less than 5 tonnes of feeding stuff and every heap or bay contains less than 5 tonnes, the prescribed amount shall be the contents of the lowest number of heaps or bays which together contain not less than 5 tonnes;

(c) if all the heaps or bays together contain less than 5 tonnes or if all the feeding stuff is in one heap or bay, the prescribed amount shall be the quantity of feeding stuff present.

(iv) In relation to liquid feeding stuff in containers—

(a) if any of those containers holds not less than 5,000 litres of feeding stuff the prescribed amount shall be the contents of any such container;

(b) if all the containers together hold not less than 5,000 litres of feeding stuff and every container holds less than 5,000 litres, the prescribed amount shall be the contents of the lowest number of containers which together hold not less than 5,000 litres;

(c) if all the containers together hold less than 5,000 litres of feeding stuff the prescribed amount shall be the quantity of feeding stuff present.

2. In the case of feeding stuff to which paragraphs 1(i) and (iv) of this Part of this Schedule apply, only unopened packages or containers which appear to the inspector proposing to take the sample to be the original packages or containers of the feeding stuff shall be selected for the purpose of the sample.

3. An inspector who proposes to take a sample under section 112 of the Act on premises on which he has reasonable cause to believe that there is any feeding stuff which the occupier of the premises has purchased, not for the purpose of re-sale in the course of trade but for the purpose of use as a feeding stuff, shall satisfy himself that the conditions in which the feeding stuff is stored are not such as might cause deterioration of the feeding stuff and that the feeding stuff appears not to have been contaminated by any other material.

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 feeding stuff shall not be exposed any longer than is necessary.

5. Where a sample has been taken from a feeding stuff in a container which has been opened for the purpose of sampling at a place other than the premises

on which the feeding stuff was packed the inspector shall affix to the container a label stating, or otherwise mark the container to the effect, that a portion of the feeding stuff has been withdrawn for the purpose of sampling and the inspector shall, if so required by the person in charge of the container, close and refasten it in an appropriate manner.

6. In this Schedule:—

"incremental sample" means a quantity taken from one point in a sampled portion;

"aggregate sample" means an aggregate of incremental samples taken from the same sampled portion;

"reduced sample" means a representative part of the aggregate sample obtained from the latter by a process of reduction; and

"final sample" means a representative part of the reduced sample or, where no intermediate reduction is required, of the aggregate sample.

PART II

GENERAL INSTRUCTIONS FOR THE TAKING OF SAMPLES

1. In the case of feeding stuff 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 feeding stuff shall be selected for the purpose of the sample.

2. The sample shall be taken and prepared as quickly as possible having regard to the precautions necessary to ensure that it remains representative of the sampled portion. Instruments, surfaces and containers used in sampling shall be clean and dry.

3. No sample shall be drawn from any part of the sampled portion which appears to be damaged.

4. Where any appreciable portion of the feeding stuff appears to be mouldy, or is otherwise apparently unsuitable for feeding purposes, separate samples shall be drawn of the unsuitable portion and of the residue of the feeding stuff respectively. These shall be treated as separated sampled portions.

5. An inspector who intends to take a sample on premises on which he has reasonable cause to believe that there is any feeding stuff which the occupier of the premises has purchased, shall:—

(a) satisfy himself that the conditions in which the feeding stuff is stored are not such as might cause undue deterioration of the said feeding stuff, and that the feeding stuff appears not to have been contaminated by any other material;

(b) where he has reasonable cause to believe that feeding stuff in packages or containers is only part of an original consignment, select the number of

packages or containers to be sampled as if not less than the whole consignment were still present, except that sampling shall not take place if fewer than the minimum number of packages or containers prescribed in sub-paragraph 2(b)(i) of Part III of this Schedule are present. The provisions of this paragraph shall not apply as respects any feeding stuff purchased for the purpose of resale in the course of trade.

6. The sampling apparatus shall be made of materials which cannot contaminate the feeding stuffs to be sampled.

7. In the case of a sampling spear its dimensions shall be appropriate to the characteristics of the sampled portion in all respects including dimensions of the container and particle size of the feeding stuff.

8. Notwithstanding anything in this Schedule a sampling spear shall not be used if objection is raised thereto, prior to the taking of the sample, on the ground that the feeding stuff is unsuitable.

9. Mechanical apparatus may be used for the sampling of moving feeding stuffs, provided that such equipment is capable of taking samples right across the flow of the product.

10. Apparatus designed to divide the sample into approximately equal parts may be used for taking incremental samples and for the preparation of reduced and final samples.

11. A sample taken according to the methods described below shall be considered as representative of the sampled portion.

PART III

QUANTITATIVE REQUIREMENTS

1. Sampled Portion

The sampled portion in compliance with regulation 1 shall be such that each of its constituent parts can be sampled in accordance with the requirements of this Schedule.

2. Incremental Sample

(a) in the case of feed blocks or mineral licks, a number of blocks or licks shall be selected in accordance with the following table:—

Number of blocks or mineral licks in the sampled portion	*Number to be selected for sampling*
1 to 25	not less than 1
26 to 50	not less than 2
51 to 75	not less than 3
76 or more	not less than 4

(b) in respect of any feeding stuffs other than those mentioned in sub-paragraph *(a)* above:—

(i) in the case of feeding stuffs in packages or containers, the number of packages or containers shall be selected in accordance with table 1 of Part VI of this Schedule, except that—

(a) the number of packages or containers need not exceed 20;

(b) where the contents of the package or container do not exceed 1 kg or 1 litre, the number of packages or containers selected shall be not less than 4;

(ii) in the case of loose feeding stuffs the number of incremental samples shall be selected in accordance with table 2 of Part VIII of this Schedule.

3. Aggregate Sample

(a) In respect of any feeding stuffs a single aggregate sample per sampled portion is required. The weight or volume, as appropriate, of the aggregate sample shall be not less than the following:—

(i)	packaged feeding stuffs	
	(a) packages of more than 1 kg	4 kg
	(b) packages not exceeding 1 kg	2 kg
(ii)	loose feeding stuffs	4 kg
(iii)	liquid or semi-liquid feeding stuffs	
	(a) containers of more than 1 litre	4 litres
	(b) containers not exceeding 1 litre	2 litres
(iv)	feed blocks or mineral licks	
	(a) feed block or mineral lick of more than 1 kg	4 kg
	(b) feed block or mineral lick not exceeding 1 kg	2 kg

4. Final Sample

The amounts of material in each final sample shall be not less than 500g in the case of solid feeding stuffs or 500ml in the case of liquid or semi-liquid feeding stuffs.

PART IV

TAKING AND PREPARATION OF SAMPLES

1. Incremental samples

Incremental samples of approximately equal sizes shall be taken at random throughout the whole sampled portion in the following manner:—

(a) in the case of packaged feeding stuffs to which paragraph 2(b)(i) of Part III of this Schedule applies—

(i) the required number of packages having been selected part of the content of each selected package shall be taken as the incremental sample;

(ii) where necessary each selected package shall be emptied and worked up with a shovel separately and one shovelful taken as the incremental sample;

(iii) when the material is of a suitable nature the incremental sample may be taken from each selected package by means of a sampling spear or by divider;

(iv) where the feeding stuff is in the state of small lumps or meal, the sample shall be taken in accordance with paragraph (ii) or (iii) of this sub-paragraph;

(v) where the feeding stuff consists of particles of grossly differing sizes the incremental sample shall be taken in accordance with paragraph (ii) of this sub-paragraph. Any lumps shall be crushed (and for this purpose may be separated from other material) and the whole then thoroughly remixed;

(vi) when the material is so packed or of such a nature that a shovel or spear or divider cannot be used, or where the content of the package does not exceed 1 kg, the whole package shall be taken as the incremental sample.

(b) in the case of loose feeding stuffs—

(i) an imaginary division shall be made of the sampled portion into a number of approximately equal parts, corresponding to the number of incremental samples required in accordance with paragraph 2*(b)*(ii) of Part III of this Schedule, and an equal number of incremental samples shall be taken at random from each of these parts;

(ii) when sampling is being carried out while the material comprising the sampled portion is in motion, the incremental samples shall be taken from the randomly selected parts as required in paragraph (i) of this sub-paragraph;

(iii) when a sampling spear is used the sample shall be taken in an angle to the base of the heap;

(iv) where the feeding stuff consists of particles of grossly differing sizes the incremental samples shall be taken and treated in accordance with the relevant provisions of paragraph 1*(a)*(v).

(c) in the case of feed blocks and mineral licks the number of blocks or licks to be selected shall be taken in accordance with sub-paragraph 2*(a)* of Part III of this Schedule. Where the block or lick does not weigh more

than 1 kg, the whole block or lick shall be taken as the incremental sample; otherwise, sufficient shall be taken in any manner to give the aggregate sample, approximately equal portions being taken from each block;

(d) in the case of liquid or semi-liquid feeding stuff in containers the number of containers to be selected shall be taken in accordance with paragraph 2*(b)*(i) of Part III of this Schedule, and

(i) where the containers each contain not more than 1 litre the entire contents of the selected containers shall be transferred into a clean dry vessel of suitable material;

(ii) where the containers each contain more than 1 litre and not more than 200 litres—

(a) if the liquid is homogenisable the selected containers shall be well shaken or the contents agitated or otherwise treated to ensure uniformity. An approximately equal proportion of fluid shall then be taken immediately from each of the selected containers and transferred into a clean dry vessel of suitable material; but

(b) if the liquid is non-homogenisable or if the inspector considers that the procedure in sub-paragraph *(a)* may not be appropriate, the contents shall be sampled by lowering an open tube (which must be long enough to reach the bottom of the container) perpendicularly, into the container. One or both ends of the tube shall be closed and the contents transferred into a clean dry vessel of suitable material. If sampling by tube is impracticable, portions shall be taken from various levels of the container with a sampling bottle so as to obtain a quantity fairly representative of the whole. An approximately equal proportion of fluid shall be taken from each container. The appropriate process shall be repeated until a total quantity of not less than 10 litres has been withdrawn;

(iii) in the case of liquid or semi-liquid feeding stuff in containers each containing more than 200 litres—

(a) when a consignment is being withdrawn from the container and there is a tap in the outlet pipe from which it is suitable to draw a sample, a quantity of not less than 4 litres shall be drawn from the tap (after first withdrawing sufficient to remove any residues in the pipe) into a clean dry vessel of suitable material, made up of portions of not less than 0·5 litre and of approximately equal size taken at regular intervals; otherwise

(b) if the liquid is homogeneous, not less than 4 litres shall be drawn from a convenient outlet in the container (after first withdrawing sufficient to remove any residues in the outlet) into a clean dry vessel of suitable material; or

(c) if the liquid is homogenisable it shall be well stirred or otherwise agitated; sampling shall then proceed as in sub-paragraph *(b)*; but

(d) if the liquid is non-homogenisable or if the inspector considers that the procedure in sub-paragraphs *(a)*, *(b)* and *(c)* may not be appropriate, the contents shall be sampled as in sub-

paragraph (ii)*(b)*. The appropriate process shall be repeated until a total quantity of not less than 10 litres has been withdrawn;

(e) where a sampled portion consists of two or more containers, incremental samples of approximately equal size shall be taken from each, drawn in the manner described in sub-paragraphs *(a), (b), (c)* or *(d),* as appropriate, until a quantity of not less than 10 litres has been withdrawn.

2. Aggregate sample

The incremental samples shall be thoroughly mixed to form a single aggregate sample. In the case of solid feeding stuff, the material in the aggregate sample shall be carefully mixed to obtain an homogenised sample. Any lumps inconsistent with the nature of the material shall be broken up (if need be by separating them out and returning them to the aggregate sample). In the case of feed blocks and mineral licks not exceeding 1 kg the incremental samples shall be crushed or otherwise broken up and then thoroughly mixed to form a single aggregate sample.

3. Reduced sample

(a) In the case of solid feeding stuff the aggregate sample shall, if necessary, be reduced to not less than 2 kg in the following manner:—

(i) the material shall be heaped to form a "cone" which shall then be flattened and quartered. Two diagonally opposite quarters shall be rejected, and the remainder shall then be mixed and the quartering and rejection continued as necessary or

(ii) a reduction method effected by the use of a mechanical device;

(b) in the case of liquid or semi-liquid feeding stuff:—

(i) where the aggregate sample consists of approximately 2 litres, it shall be taken as the final sample;

(ii) in all other cases, the aggregate sample shall be thoroughly mixed and a quantity of at least 2 litres transferred immediately into a clean dry vessel of suitable material;

(c) in the case of feed blocks and mineral licks, the aggregate sample shall if necessary, be reduced to not less than 2 kg in the manner described in sub-paragraph *(a).*

4. Final samples

The final samples shall be obtained in the following manner:—

(a) in the case of solid feeding stuff, including feed blocks and mineral licks, the reduced sample, or where necessary the aggregate sample, shall be thoroughly mixed and divided into four similar and approximately equal parts and each part placed in an appropriate airtight container;

(b) in the case of liquid or semi-liquid feeding stuff, the reduced sample, or where necessary the aggregate sample, shall be thoroughly mixed and at once divided into four similar and approximately equal parts by pouring successive portions into appropriate airtight containers.

(c) Where a sample consists of animal feeding stuffs enclosed in unopened containers, and it appears to the inspector that to open the containers and divide their contents into parts is not reasonably practicable, or may affect the composition, or impede the proper analysis or other appropriate examination of the contents the inspector may divide the containers into four lots without opening them. Each of the said lots shall be regarded as a part of the sample and shall be placed in a bag made of plastic or other suitable material. The bag shall then be secured and sealed in such a manner that the part of the sample cannot be removed without breaking the seal or the bag.

The containers used shall be such that the characteristics of the feeding stuff at the time of sampling are preserved.

PART V

MARKING, SEALING AND FASTENING OF THE FINAL SAMPLE

1. Each container of a final sample shall be so secured and sealed by the person taking the sample that the container cannot be opened without breaking the seal; alternatively the container may be placed in a stout envelope or in a linen, cotton or plastic bag, and this further receptacle then secured and sealed in such a manner that the contents cannot be removed without breaking the seal or the receptacle.

2. A label shall be attached to the container or receptacle containing the final sample and sealed in such a manner that it cannot be removed without the seal being broken. The label shall be marked with the following particulars, which shall be visible without the seal being broken:—

(a) name of the inspector and the authority to which he belongs;

(b) identification mark given by the inspector to the sample;

(c) place of sampling;

(d) date of sampling;

(e) name of the material; and

(f) identification code, batch reference number or consignment identification of the material sampled, where readily available.

3. The container or receptacle may also be sealed, or the label also signed or initialled, by the holder of the material sampled or person acting on his behalf.

PART VI

DISPOSAL OF PARTS OF SAMPLE

1. The inspector shall dispose of the four parts of any sample by:

(a) supplying one part to the agricultural analyst for the inspector's area;

(b) retaining for 15 months one part for use, if required, by the Government Chemist;

(c) in the case of a sample taken from an animal feeding stuff in transit from outside the United Kingdom, supplying one part of the sample to the consignee, or in the case of a sample taken from an animal feeding stuff in transit within the United Kingdom, supplying one part of the sample to the consignor;

(d) in the case of a sample taken from an animal feeding stuff which is not in transit, supplying one part of the sample to the person on whose premises the sample was taken;

(e) in any case in which the enforcement authority contemplates bringing proceedings against any person for an offence against the Act, supplying one part of the sample to the person against whom proceedings are contemplated (unless he has already received a part under the provisions of sub-paragraph *(c)* or *(d)* of this paragraph) and pending disposal (if required) in accordance with this paragraph retaining such part for 15 months.

2. In every case the inspector shall inform the person to whom the part of the sample is supplied that the sample has been obtained for the purpose of analysis or other appropriate examination.

3. In any case in which a part of a sample is supplied to any person pursuant to the provisions of paragraph 1*(e)* above, the inspector shall when sending it—

(a) state that the sample has been obtained by him; and

(b) specify the person from whom and the place from which he obtained it.

4. Section 127 of the Act shall have effect in relation to supplying any part of a sample in pursuance of the preceding paragraphs as it has effect in relation to the service of a document.

5. If after reasonable inquiry the inspector is unable to ascertain the name of a person to whom, or the address at which, a part of a sample ought to be supplied in pursuance of the preceding provisions of this part of this Schedule, he may retain that part of the sample instead of supplying it.

Analysis or examination of sample

6. An inspector who submits a sample for analysis to the agricultural analyst for the inspector's area shall supply with the part of the sample—

(a) a copy of the label or leaflet relating to the feeding stuff from which the sample was taken, or where this is not reasonably practicable, a copy of each of the particulars as may be necessary for the purposes of analysis by the agricultural analyst as may appear on the label or leaflet; and

(b) a statement signed by the inspector that the sample was taken or otherwise obtained in the manner prescribed by these Regulations, and, where he requires an analysis to be carried out in respect of some only of the medicinal ingredients stated on the label or leaflet to be present or of any other medicinal ingredient which may be present, he shall also supply instructions appropriate to such an analysis.

7.—(1) Subject to the following sub-paragraphs the agricultural analyst shall analyse the sample, or cause the sample to be analysed by some other person under his direction, as soon as practicable.

(2) If the agricultural analyst determines that for any reason an effective analysis of the sample cannot be performed by him or under his direction, he shall send it to the agricultural analyst for some other area together with any documents received by him with the sample, and that other agricultural analyst shall as soon as practicable analyse the sample or cause it to be analysed by some other person under his direction.

(3) If the agricultural analyst determines that for any reason it is necessary to carry out an analysis or other appropriate examination which he is not in a position to undertake he shall submit the sample for analysis or for such examination to the person having the management or control of any laboratory available for the purpose in accordance with any arrangements made in that behalf by the relevant enforcement authority.

(4) If the agricultural analyst determines that for any reason he is not in a position to undertake the whole of an analysis he shall submit a suitable proportion of the sample for the appropriate part of the analysis to the person referred to in sub-paragraph (3) of this paragraph.

(5) Before any sample which has been unsealed by an agricultural analyst is sent to another agricultural analyst or to a laboratory under the provisions of sub-paragraph (2), (3) or (4) of this paragraph it shall be resealed and initialled by the agricultural analyst so sending it.

8.—(1) An agricultural analyst who has analysed a sample submitted to him under the preceding provisions of this part of this Schedule, or who has caused such a sample to be analysed by some other person under his direction, shall issue and send a certificate of analysis in the form prescribed by these regulations to the inspector, who shall send a copy of it to any person to whom a part of the sample has been supplied in accordance with paragraph 1 of this part of this Schedule.

(2) A person having the management or control of a laboratory in which a sample submitted to him under the preceding provisions of this part of this Schedule has been analysed or examined, or a person appointed by him for the purpose, shall issue and send a certificate of such analysis or examination in the form prescribed by these regulations to the inspector, who shall send a copy of it to any person to whom a part of the sample has been supplied under paragraph 1 of this part of this Schedule.

(3) A person having the management or control of a laboratory in which a proportion only of the sample submitted to him by an agricultural analyst has been analysed, or a person appointed by him for the purpose, shall issue and send a certificate of such analysis in the form prescribed by these regulations to the agricultural analyst, who shall send it, together with his own certificate of analysis in the form prescribed by these regulations, to the inspector; and the inspector shall send a copy of each certificate to any person to whom a part of the sample has been supplied under paragraph 1 of this part of this Schedule.

9. Any certificate issued under the last preceding paragraph shall be signed by the person who issues the certificate.

Provisions as to evidence

10. In any proceedings for an offence under the Act a document produced by one of the parties to the proceedings and purporting to be a certificate issued under paragraph 8 of this part of this Schedule or a certificate of the Government Chemist, shall be sufficient evidence of the facts stated in the document, unless the other party requires that the person who issued the certificate shall be called as a witness; and, in any proceedings in Scotland, if that person is called as a witness, his evidence shall be sufficient evidence of those facts.

11. In any proceedings for an offence under the Act a document produced by one of the parties to the proceedings, which has been supplied to him by the other party as being a copy of such a certificate, shall be sufficient evidence of the facts stated in the document.

Proof by written statement

12. In relation to England and Wales section 9 of the Criminal Justice Act 1967, and in relation to Northern Ireland any corresponding enactment of the Parliament of Northern Ireland or measure of the Northern Ireland Assembly shall not have effect with respect to any document produced as mentioned in paragraph 10 or paragraph 11 of this part of this Schedule or with respect to any certificate transmitted to a court under section 115(1J)*(b)* of the Act.

Payment for sample taken under compulsory powers

13. Where for the purpose of taking a sample of any animal feeding stuff in the exercise of any powers conferred by section 112 of the Act an inspector takes some of it from any parcel of the animal feeding stuff exposed for sale by retail which does not weigh more than fourteen pounds the owners of any such parcel may require the inspector to purchase it on behalf of the authority for whom he acts.

Application of section 64 *of the Act to samples*

14. Where an animal feeding stuff is taken as a sample by an inspector in the exercise of any power conferred by section 112 of the Act, the provisions of subsections (1) to (4) of section 64 of the Act shall have effect as if the taking of the animal feeding stuff as a sample were a sale of it to the inspector by the person from whom it is taken; and, if the animal feeding stuff was prepared in pursuance of a prescription given by a veterinary surgeon or veterinary practitioner, those provisions shall so have effect as if, in subsection (1) of that section, for the words "demanded by the purchaser", there were substituted the words "specified in the prescription".

Application to Northern Ireland

15. In the application of this part of this Schedule to Northern Ireland—

(a) for any reference to the agricultural analyst for the inspector's area there shall be substituted a reference to an agricultural analyst in Northern

Ireland, and the expression "agricultural analyst" shall not include the Chief Agricultural Analyst for Northern Ireland;

(b) for any reference to the Government Chemist there shall be substituted a reference to the Chief Agricultural Analyst for Northern Ireland.

PART VII

MODIFICATIONS TO THE FOREGOING PROVISIONS OF THIS SCHEDULE PURSUANT TO REGULATION 3(2)

The following modifications to the foregoing provisions of this Schedule shall be applicable in the case of any sample set aside from a substance or article seized in pursuance of section 112(4) of the Act, or in the case of a substance or article so seized which is itself treated as a sample—

(a) references to a feeding stuff shall be construed as references to any substance or article seized by an inspector in pursuance of section 112(4) of the Act, and the reference in paragraph 1 of Part IV to the taking of a sample in the manner prescribed by this Schedule shall be construed as a reference to the setting aside of a sample in that manner or to the treating of a substance or article as a sample pursuant to section 112(4), as the context requires;

(b) paragraphs 1, 3 and 5 of Part II of this Schedule shall not apply to a sample set aside from a substance or article seized as aforesaid;

(c) Parts I, II and III of this Schedule shall not apply in the case of a substance or article treated as a sample as aforesaid;

(d) in Part VI of this Schedule, the following sub-paragraph shall be applicable in place of sub-paragraphs *(c)* and *(d)* of paragraph 1:—

"supplying one part of the sample to the person who made the request under section 113(2) of the Act for such sample to be set aside or for such substance or article to be treated as a sample;";

and sub-paragraph *(c)* of paragraph 1 shall be read as if for the reference to sub-paragraphs *(c)* and *(d)* of paragraph 1 aforesaid there were substituted a reference to the foregoing sub-paragraph applicable in place of the said sub-paragraphs *(c)* and *(d)*;

(e) paragraphs 5, 13 and 14 of Part VI of this Schedule shall not apply.

PART VIII

SAMPLING TABLES

TABLE 1

PACKAGED FEEDING STUFFS

Number of packages or containers in the sampled portion	*Number of packages or containers to be selected*
1 to 4	All packages or containers
5 to 16	not less than 4
17 to 25	,, 5
26 to 36	,, 6
37 to 49	,, 7
50 to 64	,, 8
65 to 81	,, 9
82 to 100	,, 10
101 to 121	,, 11
122 to 144	,, 12
145 to 169	,, 13
170 to 196	,, 14
197 to 225	,, 15
226 to 256	,, 16
257 to 289	,, 17
290 to 324	,, 18
325 to 361	,, 19
362 and above	,, 20

TABLE 2

LOOSE FEEDING STUFFS

Size of sampled portion in tonnes				*Number of incremental samples required*	
Up to and including 2·5				Not less than	7
Greater than 2·5 and up to and including 3				,,	8
,, 3	,,	4		,,	9
,, 4	,,	5		,,	10
,, 5	,,	6		,,	11
,, 6	,,	7		,,	12
,, 7	,,	8		,,	13
,, 8	,,	9		,,	14
,, 9	,,	11		,,	15
,, 11	,,	12		,,	16
,, 12	,,	14		,,	17
,, 14	,,	16		,,	18
,, 16	,,	18		,,	19
,, 18				,,	20

SCHEDULE 3

METHODS OF ANALYSIS

(Section 117(3) and Regulation 4)

The main divisions in this Schedule are as follows:—

1. Introduction
2. Preparation of the sample for analysis
3. Detection and identification of antibiotics of the tetracycline group
4. Determination of acinitrazole
5. Determination of amprolium
6. Determination of avoparcin
7. Determination of buquinolate
8. Determination of chlortetracycline, oxytetracycline and tetracycline by diffusion through agar
9. Determination of chlortetracycline, oxytetracycline and tetracycline by turbidimetry
10. Determination of clopidol
11. Determination of copper by the diethyldithiocarbamate spectrophotometric method
12. Determination of copper by the atomic absorption spectrophotometric method
13. Determination of dinitolmide
14. Determination of ethopabate
15. Determination of flavophospholipol (flavomycin) (bambermycin)
16. Determination of furazolidone
17. Determination of monensin sodium
18. Determination of nicarbazin
19. Determination of nifursol
20. Determination of nitrofurazone
21. Determination of nitrovin
22. Determination of nitrovin in feed supplements
23. Determination of oleandomycin
24. Determination of sulphaquinoxaline
25. Determination of tylosin
26. Determination of virginiamycin
27. Determination of zinc bacitracin

1. INTRODUCTION

(1) *General*

When two or more methods are prescribed in this Schedule to determine a medicinal 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, culture media and apparatus*

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

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

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

(d) Aluminium oxide for chromatography must be neutral unless otherwise prescribed.

(e) Any commercial culture media of similar composition and giving the same results as those prescribed, may be used.

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

(3) *Report on the results*

The results given in the certificates of analysis shall be the average value obtained on the basis of at least two complete tests. Except for special circumstances it shall be expressed as a proportion by weight of the original sample as it reached the laboratory. The result must not include more significant figures than the accuracy of the method of analysis allows.

2. PREPARATION OF THE 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 a nominal aperture size of 1 mm square[1][2], mix thoroughly and transfer a portion of not less than 100g to a non-corrodible container provided with an air-tight closure.

(3) If the sample does not wholly pass through a sieve having a nominal aperture size of 1 mm square[1][2], and wholly passes through a sieve having apertures from 2 mm to 3 mm square[3][2], mix thoroughly and further grind a portion of not less than 100g to pass through a sieve having a nominal aperture

[1] Test sieves conforming to British Standard 410: 1969 are suitable.
[2] Where an analysis for copper has to be carried out, a stainless steel sieve should be used.
[3] Test sieves of nominal aperture sizes of 2·00, 2·36 or 2·80mm conforming to British Standard 410: 1969 are suitable.

size of 1 mm square[1][2]. Transfer the portion so prepared to a non-corrodible container provided with an air-tight closure.

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

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

3. DETECTION AND IDENTIFICATION OF ANTIBIOTICS OF THE TETRACYCLINE GROUP

1. SCOPE AND FIELD OF APPLICATION

The method is for the detection and identification of antibiotics of the tetracycline group in complete feeding stuffs, protein concentrates and feed supplements containing at least 0·1 mg/kg.

2. PRINCIPLE

The sample is extracted with acidified methanol, and the extract examined by ascending paper chromatography with standard tetracycline antibiotics for comparison. The antibiotics are detected and identified by comparison of their Rf values with those of standard substances, either by fluorescence in UV light (high levels of antibiotics), or by bio-autography on an agar medium inoculated with *Bacillus cereus.*

3. REAGENTS AND CULTURE MEDIUM

3.1 Buffer solution pH 3·5:

Citric acid monohydrate	10·256g
di Sodium hydrogen phosphate $Na_2HPO_42H_2O$	7·45g
Acetone	300ml
Water to	1000ml

3.2 Phosphate buffer solution pH 5·5:

Potassium dihydrogen phosphate KH_2PO_4	130·86g
di Sodium hydrogen phosphate $Na_2HPO_42H_2O$	6·947g
Water to	1000ml

3.3 Eluting solvent I: A mixture of nitromethane, chloroform and 1,3-dichloro-propan-2-ol, in the proportions by volume: 20 + 10 + 1·5. Prepare immediately before use.

3.4 Eluting solvent II: A mixture of nitromethane, chloroform and

[1] Test sieves conforming to British Standard 410: 1969 are suitable.
[2] Where an analysis for copper has to be carried out, a stainless steel sieve should be used.
[3] Test sieves of nominal aperture sizes of 2·00, 2·36 or 2·80mm conforming to British Standard 410: 1969 are suitable.

2-picoline, in the proportions by volume: 20 + 10 + 3. Prepare immediately before use.

3.5 Acidified methanol: mix methanol and hydrochloric acid (density 1·18g per ml) in the proportions by volume: 98 + 2.

3.6 Hydrochloric acid solution 0·1M.

3.7 Ammonia, (density 0·88g per ml).

3.8 Standard substances: chlortetracycline and tetracycline, the activities of which are expressed in terms of the hydrochlorides; oxytetracycline, the activity of which is expressed in terms of the free base.

3.8.1 Standard solutions: dissolve suitable quantities of the standard substances (3.8) in hydrochloric acid (3.6) to give solutions containing respectively 500μg per ml of chlortetracycline hydrochloride, tetracycline hydrochloride and oxytetracycline base.

3.8.2 Reference solutions for detection by UV: dilute the standard solutions (3.8.1) with phosphate buffer solution (3.2) to obtain solutions containing 100μg per ml of the antibiotics expressed as in 3.8.1

3.8.3 Reference solutions for detection by bio-autography: dilute the standard solutions (3.8.1) with phosphate buffer solution (3.2) to obtain solutions containing 5μg per ml of the antibiotics expressed as in 3.8.1.

3.9 MICRO-ORGANISM: *Bacillus cereus* ATCC 11778 (NCIB 8849; NCTC 10320; NCDO 720)

3.9.1 Maintenance of the parent strain:

Proceed as described in Division 8, Determination of Chlortetracycline, Oxytetracycline and Tetracycline by Diffusion Through Agar, Sub-Division 3.1

3.9.2 Preparation of the spore suspension:

Proceed as described in Division 8, Determination of Chlortetracycline, Oxytetracycline and Tetracycline by Diffusion Through Agar, Sub-Division 3.2.1.

3.10 Culture medium:

Glucose	1g
Tryptic peptone	10g
Meat extract	1·5g
Yeast extract	3g
Agar	20g
Water	1000ml

pH after sterilization: 5·8

3.11 2,3,5-triphenyltetrazolium chloride solution: dissolve 0·1g 2,3,5-triphenyltetrazolium chloride and 5g glucose in water and dilute to 100ml.

4. APPARATUS

4.1 Apparatus for ascending paper chromatography (height of paper: 25cm). Schleicher and Schüll paper 2040b or 2043b, or equivalent.

4.2 UV lamp for the detection of fluorescence, 350nm.

4.3 Bio-assay plates approximately 20 × 30cm.

5. PROCEDURE

5.1 *Extraction*

5.1.1 Extract, by shaking for a few minutes, the finely divided and mixed sample with acidified methanol (3.5), in suitable proportions to produce a solution containing approximately 100μg per ml of the antibiotic. Centrifuge the mixture, and retain the clear supernatant.

5.1.2 Dilute the extract from 5.1.1 with acidified methanol (3.5) to give an antibiotic concentration of approximately 5μg per ml.

5.2 *Chromatography*

Immerse the paper in the buffer solution pH 3·5 (3.1) and eliminate the excess liquid by compressing the paper between leaves of dry filter paper. Place volumes of 0·01ml reference solutions (3.8.2 and 3.8.3) and extract (5.1.1 and 5.1.2) on the paper. Adequate separation depends upon the paper having an optimum water content.

Develop by ascending chromatography, use eluting solvent I (3.3) for detection by bio-autography, and eluting solvent II (3.4) for detection by UV light. When the solvent front reaches a height of 15 to 20cm (approximately $1\frac{1}{2}$ hours), remove the paper and dry.

5.3 *Detection by UV light*

For antibiotic concentrations greater than 1μg per ml. Expose the paper to ammonia vapour (3.7) and then examine under the UV lamp (4.2). Golden yellow fluorescent spots indicate the presence of antibiotics of the tetracycline group (See para. 6).

5.4 *Detection by bio-autography*

Pour the culture medium (3.10), previously inoculated with *B. cereus* spore suspension (3.9) into a sterile antibiotic assay plate (4.3) and allow to set. Place the paper on the culture medium and allow to remain in contact for 5 minutes, then transfer it to a fresh position on the culture medium, for the duration of incubation period. Incubate at 30°C overnight. The presence of an antibiotic of the tetracycline group is shown by clear zones of inhibition in the cloudy culture medium. To enhance the contrast between the zones, spray the plate with the 2,3,5-triphenyltetrazolium solution (3.11).

6. IDENTIFICATION

The relative Rf values of antibiotics of the tetracycline group are given as follows. These values may vary slightly according to the quality of the paper and its level of humidity:

Chlortetracycline	0·60
Tetracycline	0·40
Oxytetracycline	0·20
4-epi-Chlortetracycline	0·15
4-epi-Tetracycline	0·13
4-epi-Oxytetracycline	0·10

The "epi" compounds have an antibiotic activity lower than that of normal compounds.

4. DETERMINATION OF ACINITRAZOLE

(2-acetamido-5-nitrothiazole)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of acinitrazole in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 35mg/kg.

2. PRINCIPLE

The sample is extracted with hot dimethylformamide. The extract is purified on a column of aluminium oxide, the acinitrazole is eluted with acidified methanol and the elute treated with sodium hydroxide forming a yellow colour, the absorbance of which is measured at 410nm.

3. REAGENTS

3.1 Dimethylformamide.

3.2 Methanol.

3.3 Hydrochloric acid solution, 5M.

3.4 Acidified methanol: add 2ml of 5M hydrochloric acid (3.3) to 100ml of methanol (3.2).

3.5 Sodium hydroxide, alcoholic solution: dilute 2·5ml of 10M sodium hydroxide to 100ml with ethanol; stand for two days to allow carbonates to settle.

3.6 Aluminium oxide for column chromatography: transfer 100g aluminium oxide to a suitable container, add 250ml of hydrochloric acid solution (1ml of hydrochloric acid (density 1·18g per ml) diluted to 100ml with water) and stir mechanically for fifteen minutes. Collect the slurry on filter paper in a Buchner funnel. Wash the aluminium oxide on the filter-paper with ten 50ml portions of water and dry by suction. Dry it for at least four hours at 100°C.

3.7 Acinitrazole standard solution: weight to the nearest 0·1mg, 100mg of acinitrazole (B.Vet.C.grade), dissolve in dimethylformamide (3.1) and dilute to 100ml with dimethylformamide. Dilute 5ml of this solution to 200ml with dimethylformamide. 1ml of this solution contains 25μg acinitrazole.

4. APPARATUS

4.1 Spectrophotometer with 10mm cells.

4.2 Chromatography columns: glass tubes, internal diameter: 9mm; length: 400 to 500mm, with openings 4 to 5mm diameter at the lower ends. Insert small plugs of glass wool in the lower ends of the tubes and

compress the plugs firmly with a glass rod so that a thickness of 2 to 3mm is obtained.

5. PROCEDURE

5.1 *Extraction*

Weigh to the nearest 0·001g, approximately 20g of the finely divided and mixed sample or a suitable amount expected to contain about 6mg of acinitrazole and transfer it into a 250ml beaker. Add 60ml of boiling dimethylformamide (3.1), boil for two minutes stirring continuously and then cool to room temperature. Filter the liquid through a sintered-glass funnel (porosity C3) under gentle suction. Repeat the extraction with 60ml of boiling dimethylformamide and filter through the funnel. Rinse the beaker with two 30ml portions of cold dimethylformamide and filter through the funnel. Cool the filtrate to room temperature, transfer to a 200ml graduated flask and dilute to volume with dimethylformamide.

5.2 *Purification*

Prepare a slurry of aluminium oxide (3.6) by mixing three volumes of dimethylformamide (3.1) with one volume of aluminium oxide. Heat the slurry on a hotplate (do not boil) and then cool to room temperature. Pour the slurry into glass columns (4.2) and allow it to settle to a height of approximately 270mm. Prepare a separate column for each sample. Run a 10ml aliquot of the dimethylformamide extract of the sample onto the top of the column and allow the liquid to pass through under gravity. Wash with three successive 10ml portions of dimethylformamide ensuring that the surface of the aluminium oxide is covered throughout. Discard all the dimethylformamide eluates.

Run four successive 10ml portions of acidified methanol (3.4) through the column into a 50ml graduated flask ensuring that the whole of the yellow band of acinitrazole is eluted. Leave about 2ml of the last acidified methanol portion on the top of the column. Add 0·5ml of 5N hydrochloric acid (3.3) to the flask and mix. Dilute to the mark with methanol (3.2).

5.3 *Determination*

Pipette two 20ml aliquots of the sample extract solution (5.2) into each of two 25ml graduated flasks. Dilute the first (A) to the mark with methanol (3.2) and the second (B) with alcoholic sodium hydroxide (3.5). Prepare a reagent blank by adding 5ml of alcoholic sodium hydroxide to a third 25ml graduated flask and diluting to the mark with methanol (3.2). Measure the absorbances of the solutions A and B at 410nm in 10mm cells with the reagent blank as reference. Read the absorbances immediately after the solutions have been prepared.

5.4 *Standard Test*

Repeat the entire procedure on a 10ml aliquot of the standard solution (3.7) and a new column of aluminium oxide, commencing at (5.2), "Run a 10ml aliquot of the dimethylformamide . . ."

6. CALCULATION OF RESULTS

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

$$\frac{(E_A - E_B) \times S \times 200}{(E'_A - E'_B) \times W \times 10}$$

in which:

E_A = absorbance of methanolic aliquot from sample;
E_B = „ „ alcoholic NaOH aliquot from sample;
E'_A = „ „ methanolic aliquot from standard;
E'_B = „ „ alcoholic NaOH aliquot from standard;
S = weight of acinitrazole standard run onto column (μg); and
W = weight of test portion in g.

5. DETERMINATION OF AMPROLIUM

[1-(4-amino-2-propylpyrimidin-5-ylmethyl)-2-methylpyridinium chloride hydrochloride]

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of amprolium in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 40mg/kg.

2. PRINCIPLE

The sample is extracted with diluted methanol. The extract is purified on a column of aluminium oxide and treated with a methanolic solution of 2,7-dihydroxynaphthalene, potassium ferricyanide, potassium cyanide and sodium hydroxide, forming a purple-coloured complex the absorbance of which is measured at 530nm.

3. REAGENTS

3.1 Methanol.

3.2 Diluted methanol: mix two volumes of methanol (3.1) with one volume of water.

3.3 Potassium ferricyanide solution: dissolve 0·2g potassium ferricyanide ($K_3Fe(CN)_6$) in water and dilute to 100ml. This solution is stable for two weeks.

3.4 Potassium cyanide solution: dissolve 1g potassium cyanide in water and dilute to 100ml. This solution is stable for two weeks.

3.5 Sodium hydroxide solution: dissolve 2.25g sodium hydroxide in water and dilute to 200ml.

3.6 Methanolic sodium hydroxide solution: dilute 15ml of the solution (3.5) to 200ml with methanol (3.1).

3.7 2,7-dihydroxynaphthalene solution: dissolve 25mg of 2,7-dihydroxynaphthalene in methanol (3.1) and make it up to 1000ml with methanol (3.1).

3.8 Chromogenic reagent: transfer 90ml of 2,7-dihydroxynaphthalene solution (3.7) to a 250ml conical flask, add 5ml of potassium ferricyanide solution (3.3) and mix well. Then add 5ml of potassium cyanide solution (3.4) stopper the flask and mix well. Leave to stand for 30 to 35 minutes, add 100ml of methanolic sodium hydroxide solution (3.6), mix and filter through a sintered-glass crucible (porosity G3). Use this reagent within 75 minutes of filtering.

3.9 Aluminium oxide for column chromatography: before use, stir 100g of aluminium oxide with 500ml of water for 30 minutes, filter the slurry, wash the aluminium oxide on the filter 3 times with 50ml of methanol (3.1) drying each time by suction, leave to stand overnight and then dry for 2 hours at 100°C in a vacuum oven. Put in a dessicator to cool. Check the activity by subjecting a specified quantity of standard solution (3.11) to analysis, starting from point 5.2. The recovery of the amprolium must be 100% ± 4%.

3.10 Standard substance: pure amprolium complying with the following characteristics: Melting point (decomposition): 248°C.

Molecular extinction coefficient at both 265 and 235nm in water: $11{\cdot}0 \times 10^3$.

3.11 Standard solution: weight to the nearest 0·1mg, 50mg of pure amprolium (3.10). Dissolve in diluted methanol (3.2) in a 500ml graduated flask, make up to volume with the same solvent and mix. Dilute 10·0ml to 50ml with diluted methanol (3.2) in a graduated flask and mix well. 1ml of this solution contains 20μg of amprolium.

4. APPARATUS

4.1 Glass tube for chromatography (internal diameter: 9mm; length: 400 to 500mm).

4.2 Spectrophotometer, with 10mm cells.

5. PROCEDURE

5.1 *Extraction and purification*

Weigh, to the nearest 0·001g, up to 10g of the finely divided and mixed sample. Place the test portion in a 250ml conical flask and add exactly 100ml of diluted methanol (3.2). Shake for 60 minutes and filter. Dilute with diluted methanol (3.2) as necessary to obtain a solution containing 5 to 15μg of amprolium per ml.

Insert a cotton wool plug into the lower end of a chromatographic tube (4.1) and tamp in 5g of aluminium oxide (3.9) and then run in 25·0ml of the extract. Let the liquid percolate through the column, discard the first 5ml and collect the next 12ml in a graduated test-tube.

5.2 *Determination*

Transfer 5.0 ml of the solution obtained in (5.1) into centrifuge tube A. Place 5.0ml of diluted methanol (3.2) in centrifuge tube B. Add to each tube 10.0ml of chromogenic reagent (3.8), stopper the tubes, mix and allow to stand for 20 minutes. Then centrifuge for 3 minutes or until a clear solution is obtained and decant solutions A and B into 50ml conical flasks.

Immediately measure the absorbance of solution A at 530nm against solution B as a reference. Determine the quantity of amprolium by referring to the calibration curve (5.3).

5.3 *Calibration curve*

Pipette into centrifuge tubes volumes of 1·0, 2·0, 3·0, 4·0 and 5·0ml respectively of the standard solution (3.11). Make the contents of the first four tubes up to 5·0ml with diluted methanol (3.2). Add to all five tubes 10.0ml of chromogenic reagent (3.8), stopper the tubes, mix and allow to stand for 20 minutes. Then centrifuge for 3 minutes and decant the solutions into 50ml conical flasks.

Immediately measure the absorbance of the solutions at 530nm against a mixture of 5ml diluted methanol (3.2) and 10ml of chromogenic reagent (3.8) as a reference.

Plot the calibration curve, using the absorbances as the ordinates and the corresponding quantities of amprolium in μg as the abscissae.

6. CALCULATION OF RESULTS

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

$$\frac{20 \times A \times F}{W}$$

in which:

A = quantity of amprolium in μg as determined by photometric measurement.
W = weight of the test portion in g.
F = dilution factor (from 5.1).

6. DETERMINATION OF AVOPARCIN

1. PURPOSE AND SCOPE

The method is for the determination of avoparcin in complete feedingstuffs, protein concentrates and feed supplements. The lower limit of determination is 2mg/kg. The presence of polyether antibiotics may interfere in the determination.

2. PRINCIPLE

The sample is extracted with a mixture of acetone/water/hydrochloric acid. The antibiotic activity of the extract is determined by measuring the diffusion of avoparcin in an agar medium inoculated with *Bacillus subtilis*. Diffusion is shown by the formation of zones of inhibition of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration over the range of antibiotic concentrations employed.

3. MICRO-ORGANISM: *Bacillus subtilis* ATCC 6633 (NCIB 8054)

3.1 *Maintenance of stock culture*

Inoculate tubes containing slopes of culture medium (4.1) with *Bacillus subtilis* and incubate overnight at 30°C. Store the culture in a refrigerator at about 4°C. Reinoculate every month.

3.2 *Preparation of the spore suspension*[1]

Harvest the growth from a recently prepared agar slope (3.1) by means of 2 to 3 ml of sterile water. Use this suspension to inoculate 300 ml of culture medium (4.1) contained in a Roux flask and incubate for three to five days at 30°C. Harvest the growth in 15ml of ethanol (4.2) after having checked sporulation under the microscope, and mix well. This suspension may be kept for at least five months at about 4°C.

4. CULTURE MEDIA AND REAGENTS

4.1 Culture medium[2]

Peptone	6·0g
Tryptone	4·0g
Yeast extract	3·0g
Meat extract	1·5g
Glucose	1·0g
Agar	15·0g
Water	1000ml

pH 6·5 (after sterilization)

4.2 Ethanol 20% (v/v): dilute 200ml of ethanol with 800ml of water.

4.3 Hydrochloric acid, d: 1·18 to 1·19

4.4 Sodium hydroxide, 2M solution

4.5 Phosphate buffer, 0·1M:

Potassium dihydrogen phosphate, KH_2PO_4: 13·6g
Water to 1000ml
Adjust pH to 4·5.

4.6 Mixture of acetone/water/hydrochloric acid (4.3): 65/32·5/2·5(v/v/v)

4.7 Standard substance: avoparcin sulphate of known activity.

5. STANDARD SOLUTIONS

Dissolve an accurately weighed quantity of approximately 10mg of the standard substance (4.7) in phosphate buffer (4.5) and dilute with this buffer to give a stock solution containing 100µg avoparcin per ml. Stored in a stoppered flask at 4°C, this solution is stable for up to seven days.

[1] Other methods may be used provided that it has been established that they give similar spore suspensions.

[2] Any commercial culture medium of similar composition and giving the same results may be used.

5.1 *For premixes*

From this stock solution prepare by successive dilution with buffer (4.5) the following solutions:

S_8 4·0μg/ml
S_4 2·0μg/ml
S_2 1·0μg/ml
S_1 0·5μg/ml

5.2 *For feedingstuffs*

From the stock solution prepare by successive dilution with buffer (4.5) the following solutions:

S_8 2·0μg/ml
S_4 1·0μg/ml
S_2 0·5μg/ml
S_1 0·25μg/ml

6. PREPARATION OF THE EXTRACT AND ASSAY SOLUTIONS

6.1 *Protein concentrates, premixes*

Weigh, to the nearest 10mg, sufficient sample to contain 10 to 100mg avoparcin. Transfer to a 100ml graduated flask with 60ml of the mixture (4.6) and shake for 15 minutes on a mechanical shaker. Check the pH and adjust to pH2, if necessary, with hydrochloric acid (4.3). Make up to volume with the mixture (4.6) and mix well. Filter a portion through suitable filter paper (eg Whatman No 1), discarding the first 5ml of the filtrate. Take an aliquot and adjust the pH to 4·5 with sodium hydroxide solution (4.4). Dilute this solution with buffer (4.5) to obtain an expected avoparcin concentration of 4μg/ml(= U_8).

From this solution prepare solutions U_4 (expected content: 2 μg/ml), U_2 (expected content: 1μg/ml) and U_1 (expected content: 0·5μg/ml) by means of successive dilution (1 + 1) with buffer (4.5).

6.2 *Complete feeding stuffs*

Weigh out a quantity of sample of 50g and shake 100ml of mixture (4.6) for 30 minutes on a mechanical shaker. Clarify the extract by centrifugation (using stoppered centrifuge tubes), take an aliquot of the clarified extract (see table overleaf) and adjust the pH to 4·5 with sodium hydroxide solution (4.4). Dilute this aliquot with buffer (4.5) to provide U_8 (see table below).

From this solution prepare solutions U_4 (expected content: 1μg/ml), U_2 (expected content: 0·5μg/ml) and U_1 (expected content: 0·25μg/ml) by means of successive dilution (1 + 1) with buffer (4.5).

Presumed level of avoparcin (mg/kg)	5	7·5	10	15	20	40
Weight of sample (g(±0·1g))	50	50	50	50	50	50
Volume of mixture (4.6)(ml)	100	100	100	100	100	100
Volume of clarified extract (ml)	20	15	20	15	20	10
Final volume (ml): U_8	25	25	50	50	100	100
Expected U_8 concentration (μg/ml)	2	approx 2	2	approx 2	2	2

7. ASSAY PROCEDURE

7.1 *Inoculation of the assay medium*

Inoculate the assay medium (4.1) with the spore suspension (3.2) at 50 to 60°C. By preliminary trials on plates with assay medium (4.1) determine the quantity of spore suspension required to give the largest and clearest zones of inhibition with the various concentrations of avoparcin.

7.2 *Preparation of the plates*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the assay solution (U_8, U_4, U_2, U_1). These four concentrations of extract and standard must necessarily be placed in each plate. To this effect, select plates big enough to allow at least eight holes with a diameter of 10 to 13mm and not less than 30mm between centres to be made in the agar medium. The test may be carried out on plates consisting of a sheet of glass with a faced aluminium or plastic ring placed on top, 200mm in diameter and 20mm high.

Pour into the plates a quantity of the medium (4.1), inoculated as in 7.1, to give a layer about 2mm thick (60ml for a plate of 200mm diameter). Allow to set in a level position, bore the holes and place in them exactly measured volumes of assay and standard solutions (between 0·10 and 0·15ml per hole, according to the diameter). Apply each concentration at least four times so that each determination is subject to an evaluation of 32 zones of inhibition.

7.3 *Incubation*

Incubate the plates for 16 to 18 hours at 30°C.

8. EVALUATION

Measure the diameter of the zones of inhibition to the nearest 0·1mm. Record the mean measurements for each concentration on semi-logarithmic graph paper showing the logarithm of the concentrations in relation to the diameters of the zones of inhibition. Plot the 'best fit' lines of both the standard solution and the extract, for example as opposite.

Determine the 'best fit' point for the standard lowest level (SL) using the formula:

$$\text{(a) } SL = \frac{7\,S_1 + 4\,S_2 + S_4 - 2\,S_8}{10}$$

Determine the 'best fit' point for the standard highest level (SH) using the formula:

$$\text{(b) } SH = \frac{7\,S_8 + 4\,S_4 + S_2 - 2\,S_1}{10}$$

Similarly, calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) by substituting U_1, U_2, U_4 and U_8 for S_1, S_2, S_4 and S_8 in the above formulae.

Record the calculated SL and SH values on the same graph paper and join them to give the 'best fit' line for the standard solution. Similarly record UL and UH and join them to give the 'best fit' line for the extract.

In the absence of any interference the lines should be parallel. For practical purposes the lines can be considered parallel if the values (SH–SL) and (UH–UL) do not differ by more than 10% from their mean value.

If the lines are found to be non-parallel, either U_1 and S_1 or U_8 and S_8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$\text{(a}^a\text{) } SL = \frac{5\,S_1 + 2\,S_2 - S_4}{6} \text{ or } \frac{5\,S_2 + 2\,S_4 - S_8}{6}$$

$$\text{(b}^b\text{) } SH = \frac{5\,S_4 + 2\,S_2 - S_1}{6} \text{ or } \frac{5\,S_8 + 2\,S_4 - S_2}{6}$$

and similarly for UL and UH. The alternative 'best fit' lines should be checked for parallelism as before. The fact that the result has been calculated from three levels should be noted on the final report.

When the lines are considered as being parallel, calculate the logarithm of the relative activity (log. A) by means of one of the following formulae:

For four levels

$$\text{(c) } \log A. = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

For three levels

$$\text{(d) } \log A. = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4) \times 0{\cdot}401}{U_4 + S_4 - U_1 - S_1}$$

or

$$\text{(d}^d\text{) } \log A = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0{\cdot}401}{U_8 + S_8 - U_2 - S_2}$$

Real activity = supposed activity × relative activity.

If the relative activity is found to be outside the range of 0·5 to 2·0 then repeat the assay making appropriate adjustments to the extract concentrations or, if

this is not possible, to the standard solutions. When the relative activity cannot be brought into the required range, any result obtained must be considered as approximate and this should be noted on the final report.

When the lines are considered as not being parallel, repeat the determination. If parallelism is still not achieved, the determination must be considered as unsatisfactory.

9. REPEATABILITY

The difference between the results of two determinations carried out on the same sample, by the same analyst, should not exceed:

—2mg/kg, in absolute value, for contents of avoparcin from 2 and up to 10mg/kg,

—20% related to the highest value for contents of 10 to 25mg/kg,

—5mg/kg, in absolute value, for contents of 25 to 50mg/kg,

—10% related to the highest value for contents above 50mg/kg.

7. DETERMINATION OF BUQUINOLATE

(ethyl 4-hydroxy-6,7-di-isobutoxyquinoline-3-carboxylate)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of buquinolate in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 10mg/kg. Decoquinate interferes in the determination.

2. PRINCIPLE

The sample is extracted with chloroform. The extract is evaporated to dryness, the residue dissolved in chloroform and the solution is then subjected to thin-layer chromatography. The buquinolate is eluted with ethanol and determined spectrophotofluorimetrically by comparison with standard solutions.

3. REAGENTS

3.1 Chloroform.

3.2 Ethanol 96% v/v.

3.3 Mixture of chloroform and ethanol: mix 10 volumes of chloroform (3.1) with 1 volume of ethanol (3.2).

3.4 Ethanol 80% v/v.

3.5 Silica gel G for thin-layer chromatography.

3.6 Standard substance: pure buquinolate.

3.7 Standard solutions:

3.7.1 Standard solution of 0·2mg of buquinolate per ml: Weigh to the nearest 0·1mg, 50mg of standard substance (3·6). Dissolve in

chloroform (3·1) in a 250ml graduated flask by warming in a water-bath at 50°C. Leave to cool to room temperature, make up the volume with chloroform (3·1) and mix.

3.7.2 Working standard solutions: Transfer 5·0, 10·0, 15·0, 20·0 and 25·0ml portions of the solution (3.7.1) into 25ml graduated flasks. Make up to volume with chloroform (3.1) and mix. Prepare immediately before use. These solutions contain respectively 0·04, 0·08, 0·12, 0·16 and 0·20mg of buquinolate per ml.

4. APPARATUS

4.1 Glass-plates for thin-layer chromatography, 200 × 200mm, prepared as follows. Spread on the plates a uniform layer 0·5mm thick of silica gel G (3.5) and leave to dry in the air for 15 minutes. Keep the plates in a drying oven at 100°C for 2 hours and transfer into a desiccator containing dehydrating silica gel. Ready-made plates are suitable if they give results similar to those for the plates treated as indicated above.

4.2 Zone collector for thin-layer chromatography.

4.3 Short wavelength ultraviolet lamp, 254nm.

4.4 Spectrophotofluorimeter fitted with a xenon lamp, and two monochromators.

4.5 Rotary vacuum evaporator, with 250ml flask.

5. PROCEDURE

5.1 *Extraction*

Weigh to within 0·001g, a quantity of the finely divided and mixed sample containing about 1·25mg of buquinolate. Place in a 250ml conical flask and add 100ml of chloroform (3.1). Mix, stopper the flask, and shake for one hour. Decant, filter and discard the first few millilitres of the filtrate. Transfer 80ml of the clear filtrate into a 150ml beaker, or into a 250ml flask fitted to the rotary evaporator (4.5). Evaporate nearly to dryness on a water-bath at 50°C, dissolve the oily residue with a few millilitre portions of chloroform (3.1) and transfer quantitatively the liquids into a 10ml graduated flask. Make up to volume with chloroform (3.1) and mix. If the solution is not clear, centrifuge for three minutes at 3000 rpm in a stoppered tube.

5.2 *Thin-layer chromatography*

By means of a micropipette, deposit in spots on a plate for thin-layer chromatography (4.1) at intervals of 20mm, volumes of 0·25ml of the extract obtained in 5.1 and of the five working standard solutions (3.7.2). Develop the chromatogram with chloroform (3.1) until the solvent front has nearly reached the upper edge of the plate, then dry in a current of air. Develop with the chloroform-ethanol mixture (3.3) until the solvent front has travelled about 120mm. Allow the solvents to evaporate. Examine the plate under the ultraviolet lamp (4.3) and with a needle, mark the boundary of the buquinolate spots (Rf value 0·4 to 0·6).

5.3 *Elution*

Collect the silica gel from each marked zone, by means of a zone collector (4.2), and place in centrifuge tubes. Add to each tube 10·0ml of 80% v/v ethanol (3.4), shake for 20 minutes, then centrifuge for 5 minutes at 3000 rpm. Decant the clear solutions into 50ml conical flasks.

5.4 *Measurement of fluorescence*

Set the scale of the spectrophotofluorimeter (4.4) at 100 with the aid of the eluate from the most concentrated standard solution, using the excitation wavelength between 200 and 280nm that gives the most intense fluorescence at an emission wavelength of 375nm. Under these conditions, measure the fluorescence of the other eluates (5.3). From the values obtained, determine the quantity (A) of buquinolate in mg in the 10ml of eluate from the sample.

6. CALCULATION OF RESULTS

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

$$\frac{50000 \times A}{W}$$

in which:

A = quantity in mg of buquinolate determined by spectrophotofluorimetric measurement.
W = weight of test portion in g.

8. DETERMINATION OF CHLORTETRACYCLINE, OXYTETRACYCLINE AND TETRACYCLINE BY DIFFUSION THROUGH AGAR

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of chlortetracycline, oxytetracycline and tetracycline in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of determination is 5mg/kg. Lower levels than 5mg/kg may be estimated by graphic extrapolation.

2. PRINCIPLE

For levels equal to or lower than 50mg/kg the sample is extracted by diluted formamide. For levels higher than 50mg/kg it is extracted by a mixture of acetone, water and hydrochloric acid for the determination of chlortetracycline, and a mixture of methanol and hydrochloric acid for the determination of oxytetracycline and tetracycline.

The extracts are then diluted, and their antibiotic activity is determined by measuring the diffusion of chlortetracycline, oxytetracycline or tetracycline in an agar medium inoculated with *Bacillus cereus.* Diffusion is shown by the formation of zones of inhibition in the presence of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration.

3. MICRO-ORGANISM: *Bacillus cereus* ATCC 11778 (NCIB 8849; NCTC 10320; NCDO 720)

3.1 *Maintenance of the parent strain*

Inoculate *B. cereus* onto an agar slope prepared from the culture medium (4.1.1). Incubate overnight at approximately 30°C. Keep the culture in a refrigerator at about 4°C and re-inoculate agar slopes every two weeks. At the same time prepare sub-cultures for laboratory use.

3.2 *Preparation of the spore suspension*

3.2.1 Harvest the bacteria from a recently prepared agar slope (3.1) using 2 to 3ml of sodium chloride solution (4.5). Inoculate this suspension onto 300ml of culture medium (4.1.2) in a Roux flask. Incubate for 3 to 5 days at 28 to 30°C and then confirm by examination under the microscope that sporulation has occurred. Harvest the spores in 15ml of ethanol (4.6) and mix. This suspension may be kept in a refrigerator for at least 5 months.

3.2.2 Make preliminary tests on the assay plates with the culture medium (4.1.3) to determine the quantity of inoculum needed to obtain the largest possible clear zones of inhibition with the different concentrations of antibiotic used. The quantity will usually be 0·2 to 0·3ml per 1000ml. Inoculate the culture medium at a temperature between 50 and 60°C.

4. CULTURE MEDIA AND REAGENTS

4.1.1 Culture medium I:

Glucose	1g
Tryptic peptone	10g
Meat Extract	1·5g
Yeast Extract	3g
Agar, according to the quality	10 to 20g
Tween 80	1ml
Phosphate buffer solution pH5·5(4.2)	10ml
Water	1000ml

ph after sterilization: 5·8

4.1.2 Culture medium II:

As for 4.1.1, but with an agar concentration of 3 to 4%.

4.1.3 Culture medium III:

As for 4.1.1, with the following additions:

Methylene blue solution, 0·5g per 100ml in ethanol	4ml
Boric acid solution, 5g per 100ml	15ml

4.2 Phosphate buffer solution pH5·5:

Potassium dihydrogen phosphate, KH_2PO_4	130·86g
di Sodium hydrogen phosphate, $Na_2HPO_42H_2O$	6.947g
Water	1000ml

4.3 Phosphate buffer solution pH5·5 (4.2) diluted with water to 1 + 9.

4.4 Phosphate buffer solution pH8·0:

Potassium dihydrogen phosphate KH_2PO_4	1·407g
di Sodium hydrogen phosphate $Na_2HPO_42H_2O$	57·539g
Water	1000ml

4.5 Sodium chloride solution 0·9% w/v: dissolve 9g of sodium chloride in water and dilute to 1000ml: sterilize.

4.6 Ethanol 20% solution (v/v).

4.7 Hydrochloric acid solution 0·1M.

4.8 Formamide 70% solution (v/v): prepare freshly before use and adjust to pH4·5 using approximately 2M sulphuric acid.

4.9 A mixture of acetone, water and hydrochloric acid (density 1·18g per ml) in the proportions by volume: 65 + 33 + 2.

4.10 A mixture of methanol and hydrochloric acid (density 1·18g per ml) in the proportion by volume: 98 + 2.

4.11 Standard substances: chlortetracycline hydrochloride, oxytetracycline base, tetracycline hydrochloride of known activities.

5. STANDARD SOLUTIONS

5.1 *Chlortetracycline*

5.1.1 Prepare from the standard substance (4.11), by using hydrochloric acid (4.7), a standard solution with a concentration of 500μg chlortetracycline HCl per ml. This solution will keep for one week in a refrigerator.

5.1.2 From this solution (5.1.1) prepare a standard working solution (S_8) with a concentration of 0·2μg chlortetracycline HCl per ml. Dilution is made with the phosphate buffer solution pH 5·5 diluted to 1/10 (4.3).

5.1.3 Prepare the following concentrations by means of successive dilutions (1 + 1) with the buffer solution (4.3):

S_4 0·1μg per ml
S_2 0·05μg per ml
S_1 0·025μg per ml

5.2 *Oxytetracycline*

5.2.1 Proceed as indicated in 5.1. Prepare from a solution with a concentration of 400μg oxytetracycline per ml, a standard working solution S_8 containing 1·6μg oxytetracycline per ml, and the following concentrations:

S_4 0·8μg per ml
S_2 0·4μg per ml
S_1 0·2μg per ml

5.3 *Tetracycline*

Proceed as indicated in 5.1. Prepare from a solution with a concentration of 500μg tetracycline HCl per ml, a standard working solution S_8 containing 1·0μg tetracycline HCl per ml, and the following concentrations:

S_4 0·5μg per ml
S_2 0·25μg per ml
S_1 0·125μg per ml

6. PROCEDURE

6.1 *Extraction*

6.1.1 Levels not more than 50mg/kg:

Treat the sample for analysis with formamide (4.8) according to the indications given in the following table. Shake for 30 minutes on a mechanical shaker and then immediately dilute according to the indications given in the following table, with the phosphate buffer solution (4.3). The formamide concentration of this solution must not exceed 40 per cent. Centrifuge or decant to obtain a clear solution, U_8.

Antibiotic	Chlortetracycline		Oxtetracycline		Tetracycline	
Presumed level in mg per kg	10	50	10	50	10	50
Weight of sample in g	10	10	24	9·6	20	10
Volume of formamide (4.8) (ml)	100	100	80	100	80	100
Volume of phosphate buffer solution (4.3) (ml)	dilution 1:5 *(a)*	dilution 1:25 *(b)*	70	200	120	dilution 1:5 *(a)*
Expected U_8 concentration in µg per ml	0·2	0·2	1·6	1·6	1·0	1·0

(a) Dilute 20ml of the extract to 100ml with the buffer solution in a graduated flask.
(b) Dilute 4ml of the extract to 100ml with the buffer solution in a graduated flask.

Prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with the phosphate buffer solution (4.3).

6.1.2 Levels higher than 50

6.1.2.1 Chlortetracycline.

According to the presumed antibiotic level of the sample, treat a sample for analysis of 2 to 10g with 20 times its volume of mixture (4.9). Shake for 30 minutes on a laboratory shaker. Check that the pH remains lower than 3 during extraction and, if necessary, adjust to pH 3 (use 10 per cent acetic acid for mineral compounds). Take an aliquot of the extract and adjust the pH to 5·5 with the phosphate buffer solution pH 8 (4.4), in the presence of bromocresol green (colour change yellow to blue). Centrifuge to obtain a clear solution. Dilute the supernatant with phosphate buffer solution pH 5·5 (4.3) to obtain the concentration U_8 (see 6.1). Prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with phosphate buffer solution (4.3).

6.1.2.2 Oxytetracycline and tetracycline.

Proceed as indicated in 6.1.2.1 replacing the mixture (4.9) by the mixture (4.10).

6.2 *Determination*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the extract (U_8, U_4, U_2, U_1). These four concentrations of standard and extract solution must be placed in each plate. To this effect, select plates large enough to allow at least eight wells with a diameter of 10 to 13mm, and not less than 30mm between centres, to be made in agar medium.

Inoculate at 50–60°C, with the spore suspension (3.2.1) a quantity of the melted culture medium (4.1.3), sufficient to give a layer approximately 2mm thick in the assay plates to be employed. Swirl to mix thoroughly and pour into the sterile assay plates which must be held in a rigorously horizontal position until the agar has set. With a sterile cork borer remove agar plugs to form wells as described above. Pipette into each well an exactly measured and equal volume (0·10 to 0·15ml) of respectively solutions S_8, S_4, S_2, S_1 and U_8, U_4, U_2, U_1.

Apply each concentration four times so that the determination is subject to an evaluation of 32 zones of inhibition.

Incubate the plates for approximately 18 hours at 28–30°C.

6.3 *Evaluation*

See *"Evaluation"* of avoparcin.

9. DETERMINATION OF CHLORTETRACYCLINE, OXYTETRACYCLINE AND TETRACYCLINE BY TURBIDIMETRY

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of chlortetracycline, oxytetracycline and tetracycline in feeding stuffs at concentrations higher than 1000mg/kg, provided that no other substance causes interference by giving rise to cloudy extracts. This method is quicker than the method of diffusion through agar.

2. PRINCIPLE

The sample is extracted by a mixture of acetone, water and hydrochloric acid for the determination of chlortetracycline, and a mixture of methanol and hydrochloric acid for the determination of oxytetracycline and tetracycline.

The extracts are then diluted and their antibiotic effect is determined by measuring the light transmission from a culture medium which has been inoculated with *Staphylococcus aureus* and to which the antibiotic has been added. The light transmission is a function of the antibiotic concentration.

3. MICRO-ORGANISM: *Staphylococcus aureus* K 141. (NCIB 11182; NCTC 10988)

3.1 *Maintenance of the parent strain*

Inoculate *S. aureus* onto an agar slope of the culture medium II (4.1.2). Incubate overnight at 37°C. Keep the culture in a refrigerator at

about 4°C and re-inoculate agar slopes every 4 weeks. At the same time prepare sub-cultures for laboratory use.

3.2 *Preparation of the inoculum*

Approximately 24 hours before use, re-inoculate a sub-culture onto a fresh agar slope and incubate overnight at 37°C. Harvest the bacterial growth from the agar slope in approximately 2ml culture medium (4.1), and then decant the suspension into approximately 100ml of the same medium (4.1). Incubate in a water-bath at 37°C for $1\frac{1}{2}$ to 2 hours.

4. CULTURE MEDIA AND REAGENTS

4.1.1 Culture medium I:

Peptone	5·0g
Yeast extract	1·5g
Meat extract	1·5g
Sodium chloride	3·5g
Glucose	1·0g
Potassium dihydrogen phosphate, KH_2PO_4	1·32g
di Potassium hydrogen phosphate, K_2HPO_4	3·68g
Water	1000ml

pH after sterilisation: 6·8 to 7·0.

4.1.2 Culture medium II-as for 4.1.1 with 1·5 to 3·0% agar.

4.2 Phosphate buffer solution pH 4·5:

Potassium dihydrogen phosphate KH_2PO_4	13·6g
Water	1000 ml

4.3 Hydrochloric acid solution, 0·1M.

4.4 A mixture of acetone, water and hydrochloric acid (density: 1·18g per ml) in the proportions by volume: 65 + 33 + 2.

4.5 Acidified methanol: mix methanol and hydrochloric acid (density: 1·18g per ml) in the proportions by volume: 98 + 2.

4.6 Formaldehyde solution (approximately 10% formaldehyde).

4.7 Standard substances: chlortetracycline hydrochloride, oxytetracycline base, and tetracycline hydrochloride of known activities.

5. STANDARD SOLUTION

By using hydrochloric acid (4.3), prepare from the standard substance (4.7) a solution with accurately known concentration of 400 to 500μg of the antibiotic per ml. This solution will keep for one week in a refrigerator at about 4°C.

6. PROCEDURE

6.1 *Extraction*

6.1.1 Chlortetracycline:

Place 1 to 2g (weighed to the nearest 0·001g) of the sample for analysis in a 200 or 250ml graduated flask. Add approximately 100ml of the mixture (4.4) and shake for 30 minutes on a mechanical shaker. Make up to volume with the phosphate buffer solution pH 4·5 (4.2). Mix and allow to settle.

6.1.2 Oxytetracycline and tetracycline:

Place 1 to 2g (weighed to the nearest 0·001g) of the sample for analysis in a 200 or 250ml graduated flask. Add approximately 100ml of the mixture (4.5) and shake for 30 minutes on a mechanical shaker. Make up to volume with the phosphate buffer solution pH 4·5 (4.2). Mix and allow to settle.

6.2 *Determination*

6.2.1 Preparation of standard and sample extract series:

Use phosphate buffer solution pH 4·5 (4.2) to dilute the standard solution (5) and the extract (6.1) so as to obtain a series of concentrations making it possible to draw a calibration curve for each determination with the interpolation on this curve of at least two values relative to the extract. The strength of the dilutions must be chosen according to the growth conditions of the strain, which may vary from one laboratory to another. It is usual to proceed as follows:

6.2.2 Chlortetracycline:

Use phosphate buffer solution (4.2) to dilute the standard solution (5) to obtain a standard working solution with a concentration corresponding to 0·2μg chlortetracycline HCl per ml. Then, with phosphate buffer solution (4.2), prepare 6 dilutions in the tubes in which the determinations are to be made, repeating each dilution, as indicated below.

Volume of standard working solution (ml)	Volume of phosphate buffer solution (4.2) (ml)	Concentration of Chlortetracycline HCl (μg per ml)
0·7	0·3	0·14
0·6	0·4	0·12
0·55	0·45	0·11
0·45	0·55	0·09
0·4	0·6	0·08
0·3	0·7	0·06

Use the phosphate buffer solution (4.2) to dilute the extract (6.1) to obtain an expected concentration of chlortetracycline HCl of 0·12μg per ml. Place 1ml of this solution in 2 tubes and 0·75ml (=0·09μg) in 2 other tubes. Make up the volume of the last 2 tubes to 1ml with the phosphate buffer solution (4.2).

6.2.3 Oxytetracycline and tetracycline:

Use the phosphate buffer solution (4.2) to dilute the solution (5) to obtain a standard working solution with a concentration corresponding to 0·6μg oxytetracycline or tetracycline HCl per ml. Then, with the phosphate buffer solution (4.2), prepare 7 dilutions in the tubes in which the determinations are to be made, repeating each dilution, as indicated opposite.

Volume of standard working solution (ml)	Volume of phosphate buffer solution (4.2) (ml)	Concentration of Oxytetracycline or Tetracycline HCl (μg per ml)
0·9	0·1	0·54
0·8	0·2	0·48
0·7	0·3	0·42
0·6	0·4	0·36
0·4	0·6	0·24
0·3	0·7	0·18
0·2	0·8	0·12

Use the phosphate buffer solution (4.2) to dilute the extract (6.1) to obtain an expected concentration of oxytetracycline or tetracycline HCl of 0·48μg per ml. Place 1ml of this solution in 2 tubes and 0·5ml (=0·24μg) in 2 other tubes. Make up the volume of the last 2 tubes to 1ml with the phosphate buffer solution (4.2).

6.3 *Inoculation of the culture medium*

Inoculate the culture medium (4.1.1) with the inoculum (3.2) so as to obtain, with the spectrophotometer at 590nm, a light transmission of 85% in a 50mm cell or 92% in a 20mm cell, having first adjusted the apparatus to give 100% transmission on the culture medium (4.1.1) before inoculation.

6.4 *Seeding*

Place in each tube (6.2.2 or 6.2.3) 9ml of inoculated culture medium (6.3). The tubes must be filled in hygienic, but not necessarily sterile conditions.

6.5 *Incubation*

Incubation must be carried out in a stirred water-bath where the temperature is kept at 37°C ± 0·1°C. The time of incubation (generally $2\frac{1}{2}$ to 3 hours) should be chosen so as to obtain transmission curves, the slope of which enables precise measurements to be made. Further growth is then prevented rapidly injecting 1ml formaldehyde solution (4.6) into each tube.

6.6 *Measurement of the growth*

Measure the transmissions with the spectrophotometer at 590nm, having first adjusted the apparatus to give 100% transmission on the clearest standard solution (corresponding to the highest antibiotic level). Owing to slight differences of turbidity in the different tubes, cells of at least 20mm, and preferably 50mm, are recommended.

7. CALCULATION OF RESULTS

Plot a graph of the photometric transmissions against the antibiotic concentrations. Interpolate the transmissions relative to the extract on the curve. Calculate the antibiotic content of the sample.

10. DETERMINATION OF CLOPIDOL

(3,5-dichloro-2,6-dimethylpyridine-4-ol)

1. SCOPE AND FIELD OF APPLICATION

The method for the determination of the quantity of clopidol in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 60mg/kg.

2. PRINCIPLE

Clopidol is extracted from the feed with methanolic ammonia solution, and a portion of the extract is passed through a column of aluminium oxide onto a column of ion-exchange resin. The clopidol is retained on the resin and interfering substances are removed by washing with 80% methanol. The clopidol is eluted from the resin with 40% acetic acid and the absorbance is measured at 267nm.

3. REAGENTS

Note: The suitability of a batch of aluminium oxide and of the other reagents should be tested before use by analysing a blank feed to which a known amount of clopidol has been added.

3.1 Aluminium oxide for column chromatography, 100 to 250 mesh, alkaline, Brockman activity 1.

3.2 Ammonia (density 0·88g per ml).

3.3 Anion exchange resin, AG1–X8 or Dowex 1–X8, 100 to 200 mesh—

To convert Dowex resin in the chloride form to the acetate form add 1 litre of 6M hydrochloric acid to 350g of resin in a 3 litre beaker, and heat the mixture on a steam bath for 2 to 3 hours. Pour the slurry into a glass Buchner funnel, and wash the resin with water until the washings are free from chloride (about 6 litres of water are required). Transfer the resin to a 50mm diameter glass column having a coarse sintered-glass disc at the bottom end, and wash with sodium acetate solution (5g sodium acetate, anhydrous, dissolved in water and diluted to 100ml) until the column effluent gives only a cloudy solution on addition of silver nitrate solution. Return the resin to the glass Buchner funnel, and wash with water. Transfer the resin to a 3 litre beaker, add 1 litre of 40% v/v acetic acid solution (3.4) and heat on a steam bath for 3 hours or longer. Filter, and wash the resin again with water until the washings are free from chloride. Store the resin in water.

3.4 Acetic acid solution 40% v/v.

3.5 Methanol.

3.6 Methanol solution 80% v/v.

3.7 Ammoniacal methanol solution: dilute 1 volume of ammonia (3.2) with 19 volumes of methanol (3.5).

3.8 Clopidol standard solution: weigh, to the nearest 0·1mg, 125mg of clopidol into a beaker, add 25ml of sodium hydroxide solution (2g sodium hydroxide dissolved in water and diluted to 100ml) to dissolve the clopidol, transfer the solution to a 500ml graduated flask, and

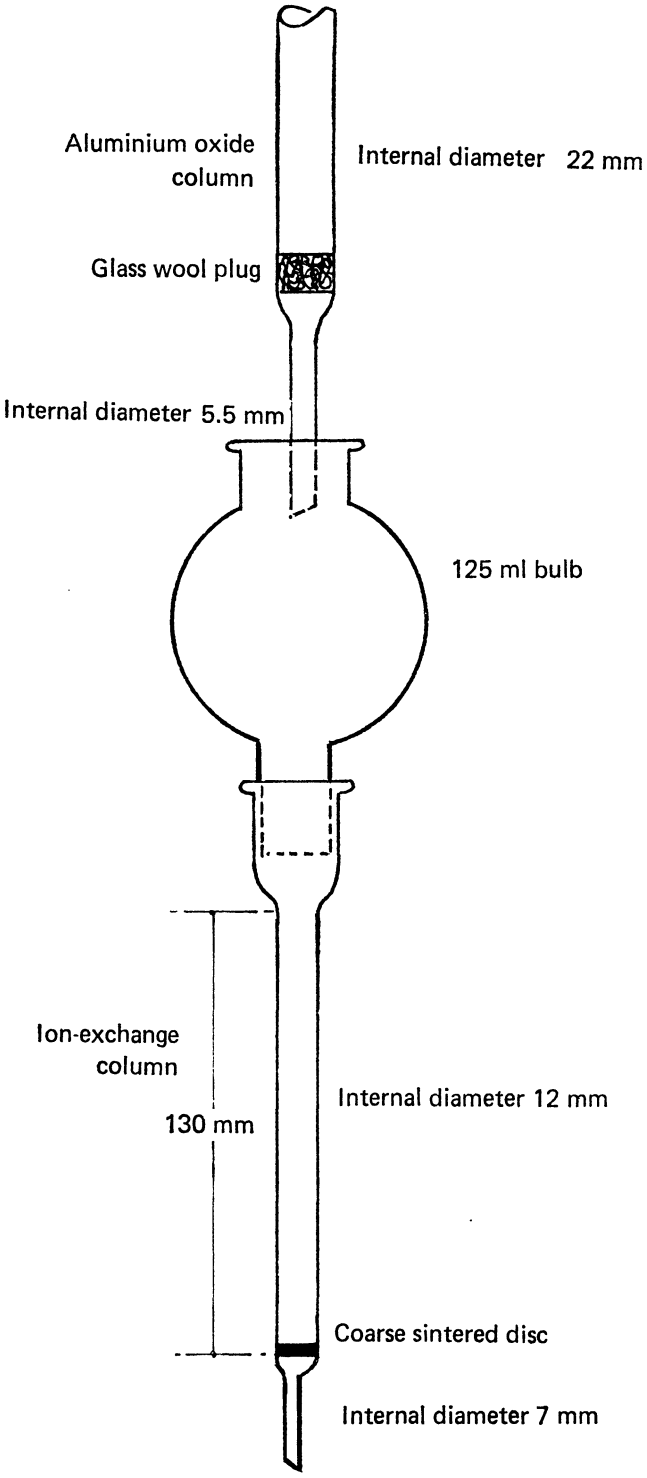
dilute to the mark with water. This solution contains 250μg per ml clopidol.

4. APPARATUS

4.1 Aluminium oxide column: constructed as indicated in the diagram overleaf.

4.2 Ion exchange column: constructed as indicated in the diagram overleaf.

4.3 Spectrophotometer, recording, with 10mm silica cells.


Aluminium oxide column
Internal diameter 22 mm
Glass wool plug
Internal diameter 5.5 mm
125 ml bulb
Ion-exchange column
130 mm
Internal diameter 12 mm
Coarse sintered disc
Internal diameter 7 mm

5. PROCEDURE

5.1 *Extraction of clopidol*

Weigh, to the nearest 0·001g, approximately 50g of the finely divided and mixed sample, or a suitable amount expected to contain about 12mg of clopidol, transfer to a 500ml graduated flask, and add 400ml of ammoniacal methanol solution (3.7). Place a magnetic stirring bar in the flask and stir the mixture on a magnetic stirrer for 20 minutes. Remove the stirring bar from the flask, dilute to the mark with ammoniacal methanol solution (3.7), mix the contents well, and set aside for 20 to 30 minutes.

5.2 *Purification*

5.2.1 Aluminium oxide column: For each column required weigh approximately 25g of aluminium oxide (3.1) into an aluminium foil dish and place it in an oven at 105 ± 5°C for 1 hour. Remove the dish from the oven and cool to room temperature in a desiccator. Make a slurry of the aluminium oxide with 25ml of ammoniacal methanol solution (3.7) and filter on a Buchner funnel. Wash the aluminium oxide with methanol (3.5) until the washings are neutral. Form a slurry of the aluminium oxide with 50ml of methanol (3.5) and pour the slurry into the column (4.1). Allow the methanol to drip through the column. Place a plug of glass wool lightly on top of the aluminium oxide and then wash with 25ml of methanol (3.5). Do not allow the liquid in the column to fall below the top of the aluminium oxide. Discard the eluate.

5.2.2 Anion exchange column: Form a slurry in acetic acid (3.4) of sufficient resin (3.3) to fill the columns required. Filter on a Buchner funnel, wash the resin with twice its own volume of acetic acid (3.4), and then with aqueous methanol (3.6) until the washings are neutral. Form a slurry of the resin with aqueous methanol (3.6) and add sufficient to a column (4.2) to give a resin bed 20 to 30mm deep after settling. Place a small plug of glass wool on top of the resin and wash the column with two 13ml portions of aqueous methanol (3.6). Do not allow the liquid level in the column to fall below the top of the resin. Discard the eluate.

5.2.3 Chromatographic procedure: By pipette transfer 10·0ml of the extract of the feed sample (5.1) directly onto an aluminium oxide column and at the same time transfer the same volume of ammoniacal methanol solution (3.7) directly onto a second aluminium oxide column (reagent blank). Allow the solutions to drain to the top of the aluminium oxide and then wash each column with three 12ml portions of aqueous methanol (3.6), allowing the liquid to drain to the top of the aluminium oxide each time. Let all the eluate from each column drain directly into separate ion-exchange columns, and then remove the aluminium oxide columns. Allow the liquid to drain to the top of the ion-exchange resin and then wash each column with four 13ml portions of aqueous methanol (3.6). Discard all eluate.

Elute each column with two 10ml and then one 4ml portions of acetic acid (3.4). Collect the eluates from each column in separate 25ml graduated flasks and dilute the contents of each to the mark with acetic acid (3.4).

5.3 *Determination*

Record the absorption spectrum of the sample extract between 350 and 245nm in 10mm silica cells with the reagent blank solution (5.2.3) as reference. Measure the absorbance of the sample extract at 267nm above a baseline obtained by drawing a line through the absorbance at 327 and 297nm and extending it through 267nm.

(Note: Background absorption due to the feed approaches a linear function that can be described by the points on the curve at 296 and 327nm. Occasionally this is not the case, as can be detected by absorption peaks in the region between 350 and 297nm.)

Determine the concentration of clopidol in the sample by reference to the calibration curve (5.4).

5.4 *Calibration curve*

By pipette transfer 1, 5, 7·5, 10, 12·5 and 15ml portions of clopidol standard solution (3.8) to separate 250ml graduated flasks. Dilute the contents of each flask to the mark with acetic acid (3.4). Record the absorption spectra of these solutions in 10mm silica cells between 350 and 245nm with acetic acid (3.4) as reference. Construct a calibration curve using the absorbances at 267nm as ordinates and the corresponding concentrations of clopidol in μg per ml as abscissae.

6. CALCULATION OF RESULTS

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

$$\frac{23{\cdot}23 \times C \times 50}{W}$$

in which:

C = concentration of clopidol, in μg per ml, read from the calibration curve equivalent to the absorbance of the test solution;
23·23 = a factor that makes allowance for the volume of the feed sample in the flask; and
W = weight of test portion in g.

Absorbance at 327 and 297nm should not differ by more than 0·05 units and both points should be below 0·2. Results should be satisfactory as long as these criteria are kept in mind along with any obvious distortion in the appearance of the curve. No maximum other than that of clopidol should be present.

11. DETERMINATION OF COPPER BY THE DIETHYLDITHIOCARBAMATE SPECTROPHOTOMETRIC METHOD

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of copper in complete feeding stuffs, protein concentrates and feed supplements.

2. PRINCIPLE

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

3. REAGENTS

The water used should be free from copper.

3.1 Carbon tetrachloride, redistilled.

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

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

3.4 Ammonium hydroxide solution approximately 6M: 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, 2M.

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

3.7 Hydrochloric acid solution, 2M.

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

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

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

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

4. APPARATUS

4.1 Spectrophotometer with 10mm cells.

5. PREPARATION OF THE TEST SAMPLE

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

6. PROCEDURE

6.1 *Dissolution of sample*

Weigh, to the nearest 0·001g, approximately 10g of the sample prepared in accordance with (5) into a silica dish or basin, and place a silica cover on top. Transfer to a cool muffle furnace. Raise the temperature to 450° ± 10°C and allow to ash until all the carbonaceous matter has disappeared; a slow current of air through the furnace during the initial stages of ashing is desirable. In the case of high-fat content materials, care must be taken to avoid ignition of the sample. When all the organic matter has been destroyed, cool, add 10ml 50% v/v hydrochloric acid solution (3.6) and evaporate to dryness on a water-bath. Extract the soluble salts from the residue with two successive 10ml portions of boiling 2M hydrochloric acid solution (3.7) decanting the solution each time through the same suitable filter-paper[1] into a 50ml graduated flask. Then add 5ml of 50% v/v hydrochloric acid solution (3.6) and about 5ml of 30% v/v nitric acid solution (3.8) to the residue in the basin, and evaporate the mixture to dryness on a hot-plate at low heat. Finally, add a further 10ml of boiling 2M 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 M. hydrochloric acid) containing not more than 50 μg of copper. Add 10ml EDTA-citrate solution (3.3), two drops of thymol blue indicator solution (3.11) and ammonium hydroxide solution (3.4) until the mixture is coloured green or bluish-green. Cool the mixture, add 1ml of sodium diethyldithiocarbamate solution (3.2) and, from a burette, 15ml of carbon tetrachloride (3.1).

Stopper the funnel, shake vigorously for two minutes and allow the layers to separate. Place a piece of cotton-wool in the stem of the funnel and run off the carbon tetrachloride layer into a dry 10mm spectrophotometer cell (4.1). Avoid undue exposure of the solution to light.

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

6.3 *Blank test*

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

6.4 *Calibration curve*

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

---

[1] Whatman No. 541 or equivalent.

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

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 = column of aliquot taken for colour development (ml);
W = weight of test portion in g;
F = dilution factor (from 6.2).

12. DETERMINATION OF COPPER BY THE ATOMIC ABSORPTION SPECTROPHOTOMETRIC METHOD

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of copper in complete feeding stuffs, protein concentrates and feed supplements.

2. PRINCIPLE

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

3. REAGENTS

The water used should be free from copper.

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

3.2 Hydrochloric acid solution 2M.

3.3 Hydrochloric acid solution 0·5M.

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

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

4. APPARATUS

4.1 Atomic absorption spectrophotometer with a copper hollow cathode lamp.

5. PREPARATION OF THE TEST SAMPLE

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

6. PROCEDURE

6.1 *Dissolution of sample*

Weigh, to the nearest 0·001g, approximately 10g of the sample as prepared under (5) into a silica dish or basin, and place the silica cover on top. Transfer to a cool muffle furnace. Raise the temperature to 450° ± 10°C and heat until no carbonaceous material remains. A slow current of air through the furnace during the initial stages of the ashing is advantageous. Care must be taken with high-fat content material to avoid ignition of the sample. When all the organic matter has been destroyed, cool, add 10ml 50% v/v hydrochloric acid solution (3.1) and evaporate to dryness on a water-bath. Extract the soluble salts from the residue with two successive 10ml portions of boiling 2M hydrochloric acid solution (3.2), decanting the solution each time through the same suitable filter-paper[1] into a 50ml graduated flask. Then add 5ml 50% v/v hydrochloric acid solution (3.1) and about 5ml 30% v/v nitric acid solution (3.4) to the residue in the basin, and take the mixture to dryness on a hot-plate at low heat. Finally, add a further 10ml of boiling 2M 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 the water and mix.

6.2 *Blank test*

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

6.3 *Determination*

6.3.1 Preparation of sample and blank test solutions:

Take an aliquot of the extract prepared as in 6.1 and dilute with 0·5M hydrochloric acid solution (3.3) to a known volume containing between 0 and 10μg per ml of copper. Treat the blank test solution (6.2) identically.

6.3.2 Preparation of standard solutions for calibration:

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

6.3.3 Measurement:

[1] Whatman No. 541 or equivalent.

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

7. CALCULATION OF RESULTS

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

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

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.

13. DETERMINATION OF DINITOLMIDE

(3,5-dinitro-2-toluamide)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of dinitolmide in complete feeding stuffs, protein concentrates and feed supplements. Nitrofuran derivatives may interfere. The lower limit of the determination is 40mg/kg.

2. PRINCIPLE

The sample is extracted with acetonitrile. The extract is purified on aluminium oxide and filtered. An aliquot of the filtrate is evaporated to dryness. The residue is dissolved in dimethylformamide and treated with diaminoethane forming a purple-coloured complex, the absorbance of which is measured at 560nm.

3. REAGENTS

3.1 Acetonitrile 85% (v/v): mix 850ml of acetonitrile, and 150ml of water. Before use distil the mixture and collect the fraction which boils between 75° and 77°C.

3.2 Aluminium oxide for column chromatography: heat at 750°C for at least 2 hours, cool in a desiccator and keep in an amber glass bottle with a ground-glass stopper. Before use de-activate as follows: place in an amber glass bottle 10g of aluminium oxide and 0·7ml of water, stopper, heat for 5 minutes in a bath of boiling water, with occasional shaking and allow to cool, shaking from time to time. Check the

activity by subjecting to analysis, starting from point 5.1, a measured quantity of standard solution (3.6). The recovery of the dinitolmide must be 100% ± 2%.

3.3 Dimethylformamide 95% (v/v): mix 95·0ml of dimethylformamide and 5·0ml of water.

3.4 Diaminoethane, colourless, maximum water content: 2·0%.

3.5 Standard substance: pure 3,5-dinitro-2-toluamide complying with the following characteristics:

Melting point: 177°C;
Molecular extinction coefficient at 248nm in acetonitrile: $13{\cdot}1 \times 10^3$;
Molecular extinction coefficient at 266nm in dimethylformamide: $10{\cdot}1 \times 10^3$.

3.6 Standard solution: weigh to the nearest 0·1mg, 40mg of pure dinitolmide (3.5), dissolve in acetonitrile (3.1) in a 200ml graduated flask, make up to volume with the same solvent and mix. Dilute 20·0ml to 100ml with acetonitrile (3.1) in a graduated flask and mix. 1ml of this solution contains 40μg of dinitolmide.

4. APPARATUS

4.1 Sintered-glass crucible, porosity G3, diameter 60mm.

4.2 Spectrophotometer, with 10mm cells.

5. PROCEDURE

5.1 *Extraction and purification*

Weigh, to within 0·001g, approximately 10g of the finely divided and mixed sample. For protein concentrates and feed supplements, weigh, to within 0·001g, approximately 1g. Place the test portion in a 250ml conical flask and add 65ml of acetonitrile (3.1). Mix, fit a reflux condenser to the flask and heat in a water-bath maintained at 50°C for 30 minutes, shaking frequently. Cool under a stream of cold water. Add 20g of aluminium oxide (3.2), shake for 3 minutes, allow to settle.

Filter the solution under suction through the sintered-glass crucible (4.1) transferring as much of the solid material as possible. Transfer the remaining solids to the sintered-glass crucible with a few ml of acetonitrile (3.1) and suck the residue dry. Release the partial vacuum and suspend the filter cake by stirring with a few drops of acetonitrile (3.1). Remove the liquid by applying suction, then repeat the suspension and filtration steps until a total volume of about 90ml has been collected. Transfer this filtrate to a 100ml graduated flask. Rinse the collecting vessel with a few millilitres of acetonitrile (3.1) and add this to the flask. Finally make up to volume and mix. If necessary, dilute an aliquot with acetonitrile (3.1) to obtain a solution containing 5 to 15μg of dinitolmide per ml.

5.2 *Determination*

Pipette into three 50ml beakers A, B and C respectively, 4·0ml of the solution obtained in 5.1. Add to beaker C 1·0ml of standard solution (3.6). Place the three beakers on the water-bath under a well-ventilated

hood, and evaporate until dry in a current of dry air. Cool the three beakers to room temperature.

Add 10·0ml of dimethylformamide (3.3) in beaker A and 2·0ml in beakers B and C respectively, leave in contact for a few minutes, stirring a little, until the residue completely dissolves. Then add 8·0ml of diaminoethane (3.4) in beakers B and C and mix. Exactly 5 minutes after adding the diaminoethane measure the absorbance of the three solutions at 560nm against the dimethylformamide (3.3) as a reference.

6. CALCULATION OF RESULTS

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

$$\frac{1000 \times F \times (E_B - E_A)}{W \times (E_C - E_B)}$$

in which:

E_A = absorbance of solution A (blank);
E_B = absorbance of solution B (sample);
E_C = absorbance of solution C (internal standard);
W = weight of test portion in g; and
F = dilution factor (from 5.1).

14. DETERMINATION OF ETHOPABATE

(methyl 4-acetamido-2-ethoxybenzoate)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of ethopabate in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 2mg/kg.

2. PRINCIPLE

The sample is extracted with diluted methanol. The solution is acidified and extracted with chloroform. The chloroform extract is washed first with an alkaline solution and then with water. The purified extract is concentrated, the ethopabate is hydrolysed with diluted hydrochloric acid. The amino derivative thus formed is diazotised and coupled with N-2-aminoethyl-1-naphthylamine. The coloured complex is extracted with butanol and the absorbance of the solution is measured at 555nm.

3. REAGENTS

3.1 Methanol.

3.2 Methanol, 50% (v/v): mix equal volumes of methanol (3.1) and water.

3.3 Hydrochloric acid (density 1·18g per ml).

3.4 Dilute hydrochloric acid: dilute 10·0ml of hydrochloric acid (3.3), to 100ml with water.

3.5 Hydrochloric acid, approximately 0·3M: dilute 25ml of hydrochloric acid (3.3), to 1000ml with water.

3.6 Chloroform.

3.7 Sodium carbonate solution: dissolve 4g sodium carbonate (anhydrous) in water and dilute to 100ml.

3.8 Sodium nitrite solution: dissolve 100mg of sodium nitrite in water and make up to 50ml with water in a graduated flask. Prepare immediately before use.

3.9 Ammonium sulphamate solution: dissolve 500mg of ammonium sulphamate in water and make up to 50ml in a graduated flask. Prepare immediately before use.

3.10 N-2-aminoethyl-1-naphthylamine solution: dissolve 100mg of N-2-aminoethyl-1-naphthylamine dihydrochloride in water and make up to 50ml with water in a graduated flask. Prepare immediately before use.

3.11 Sodium chloride.

3.12 Butan-1-ol.

3.13 Standard substance: pure ethopabate.

3.14 Standard solutions:

3.14.1 Solution of 0·040mg of ethopabate per ml: weigh to the nearest 0·1mg, 40mg of pure ethopabate (3.13). Dissolve in 50% v/v methanol (3.2) in a 100ml graduated flask; make up to volume with the same solvent and mix. Dilute 10·0ml to 100ml with 50% v/v methanol (3.2) in a graduated flask and mix. This solution is stable for a month.

3.14.2 Solution of 0·016mg of ethopabate per 20ml: dilute 5·0ml of the solution (3.14.1) to 250ml with 50% v/v methanol (3.2) in a graduated flask and mix well. Prepare immediately before use.

4. APPARATUS

4.1 Rotary vacuum evaporator, with 250ml flasks.

4.2 Spectrophotometer, with 10mm cells.

5. PROCEDURE

5.1 *Extraction*

Weigh to the nearest 0·001g, a quantity of the finely divided and mixed sample, containing about 80μg of ethopabate. Place the test portion in a 250ml conical flask and add 100·0ml of 50% v/v methanol (3.2). Mix, stopper the flask and shake for 1 hour with the aid of a mechanical shaker. Decant, filter and discard the first 5ml of the filtrate.

5.2 *Purification*

Note: All operations in this sub-section must be carried out rapidly. Transfer 20·0ml of the clear extract into a 100ml separating funnel, add 5·0ml of dilute hydrochloric acid (3.4) and 20·0ml of chloroform (3.6).

Shake, first carefully and then vigorously, for 3 minutes. Allow to stand until the layers separate and collect the chloroform phase in a second 100ml separating funnel.

Extract the acid layer twice more with 20·0ml of chloroform (3.6). Collect the chloroform extracts in the second separating funnel and discard the acid layer. Add to the combined chloroform solution 10ml of sodium carbonate solution (3.7), shake for 3 minutes and allow to stand until the layers separate. Collect the chloroform phase in a third 100ml separating funnel and discard the aqueous layer. Add to the chloroform solution 10ml of sodium carbonate solution (3.7), shake for 3 minutes and allow to stand until the layers separate.

Collect the chloroform phase in a fourth 100ml separating funnel, wash twice with 25·0ml of water each time, separate the aqueous layer and quantitatively collect the chloroform extract in a 250ml round bottom flask. Combine the aqueous layers in one of the separating funnels; rinse each empty funnel with a few millilitres of chloroform; shake the aqueous layer with the chloroform used for rinsing, allow layers to separate, and transfer the chloroform phase to the chloroform extract collected in the flask.

5.3 *Hydrolysis*

Evaporate the chloroform extract down to about 2ml on a water-bath at 50°C with the aid of the rotary vacuum evaporator (4.1). Dissolve the residue in 2 to 3ml of methanol (3.1), and transfer quantitatively the solution to a 50ml centrifuge tube with the aid of two 10ml portions and one 5ml portion of 0·3M hydrochloric acid (3.5). Add a few glass beads, shake well, and place the tube, un-stoppered, in a bath of boiling water for 45 minutes. Then cool under a stream of cold running water.

5.4 *Determination*

Add 1·0ml of sodium nitrite solution (3.8), stir and allow to stand for 2 minutes. Add 1·0ml of ammonium sulphamate solution (3.9), shake and allow to stand for 2 minutes. Add 1·0ml of N-2-aminoethyl-1-naphthylamine solution (3.10), stir and allow to stand for 10 minutes. Add 5·0g of sodium chloride (3.11) and 10·0ml of butan-1-ol (3.12), and shake vigorously until the sodium chloride has completely dissolved.

Draw off the supernatant butanolic solution with the aid of a pipette, and transfer it to a 15ml centrifuge tube and centrifuge. Then measure the absorbance E_A at 555nm against butan-1-ol (3.12) as reference.

5.5 *Reagent blank*

Carry out a blank test, using the same procedure, starting from point 5.2, on 20·0ml of diluted methanol (3.2). Measure the absorbance E_B at 555nm against butan-1-ol (3.12) as reference.

5.6 *Standard test*

Carry out a test, using the same procedure, starting from point 5.2 on 20·0ml of standard solution (3.14.2). Measure the absorbance E_C at 555nm against butan-1-ol (3.12) as reference.

6. CALCULATION OF RESULTS

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

$$\frac{80 \times (E_A - E_B)}{W \times (E_C - E_B)}$$

in which:

E_A = absorbance of the solution from the sample;
E_B = absorbance of the solution resulting from the reagent blank;
E_C = absorbance of the solution resulting from the standard test; and
W = weight of test portion in g.

15. DETERMINATION OF FLAVOPHOSPHOLIPOL (FLAVOMYCIN) (BAMBERMYCIN)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of flavophospholipol in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of determination is 1 mg/kg.

2. PRINCIPLE

The sample is extracted with diluted methanol by heating under reflux. After centrifuging, the extract is purified (where necessary) by treatment with ion exchange resins and diluted. Its antibiotic activity is determined by measuring the diffusion of flavophospholipol in an agar medium inoculated with *Staphylococcus aureus.* Diffusion is shown by the formation of zones of inhibition in the presence of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration.

3. MICRO-ORGANISM: *Staphylococcus aureus* ATCC 6538 P (NCIB 8625; NCTC 7447; NCDO 1963)

3.1 *Maintenance of the parent strain*

Inoculate *S. aureus* onto an agar slope prepared from the culture medium (4.1). Incubate for 24 hours at 37°C. Keep the culture in a refrigerator and re-inoculate agar slopes every month.

3.2 *Preparation of the bacterial suspension*

Set aside two tubes containing the stock culture (3.1) and re-inoculate them weekly. Incubate for 24 hours at 37°C and store in a refrigerator at about 4°C. Twenty-four hours before the assay, inoculate with this growth two to four tubes containing slopes of culture medium (4.1). Incubate for 16 to 18 hours at 37°C. Make a suspension of the growth in the sodium chloride solution (4.3). The light transmission of the suspension must be about 40%, measured at 578nm in a 10mm cell against sodium chloride solution (4.3) as a reference.

Make preliminary tests on assay plates using the culture medium (4.2.2) to determine the quantity of inoculum required to give the largest possible clear zones of inhibition with the various concentra-

tions of flavophospholipol used. The quantity will usually be 0·2 to 0·3ml per 1000ml. Inoculate the culture medium at a temperature between 48 and 50°C.

4. CULTURE MEDIA AND REAGENTS

4.1 Culture medium I

Peptone	6g
Tryptone	4g
Yeast extract	3g
Meat extract	1·5g
Glucose	1g
Agar	15g
Water	1000ml

pH after sterilisation: 6·5

4.2 Culture medium II

4.2.1 Base layer

Peptone	6g
Yeast extract	3g
Meat extract	1·5g
Agar	10g
Water	1000ml

pH after sterilisation: 6.5

4.2.2 Seed layer

As for culture medium I (4.1) with the addition of 2·0g of silicone anti-foaming emulsion (eg SE 2, Wacker Chemicals UK Ltd).

4.3 Sodium chloride solution: dissolve 4g of sodium chloride in water and dilute to 1000ml; sterilise.

4.4 Methanol.

4.5 Methanol, 50% solution (v/v).

4.6 Methanol, 80% solution (v/v).

4.7 Tris (hydroxymethyl) aminomethane.

4.8 Methanolic potassium chloride solution: dissolve 1·5g of potassium chloride in 20ml of water and make up the volume to 100ml with methanol (4.4).

4.9 Cation exchanger: Dowex 50 WX8 (HCR-W), 20 to 50 mesh, Na form or equivalent.

4.10 Anion exchanger: Dowex 1X2, 50 to 100 mesh, Cl form or equivalent. Before use, keep for 12 to 14 hours in 80% methanol (4.6).

4.11 Glass wool.

4.12 pH indicator paper (pH 6·6 to 8·1).

4.13 Ascorbic acid.

4.14 Standard substance: flavophospholipol of known activity.

5. APPARATUS

5.1 Glass tube for chromatography, internal diameter: 9mm; length; 150 to 200mm, fitted with a stopcock at the narrowed part of the lower end and a ground-glass joint (to connect with the dropping funnel (5.2)) at the upper end.

5.2 Dropping funnel 250ml, fitted with a stopcock and a ground-glass joint.

6. STANDARD SOLUTION

Dissolve an accurately weighed quantity of the standard substance (4.14) in 50% methanol (4.5) and dilute to give a stock solution containing 100μg flavophospholipol per ml. Stored in stoppered flasks at 4°C this solution is stable for up to two months.

Prepare from this solution, diluting with solution (4.5), a standard working solution S_8 containing 0·2μg flavophospholipol per ml. Then prepare the following concentrations by means of successive dilutions (1 + 1) using with the solution (4.5):

S_4 0·1μg per ml
S_2 0·05μg per ml
S_1 0·025μg per ml

7. PROCEDURE

7.1 *Extraction*

For concentrates, premixes and mineral feeds take a sample for analysis of 2 to 5g, weighed to the nearest 0·01g, and add about 150mg of ascorbic acid (4.13); for other feeds take a sample for analysis of 5 to 30g, weighed to the nearest 0·01g, and containing at least 30μg of flavophospholipol. Homogenize with 150ml of 50% methanol (4.5) in a conical flask and adjust the pH to 8·1 to 8·2 with about 400mg of tris (hydroxymethyl) aminomethane (4.7). Check the pH with indicator paper (4.12). Allow to stand for 15 minutes, then readjust the pH to 8·1 to 8·2 with tris (hydroxymethyl) aminomethane (4.7) and boil for 10 minutes under reflux with constant stirring. Allow to cool, centrifuge the mixture and decant the extract.

7.2 *Purification (this step may be omitted for concentrates, premixes and mineral feeds)*

Mix 110ml of the extract with 11g of the cation exchanger (4.9), boil for one minute under reflux with constant stirring. Separate the cation exchanger by centrifugation or filtration. Mix 100ml of the extract with 150ml of methanol (4.4) and store the solution for 12 to 15 hours at 4°C. Filter off the flocculent mass while cold.

Insert a glass wool plug (4.11) at the bottom end of a glass tube (5.1), pour into the tube 5ml of the anion exchanger (4.10) and wash the column with 100ml of 80% methanol (4.6). Using the funnel (5.2), transfer to the column a volume of filtrate of at least 100ml which is expected to contain 16μg of flavophospholipol (200ml for a 30g sample of feeding stuff at 1mg/kg). Where necessary, before application to the column, dilute the filtrate with 80% methanol (4.6) to obtain an expected flavophospholipol content of 16μg per 100ml. Adjust the flow

rate to about 2ml per minute. Discard the eluent. Then wash the column with 50ml of 80% methanol (4.6) and discard the eluent.

Elute the flavophospholipol and the methanolic solution of potassium chloride (4.8) keeping the flow rate to about 2ml per minute. Collect 50ml of the eluate, add 30ml of water and mix to obtain an expected concentration of flavophospholipol of 0·2μg per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with solution (4.5).

Where the purification step has been omitted dilute the extract with 50% methanol solution (4.5) to obtain an expected concentration of flavophospholipol of 0·2μg per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with solution (4.5).

7.3 *Determination*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the extract (U_8, U_4, U_2, U_1). These four concentrations of standard solution and extract must be placed in each plate. To this effect, select plates large enough to allow at least 8 wells with a diameter of 10 to 13mm, and not less than 30mm between centres, to be made in the agar medium.

Pour into the levelled plates a quantity of the melted culture medium (4.2.1) sufficient to give a base layer approximately 1·5mm thick, and allow to set.

Inoculate at 48–50°C with the bacterial suspension (3.2) a quantity of the melted culture medium (4.2.2) sufficient to give a seed layer approximately 1mm thick, swirl to mix thoroughly and pour onto the base layer, maintaining a level position.

When set remove agar plugs with a sterile cork borer to form wells as described above.

Pipette into each well an exactly measured and equal volume (0·10 to 0·15ml) of respectively solutions S_8, S_4, S_2, S_1 and U_8, U_4, U_2, U_1. Apply each concentration 4 times so that the determination is subject to an evaluation of 32 zones of inhibition.

Incubate the plates for approximately 18 hours at 28–30°C.

7.4 *Evaluation*

See *"Evaluation"* of avoparcin.

7.5 *Repeatability*

The difference between the results of two determinations carried out on the same sample by the same analyst should not exceed:

(a) 0·5mg/kg, in absolute value, for contents of flavophospholipol from 1 and up to 2mg/kg;

(b) 25% related to the highest value for contents greater than 2 and up to 10mg/kg;

(c) 20% related to the highest value for contents greater than 10 and up to 25mg/kg;

(d) 5mg/kg, in absolute value, for contents greater than 25 and up to 50mg/kg;

(e) 10% related to the highest value for contents above 50mg/kg.

16. DETERMINATION OF FURAZOLIDONE

[3-(5-nitrofurfurylideneamino)oxazolidin-2-one]

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of furazolidone in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 10mg/kg.

2. PRINCIPLE

After a preliminary extraction with light petroleum to remove fat, the sample is extracted with acetone. The extract is purified on a column of aluminium oxide and the furazolidone is eluted with acetone. The acetone eluate is evaporated to dryness and the residue dissolved in pentanol. Furazolidone is then extracted from the pentanol with aqueous urea solution the absorbance of which is measured at 375nm.

3. REAGENTS

3.1 Acetone.

3.2 Aluminium oxide for chromatography, 100–240 mesh, prepared as follows: stir 500g of the aluminium oxide with 1 litre of hot water and decant the supernatant liquid. Repeat this procedure twice more, and finally filter through a Buchner funnel. Dry the aluminium oxide at 105°C to constant weight.

3.3 Pentyl acetate.

3.4 Pentan-1-ol. (Material containing mixed isomers is acceptable).

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

3.6 Urea solution. Mix 90g of urea with 100ml of water, dissolve completely by warming gently.

3.7 Standard substance: pure furazolidone.

3.8 Standard solution: Weigh, to the nearest 0·1mg, 25mg of standard substance (3.7) dissolve in acetone (3.1) in a 250ml graduated flask (4.1), make up to volume with acetone (3.1) and mix. 1ml of this solution contains 100μg of furazolidone.

4. APPARATUS

4.1 Amberglass 100 and 250ml volumetric flasks.

4.2 Amberglass 100ml separating funnels.

4.3 Glass tubes for chromatography, internal diameter 10mm, length 300mm.

4.4 Spectrophotometer with 10mm cells.

5. PROCEDURE

Note: All procedures should be carried out in subdued light.

5.1 *Extraction*

Weigh, to within 0·001g, 5 to 20g of the finely divided and mixed sample (containing not more than 1mg of furazolidone) into an extraction thimble and transfer it to an extraction apparatus. Extract with light petroleum (3.5), ensuring, in the case of a Soxhlet apparatus, 13 to 17 cycles of solvent; if other extractors are used, allow not less than 30 minutes for this stage. Remove the thimble from the apparatus, drain off the residual solvent and dry the thimble and the extracted feed in a current of warm air. Place the dried thimble and contents in a clean extraction apparatus and extract with acetone (3.1), allowing at least 25 cycles of solvent when a Soxhlet apparatus is being used. The exact conditions for achieving complete extraction with any particular apparatus should be pre-determined. Evaporate the acetone extract to a volume of 5–10ml on a steam bath, and cool to room temperature.

5.2 *Chromatography*

Insert a plug of glass wool into the lower end of a chromatography tube (4.3) and tamp it down with a suitable rod to a thickness of 2 to 3mm. Prepare a slurry of aluminium oxide (3.2) with acetone (3.1), pour into the tube and allow to settle. The prepared column should be about 200mm in height. Allow the acetone layer to drain down to the top of the column.

Transfer the acetone extract obtained in 5.1 from the flask to the column, rinse the flask several times with acetone (3.1) and transfer the liquid onto the column. Place a suitable flask under the column and elute the furazolidone with acetone (3.1); the total volume of acetone used, including that used for rinsing, should be about 150ml.

5.3 *Extraction and measurement of the absorbance*

Evaporate the acetone eluate (5.2) just to dryness on a steam bath. (On occasions a small quantity of diacetone alcohol, produced by condensation of acetone on the aluminium oxide may be left but this will not interfere with the subsequent extractions.) Dissolve the residue in 10ml of pentan-1-ol (3.4) and transfer the solution to a separating funnel. Repeat the process using 10ml of pentyl acetate (3.3) as a rinse liquid. Finally rinse the vessel which contained the extract residue with 10ml of urea solution (3.6), add this to the separating funnel and shake fairly vigorously for two minutes.

Allow the phases to separate for a period of three to four minutes before transferring the aqueous extract to a 100ml graduated flask (4.1). Repeat the rinsing and extraction stages with four further 10ml aliquots of urea solution (3.6) and transfer the aqueous extracts to the graduated flask. Dilute the contents of the graduated flask to 100ml with urea solution (3.6) and mix. Measure the absorbance of the solution in the spectrophotometer (4.4) at 375nm against urea solution (3.6) in the reference cell. Determine the quantity of furazolidone by referring to the calibration curve (5.4).

5.4 *Calibration curve*

Prepare four chromatographic columns as described in 5.2. Transfer into separate columns volumes of 2·5, 5·0, 7·5 and 10·0ml respectively of the standard solution (3.8). Wash each of the four columns with 150ml of acetone (3.1) and continue as in paragraph 5.3. Plot the calibration curve, using the absorbance values as ordinates and the corresponding quantities of furazolidone in μg as abscissae.

6. CALCULATION OF RESULTS

The furazolidone content in mg/kg is given by the formula:

$$\frac{A}{W}$$

in which:

A = quantity of furazolidone in μg as determined by photometric measurement.

W = weight of test portion in g.

17. DETERMINATION OF MONENSIN SODIUM BY DIFFUSION IN AN AGAR MEDIUM

1. PURPOSE AND SCOPE

The method is for the determination of monensin sodium in complete feedingstuffs, protein concentrates and feed supplements. The lower limit of determination is 10mg/kg.

2. PRINCIPLE

The sample is extracted with 90% methanol. The extract is submitted to appropriate procedures according to the monensin sodium content of the sample. The antibiotic activity is determined by measuring the diffusion of monensin sodium in an agar medium inoculated with *Bacillus subtilis*. Diffusion is shown by the formation of zones of inhibition of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration over the range of antibiotic concentrations employed. The sensitivity of this assay system is reduced in the presence of sodium ions.

3. MICRO-ORGANISM: *Bacillus subtilis* ATCC 6633 (NCIB 8054)

3.1 *Maintenance of stock culture*

Inoculate tubes containing slopes of culture medium (4.1) with *Bacillus subtilis* and incubate overnight at 30°C. Store the culture in a refrigerator at about 4°C. Reinoculate every month.

3.2 *Preparation of the spore suspension*[1]

Harvest the growth from a recently prepared agar slope (3.1) by means of 3 to 3 ml of sterile water. Use this suspension to inoculate 300ml of culture medium (4.1) contained in a Roux flask and incubate for three to five days at 30°C. Harvest the growth in 15ml of 20% ethanol (4.3), after having checked sporulation under the microscope and mix well. This suspension may be kept for at least five months at about 4°C.

4. CULTURE MEDIA AND REAGENTS

4.1 Culture medium[2]

Tryptone	10·0g
Yeast extract	3·0g
Meat extract	1·5g
Glucose	1·0g
Agar (according to quality)	10·0 to 20·0g
Water	1000ml

pH 6·5 (after sterilization).

4.2 Assay medium

Glucose	10·0g
Yeast extract	2·5g
Dipotassium hydrogen phosphate, K_2HPO_4	0·69g
Potassium dihydrogen phosphate, KH_2PO_4	0·45g
Agar (according to quality)	10·0 to 20·0g
Water	1000ml

pH6 (after sterilization).

4.3 Ethanol 20% (v/v): dilute 20ml of ethanol with 800ml of water.

4.4 Methanol, anhydrous.

4.5 Methanol 90% (v/v): dilute 900ml of methanol (4.4) with 100ml of water.

4.6 Methanol 50% (v/v): dilute 500ml of methanol (4.4) with 500ml of water.

4.7 Aluminium oxide, granulated (alcoa F, 20 mesh; activated alumina UG1: F. Lancaster and Co., or equivalent).

4.8 Standard substances: monensin sodium of known activity (eg from the International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, UK-Surrey KT15 3NB).

5. APPARATUS

5.1 Rotary vacuum evaporator, with a 250ml round-bottom flask.

5.2 Glass tube for chromatography, internal diameter: 25mm, length: 400mm, with an open end of 2mm diameter.

5.3 Glass tube for chromatography, internal diameter: 11mm, length: approximately 300mm, with an open end of 2mm diameter.

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[1] Other methods may be used provided that it has been established that they give similar spore suspensions.

[2] Any commercial culture medium of similar composition and giving the same results may be used.

6. STANDARD SOLUTIONS

Dissolve an accurately weighed quantity of the standard substance (4.8) in methanol (4.4) and dilute to give a stock solution containing 800μg monensin sodium per ml. Stored in stoppered flasks at 4°C, this solution is stable for up to two weeks.

From this stock solution prepare by successive dilution with 50% methanol (4.6) the following solutions:

S_8 8·0μg/ml
S_4 4·0μg/ml
S_2 2·0μg/ml
S_1 1·0μg/ml

7. PREPARATION OF THE EXTRACT

7.1 *Extraction*

7.1.1 *Protein concentrates, premixes*

Weigh out a quantity of sample of 2g, and 100ml of 90% methanol (4.5), homogenize and centrifuge for a few minutes. Dilute the supernatant solution with 50% methanol (4.6) to obtain an expected monensin sodium content of 8μg/ml ($=U_8$).

7.1.2 *Complete feedingstuffs with a level of monensin sodium not lower than 50mg/kg*

Weigh out a quantity of sample of 10 to 20g, add 100ml of 90% methanol (4.5), homogenize for 15 minutes and leave to settle.

Insert a cotton-wool plug at the narrow end of a glass tube (5.2) and add aluminium oxide (4.7) with gentle tapping until the column reaches 75 to 80mm high.

Decant the extract on to the aluminium oxide column and collect the filtrate. Dilute 30ml of the filtrate to 50ml with water. Make subsequent dilutions with 50% methanol (4.6) to obtain an expected monensin sodium content of 8μg/ml ($=U_8$).

7.1.3 *Complete feedingstuffs with a level of monensin sodium lower than 50mg/kg (up to the limit of 10mg/kg)*

Weigh out a quantity of sample of 10 to 20g, add 100ml of 90% methanol (4.5) and homogenize for 15 minutes. Centrifuge till clear.

For a sample containing 20mg/kg of monensin sodium, take 40ml of the supernatant liquid. For a sample containing 10mg/kg, take 80ml and evaporate to dryness under vacuum on a rotary evaporator (5.1) at not more than 40°C. Dissolve the residue in 10ml of 90% methanol (4.5).

Insert a cotton-wool plug at the narrow end of a glass tube (5.3) and add aluminium oxide (4.7) with gentle tapping until the column reaches 75 to 80mm high.

Decant the methanolic solution of the residue on to the aluminium oxide column and collect the filtrate. Wash the column with 10ml of 90% methanol (4.5) and combine the washings with the filtrate.

Evaporate the solution to dryness under vacuum on a rotary evaporator (5.1) at less than 40°C. Dissolve the residue in 10ml of anhydrous methanol (4.4) and make up to 20ml with water. Centrifuge the solution at at least 4000 r/min for at least five minutes. Make subsequent dilutions with 50% methanol (4.6) to obtain an expected monensin sodium content of 8μg/ml (= U_8).

7.2 *Assay solutions*

From solution U_8 prepare solutions U_4 (expected content: 4μg/ml), U_2 (expected content: 2μg/ml) and U_1 (expected content: 1μg/ml) by means of successive dilution (1 + 1) with 50% methanol (4.6).

8. ASSAY PROCEDURE

8.1 *Inoculation of the assay medium*

Inoculate the assay medium (4.2) with the spore suspension (3.2) at 50 to 60°C. By preliminary trials on plates with assay medium (4.2) determine the quantity of spore suspension required to give the largest and clearest zones of inhibition with the various concentrations of monensin sodium.

8.2 *Preparation of the plates*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the assay solution (U_8, U_4, U_2, U_1). These four concentrations of extract and standard must necessarily be placed in each plate. To this effect, select plates big enough to allow at least eight holes with a diameter of 10 to 13mm and not less than 30mm between centres to be made in the agar medium. The test may be carried out on plates consisting of a sheet of glass with a faced aluminium or plastic ring placed on top, 200mm in diameter and 20mm high.

Pour into the plates a quantity of the medium (4.2), inoculated as in 8.1, to give a layer about 2mm thick (60ml for a plate of 200mm diameter). Allow to set in a level position, bore the holes and place in them exactly measured volumes of assay and standard solutions (between 0·10 and 0·15ml per hole, according to the diameter). Apply each concentration at least four times so that each determination is subject to an evaluation of 32 zones of inhibition.

8.3 *Incubation*

Incubate the plates for approximately 18 hours at 35 to 37°C.

9. EVALUATION

See "*Evaluation*" of avoparcin.

10. REPEATABILITY

The difference between the results of two determinations carried out on the same sample, by the same analyst, should not exceed:

—20% related to the highest value for contents of monensin sodium from 10 to 25mg/kg;

—5mg/kg, in absolute value, for contents of 25 to 50mg/kg;

—10% related to the highest value for contents above 50mg/kg.

18. DETERMINATION OF NICARBAZIN

(equimolecular mixture of 1,3-bis(4-nitrophenyl)urea and 4,6-dimethylpyrimidin-2-ol)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of nicarbazin in complete feeding stuffs, protein concentrates and feed supplements containing not more than 5% grassmeal. Nitrofuran derivatives, acinitrazole and carbadox may interfere. The lower limit of the determination is 20mg/kg.

2. PRINCIPLE

The sample is extracted with dimethylformamide. The extract is purified by chromatography on a column of aluminium oxide; the nicarbazin is eluted with ethanol. The eluate is treated with ethanolic sodium hydroxide forming a yellow colour, the absorbance of which is measured at 430nm.

3. REAGENTS

3.1 Dimethylformamide.

3.2 Aluminium oxide for column chromatography: heat at 750°C for at least 2 hours, cool in desiccator and keep in an amber glass bottle with a ground-glass stopper. Before use, check activity by subjecting to analysis, starting from point 5.2, a measured quantity of standard solution (3.8.3). The recovery of the nicarbazin must be 100% ± 2%.

3.3 Ethanol, 95% v/v.

3.4 Ethanol, 80% v/v.

3.5 Sodium hydroxide solution: dissolve 50g sodium hydroxide in water and dilute to 100ml.

3.6 Ethanolic sodium hydroxide: measure 1ml of sodium hydroxide solution (3.5) into a 50ml graduated flask; make up to volume with 80% ethanol (3.4). The solution must be freshly prepared.

3.7 Standard substance: pure nicarbazin, molecular extinction coefficient at 362nm in dimethylformamide: $37{\cdot}8 \times 10^3$.

3.8 Standard solutions:

Note: Nicarbazin solutions should be protected from light.

3.8.1 Solution of 1·25mg of nicarbazin per ml: weigh to the nearest 0·1mg, 125mg of pure nicarbazin (3.7). Dissolve in 75ml of dimethylformamide (3.1) in a 100ml graduated flask with gentle heat. Allow to cool, make up to volume with the same solvent and mix.

3.8.2 Solution of 0·125mg of nicarbazin per ml: dilute 10·0ml of the solution (3.8.1) to 100ml with dimethylformamide (3.1) in a graduated flask and mix.

3.8.3 Solution of 0·025mg of nicarbazin per ml: dilute 20·0ml of the solution (3.8.2) to 100ml with dimethylformamide (3.1) in a graduated flask and mix.

4. APPARATUS

4.1 Glass tube for chromatography (internal diameter: 25mm; length: 300mm).

4.2 Spectrophotometer, with 10mm cells.

5. PROCEDURE

5.1 *Extraction*

Weigh, to within 0·001g, approximately 10g of the finely divided and mixed sample. For protein concentrates and feed supplements, weigh to the nearest 0·001g approximately 1g. Transfer the test portion to a 250ml conical flask and add exactly 100ml of dimethylformamide (3.1). Mix, fit a reflux condenser to the flask and heat on a water-bath for 15 minutes, shaking from time to time. Cool under a stream of cold water. Pour the supernatant layer into a centrifuge tube and centrifuge for about 3 minutes. If necessary, dilute 25·0ml of the supernatant layer with dimethylformamide (3.1), to obtain a solution containing 2·0 to 10μg of nicarbazin per ml.

5.2 *Chromatography*

Pour into the chromatographic tube (4.1) a slurry of 30g of aluminium oxide (3.2) in dimethylformamide (3.1). Allow the liquid level to fall to 10mm above the column of aluminium oxide and then put onto the column 25·0ml of the extract obtained in 5.1. Allow the liquid to flow through, not allowing the column to become dry, and wash the column with three 10ml portions of dimethylformamide (3.1). Then elute with 70ml of 95% ethanol (3.3). Discard the first 10ml of the eluate and collect the three fractions in the following order:

(a) a 5ml fraction;

(b) a 50ml fraction in a graduated flask;

(c) a 5ml fraction.

Check that fractions *(a)* and *(c)* do not turn yellow when ethanolic sodium hydroxide (3.6) is added. Continue the operations on fraction *(b)* as shown in 5.3.

5.3 *Determination*

Pipette two 20·0ml portions of fraction *(b)* into separate 25ml graduated flasks A and B. Add to flask A 5·0ml of ethanolic sodium hydroxide (3.6) and to flask B 5·0ml of 95% ethanol (3.3). Mix well.

Within five minutes measure the absorbance of both solutions at 430nm, against a mixture of 20·0ml of 95% ethanol (3.3) and 5·0ml of ethanolic sodium hydroxide (3.6) as a reference.

Subtract the value of the absorbance of solution B from that of solution A. From this value determine the quantity of nicarbazin by reference to the calibration curve (5.4).

5.4 *Calibration curve*

Chromatograph 25·0ml of the standard solution (3.8.3) as described in 5.2. Transfer 2·0, 4·0, 6·0, 8·0 and 10·0ml portions (corresponding to 25, 50, 75, 100 and 125μg of nicarbazin respectively) of fraction *(b)* into 25ml graduated flasks from a burette. To each flask add 5·0ml of ethanolic sodium hydroxide (3.6), make up to volume with 95% ethanol (3.3) and mix well.

Within five minutes measure the absorbance of the solutions at 430nm, against a mixture of 20·0ml of 95% ethanol (3.3) and 5·0ml of ethanolic sodium hydroxide (3.6) as a reference.

Plot the calibration curve, using the absorbance values as the ordinates and the corresponding quantities of nicarbazin in μg as the abscissae.

6. CALCULATION OF RESULTS

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

$$\frac{10 \times A \times F}{W}$$

in which:

A = quantity of nicarbazin in μg as determined by photometric measurement;
W = weight of test portion in g; and
F = dilution factor (from 5.1).

19. DETERMINATION OF NIFURSOL

[3,5-dinitro-2-(5-nitrofurfurylidene)salicylohydrazide]

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of nifursol in complete feeding stuffs, protein concentrates and feed supplements. Other substances that will provide a nitro group under the conditions of the method, eg. nitrofurazone and furazolidone, will interfere. The lower limit of the determination is 20mg/kg.

2. PRINCIPLE

The sample is extracted with dimethylformamide and the extract is purified on a column of aluminium oxide. A portion of the purified extract containing the nifursol is treated with phenylhydrazine hydrochloride and the resulting phenylhydrazone extracted into toluene. The addition of hyamine hydroxide to the toluene solution produces a blue colour, the absorbance of which is measured at 515nm.

3. REAGENTS

3.1 Toluene.

3.2 Aluminium oxide for column chromatography, 80 to 200 mesh,

alkaline, Brockman activity 1. To 100 parts of the aluminium oxide add 6 parts of powdered magnesium hydroxide. Shake in a screw-cap bottle to mix, add 8 parts of water, and mix until free from lumps.

3.3 Sand: acid washed.

3.4 Dimethylformamide solution, 95% v/v.

3.5 Dimethylformamide solution, 50% v/v.

3.6 Phenylhydrazine hydrochloride solution: shake 0·25 ± 0·005g of phenylhydrazine hydrochloride in 25ml of water, add 25ml of concentrated hydrochloric acid, and shake to dissolve the solid, filtering if necessary. Prepare this reagent immediately before use.

3.7 Hyamine 10-X hydroxide solution: about 10% in methanol. (Note: Hyamine is a trade mark of the Rohm & Haas Company. The reagent solution can be obtained from BDH Chemicals Ltd.)

3.8 Nifursol standard solution: weigh, to the nearest 0·1mg, 25mg of pure nifursol into a 100ml graduated flask, add 5ml of 95% v/v dimethylformamide solution (3.4), and mix until all the solid has dissolved. Dilute to the mark with methanol. Prepare this solution freshly each day.

4. APPARATUS

4.1 Chromatographic column—A glass column, internal diameter: 20 to 25mm; length: 100 to 150mm plugged at the lower end with glass wool.

4.2 Spectrophotometer, with 10mm cells.

5. PROCEDURE

5.1 *Extraction*

Weigh to the nearest 0·001g, approximately 5g of the finely divided and mixed sample, or a suitable amount expected to contain about 350μg of nifursol and transfer to a 125ml conical flask. Add 50·0ml of 95% v/v dimethylformamide solution (3.4), insert a stopper loosely, and place the flask in a water-bath at 60° ± 5°C for 30 minutes. Swirl the contents of the flask occasionally during this period. Shake the flask on a mechanical shaker for 30 minutes and then filter the contents through a rapid filter-paper, preferably under reduced pressure on a Buchner funnel. Transfer 40·0ml of the filtrate to a beaker, add 40·0ml of water, and stir. Set the beaker aside, protected from light, for 30 minutes.

5.2 *Purification*

Pack the chromatographic column (4.1) to a depth of 70mm with the prepared aluminium oxide (3.2) and on top of the aluminium oxide add a layer of sand (3.3) 15mm deep. Wash the column with 50ml of 50% v/v dimethylformamide solution (3.5) and then pass the dimethylformamide extract of the test sample through the column; reject the first 45ml of eluate and collect the next 17ml.

5.3 *Determination*

Pipette 5·0ml of the eluate to a 20ml centrifuge tube, add 5ml of phenylhydrazine hydrochloride solution (3.6), mix, and place the tube

in a water-bath at 40° ± 2°C for 20 minutes. Remove the tube from the water-bath and cool it in running water for 5 minutes. Add 5·0ml of toluene (3.1) to the contents of the tube, insert a glass or plastic stopper (a rubber stopper must not be used), and shake vigorously 40 times. Centrifuge for 5 minutes to clear the toluene layer, and transfer 3·0ml of the toluene layer to a 10mm spectrophotometer cell. Add 0·2ml of hyamine hydroxide solution (3.7), mix immediately, and measure the absorbance of the solution within one minute at 515nm with toluene as reference. Determine the quantity of nifursol by reference to the calibration curve (5.4).

5.4 *Calibration curve*

Pipette 5·0ml of nifursol standard solution (3.8) to a 200ml graduated flask, add 100ml of 95% v/v dimethylformamide solution (3.4), dilute to the mark with water and mix. Into separate 20ml centrifuge tubes transfer by pipette 1, 2, 3, 4 and 5ml portions of this solution and dilute the contents of each tube to 5ml with 50% v/v dimethylformamide solution (3.5).

Treat the contents of each tube as described under "Determination" (5.3) beginning at ". . . add 5ml of phenylhydrazine hydrochloride solution (3.6) . . .". Plot the calibration curve using the absorbances as the ordinates and the corresponding quantities of nifursol in μg as abscissae.

6. CALCULATION OF RESULTS

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

$$\frac{20 \times A}{W}$$

in which:

A = μg of nifursol read from the calibration curve; and
W = weight of the test portion in g.

20. DETERMINATION OF NITROFURAZONE

(5-nitro-2-furaldehyde semicarbazone)

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of nitrofurazone in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 30mg/kg.

2. PRINCIPLE

The sample is treated with light petroleum in order to remove interfering substances. The sample is then extracted with acetone and the extract then evaporated to dryness. The residue containing the nitrofurazone is treated with alkaline dimethylformamide to form a coloured solution the absorbance of which is measured at 530nm.

3. REAGENTS

3.1 Acetone.

3.2 Dimethylformamide: test the suitability of the reagent by developing the colour from nitrofurazone with solutions of phenol and sodium hydroxide (see 5.2 and 5.3 below); the colour should remain stable for at least two hours.

3.3 Light petroleum, boiling range 40° to 60°C.

3.4 Phenol solution: Dissolve 5g of phenol in dimethylformamide (3.2) and dilute to 100ml with dimethylformamide.

3.5 Potassium permanganate, 0·1M solution.

3.6 Sodium dithionite solution: dissolve 1g of sodium dithionite, $Na_2S_2O_4$ in M sodium hydroxide solution and dilute to 100ml with M sodium hydroxide solution. Prepare this solution immediately before use.

3.7 Sodium hydroxide, M solution.

3.8 Nitrofurazone standard solution: weigh to the nearest 0·1mg, 100mg of nitrofurazone (B.Vet C grade) dissolve in dimethylformamide and dilute to 100ml with dimethylformamide. Dilute 10ml of this solution to 100ml with dimethylformamide. 1ml of this solution contains 100μg nitrofurazone. Prepare the dilute solution immediately before use.

4. APPARATUS

4.1 Spectrophotometer with 10mm glass cells.

4.2 Rotary evaporator.

5. PROCEDURE

5.1 *Extraction of interfering substances*

Weigh to the nearest 0·001g an amount of the finely divided and mixed sample expected to contain 1mg of nitrofurazone into an extraction thimble; cover the sample with a small pad of cottonwool. Insert the packed thimble into an extraction apparatus and extract the sample with light petroleum (3.3); use an electric heating mantle as the source of heat, so adjusted that the solvent cycles twenty times in about 45 minutes, and sufficient solvent so that the volume in the flask throughout the operation is not less than 25ml.

Remove the packed thimble, allow the solvent to drain, and carefully remove any residual solvent in a current of warm air at a temperature not exceeding 60°C.

5.2 *Extraction of the nitrofurazone*

Transfer the packed thimble to a clean extraction apparatus, and extract the sample with acetone (3.1); use an electric heating mantle as a source of heat so that the solvent cycles twenty times in about one hour, and sufficient solvent so that the volume in the flask throughout the operation is not less than 25ml. During the extraction shield the apparatus from light with a cardboard cylinder containing a small inspection window, or by any other suitable means. When the extraction is complete, rapidly cool the flask containing the extract to

20°C, and add 0·1M potassium permanganate solution (3.5), drop by drop, until a faint pink colour is obtained that persists for about two seconds (about four drops are required). Evaporate the extract on a water-bath to a volume of about 5ml, shielding the extract from light. It is important at this stage to avoid evaporating to dryness.

Remove the flask from the water-bath, place an externally ribbed conical filter funnel into the neck of the flask, and evaporate off the residual acetone under vacuum or by blowing a current of warm air (temperature not exceeding 60°C) across the top of the funnel in such a way that a slight turbulance is produced on the surface of the liquid in the flask. A rotary evaporator (4.2) may be used for the evaporation stages.

Dissolve the residue in dimethylformamide (3.2), transfer the solution quantitatively to a 50ml graduated flask, suitably shielded from light, and dilute to the mark at 20°C with dimethylformamide. Transfer a suitable portion containing about 200μg of nitrofurazone to each of two 50ml graduated flasks containing 5ml of phenol solution (3.4).

5.3 *Determination*

To the contents of one flask add 2·5ml of M sodium hydroxide solution (3.7) and dilute to the mark at 20°C with dimethylformamide (3.2); this is the sample solution. To the contents of the other flask add 2·5ml of sodium dithionite solution (3.6), and dilute to the mark at 20°C with dimethylformamide; this is the blank solution and it should be a pale lemon-yellow colour free from any red or purplish tinge. Centrifuge until clear solutions are obtained. Measure the absorbance of the sample solution in a 10mm cell at 530nm against the blank solution as reference. Determine the quantity of nitrofurazone by reference to the calibration curve (5.4).

5.4 *Calibration curve*

Transfer amounts of nitrofurazone standard solution (3.8) corresponding to 100, 150, 200, 250 and 300μg of nitrofurazone into two series of 50ml graduated flasks each containing 5ml of phenol solution (3.4), and proceed as described under 5.3. Plot the calibration curve using the absorbances as the ordinates and the corresponding quantities of nitrofurazone in μg as abscissae.

6. CALCULATION OF RESULTS

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

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

in which:

A = weight of nitrofurazone in aliquot taken for colour development as read from the calibration curve (μg);
V = volume of aliquot taken for colour development (ml); and
W = weight of test portion in g.

21. DETERMINATION OF NITROVIN

1-3(-(5-nitro-2-furyl)-1-[2-(5-nitro-2-furyl)vinyl]allylideneamino) guanidine hydrochloride

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of nitrovin in complete feeding stuffs and protein concentrates. The lower limit of the determination is 5mg/kg.

2. PRINCIPLE

After a preliminary extraction with hexane the sample is extracted with dimethylformamide. This extract is cleaned up on a column of aluminium oxide and the nitrovin is eluted with dimethylformamide containing ammonium hydroxide. The absorbance of the eluate is measured at 495nm, a portion of the eluate is acidified and the absorbance of this solution is subtracted from the absorbance of the alkaline solution to give the corrected absorbance, from which the nitrovin content is calculated by reference to a standard curve.

3. REAGENTS

3.1 Aluminium oxide, for column chromatography: heat overnight in a muffle furnace at 600°C. Test the suitability of each batch of aluminium oxide by adding 15ml of dilute nitrovin standard solution (3.7) to the column and eluting as described in 5.3. At least 95% recovery should be indicated.

3.2 Dimethylformamide.

3.3 Eluting solution: dilute 20ml of ammonium hydroxide solution (density 0·88g per ml) to 1 litre with dimethylformamide.

3.4 Hexane: fraction from petroleum.

3.5 Hydrochloric acid solution: dilute a suitable volume of hydrochloric acid (density 1·18g per ml) with an equal volume of water.

3.6 Dichloromethane.

3.7 Nitrovin standard solution: weigh to the nearest 0·1mg, 100mg of pure nitrovin and transfer it to a 100ml graduated flask. Dissolve in, and dilute to volume with the eluting solution (3.3), and mix well. Transfer by pipette 10·0ml of this solution to a 100ml graduated flask, dilute to volume with the eluting solution (3.3) and mix well. This gives a solution containing 100μg per ml. Transfer by pipette 10·0ml of the 100μg per ml solution to a 100ml calibrated flask, dilute to volume with the eluting solution (3.3) and mix well. This gives a solution containing 10μg per ml (solution 3.7).

4. APPARATUS

4.1 Chromatographic column—diameter: 15mm; length: 250mm, with polytetrafluoroethylene stopcock and 250ml reservoir.

4.2 Filter-paper, glass fibre (Whatman GF/A or equivalent).

4.3 Spectrophotometer with 40mm cells.

5. PROCEDURE

Warning: It is necessary that the glassware used should be completely dry, as a small amount of water in the feed extract will adversely affect the aluminium oxide chromatography by causing the band to become diffuse, which could lead to low recovery.

5.1 *Extraction of interfering substances*

Weigh, to the nearest 0·001g, approximately 50g of the finely divided and mixed sample, transfer to a bottle of not less than 350ml capacity fitted with a polyethylene lined screw cap. Add 5ml of hexane (3.4) for each gram of feed taken, close the bottle and shake for 1 hour. Filter the contents of the bottle through two layers of glass fibre filter-paper (4.2) on a Buchner funnel, and wash with an equal amount of fresh hexane (3.4). Continue to suck air through the filter for 15 minutes. Transfer the filter-paper and sample to an aluminium tray and dry in a vacuum oven at 65°C for 1 hour.

5.2 *Extraction of nitrovin*

Transfer the dried sample and glass fibre filter-paper (5.1) to a bottle, add 2·5ml of dimethylformamide (3.2) for each gram of feed present, cap with a polyethylene lined screw cap and shake for 1 hour. Filter the extract through two layers of glass fibre paper (4.2) on a Buchner funnel.

5.3 *Purification*

Place a plug of glass wool in the bottom of the 15mm diameter glass tube (4.1), close the stopcock and fill the barrel of the column with dichloromethane (3.6). Tamp with a glass rod to remove air bubbles, and add 15g of aluminium oxide (3.1). Allow the aluminium oxide to settle by gravity, and drain the solvent until the surface reaches the level of the aluminium oxide bed. Close the stopcock. Transfer by pipette 20·0ml of the feed extract to the column. Allow the extract and subsequent washes to percolate through the column at a rate of 30 drops per minute. When the surface of the extract has reached the level of the aluminium oxide bed, wash the column with two 5ml portions of dimethylformamide (3.2). When the surface of the second wash has reached the level of the aluminium oxide bed, wash the column with sufficient dimethylformamide to wash out the rapidly descending yellow band without eluting any of the slower moving red nitrovin band. When the surface of the final wash reaches the surface of the aluminium oxide bed, discard the effluent that has been collected and place a 100ml graduated flask under the column. Add about 125ml of the eluting solution (3.3) to the reservoir and allow it to percolate through the column at a rate of 30 drops per minute. Collect 100ml of the eluate.

5.4 *Determination*

Mix the eluate well and measure its absorbance at 495nm with the eluting solution as reference. Withdraw a further 25ml of the eluate from the flask and add to this 2ml of hydrochloric acid solution (3.5) (no correction is made for the additional volume as the reading is small). Mix well, measure the absorbance of the acidified eluate at 495nm against the eluting solution, and subtract this value from the absorbance of the alkaline eluate to determine the corrected absor-

bance. Determine the concentration of nitrovin in the sample solution by reference to the calibration curve (5.5).

5.5 *Calibration curve*

Transfer by pipette 2·0, 4·0, 6·0 and 8·0ml portions of nitrovin standard solution (3.7) to separate 50ml graduated flasks. Dilute the contents of each to volume with the eluting solution (3.3), and mix well. These solutions have concentrations of 0·4, 0·8, 1·2 and 1·6μg of nitrovin per ml, respectively. Measure the absorbances of these solutions at 495nm against the eluting solution as reference.

Withdraw 25ml of each of these standard solutions, place in separate flasks, acidify by the addition of 2ml of hydrochloric acid solution (3.5) and mix well. If any of these solutions become turbid clarify them by spinning in a centrifuge. Measure the absorbances of the acidified solutions in the same manner as for the alkaline solutions and subtract the reading from the absorbance of the appropriate alkaline solution to give the corrected absorbance. Plot the calibration curve using the absorbances as the ordinates and the corresponding concentrations of nitrovin in μg per ml as abscissae.

6. CALCULATION OF RESULTS

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

$$\frac{5 \times C \times V}{W}$$

in which:

C = concentration of nitrovin in eluate μg per ml;
V = volume of extract, ml;
W = weight of the test portion in g.

22. DETERMINATION OF NITROVIN IN FEED SUPPLEMENTS

1-3(-(5-nitro-2-furyl)-1-[2-(5-nitro-2-furyl)vinyl]allylideneamino) guanidine hydrochloride

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of nitrovin in feed supplements, made with inert or cereal carriers.

2. PRINCIPLE

Nitrovin is extracted from the sample with dimethylformamide containing ammonium hydroxide and the absorbance of this solution before and after acidification is measured at 495nm.

3. REAGENTS

3.1 Dimethylformamide solvent: dilute 20ml of ammonia solution (density 0·88g per ml) to 1 litre with dimethylformamide.

3.2 Hydrochloric acid solution: dilute a suitable volume of hydrochloric acid (density 1·18g per ml) with an equal volume of water.

3.3 Nitrovin standard solution: weigh to the nearest 0·1mg, 100mg of pure nitrovin and transfer it to a 200ml graduated flask. Dissolve in and dilute to volume with the dimethylformamide solvent (3.1), and mix well. Transfer by pipette 10·0ml of this solution to a 100ml graduated flask, dilute to volume with the dimethylformamide solvent, and mix well. This solution contains 50μg of nitrovin per ml.

4. APPARATUS

4.1 Spectrophotometer with 10mm cells.

5. PROCEDURE

5.1 *Extraction of nitrovin*

Weigh to the nearest 0·001g about 1g of the finely divided and mixed sample and transfer it to a 200ml graduated flask. Add 100ml of the dimethylformamide solvent (3.1), and place on a mechanical shaker and shake for 20 minutes. Dilute to the mark with the dimethylformamide solvent (3.1) and mix well. Filter the solution through a suitable filter-paper (Whatman No. 41 or equivalent), discarding the first 20ml of filtrate. By pipette transfer 2·0ml of filtrate to a 100ml graduated flask, dilute to the mark with the dimethylformamide solvent, and mix well. Measure the absorbance of this solution at 495nm with dimethylformamide solvent as reference. Withdraw 25ml of the solution from the flask and add to this 2ml of hydrochloric acid solution (3.2) and mix well (no correction is made for the additional volume as the reading is small). Measure the absorbance of the acidified solution at 495nm against dimethylformamide solvent.

Subtract the absorbance of the acidified solution from that of the untreated solution to give the corrected absorbance. Determine the concentration of nitrovin in the sample by reference to the calibration curve (5.2).

5.2 *Calibration curve*

Transfer by pipette, 1·0, 2·0, 4·0, 6·0, 8·0 and 10·0ml of nitrovin standard solution (3.3) to separate 50ml graduated flasks. Dilute the contents of each to volume with the dimethylformamide solvent, (3.1) and mix well. These solutions will have concentrations of 1·0, 2·0,4·0, 6·0, 8·0, 10·0μg of nitrovin per ml, respectively. Measure the absorbance of these solutions at 495nm with dimethylformamide solvent as reference. Withdraw 25ml of each of these standard solutions, place in separate flasks, acidify by the addition of 2ml of hydrochloric acid solution (3.5) and mix well. If any of these solutions has become turbid clarify it by spinning in a centrifuge. Measure the absorbances of the acidified solutions in the same manner as for the alkaline solutions and subtract the reading from the absorbance of the appropriate alkaline solution to give the corrected absorbance. Plot the calibration curve using the absorbances as the ordinates and the corresponding concentrations of nitrovin in μg per ml as the abscissae.

6. CALCULATION OF RESULTS

The nitrovin content per cent of sample is given by the formula:

$$\frac{C}{W}$$

in which:

C = concentration of nitrovin in the final solution, μg per ml; and
W = weight of the test portion in g.

23. DETERMINATION OF OLEANDOMYCIN

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of oleandomycin in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of determination is 0·5mg/kg.

2. PRINCIPLE

The sample is extracted with a solution of aqueous methanol containing "Tris-buffer". After centrifuging, the extract is diluted and its antibiotic activity is determined by measuring the diffusion of the oleandomycin in an agar medium inoculated with *Bacillus cereus*. Diffusion is shown by the formation of zones of inhibition in the presence of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration.

3. MICRO-ORGANISM: *Bacillus cereus* K 250 TR (RESISTANT TO TETRACYCLINES) (NCIB 11183; NCTC 10989)

3.1 *Maintenance of the parent strain*

Inoculate *B. cereus* onto an agar slope prepared from the culture medium (4.1.1) to which has been added 100μg oxytetracycline per 5ml. Incubate overnight at approximately 30°C. Keep the culture in a refrigerator at about 4°C and re-inoculate agar slopes every 4 weeks.

3.2 *Preparation of the spore suspension*

Harvest the bacteria from the agar slope (3.1) in 2 to 3ml of sodium chloride solution (4.2). Inoculate this suspension onto 300ml of culture medium (4.1.2) in a Roux flask. Incubate for 3 to 5 days at 28 to 30°C and then confirm by examination under the microscope that sporulation has occurred. Harvest the spores in 15ml of ethanol (4.3) and mix. This suspension may be kept in a refrigerator at about 4°C for at least 5 months.

Make preliminary tests on the assay plates with the culture medium (4.1.3) to determine the quantity of inoculum needed to obtain the largest possible clear zones of inhibition with the different concentrations of antibiotic used. The quantity will usually be 0·2 to 0·3ml per 1000ml. Inoculate the culture medium at a temperature between 50 and 60°C.

4. CULTURE MEDIA AND REAGENTS

4.1.1 Culture Medium I:

Glucose	1g
Tryptic peptone	10g
Meat extract	1·5g
Yeast extract	3g
Agar, according to quality	10 to 20g
Water	1000ml

pH after sterilization: 6·5

4.1.2 Culture Medium II:

As for 4.1.1 with agar concentration of 3 to 4%.

4.1.3 Culture Medium III:

As for 4.1.1 with pH 8·8.

4.2 Sodium chloride solution 0·9% w/v: dissolve 9g of sodium chloride in water and dilute to 1000ml; sterilize.

4.3 Ethanol, 20% solution (v/v).

4.4 Methanol.

4.5 "Tris-buffer" solution: dissolve 0·5g 2-amino-2-hydroxymethylpropanediol in water and dilute to 100ml.

4.6 *Extraction solution*

Methanol	50ml
Water	50ml
2-amino-2-hydroxymethylpropanediol	0·5g

4.7 Standard substance: oleandomycin chloroform adduct of known activity.

5. STANDARD SOLUTION

Dissolve an accurately weighed quantity of between 10 and 50mg of the standard substance (4.7) in about 5ml methanol (4.4) and dilute with solution (4.5) to obtain an oleandomycin concentration of 100μg per ml.

Prepare from this solution diluting with solution (4.5) a standard working solution S_8 containing 0·1μg oleandomycin per ml. Then prepare the following concentrations by means of successive dilutions (1 + 1) with solution (4.5):

S_4 0·05μg per ml
S_2 0·025μg per ml
S_1 0·0125μg per ml

6. PROCEDURE

6.1 *Extraction*

According to the presumed level of oleandomycin in the sample, take a sample for analysis of 2 to 10g, weighed to the nearest 0·001g, add 100ml of solution (4.6) and shake for 30 minutes on a mechanical shaker.

Centrifuge, and dilute a suitable volume of the clear supernatant

with the solution (4.5) to obtain an expected oleandomycin content of 0·1μg per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with solution (4.5).

6.2 *Determination*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the extract (U_8, U_4, U_2, U_1). These four concentrations of standard and extract solution must be placed in each plate. To this effect, select plates large enough to allow at least 8 wells with a diameter of 10 to 13mm, and not less than 30mm between centres, to be made in the agar medium.

Inoculate at 50–60°C with the spore suspension (3.2) a quantity of the melted culture medium (4.1.3), sufficient to give a layer approximately 2mm thick in the assay plates to be employed. Swirl to mix thoroughly, and pour into the sterile assay plates which must be held in a rigorously horizontal position until the agar has set. With a sterile cork borer remove agar plugs to form wells as described above.

Pipette into each well an exactly measured and equal volume (0·10 to 0·15ml) of respectively solutions S_8, S_4, S_2, S_1, and U_8, U_4, U_2, U_1.

Apply each concentration 4 times so that the determination is subject to an evaluation of 32 zones of inhibition.

Incubate the plates for approximately $1\frac{3}{4}$ hours at 28–30°C.

6.3 *Evaluation*

See *"Evaluation"* of avoparcin.

24. DETERMINATION OF SULPHAQUINOXALINE

*N*¹-quinoxalin-2-ylsulphanilamide

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of sulphaquinoxaline in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of the determination is 20mg/kg.

2. PRINCIPLE

The sample is extracted with a mixture of dimethylformamide and chloroform and the extract treated with an alkaline brine solution. After acidifying the aqueous phase the sulphaquinoxaline is diazotised and coupled with N-2-aminoethyl-1-naphthylamine, forming a red colour, the absorbance of which is measured at 545nm.

3. REAGENTS

3.1 Dimethylformamide.

3.2 Chloroform.

3.3 Ethanol absolute.

3.4 Alkaline brine: dissolve 10g sodium hydroxide and 25g sodium chloride in water. Make up to 500ml with water.

3.5 Hydrochloric acid (density 1·18g per ml).

3.6 Sodium nitrite solution: dissolve 0·1g sodium nitrite in water and dilute to 100ml. Prepare immediately before use.

3.7 Ammonium sulphamate solution: dissolve 0·5g ammonium sulphamate in water and dilute to 100ml. Prepare immediately before use.

3.8 N-2-aminoethyl-1-naphthylamine dihydrochloride solution: dissolve 0·1g of N-2-aminoethyl-1-naphthylamine dihydrochloride in dilute hydrochloric acid (0·2ml hydrochloric acid (3.5) diluted to 200ml with water) and make up to 100ml with the same acid. Prepare immediately before use.

3.9 Sulphaquinoxaline standard solution: weigh to the nearest 0·1mg, 250mg of pure sulphaquinoxaline, dissolve in 50ml sodium hydroxide solution (25ml 0·1M sodium hydroxide solution + 25ml water) and make up to 500ml with water. Dilute 5·0ml to 100ml with water. 1ml of this solution contains 25μg of sulphaquinoxaline.

4. APPARATUS

4.1 Sintered-glass funnel, porosity: G3, diameter 80mm, with filter flask.

4.2 Spectrophotometer, with 20mm cells.

5. PROCEDURE

5.1 *Extraction*

Weigh, to the nearest 0·001g, a quantity of the finely divided and mixed sample expected to contain between 0·25 and 1·25mg of sulphaquinoxaline. Place the test portion in a 250ml conical flask and add 20ml dimethylformamide (3.1). Mix and heat the flask on a steam-bath for 20 minutes, then allow to cool under a stream of cold water. Add 60ml of chloroform (3.2), stopper the flask and shake mechanically for 30 minutes.

Filter the liquid through the sintered-glass funnel (4.1) under mild suction. Rinse the conical flask with four 5ml portions of chloroform (3.2) and pass the rinsings through the funnel. Transfer the filtrate to a separating funnel, rinse the filter flask with about 15ml chloroform (3.2) and transfer the rinsings to the funnel.

Add to the funnel 50ml of alkaline brine (3.4) and 5ml ethanol (3.3). Mix the layers thoroughly, avoiding emulsion formation, either by slow inversion of the funnel about twenty times or by rotating it about the horizontal axis of the stem and the stopper. Allow the layers to separate (separation is usually complete in about 15 minutes).

Transfer the upper (aqueous) layer to a 250ml graduated flask. Repeat the extraction of the chloroform layer with three further 50ml portions of alkaline brine (3.4), adding each aqueous extract to the contents of the graduated flask.

Make up the volume with water and mix.

Transfer 25·0ml of the solution to a 50ml graduated flask, add 5ml

hydrochloric acid (3.5), make up the volume with water and mix. Filter if necessary, discarding the first 15ml of filtrate. Transfer 10·0ml aliquots of the solution to two boiling tubes A and B.

5.2 *Determination*

To each tube add 2·0ml of sodium nitrite solution (3.6), mix and allow to stand for three minutes. Add 2·0ml of ammonium sulphamate solution (3.7), mix and allow to stand for two minutes. Add 1·0ml of N-2-aminoethyl-1-naphthylamine dihydrochloride solution (3.8) to tube A and 1·0ml water to tube B. Mix thoroughly the contents of each tube. By means of a water pump apply a partial vacuum to the tubes through rubber connections in order to remove dissolved nitrogen. After 10 minutes measure the absorbances E_A and E_B of the solutions in 20mm cells at 545nm against water as reference. From the value $E_A - E_B$ determine the amount (A) of sulphaquinoxaline present in the sample solution by reference to the calibration curve (5.3).

5.3 *Calibration curve*

Transfer into a series of 100ml graduated flasks volumes of 2·0, 4·0, 6·0, 8·0 and 10·0ml of the standard solution (3.9) corresponding to 50, 100, 150, 200, and 250 μg of sulphaquinoxaline. Add 8ml hydrochloric acid (3.5) to each flask, make up the volume with water and mix. Transfer by pipette 10·0ml of each solution (equivalent to 5, 10, 15, 20 and 25 μg sulphaquinoxaline) into boiling tubes. Develop the colour reaction as indicated under (5.2). Measure the absorbances in 20mm cells at 545nm against water as reference. Plot the calibration curve, using the absorbances as the ordinates and the corresponding quantities of sulphaquinoxaline in μg as the abscissae.

6. CALCULATION OF RESULTS

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

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

in which:

A = quantity of sulphaquinoxaline in μg as determined by photometric measurement;
W = weight of test portion in g.

25. DETERMINATION OF TYLOSIN

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of tylosin in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of determination is 2mg/kg.

2. PRINCIPLE

The sample is treated with pH 8·0 phosphate buffer solution which has been heated to a temperature of 80°C, and then extracted by methanol. After

centrifuging, the extract is diluted and its antibiotic activity is determined by measuring the diffusion of the tylosin in an agar medium inoculated with *Micrococcus luteus.* Diffusion is shown by the formation of zones of inhibition in the presence of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration.

3. MICRO-ORGANISM: *Micrococcus luteus* ATCC 9341 (NCIB 8553; NCTC 8340; NCDO 758)

3.1 *Maintenance of the parent strain*

Inoculate *M luteus* on to an agar slope prepared from the culture medium (4.1.1). Incubate overnight at approximately 35°C. Keep the culture in a refrigerator at about 4°C and re-inoculate agar slopes every two weeks.

3.2 *Preparation of the bacterial suspension*

Harvest the bacteria from a recently-prepared agar slope (3.1) with 2 to 3ml of sodium chloride solution (4.4). Inoculate this suspension into 250ml of culture medium (4.1.1) contained in a Roux flask. Incubate for 24 hours at 35°C, and harvest the bacteria in 25ml of sodium chloride solution (4.4). Mix and dilute this suspension to obtain a light transmission of approximately 75% at 650nm, in a 10mm cell using sodium chloride solution as reference. This suspension is usable for one week if kept in a refrigerator.

Make preliminary tests on the assay plates with the culture medium (4.1.2) to determine the quantity of inoculum needed to obtain the largest possible clear zones of inhibition with the different concentrations of antibiotic used. The quantity will usually be 0·2 to 0·3ml per 1000ml. Inoculate the culture medium at a temperature between 48 and 50°C.

4. CULTURE MEDIA AND REAGENTS

4.1.1 Culture Medium I:

Glucose	1g
Tryptic peptone	10g
Meat extract	1·5g
Yeast extract	3g
Agar, according to quality	10 to 20g
Water	1000ml

pH after sterilisation: 7·0

4.1.2 Culture Medium II:

As for 4.1.1 but pH after sterilisation of 8·0.

4.2 Phosphate buffer solution pH 8·0:

Potassium dihydrogen phosphate KH_2PO_4	0·523g
di Potassium hydrogen phosphate K_2HPO_4	16·730g
Water	1000ml

4.3 Phosphate buffer solution pH 7·0:

Potassium dihydrogen phosphate KH_2PO_4	5·5g
di Potassium hydrogen phosphate K_2HPO_4	13·6g
Water	1000ml

4.4 Sodium chloride solution 0·9% w/v: dissolve 9g of sodium chloride in water and dilute to 1000ml; sterilize.

4.5 Methanol.

4.6 Methanol, 10% solution (v/v).

4.7 A mixture of phosphate buffer solution (4.2) and methanol, in the proportions by volume 60 + 40.

4.8 Standard substance: tylosin base of known activity.

5. STANDARD SOLUTION

Dry the standard substance (4.8) for 3 hours at 60°C in a vacuum oven (5mm mercury). Accurately weigh 10 to 50mg, dissolve in 5ml methanol (4.5) and dilute the solution with the phosphate buffer solution pH 7·0 (4.3) to obtain a concentration of tylosin base of 1000μg per ml.

Diluting with the mixture (4.7), prepare from this solution a standard working solution S_8 containing 2μg tylosin base per ml.

Then by means of successive dilutions (1 + 1) with the mixture (4.7), prepare the following concentrations:

S_4 1μg per ml
S_2 0·5μg per ml
S_1 0·25μg per ml

6. PROCEDURE

6.1 *Extraction*

For feed supplements take a sample for analysis of 10g weighed to the nearest 0·01g; for protein concentrates and complete feeding stuffs, take a sample for analysis of 20g, weighed to the nearest 0·01g. Add 60ml phosphate buffer solution pH 8 (4.2), heat to a temperature of 80°C, and homogenise for 2 minutes in a blender.

Allow to stand for 10 minutes, add 40ml methanol (4.5) and homogenise again for 5 minutes. Centrifuge, take an aliquot of the clear supernatant and dilute with the mixture (4.7) to obtain an expected tylosin content of 2 per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with the mixture (4.7). For levels lower than 10mg per kg, concentrate the extract in a rotary evaporator at 35°C, dissolve the residue in 10% methanol (4.6) and dilute to a known volume to give an expected concentration of 2μg per ml.

6.2 *Determination*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the extract (U_8, U_4, U_2, U_1). These four concentrations of standard and extract solution must be placed in each plate. To this effect, select plates large enough to allow at least 8 wells with a diameter of 10 to 13mm, and not less than 30mm between centres, to be made in the agar medium.

Inoculate at 48–50°C with the bacterial suspension (3.2) a quantity of the melted culture medium (4.1.2), sufficient to give a layer approximately 2mm thick in the assay plates to be employed. Swirl to mix thoroughly, and pour into the sterile assay plates which must be held in a rigorously horizontal position until the agar has set. With a sterile cork borer remove agar plugs to form wells as described above.

Pipette into each well an exactly measured and equal volume (0·10 to 0·15ml) of respectively solutions S_8, S_4, S_2, S_1 and U_8, U_4, U_2, U_1.

Apply each concentration 4 times so that the determination is subject to an evaluation of 32 zones of inhibition.

Incubate the plates for approximately 18 hours at 35–37°C.

6.3 *Evaluation*

See *"Evaluation"* of avoparcin.

26. DETERMINATION OF VIRGINIAMYCIN

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of virginiamycin in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of determination is 2mg/kg.

2. PRINCIPLE

The sample is extracted with a methanol solution of a non-ionic surfactant. After centrifuging or filtering, the extract is diluted and its antibiotic activity is determined by measuring the diffusion of the virginiamycin in an agar medium inoculated with *Micrococcus luteus.* Diffusion is shown by the formation of zones of inhibition in the presence of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration.

3. MICRO-ORGANISM: *Micrococcus luteus ATCC 9341* (NCIB 8553; NCTC 8340)

3.1 *Maintenance of the parent strain*

Inoculate *M. luteus* onto an agar slope prepared from the culture medium (4.1). Incubate overnight at approximately 35°C. Keep the culture in a refrigerator at about 4°C and re-inoculate agar slopes every two weeks.

3.2 *Preparation of the bacterial suspension*

Harvest the bacteria from a recently-prepared agar slope (3.1) with 2 to 3ml sodium chloride solution (4.3). Inoculate this suspension into 250ml of culture medium (4.1) contained in a Roux flask. Incubate for 24 hours at 35°C, and harvest the bacteria using 25ml of sodium chloride solution.

Mix and dilute this suspension to obtain a light transmission of

approximately 75% at 650nm, in a 10mm cell using sodium chloride solution as reference. This suspension is usable for one week if kept in a refrigerator.

Make preliminary tests on the assay plates using the culture medium (4.1) to determine the quantity of inoculum needed to obtain the largest possible clear zones of inhibition with the different concentrations of antibiotic used. Inoculate the culture medium at a temperature between 48 and 50°C.

4. CULTURE MEDIUM AND REAGENTS

4.1 Culture medium:

Glucose	1g
Tryptic peptone	10g
Meat extract	1·5g
Yeast extract	3g
Agar, according to quality	10 to 20g
Water	1000ml

pH after sterilization: 6·5

4.2 Phosphate buffer solution pH 6·0:

Potassium dihydrogen phosphate, KH_2PO_4	8·0g
di Potassium hydrogen phosphate, K_2HPO_4	2·0g
Water to	1000ml

4.3 Sodium chloride solution 0·9% w/v: dissolve 9g of sodium chloride in water and dilute to 1000ml; sterilize.

4.4 Methanol.

4.5 A mixture of phosphate buffer solution (4.2) and methanol (4.4) in the proportions by volume: 80 + 20.

4.6 Tween 80 0·5% (w/v): dissolve 1g in methanol and dilute to 200ml.

4.7 Standard substance: virginiamycin of known activity.

5. STANDARD SOLUTION

Dissolve an accurately weighed quantity of the standard substance (4.7) in methanol (4.4) to give a solution containing 800μg virginiamycin per ml. Prepare from this solution, diluting with solution (4.5) a standard working solution S_8 containing 1μg virginiamycin per ml. Then prepare the following concentrations by means of successive dilutions (1 + 1) with the solution (4.5):

S_4 0·5 μg per ml
S_2 0·25 μg per ml
S_1 0·125 μg per ml

6. PROCEDURE

6.1 *Extraction*

6.1.1 Levels of virginiamycin not more than 50mg/kg:

Take 10 to 20g of the sample, weighed to the nearest 0·01g add 100ml of solution (4.6) and shake for 30 minutes. Centrifuge or filter, take 20ml of the clear solution and evaporate to dryness in

a rotary evaporator. Take up the residue with 20ml or more of the mixture (4.5) to obtain an expected virginiamycin content of 1μg per ml (U_8).

Then prepare the concentrations U_4, U_2, and U_1 by means of successive dilutions (1 + 1) using the mixture (4.5).

6.1.2 Levels greater than 50mg/kg:

Take 1 to 10g of the sample, weighed to the nearest 0·001gm add 100ml of solution (4.6) and shake for 30 minutes. Centrifuge or filter, and then dilute with the mixture (4.5) to obtain an expected virginiamycin content of 1μg per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 as described in 6.1.1.

6.2 *Determination*

Diffusion through agar is carried out in plates with the four concentrations of the standard (S_8, S_4, S_2, S_1) and the four concentrations of the extract (U_8, U_4, U_2, U_1). These four concentrations of standard and extract solution must be placed in each plate. To this effect, select plates large enough to allow at least 8 wells with a diameter of 10 to 13mm, and not less than 30mm between centres, to be punched out of the agar medium.

Inoculate 48–50°C with the bacterial suspension (3.2) a quantity of the melted culture medium (4.1), sufficient to give a layer approximately 2mm thick in the assay plates to be employed. Swirl to mix thoroughly, and pour into the sterile assay plates which must be held in a rigorously horizontal position until the agar has set. With a sterile cork borer remove agar plugs to form wells as described above.

Pipette into each well an exactly measured and equal volume (0·10 to 0·15ml) of respectively solutions S_8, S_4, S_2, S_1 and U_8, U_4, U_2, U_1.

Apply each concentration 4 times so that the determination is subject to an evaluation of 32 zones of inhibition.

Incubate the plates for approximately 18 hours at 28–30°C.

6.3 *Evaluation*

See *"Evaluation"* of avoparcin.

27. DETERMINATION OF ZINC BACITRACIN

1. SCOPE AND FIELD OF APPLICATION

The method is for the determination of the quantity of zinc bacitracin in complete feeding stuffs, protein concentrates and feed supplements. The lower limit of determination is 5mg/kg. (210 iu)[1]. The method is not valid in the presence of large amounts of copper and ascorbic acid.

2. PRINCIPLE

The sample is extracted at pH 2 with acidified aqueous methanol. The extract is brought to pH 6·5, concentrated (where necessary) and diluted. Its

[1] When used in feedingstuffs 1mg zinc bacitracin is equivalent to 42 international units (iu).

antibiotic activity is determined by measuring the diffusion of the zinc bacitracin in an agar medium inoculated with *Micrococcus luteus* (syn. *M. flavus*). Diffusion is shown by the formation of zones of inhibition in the presence of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration.

3. MICRO-ORGANISM: *Micrococcus luteus* ATCC 10240 (NCIB 8166; NCTC 7743; NCDO 982)

3.1 *Maintenance of the parent strain*

Inoculate *M. luteus* on to an agar slope prepared from the culture medium (4.1.1). Incubate overnight at approximately 35°C. Keep the culture in a refrigerator at about 4°C and re-inoculate agar slopes every two weeks.

3.2 *Preparation of the bacterial suspension*

Harvest the bacteria from a recently-prepared agar slope (3.1) with 2 to 3ml of sodium chloride solution (4.3). Inoculate this suspension into 250ml of culture medium (4.1.1) contained in a Roux flask. Incubate for 24 hours at 35°C, and harvest the bacteria in 25ml of sodium chloride solution (4.3).

Mix and dilute this suspension to obtain a light transmission of approximately 75% at 650nm in a 10mm cell, using sodium chloride solution (4.3) as reference. This suspension is usable for one week if kept in a refrigerator.

Make preliminary tests on assay plates using the culture medium (4.1.2) to determine the quantity of inoculum required to give the largest possible clear zones of inhibition with the various concentrations of zinc bacitracin used. The quantity will usually be 0·2 to 0·3ml per 1000ml. Inoculate the culture medium at a temperature between 48 and 50°C.

4. CULTURE MEDIA AND REAGENTS

4.1.1 Culture medium I

Peptone	6g
Tryptone	4g
Yeast extract	3g
Meat extract	1·5g
Glucose	1g
Agar	15g
Water	1000ml

pH after sterilisation: 6·5

4.1.2 Culture medium II

Tryptone	10g
Yeast extract	3g
Meat extract	1·5g
Glucose	1g
Agar	20g
Tween 80	1ml
Water	1000ml

pH after sterilisation: 6·5

4.2 Phosphate buffer solution pH 6·5:

Potassium dihydrogen phosphate KH_2PO_4	27·85g
di Potassium hydrogen phosphate K_2HPO_4	22·15g
Water to	1000ml

4.3 Sodium chloride solution 0·9% (w/v): dissolve 9g of sodium chloride in water and dilute to 1000ml; sterilize.

4.4 Hydrochloric acid solution 0·1M.

4.5 Sodium hydroxide solution 1M.

4.6 A mixture of methanol, water and hydrochloric acid (density 1·18g per ml) in the proportions by volume: 80 + 17·5 + 2·5.

4.7 Bromocresol purple solution: dissolve 0·1g of bromocresol purple in 18·5ml of 0·01M sodium hydroxide solution and make up the volume to 250ml with water and mix.

4.8 Standard substance: zinc bacitracin of known activity.

5. STANDARD SOLUTION

Dissolve an accurately weighed quantity of standard zinc bacitracin (4.8) corresponding to 1050iu in 5ml of 0·1M hydrochloric acid solution (4·4) in a 50ml graduated flask and leave to stand for 15 minutes. Add 30ml of water, adjust the pH to 4·5 with phosphate buffer solution pH 6·5 (4.2) (about 4ml), and dilute with water to 50ml to obtain a concentration of zinc bacitracin of 21iu per ml.

Prepare from this solution, diluting with solution (4.2), a standard working solution S_8 containing 0·42iu zinc bacitracin per ml. Then prepare the following concentrations by means of successive dilutions (1 + 1) using the solution (4.2):

S_4 0·21iu per ml
S_2 0·105iu per ml
S_1 0·0525iu per ml

6. PROCEDURE

6.1 *Extraction*

6.1.1 Concentrates, premixes and mineral feeds:

Take 2 to 5g of the sample, weighed to the nearest 0·01g, add 30ml of the mixture (4.6) and shake briefly. Check that the pH is about 2. Shake for 10 minutes, add 30ml of phosphate buffer pH 6·5 (4.2) and shake for 15 minutes. Dilute with phosphate buffer (4.2) to obtain an expected zinc bacitracin content of 0·42iu per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 by means of successive dilutions (1 + 1) with solution (4.2).

6.1.2 Protein concentrates:

Take 10g of the sample, weighed to the nearest 0·01g, and 50ml of the mixture (4.6) and shake briefly. Check that the pH is about 2. Shake for 10 minutes, add 50ml of phosphate buffer pH 6·5 (4.2) and shake for 15 minutes. Dilute with phosphate buffer (4.2) to obtain an expected zinc bacitracin content of 0·42iu per ml (U_8). Then prepare the concentrations U_4, U_2, and U_1 as described in 6.1.1.

6.1.3 Other feeds:

Take 10g of the sample, weighed to the nearest 0·01g (20g for an expected zinc bacitracin content of 5mg/kg), add 25ml of the mixture (4.6) and homogenize for about 10 minutes. Add 25ml of phosphate buffer pH 6·5 (4.2), shake for 15 minutes and centrifuge. Take 20ml of the supernatant solution and adjust the pH to 6·5 by means of 1M sodium hydroxide solution (4.5) with the bromocresol purple solution (4.7) as indicator. Evaporate to about 4ml in a rotary evaporator at a temperature not exceeding 35°C. Dilute the residue with water to obtain an expected zinc bacitracin content of 0·42iu per ml (U_8). Then prepare the concentrations U_4, U_2 and U_1 as described in 6.1.1.

6.2 *Determination*

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the extract (U_8, U_4, U_2, U_1). These four concentrations of standard solution and extract must be placed in each plate. To this effect, select plates large enough to allow at least 8 wells with a diameter of 10 to 13mm, and not less than 30mm between centres, to be made in the agar medium.

Inoculate at 48–50°C with the bacterial suspension (3.2) a quantity of the melted culture medium (4.1), sufficient to give a layer approximately 2mm thick in the assay plates to be employed. Swirl to mix thoroughly, and pour into the sterile levelled assay plates until the agar has set. With a sterile cork borer remove agar plugs to form wells as described above.

Pipette into each well an exactly measured and equal volume (0·10 to 0·15ml) of respectively solutions S_8, S_4, S_2, S_1 and U_8, U_4, U_2, U_1.

Apply each concentration 4 times so that the determination is subject to an evaluation of 32 zones of inhibition.

Incubate the plate for approximately 18 hours at 28–30°C.

6.3 *Evaluation*

See *"Evaluation"* of avoparcin.

6.4 *Repeatability*

The difference between the results of two determinations carried out on the sample by the same analyst should not exceed:

(a) 20% related to the highest value for contents of zinc bacitracin from 5 and up to 25mg/kg;

(b) 5mg/kg, in absolute value, for contents greater than 25 and up to 50mg/kg;

(c) 10% related to the highest value for contents above 50mg/kg.

SCHEDULE 4

FORMS OF CERTIFICATE OF ANALYSIS OF EXAMINATION

(Regulation 5)

PART I

CERTIFICATE OF ANALYSIS OF ANIMAL FEEDINGSTUFF (1)

I, the undersigned, agricultural analyst for the (2)
in pursuance of the provisions of the Medicines Act 1968, hereby certify that I received on the day of , 19 , from (3)
one part of a sample of (4) for analysis; which was duly sealed and fastened up and marked (5)
and was accompanied by a (6)
as follows:— (7)

and also by a signed statement that the sample was taken in the prescribed manner by the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983; and that the said part has been analysed by me or under my direction, and I declare the results of the analysis to be as follows:— (8)
(9)
and I am of the opinion that (10)
The analysis was made in accordance with the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983.

Signature of analyst

Address

Date

GUIDANCE NOTES

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.

(1) Statements made in certificates are to be confined to matters which are relevant to the presence in the feeding stuff of a medicinal product, as defined in section 130(1) of the Act, or of a substance used for a medicinal purpose, being a purpose defined in section 130(1) of the Act, or of a substance used for medicinal purpose, being a purpose defined in section 130(2) of the Act.

(2) Insert the name of the local authority.

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

(4) Insert the name or description of the feeding stuff.

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

(6) Insert either "copy of the label of leaflet" or "copy of such particulars as

may be necessary for the purpose of analysis", or otherwise as the case may be.

(7) Insert the relevant particulars for which analyses have been made and which are contained in the items indicated at (6) above.

(8) Insert relevant results including, if appropriate, the identification and quantity of any medicinal ingredient not specified in the items indicated at (6) above and an indication of any medicinal ingredient in respect of which it has not been possible to carry out a satisfactory determination.

(9) Indicate the method of analysis used, where alternative methods are prescribed.

(10) (a) If a full analysis has been performed, ie one where the work is not shared with a person having the management or control of a laboratory, here enter information as follows:—

(i) If the composition of the feeding stuff agrees with or does not differ by more than the limits of variation from the statement of particulars contained in the copy of the label or leaflet or other relevant particulars indicated at item (6) above, state that the particulars are correct within the limits of variation.

(ii) If the composition of the feeding stuff differs by more than the limits of variation from the statement of particulars contained in the copy of the label or leaflet or other relevant particulars indicated at item (6) above, state the difference between the amount found and the amount stated.

(b) If a part analysis only has been performed, ie one where the work is shared with a person having the management or control of a laboratory, here enter the information as under (i) or (ii) above which is appropriate to the part analysis.

PART II

CERTIFICATE OF ANALYSIS OR EXAMINATION OF ANIMAL FEEDINGSTUFF (1)

I, the undersigned, having management or control of the (2)
with which arrangements have been made in pursuance of the provisions of the Medicines Act 1968 hereby certify that I received on the day of 19 from (3)
one part of a sample of (4) for *analysis/examination; which was duly sealed and fastened up and marked (5)
and was accompanied by a (6)
as follows:— (7)

and also by a signed statement that the sample was taken in the prescribed manner by the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983 (8)†, and that the said part has been *analysed/examined by me or under my direction. I further certify the results of *analysis/examination to be as follows:— (9)

(10)
and I am of the opinion that (11)
The *analysis/examination was made in accordance with the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983.

Signature of analyst

Address

Date

*Delete as necessary

†Delete if not required.

GUIDANCE NOTES

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.

(1) Statements made in certificates are to be confined to matters which are relevant to the presence in the feedingstuff of a medicinal product, as defined in section 130(1) of the Act, or of a substance used for a medicinal purpose, being a purpose defined in section 130(2) of the Act.

(2) Insert the name of the laboratory.

(3) Insert the name of the agricultural analyst who submitted the sample for analysis or examination.

(4) Insert the name or description applied to the feeding stuff.

(5) Insert the distinguishing mark on the sample and the date of sampling show thereon.

(6) Insert either "copy of the label or leaflet", "copy of such particulars as may be necessary for the purpose of analysis or examination" or otherwise as the case may be.

(7) Insert the relevant particulars for which analyses or examinations have been made and which are contained in the items indicated at (6) above.

(8) A statement, signed by the inspector, that the sample was taken or otherwise obtained in the manner prescribed by the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1983, will accompany the sample only if a full analysis, ie one where the work is not shared with the agricultural analyst or an examination is to be carried out at the laboratory with which special arrangements have been made.

(9) Insert relevant results including, if appropriate, the identification and quantity of any medicinal ingredient not specified in the items indicated at (6) above and an indication of any medicinal ingredient in respect of which it has not been possible to carry out a satisfactory determination or examination.

(10) Indicate the method of analysis used, where alternative methods are prescribed.

(11) (a) If a full analysis has been performed, ie one where the work is not shared with the agricultural analyst (or an examination has been performed) enter information as follows:—

(i) If the composition of the feeding stuff agrees with or does not differ by more than the limits of variation from the statement of

particulars contained in the copy of the label or leaflet or other relevant particulars indicated at item (6) above, state that the particulars are correct within the limits of variation.

(ii) If the composition of the feeding stuff differ by more than the limits of variation from the statement of particulars contained in the copy of the label or leaflet or other relevant particulars indicated at item (6) above, state the difference between the amount found and the amount stated.

(b) If a part analysis only has been performed, ie one where the work is shared with the agricultural analyst, here enter the information as under (i) or (ii) above which is appropriate to the part analysis.

EXPLANATORY NOTE

(This Note is not part of the Regulations.)

These Regulations, which apply throughout the United Kingdom, revoke and replace the Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1976 as amended. The main provisions of the Regulations are as follows:—

1. Modifications of sections 112, 113 and 115 of the Medicines Act 1968 (which sections contain powers of inspecting, sampling, etc., medicinal products and lay down procedures for analysis of samples) are made for the purpose of the application under the Medicines Act of those sections to animal feeding stuffs (Regulation 2 and Schedule 1 Part I). (The full provisions of those sections as so modified are set out in Schedule 1 Part II).

2. The methods by which samples of animal feeding stuffs shall be taken, set aside and submitted for analysis by an agricultural analyst, and the steps to be taken by such analyst when samples are received for analysis are set out (Regulation 3 and Schedule 2).

3. The methods by which analysis of animal feeding stuffs is to be carried out are prescribed (Regulation 4 and Schedule 3), and also the forms of certificate of analysis or examination (Regulation 5 and Schedule 4).

The Regulations implement in part Council Directive 70/373/EEC of 20th of July, 1970 (O.J. No. L. 170, 3.8.70, p. 2) (SE 1970 (II), p. 535) on the introduction of Community methods of sampling and analysis for the official control of feeding stuffs; and Commission Directives 72/199/EEC (O.J. No. L. 123, 29.5.72, p. 6) (SE 1966–72, p. 74), 74/203/EEC (O.J. No. L. 108, 22.4.74, p. 7), 75/84/EEC (O.J. No. L. 32, 5.2.75, p. 26), 78/633/EEC (O.J. No. L. 206, 29.7.78, p. 43) and 81/715/EEC (O.J. No. L. 257, 10.9.81, p. 38) establishing Community methods of analysis for the official control of feeding stuffs.

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