SEROLOGICAL IDENTIFICATION OF THE SPECIES ORIGIN OF ANIMAL MEATS"

BY

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MEMORANDUM

This dissertation is submitted in accordance with the regulations for the degree of Master of Science, University of Bristol.

Except where assistance and advice has specifically been acknowledged, this dissertation is the result of my original unaided work.

Candidate Kiam Cangethe

Date 29-9-81.

Abstract

Antisera to bovine, sheep and horse serum albumins were raised in rabbits and purified by immunoadsorbent chromatography. The antigenic properties of the purified and unpurified sera were investigated using the double immunodiffusion test. This showed that the purification step enabled exclusive species identification to be made using the anti-BSA and -HSA specific fractions but not with the anti-SSA fraction isolated by the same purification procedure.

Identification of the species of origin of meat by the ELISA method using the specific antibody fractions (anti-BSA and -HSA) showed that beef could be differentiated from horse, sheep and veal meats, and horse from sheep and beef at high levels of significance ($P \leqslant 0.001$). Sheep was differentiated from horse, beef and venison at lower levels of significance but not from goat when anti-SSA specific fraction was used.

Horse meat in beef mince was differentiated from pure beef mince at levels between 10 and 50% of adulteration, and this limit could be extended to 0.5% adulteration but only in mixtures of separately prepared beef and horse meat extracts. Precise quantification of the amount of horse meat in beef mince from preconstructed standard curves using either the indirect ELISA method or the competitive antigen modification of indirect ELISA was not possible.

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INTRODUCTION

Meat is the post-mortem aspect of muscle. Animal meat is composed of muscle fibres held together by connective tissue through which blood vessels, nerves and fat cells are distributed. Proteins are second only to water as the most abundant substances in animal tissues and are without doubt the most important constituents of the edible portions of meat animals.

The muscle proteins can be divided broadly into sarcoplasmic, myofibrillar and connective tissue proteins (Lawrie, 1979). Since animal muscle has universally the same active proteins and biochemical mechanisms, any inter-species differences in muscle composition is very small and therefore differentiation of meat from different species is difficult. Identification of the species origin presents a challenging problem in food hygiene, food control and veterinary forensic medicine.

The eating habits of man differ from one society to another to the extent that certain foods held as delicacies in one society are taboo in another. For instance, horse meat is not eaten by the British population whereas in some countries in continental Europe it is an accepted food.

Addition of low cost meat such as whale or horse to beef in the preparation of meat products is not uncommon in many parts of the world. It is now widely accepted that reliable analytical methods are needed to check the specification of meat in the interests of fair trading either at the international level or simply for the protection of the consumer's rights.

A recent trend in the modern meat industry is for the abattoirs or meat cutting plants to debone carcasses whilst still "hot". As soon as dressing of a carcass has been completed, the meat is stripped from the skeleton and vacuum packed as primal joints. It is then chilled, aged and often frozen before distribution to retail outlets. This achieves a major saving in energy costs, chiller space and weight loss due to evaporation and results in a greater yield of saleable meat (Cuthbertson, 1980). Under such circumstances the anatomical differences that exist in the skeletons cannot be used as aids to the identification of the species of origin of the meat. On the other hand the organs that are sold for human consumption, mainly the liver, heart and kidney pose no problem because the anatomical features and the colour of the associated fat identify the particular species (Nickel et al, 1973).

This project was undertaken to try to develop an enzyme-linked immunosorbent assay (ELISA) as a technique for identifying the species of origin of different raw meats. The initiation of such a method at the Meat Research Institute, at the present time when there is much interest in the detection of horse meat in meat products, is particularly timely.

LITERATURE REVIEW : METHODOLOGY

Identification of the species of origin of proteins has been attempted in the past using microscopical, chemical, physico-chemical and serological techniques. Each of these techniques has certain advantages as well as disadvantages and these are summarized briefly.

Microscopy

Use of microscopy for identification of protein type has been limited to the detection of non-meat protein in meat products and in this context provides a valuable and rapid method for detection of soya. Jewell (1973) developed a technique for detecting 0.5% or less of textured soya protein in luncheon meats. Frozen sections were stained by periodic acid Schiff reagent or iodine and then examined by phase contrast or polarized light. Similar methods have been described by Coomaraswamy et al (1973) and Flint et al (1979). Use of microscopy presupposes an adequate knowledge of histology and this may perhaps be one of the reasons for its limited development and application.

Chemical Techniques

Chemical extraction methods such as that of Bailey (1942) are time consuming, so a rapid sorting test to indicate which samples are worthy of more detailed investigation is useful. Degenkolb et al (1967) described such a method based on the observation that meat proteins are rendered insoluble in physiological saline by heating to 75°C, whereas the non-meat proteins are more stable. To ensure denaturation of the meat, samples were first heated in a waterbath at 75°C for 2 h, then homogenised in saline and the pH adjusted to between 7.0 and 8.0. After centrifugation, 1 ml of the saline

extract between the fat layer and sediment is pipetted into a petri dish and a drop of concentrated nitric acid placed in the middle of the liquid. The extent and nature of the cloudiness appearing at the centre after 0.5 min indicates the amount of non-meat protein present.

Physico-chemical techniques : electrophoresis

Use of electrophoretic techniques for the identification of muscle proteins depends upon differences in their electric charge and consequently different migration rates in an electric field. These differences show up in:- (1) the number of protein bands formed, (2) the intensity of the different bands indicating differences in the concentration of the respective proteins and (3) the electrophoretic mobility of similar proteins, indicating slight differences in their physical properties (Giles, 1962). Electrophoretic techniques which have been used include:- free boundary electrophoresis (Conell, 1953), starch-gel electrophoresis (Scopes, 1968 and 1970), sodium dodecyl sulphate polyacrylamide gel electrophoresis (Cowie, 1968; Hofmann, 1974 and 1978; Hofmann and Penny, 1971 and 1973; Penny and Hofmann, 1971; Mattey, 1972) and isoelectric focusing (Kraiser et al, 1980; Mackie, 1979).

Different protein fractions have been used in electrophoretic methods of identification of meat types. Sarcoplasmic proteins were used by Giles (1962); Hamoir (1955); Hoyem and Thorson, (1970); Lawrie (1953) and Scopes (1968 and 1970). Myosin by Champion et al (1970), and tropomyosin by Parsons et al (1969). The intensity of the different bands formed indicated clear differences in the concentrations of the protein fractions. The sarcoplasmic proteins, although

they have been used to identify the origin of meat, have also been shown to vary not only between species but also within species and even between muscles of the same animal (Giles, 1962; Lawrie, 1953). The number of protein bands formed depends upon the relative electrophoretic mobilities of the proteins and the voltage applied across the field to achieve resolution of the protein zones. Complete separation is desirable so that densitometric measurements can be made to quantify the analysis. This is not always easy due to 'background staining' which increases the errors of measurement. Varying degrees of success using electrophoretic techniques have been reported (Choi et al, 1970; Codun et al, 1972; Ebermann et al, 1972; Giorgi et al, 1970; Mattey et al, 1970), but there are clearly associated problems.

Serological Methods

Serology offers an attractive alternative to microscopic and electrophoretic techniques, although serological techniques are not without their own difficulties. The two major problem areas are:- (a) the production of antisera with adequate titre and sufficient species specificity for test purposes and (b) the efficient extraction of antigens from the meat under test with only minimum alteration of their antigenic competence.

Species specificity of antisera has been found to vary according to the route of immunization and with the number of subsequent boosters (Proom, 1943). He observed that intramuscular and subcutaneous routes of immunization resulted in antisera with greater specificity than those induced by intravenous or intraperitoneal routes. Repeated boosters resulted in decreased titre and specificity. Lack

of specificity results in extensive cross-reaction (Furminger, 1964) and formation of non-specific precipitation bands which can lead to erroneous conclusions (Panetsos et al, 1972).

Use of immunoadsorbent chromatography has enabled preparation of monospecific antisera which afforded precise identification of species protein with the minimum of cross-reaction (Kamiyama et al, 1978(c); Karpas et al, 1970; Ozawa et al, 1969 and Pinto, 1961).

Immunoadsorbent chromatography is the method of choice for the isolation and purification of species specific antobodies from a cross reacting antiserum (Cuatrecasas, 1969; Omenn et al, 1970; Silman et al, 1966; Wide et al, 1966). This method exploits the reversible and specific interactions of ligands with macromolecules (Cuatrecasas et al, 1970; Cuatrecasas, 1970(a), 1970(b) and 1972). Purification is effected by chromatographing the impure antiserum on a column containing an insoluble matrix to which the specific ligand (i.e. in this case, specific antigen for wanted antibody) is covalently attached. Beaded agarose ('Sepharose') activated with cyanogen bromide (Axen et al, 1967; March et al, 1974; Porath et al, 1967) is a useful support which has been employed successfully in selective purification of antisera (Lecomte et al, 1976; Stankus et al, 1976).

Identification of meat type in cooked meat products is even more difficult because the duration and the temperature of heating affects the degree of denaturation of the species specific antigens (Katsube et al, 1968; Murakami et al, 1969). However, identification of meat species in sausages has been achieved (Anon, 1970; Karpas et al, 1970) by use of species purified immunoglobulin G heated to simulate sausage manufacture. The resulting precipitate of the partially denatured protein was used to immunize rabbits and the derived

antiserum rendered monospecific by immunoadsorption chromatography.

Verbeke et al (1979 and 1980) utilized the positional and distribution of palmitic acid and unsaturated fatty acid within the triglyceride molecule. Plotting the proportion of oleic acid in position 2 against that of palmitic acid in the same position in the total triglyceride afforded an effective way of discriminating pig fats in cooked meat products. These authors have used the position and distribution of other fatty acids in position 2 of the triglyceride molecule to identify fats from other animal species. This technique offers an attractive alternative for identifying the origin of meat in cooked meat products because of the heat stability of fat.

Serum albumin is the most abundant of the serum proteins and has a molecular weight of approximately 70,000. It is synthesized in the liver and is effective in maintaining the osmotic equilibrium between blood and tissue fluids. Separation of albumin from other serum proteins is relatively simple and can be achieved by (a) salt fractionating using ammonium sulphate at 0.50 saturation leaving albumin in solution after other fractions have been precipitated, (b) the Cohn plasma fractionation method employing ethanol at 40% concentration and pH 4.8 (Edsall, 1947) (c) Ultracentrifugation (Varley, 1967).

Antisera produced in rabbits in response to injection with purified albumins from various animal species has unfortunately shown a high degree of cross-reactivity which correlates with the species evolutional relationships (Kamiyama, 1977(a)). Kamiyama (1977(b)) investigated the location of species specific determinants on the serum albumin molecule by cleaving it with cyanogen bromide.

This cleavage yielded two fragments; an N-fragment (containing the $-NH_2$ terminal group) and a C-fragment (containing -COOH terminal group). Kamiyama showed that 25 - 35% of antibodies formed against intact serum albumin bound to an affinity chromatography column which had the N-fragment as the coupled ligand, whilst the remainder bound to one with the C-fragments as the coupled ligand. This was confirmed by immunodiffusion and a passive haemagglutination test which showed that the C-fragments had a higher degree of species specificity. However, the use of antisera to C-fragments did not offer any added advantage over antisera raised against intact albumins, when both were used for species identification.

Current slaughter techniques leave residual blood in the carcass: of the total blood, 1.5 - 2.0% remains in the musculature (Warriss, 1977). Katsube and Imaizumi (1968) suggested that serum protein retained in the muscle might be one of the important antigens involved in the precipitin reaction with antiserum, although cross-reaction between the muscle and serum components could not be ruled out. Using the precipitin reaction, Murakami et al (1969) showed that the antigenic substance of raw meat extract detectable by the test was similar to that found in serum corresponding to serum albumin. The presence of serum albumin in meat extracts was confirmed by the presence of a band having the same mobility as that of purified serum albumin in polyacrylamide gel electrophoresis (Kamiyama et al., 1978(a)) and by a precipitin line in agar-gel precipitin reactions investigated by Haydem (1978). The concentration of serum albumin was found to vary between 0.61 to 1.95mg/ml in horse and pig meat extracts respectively. Cattle, sheep and goat values fell between those of horse and pig (Kamiyama et al., 1978 (a)). This content is quite adequate to cause a response in precipitin reactions if an antiserum to serum albumin is used for serological identification of the species type.

During the last two decades, there has been a considerable increase in the number and variety of immunodiagnostic tests

available. Immunofluorescence and radioimmunoassay (RIA) have been used as the methods of choice where high sensitivity was required. Immunofluorescence however is a tedious, time consuming procedure which cannot be easily automated, so it can only be used conveniently for small batches of samples. In contrast, RIA is suitable for large scale operations but the short half-life of the reagents, the rather expensive nature of the equipment and the regulatory control on the use of isotopes tends to exclude RIA from the smaller laboratories. These considerations prompted a search for alternative detection labels for antibodies or antigens, and led to the use of enzymes and the development of the technique of enzyme-linked immunosorbent assay (ELISA) (Engvall et al., 1971 (a)). In ELISA, different techniques are presently employed:-

a) Competitive Enzyme Immunoassay (EIA) for antigens - In this technique labelled antigen competes with unlabelled antigen for binding to a limited quantity of antibody. The antibody-bound antigen is separated from the free antigen and the enzyme activity in either the bound or free fraction is determined and related to the concentration of the labelled antigen (Engvall et al., 1971 (b)).

b) 'Sandwich' EIA for antigen - The antigen must have at least two binding sites. It reacts with excess solid-phase antibody and after incubation followed by washing, the bound antigen is treated with excess labelled antibody. After further washing the bound label is assayed, and this provides a direct measure of the amount of antigen originally present (Maiolini et al., 1975 (a)).

c) EIA for antibody - The antibody binds to excess solid-phase antigen and after incubation followed by washing labelled second antibody with specificity for the first antibody is added. The bound label is assayed after washing and it provides a direct measurement of the amount of the specific antibody present (Engvall et al.,(1972) This system may also be used to assay antigens. Excellent review articles on EIA have been published by Wisdom (1976); Schuurs et al., (1977)and Voller et al., (1978)

A variety of solid-phases have been used in enzyme-immunoassay over the last few years: polystyrene tubes (Engvall et al, 1972), Silicone rubber (Hamanguchi et al., 1976), and specially treated Dipsticks (Felgner, 1977 and 1978). Although various carrier materials can be used, it is essential that each new type is thoroughly tested to find the amount and reproducibility of uptake of the antigen or antibody since these variables influence the results of the test (Bidwell et al, 1977; Lehtonen et al, 1980). The labelled antigen/or antibody/enzyme conjugates are normally coupled through a reaction with glutaraldehyde which enables them to retain a significant part of their immunological and enzymatic activity (Avrameas, 1969).

A variety of enzymes have been used and include alkaline phosphatase (Avrameas, 1969), glucose oxidase (Maiolini et al, 1975 (a) and 1975(b)) β -d-galactosidase (Kato et al., 1976) and horse radish peroxidase (Engvall et al., 1971 (a)).

Enzyme substrates are chosen to give a coloured product following enzymatic degradation. Ortho-phenylenediamine and p-nitrophenyl phosphate are suitable for use with peroxidase and alkaline phosphatase conjugates respectively (Voller et al., 1978).

ELISA has had wide application since its inception. It has been used for hormone assay (Van Weemen et al, 1971 and 1972), detection of staphylococcal enterotoxin (Kuo et al., 1980), connective tissue type assay (Rennard et al., 1980) and recently for the detection of soya protein in food (Hitchcock et al, 1981). Although ELISA may develop to the same level of automation as in RIA and may eventually supersede it, it can be used simply and economically at present.

From the foregoing, the application of ELISA to the serological identification of the origin of animal meats, seems to be a more versatile and sensitive method to replace the precipitin tests currently used. ELISA, was therefore, adopted for use in this study.

MATERIALS AND METHODS

Production of Antisera

Horse serum albumin (HSA), bovine serum albumin (BSA), and sheep serum albumin (SSA) respectively were purchased from Sigma London, Chemical Co. Ltd. Incomplete Freund's adjuvant (IFA) comprising 6 parts mineral oil (Bayol F) to 1 part emulsifying agent (Arlacel A) and Complete Freund's adjuvant (CFA) incorporating attenuated Mycobacterium tuberculosis were obtained from Difco Laboratories, Detroit, U.S.A.

An antiserum to each albumin was raised by injection of individual rabbits (New Zealand White, 8 weeks old) with either BSA, HSA or SSA. For this purpose each albumin was dissolved in sterile physiological saline and incorporated in a water-in-oil emulsion of the following composition:-

500 µg albumin.
500 µl sterile saline (0.9%).
1,500 µl CFA.

Primary immurization was effected by intradermal injection of 200 µl aliquots of the emulsion at 10 separate sites along the back. After 4 weeks, the procedure was repeated but IFA was substituted for CFA in the emulsion. Four weeks later, the rabbits were anaesthetized and exsanguinated by cardiac puncture. Approximately 60 ml of whole blood was collected and allowed to clot at room temperature. Serum was separated by centrifugation (2,500 rev/min, for 5 min at room temperature) frozen rapidly in small aliquots and stored at -20°C.

Separation of anti-BSA, -HSA and -SSA specific antibody fractions

Preparation of the immunoadsorbent

Cyanogen bromide activated Sepharose 4B was purchased from Sigma London Co. Ltd. Buffers used were all prepared from Analar reagents or reagents of an equivalent grade.

5g of activated Sepharose 4B (with a swelling capacity of 3.5 ml/g of lyophilized powder) was reswollen for 15 min in 100 ml of 1 mM HCl and washed with 1 l of the same solution on a sintered glass filter (porosity G3). The gel was further washed with 25 ml of bicarbonate buffer (0.1 M, pH 8.3) containing 0.5 M NaCl.

Approximately 131.00 mg of ligands (BSA, HBA and SSA) were dissolved separately in 17.5 ml of bicarbonate buffer. Each albumin solution was mixed with 17.5 ml of the reconstituted washed gel and allowed to shake gently for 12 h at 4°C.

Any residual uncoupled active groups on the Sepharose were then blocked by allowing the gel to stand in glycine buffer (0.2 M, pH 8.0) for 2 h at room temperature. The blocking agent was then removed by washing with 200 ml of the bicarbonate buffer. The immunoadsorbent was further washed five times with alternate high and low pH buffers (bicarbonate and acetate (0.1 M, pH 8.0), containing (0.5 M) NaCl) respectively, to ensure that no free ligand remained ionically bound to the immobilized ligand. The immunoadsorbent was stored in phosphate buffered saline (PBS) (0.15 M, pH 7.2, containing 0.02% sodium azide) at 4°C until required.

Use of immunoadsorbent for antibody purification

Chromatography column size C10 were purchased from Pharmacia Fine Chemicals Co. Ltd. A peristaltic pump (LKB) and a spectrophotometer (SP500, Unicam Instruments Ltd.) were also used.

The whole procedure described below was carried out at 4°C. Immuncadsorbents were poured into the chromatography columns and equilibrated with PBS (0.15 M, pH 7.2). Rabbit anti-BSA serum was diluted 1:2 in PBS (0.3 M, pH 7.2) and passed through SSA- and HSA-linked columns, connected in series. Circulation of the antiserum through the two columns for 24 h was maintained by means of the peristaltic pump. The non-adsorbed antibody fraction, (BSA specific) was further purified by circulating through a column linked with BSA for 24 h. The unbound proteins from the columns were washed off, using PBS (0.15 M, pH 7.2), until the absorbance of the eluate was less than 0.1 units measured at 280 nm against the appropriate blank. The antibody fractions were eluted in 2 stages. The first fraction was eluted with glycine-HCl buffer (0.1 M, pH 2.5). Small tubes containing 0.5 ml trichloroacetic acid (10% aqueous) were used to sample for protein during elution and collection of the eluate started after protein was first detected and stopped when protein was no longer detectable. The second fraction was eluted with glycine-HCl buffer containing 10% dioxane, samples being treated in the same way as before. The solutions were adjusted to pH 8.5 by titrating with solid Tris (hydroxy methyl) methylamine. The antibody fractions (BSA-SSA and BSA-HSA common fractions, and BSA specific) were concentrated by negative pressure dialysis to 5 to 10 ml and then dialysed against PBS (0.15 m, pH 7.2) for 12 h. The dialysate was centrifuged (2,500 rev/min, for 3 min at room temperature) and the antibody fractions collected and stored at -20°C.

Regeneration of the immunoadsorbents

Immunoadsorbents were regenerated by washing the columns with 10 column volumes of Tris-HCl buffer (0.1 M, pH 8.5 containing (0.5 M) NaCl) followed by 10 volumes of acetate buffer (0.1 M, pH 4.5, containing (0.5 M) NaCl). The columns were then equilibrated with PBS (0.15 M, pH 7.2).

Rabbit anti-HSA and -SSA sera were then purified in the same way using the appropriate combination of immunoadsorbent columns.

Extraction of meat antigens

100g of meat samples (beef, horse, sheep, goat, venison and veal) trimmed of fat and connective tissue were cut into small pieces and homogenised in 100 ml NaCl (0.85%). The homogenate was centrifuged (10,000 rev/min for 30 min at 4°C) and then filtered through Whatman No. 3 paper to remove the fat residue. Extracts were stored at 4°C after addition of a bacteriostatic agent, sodium azide (0.1g/l).

Double immunodiffusion test

Test plates were made by covering dry pre-coated glass slides (purified agar, Oxoid, (1.5%)) with a 3 mm thick layer of agarose (BDH, 1.5%)) containing sodium azide (0.1g/l). A hexagonal 'Ouchterlony' pattern of wells (6 mm diameter) were cut around a central well (8 mm diameter). The outer wells were filled with respective antigen solutions, serum albumin (1 mg/ml) or undiluted meat extracts, while the central well was filled with undiluted unpurified antiserum or the specific antibody fractions.

The plates were incubated in a humid box for 4 days at 4°C to allow the precipitin lines to develop. They were then soaked in saline (NaCl, 0.85%) for 24 h and stained with Napthalene black (1.5% in 7% acetic acid). Staining was done for 3 min and excess dye removed by

soaking the plates in 7% acetic acid until a sharp contrast between the precipitin lines and background was obtained. Permanent records were prepared by photography.

Enzyme-linked immunosorbent assay (ELISA)

Optimization of antiserum dilution

Goat anti-rabbit immunoglobulin G (Ig G) peroxidase, conjugated by the method of Nakane, et al, (1974), and o-phenylene-diamine were purchased from Sigma London Chemicals Co. Ltd., and 96 well micro-ELISA plates (12 columns x 8 rows), a micro-ELISA reader and mini-wash (Dynatech) were used.

Beef extract prepared as described above (see page 18) was diluted in carbonate-bicarbonate 'coating' buffer (0.05 M, pH 9.6) to give a range of 12 serial dilutions from 1:2 to 1:4096. Aliquots (100 µl) of each dilution were added to eight wells (1 column) of the micro-ELISA plate and incubated in a humid box (at 4°C, for 3 h) to allow the antigen to adhere to the solid phase (plate). The plates were washed 4 times with phosphate buffered saline containing 0.05' Tween-20 (PBS-Tween).

Anti-BSA specific antibody fraction was diluted serially in PBS-Tween to give a range of 8 serial dilutions from 1:10 to 1:1280. Aliquots (100 µl) of each dilution were added to 12 wells (1 row) of a micro-ELISA plate, and incubated at room temperature for 2 h in a humid box. The plates were washed with PBS-Tween again. 100 µl of the Ig G peroxidase conjugate diluted 1:1000 in PBS-Tween was added to each well and incubated (4°C, 12 h) in a humid box.

After washing (PBS-Tween), 200 µl of the substrate solution was added to each well and incubated in the dark at room temperature for 30 min. The substrate solution was prepared immediately before use by

dissolving o-phenylene-diamine (34 mg) in 100 ml of citrate phosphate buffer (0.15 M, pH 5.0) and adding 50 µl of hydrogen peroxide (20 vol). After incubation the enzyme reaction was stopped by adding 50 µl of sulphuric acid (12.5%). The absorbance of each well was then read at 488 nm using a micro-ELISA reader.

Analysis of the data was carried out by plotting antiserum dilution against absorbance values for selected antigen dilutions. The optimum antiserum dilution was obtained from those dilutions occurring on the straight part of the curve, where the assay was most sensitive. The procedure was repeated to determine the optimum dilutions for the anti-HSA and -SSA specific sera.

Optimisation of antigen dilution

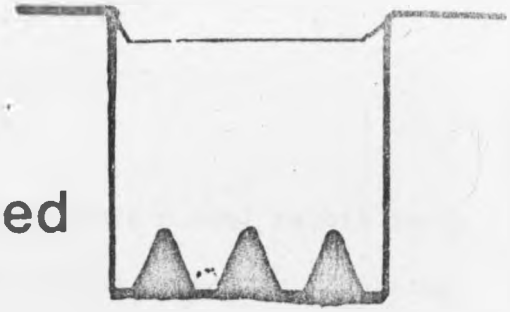
The same ELISA procedure (see Fig. 1) was carried out employing the optimum antiserum dilution previously determined. The range of antigen dilution used was 1:2 to 1:4096. The optimum antigen dilution was chosen from the mean absorbance value which differed significantly from that of the control (same antigen dilution tested using normal rabbit serum diluted in PBS-Tween at the same dilution as the test serum). This was the region of minimum interference from the other proteins extracted from the meat.

Identification of the species of origin of raw meat

Meat extracts of beef, sheep, veal and horse prepared as described (p 18) were diluted in carbonate-bicarbonate buffer 1:64 and then serially to 1:8192. Aliquots (100 µl) of each dilution were added to eight wells (1 column) of the micro-ELISA plate and incubated (4°C for 3 h) in a humid box. The plates were then washed 4 times in PBS-Tween. 100 µl of anti-BSA specific antibody fraction diluted 1:200 in PBS-Tween, was

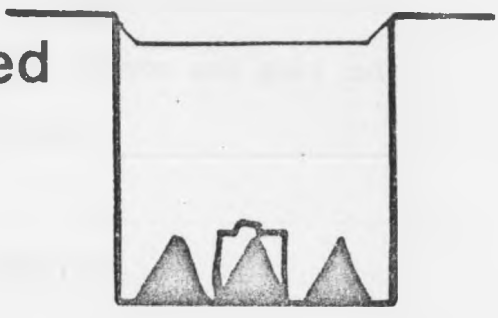
Fig. 1. Procedure for the Indirect ELISA test.

1. Antigen adsorbed to plate



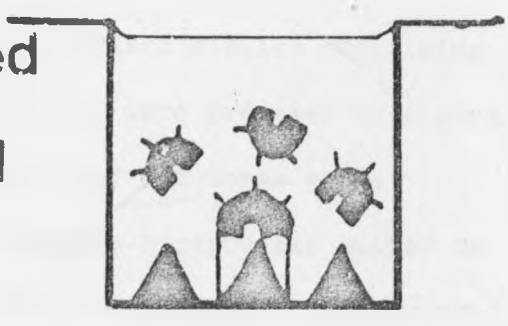
Wash

2. Antiserum added



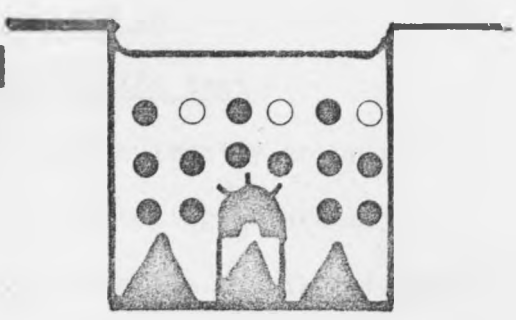
Wash

3. Enzyme labelled antibody added



Wash

4. Substrate added ○ → ●



Indirect ELISA

added to 4 rows of wells while to the other 4 rows normal rabbit serum diluted 1:200 in PBS-Tween was added. The plates were then incubated in a humid box (room temperature, 2 h) and the determination completed as previously described (p 19).

Other assays were performed to discriminate horse meat from beef and sheep using anti-HSA specific fraction (1:400) in PBS-Tween and to differentiate sheep meat from beef, horse, venison and goat using anti-SSA specific fraction (1:400) in PBS-Tween.

Qualitative detection of horse meat in beef/horse mince

Minced beef and horse meats were mixed together to give the following per cent horse meat in beef mince:- 0.2, 0.5, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 80. Antigen extraction from these mixtures were carried out as previously described (p 18). Further samples comprising of 0.2, 0.5, 2 and 5 per cent horse meat in beef were prepared by mixing appropriate aliquots from extracts of purely beef and horse meats. These extracts were diluted serially in carbonate-bicarbonate buffer to provide a range of antigen dilution from 1:512 to 1:16384. ELISA method described above was used employing anti-HSA specific antibody fraction (1:400) in PBS-Tween.

Quantitative determination of horse meat in beef mince : Using competitive indirect ELISA test

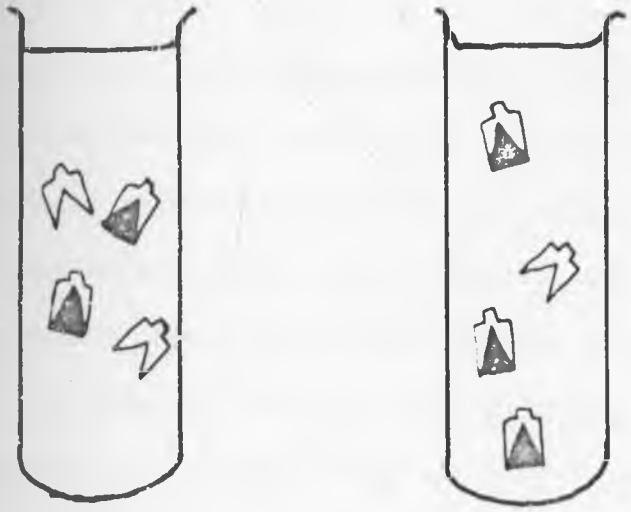
The principle of this test (Fig. 2) is to assay indirectly an unknown antigen, for example, a meat extract, by pre-incubating separately a mixture of the reference antibody with the test sample. This inhibits the activity of the antibody. The resulting solution is then assayed

Fig. 2. Procedure for the competitive antigen modification of the indirect ELISA test.

In the test sample (a) has low antigen content while (b) has high antigen content.

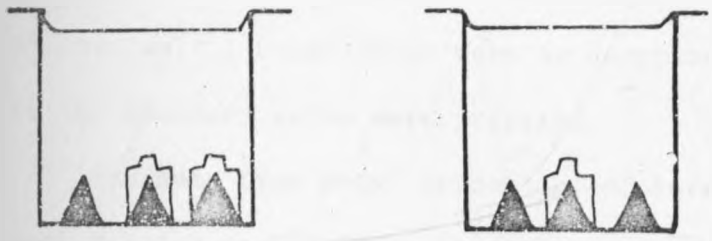
1· Plate coated with antigen

2· Wash



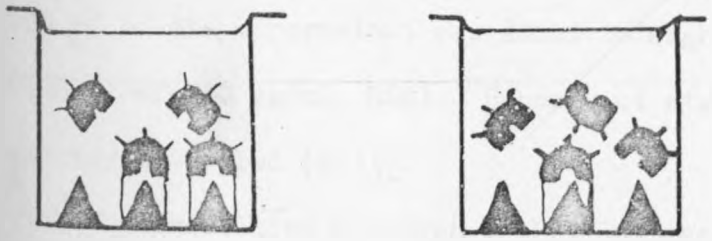
3· Antiserum incubated with antigen

4· Wash



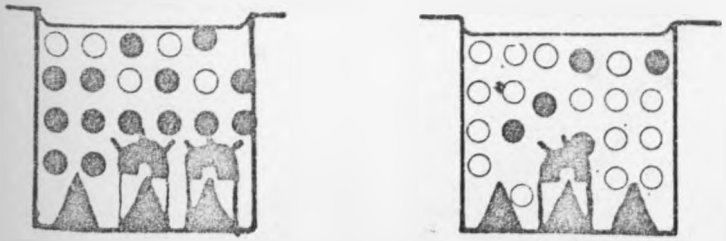
5· Excess antibody reacts with antigen

6· Wash



7· Conjugate added

8· Wash



9· Substrate added

○ → ●

(a) (b)

as before against a constant coating of reference antigen on a micro-ELISA plate. Test samples can be made to ascertain a standard 'inhibition' curve of antigen effect and then unknown samples quantified by direct comparison. High absorbance values indicate high antibody activity and low test antigen concentration, whereas low absorbance values indicate that the reference antibody has reacted (inhibited) with higher amounts of the antigen under test. The test can be modified to ascertain the extent of cross-reactivities between antisera.

A standard curve was constructed by incubating separately anti-HSA specific antibody fraction (1:400) in PBS-Tween with an equal volume of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 and 40 µg/ml HSA at room temperature for 1 h. The mixtures were then centrifuged (3,000 rev/min, for 10 min at 4°C) and 100 µl of the supernatant added to eight wells of a plate precoated with 100 ul aliquots of 250 µg/ml HSA per well. Other steps were as described (p 19). Six replicates of the standard curve were prepared.

Extracts from known percentage of horse meat in beef mince were each diluted in PBS-Tween (1:200) and incubated (room temperature for 1 h) with an equal volume of anti-HSA specific antibody fraction (1:400) in PBS-Tween. After centrifugation (3,000 rev/min, for 10 min, at 4°C) 100 µl of the supernatant was added to eight wells of a precoated plate (100 µl of 250 µg/ml, HSA). Subsequent steps were similar to those already described (p 19).

Pre-incubation mixtures of BSA and the antibody solution over a similar range of concentrations as in the standard were prepared to assess the inhibition effect of BSA on the anti-HSA specific fraction, as would be anticipated in beef extracts.

Statistical analysis of the data

Tests of significance were carried out using a Compucorp 327 Scientist Calculator programmed with paired t-test (M.R.I. programmes). The overall student's t-value were calculated between the absorbance values of:- (a) beef as differentiated from horse, sheep and veal, (b) horse as differentiated from beef and sheep and (c) sheep as differentiated from horse, beef, venison and goat over the appropriate range of antigen dilutions used.

Common curves were fitted using the Maximum Likelihood Program (MLP) Rothamsted Experimental Station (Ross, 1975) with the equation:

$$Y = A + B_e^{-kx}$$ where, $\frac{\log_e 2}{K}$ is the time for Y-A to halve,

A is the lower asymptote (value of Y as X tends to ∞) and B is the distance between the value of Y and X = 0 and X = $+\infty$. Analysis of variance to test the significance of parallelism (when the curves have the same K value but different A and B values) and the displacement within the curves used was calculated. A common curve was fitted when parallelism and displacement were found not to be significant.

RESULTS

Double immunodiffusion precipitin test

Unpurified anti-BSA serum gave strong reactions of identity with BSA and SSA but not with HSA. With meat extracts, similar reactions of identity were observed with beef, veal, goat, venison and sheep (Plate 1). Purification reduced the range of cross reactivity, as the specific antibody fraction gave a reaction of identity only with beef and veal meat extracts. No reaction was observed with extracts of horse, sheep, goat or venison meats. Reaction with the individual species albumins confirmed that the purified antibody fractions had complete specificity for BSA.

Unpurified anti-SSA serum gave strong reactions of identity with HSA and PSA (pig serum albumin) (Plate 4). Reactions of identity were also recorded between sheep meat extracts and those of beef, veal, goat and venison against the same antiserum showing the presence of common antigenic determinants in each species (Plate 2). After purification, the specific fraction showed specificity for SSA as no precipitin lines were formed around the wells containing BSA or HSA. However, with the meat extracts reactions of identity were still observed between sheep meat and beef, venison and goat (Plate 6).

Unpurified anti-HSA reacted with HSA and horse meat extract showing an exclusive reaction of identity. No reactions with BSA, SSA or with meat extracts of beef, sheep, veal, goat and venison were observed (Plate 3). No differences were noted in the reactions of the species specific fraction obtained by the same purification procedure (Plate 5).

Plate 1. Shows the antigenic properties of unpurified anti-BSA serum. In the 4 tests, wells a, c and e contain beef extract while b, d, and f in 1, 2, 3 and 4 contain sheep, goat, venison or veal extracts respectively.

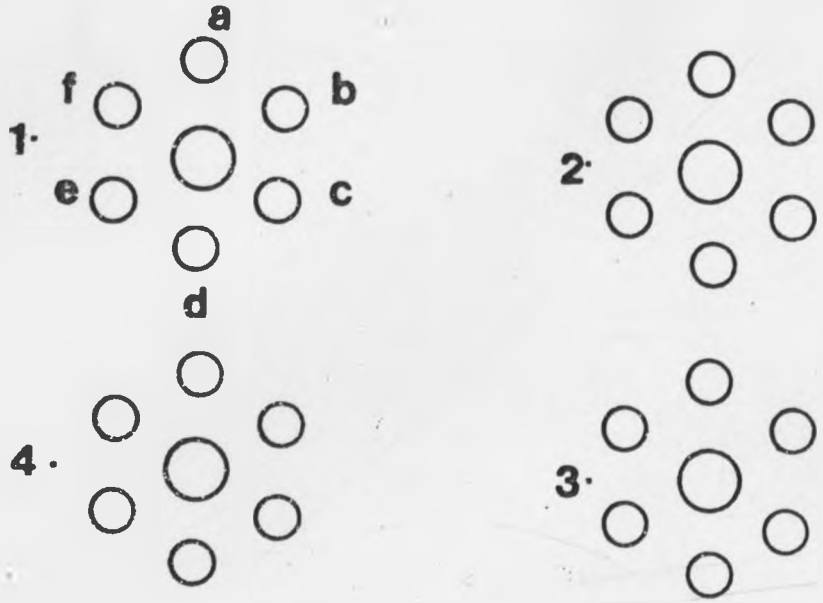
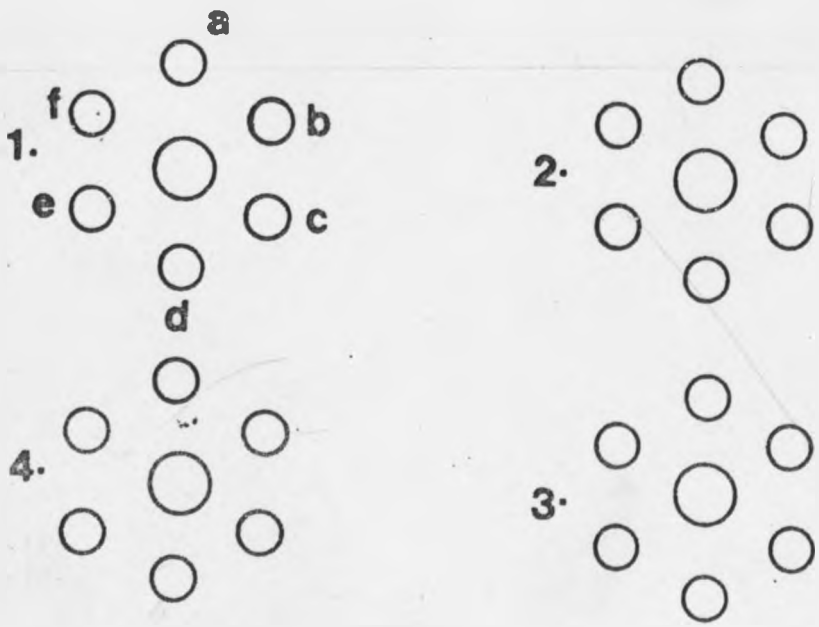
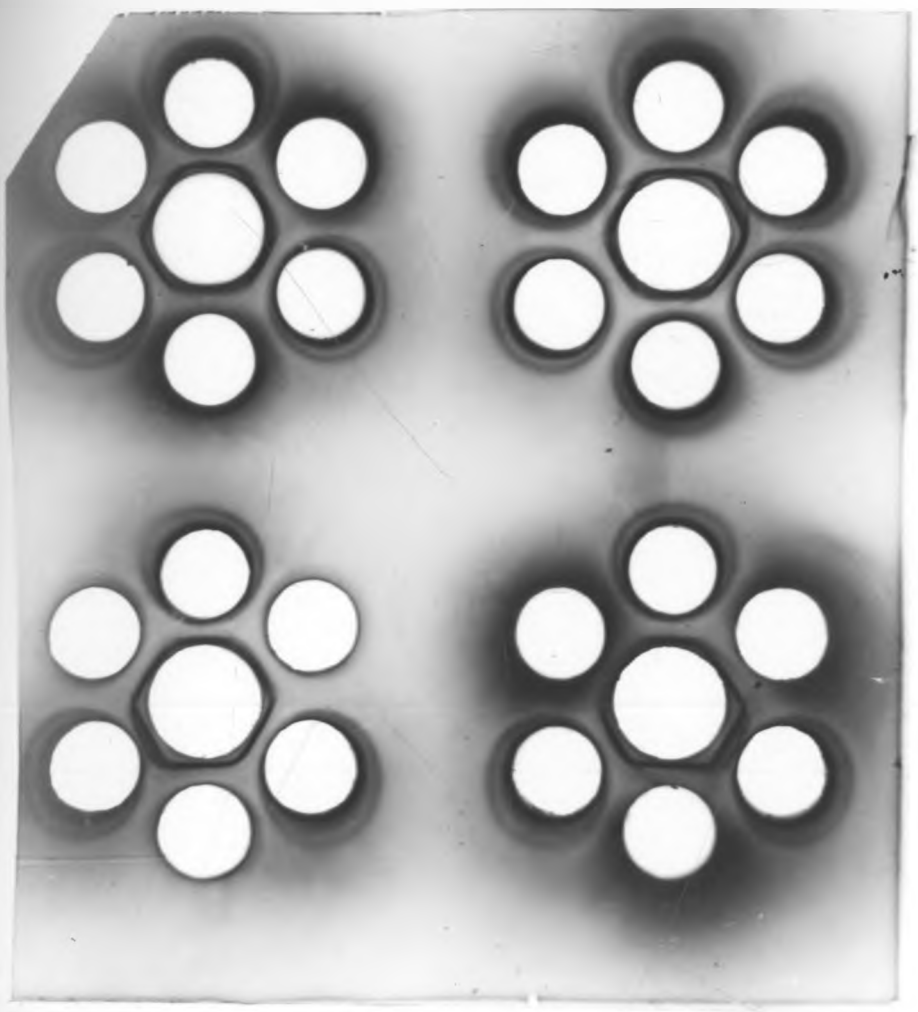


Plate 2. Shows the antigenic relationship of unpurified anti-SSA serum. Wells a, c and e contain sheep meat extracts while b, d and f in 1, 2, 3 and 4 contain beef, goat, venison or veal extracts respectively.





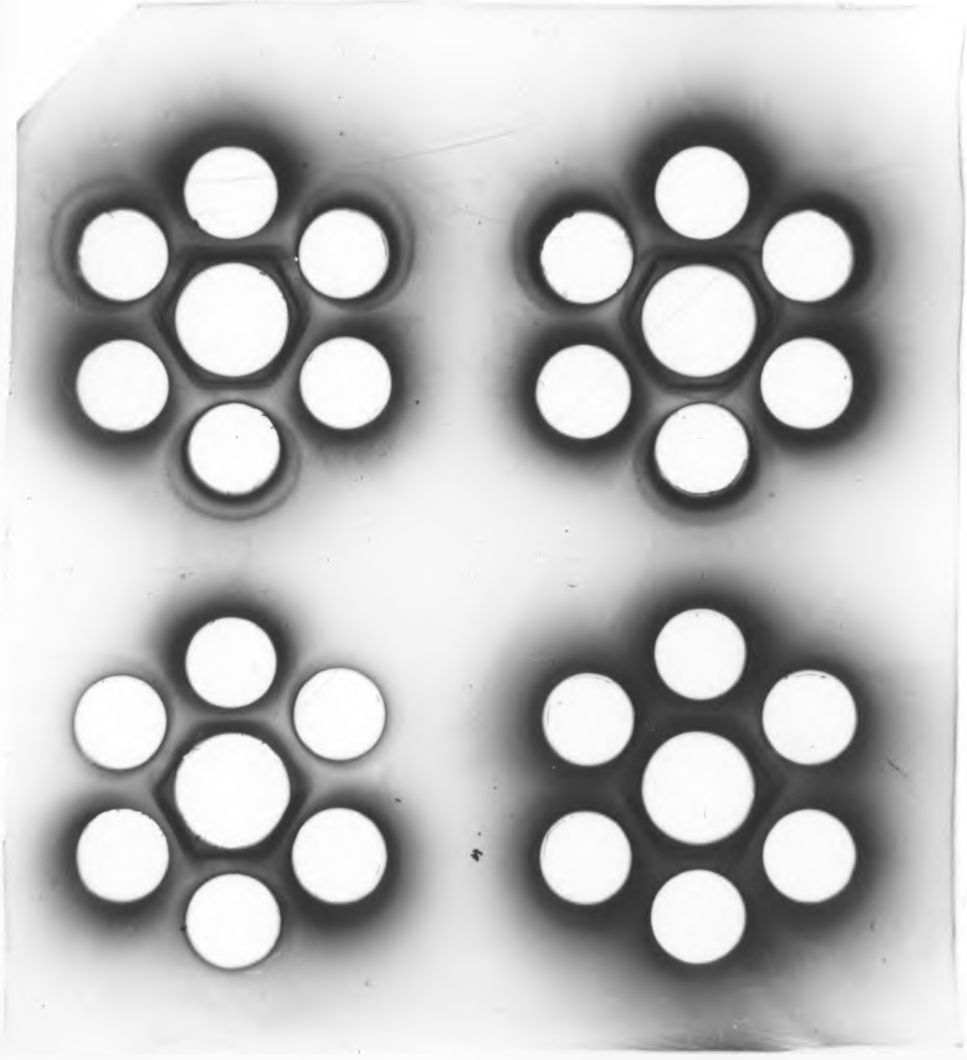


Plate 3. Shows the antigenic relationship of unpurified anti-HSA serum. Wells a, c and e in all tests contain horse meat extract while b, d and f in 1, 2, 3 and 4 contain beef, sheep, venison or veal extracts respectively.

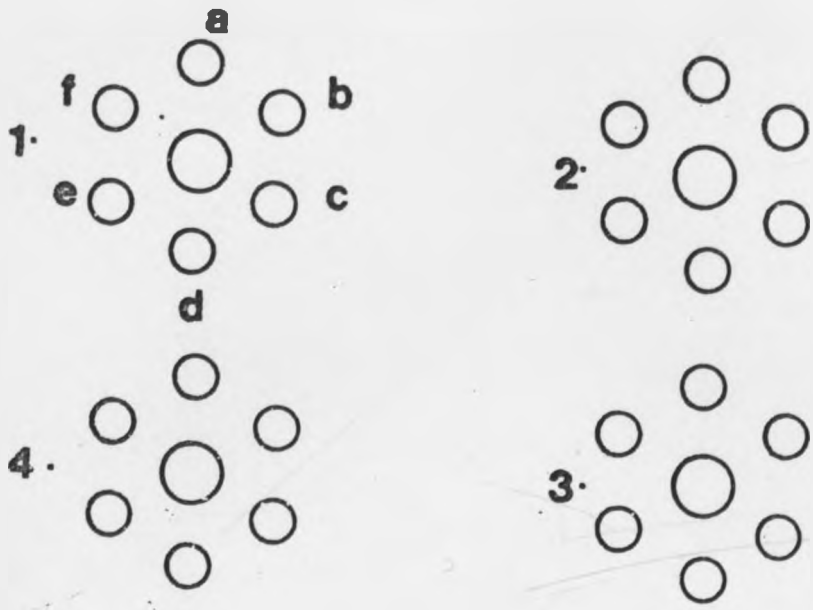
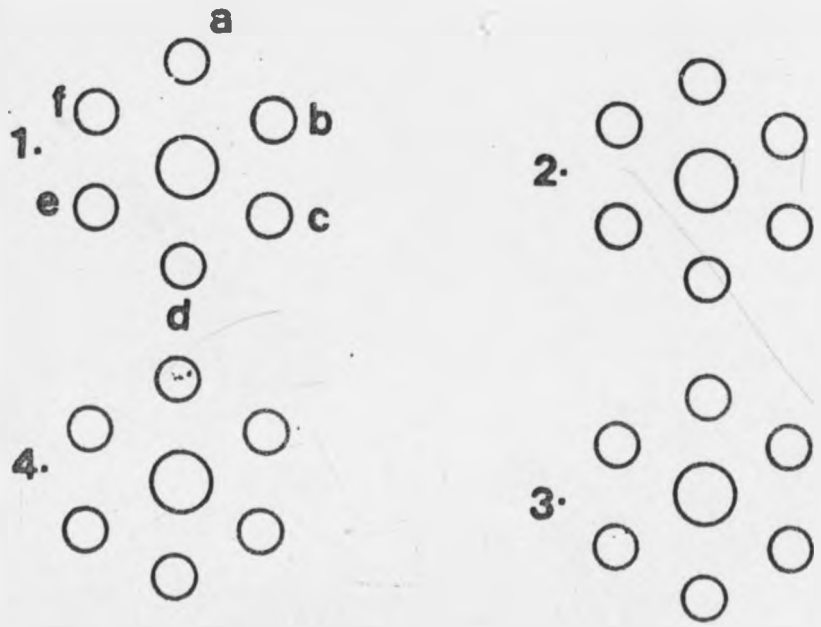
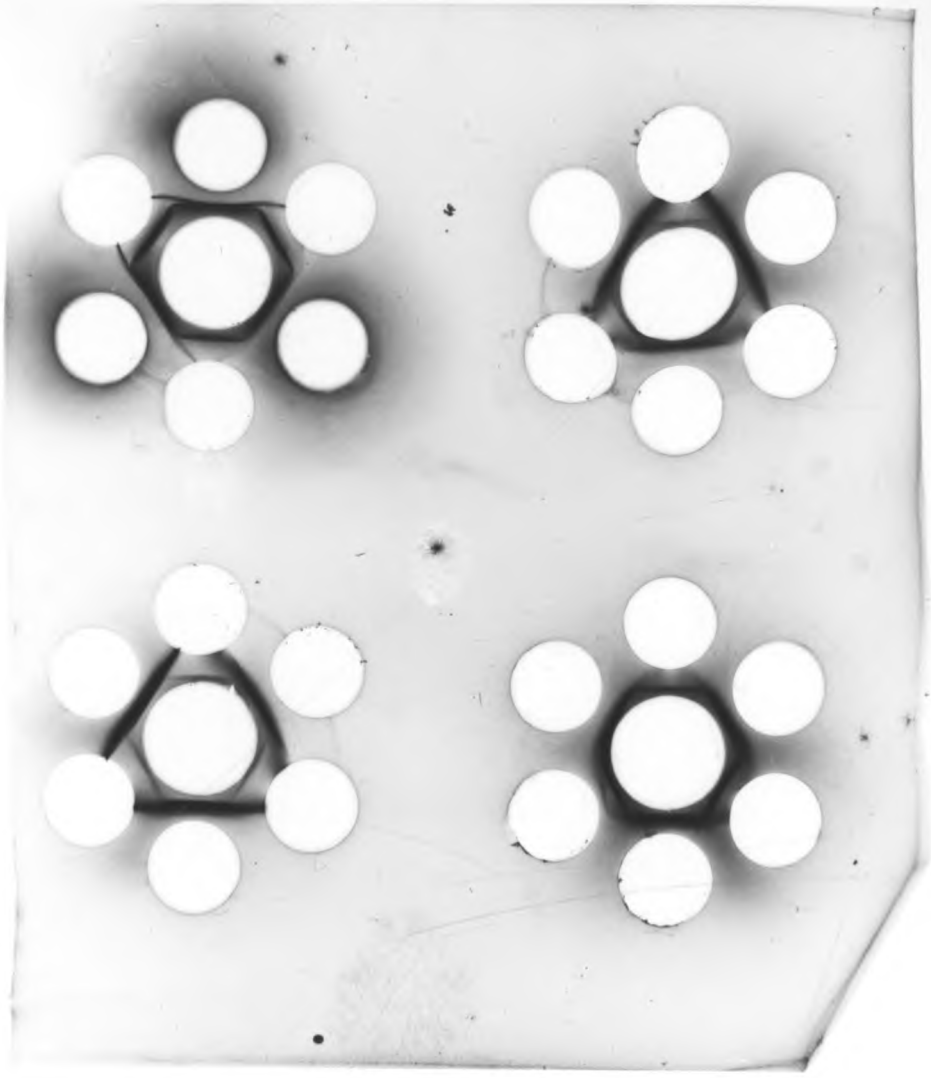


Plate 4. Shows the antigenic properties of unpurified anti-SSA serum. Wells a, c and e in all tests contain 1 mg/ml SSA while b, d and f in 1, 2, 3 and 4 contain BSA (1 mg/ml), HSA (1 mg/ml), sheep meat extract or PSA (1 mg/ml) respectively.





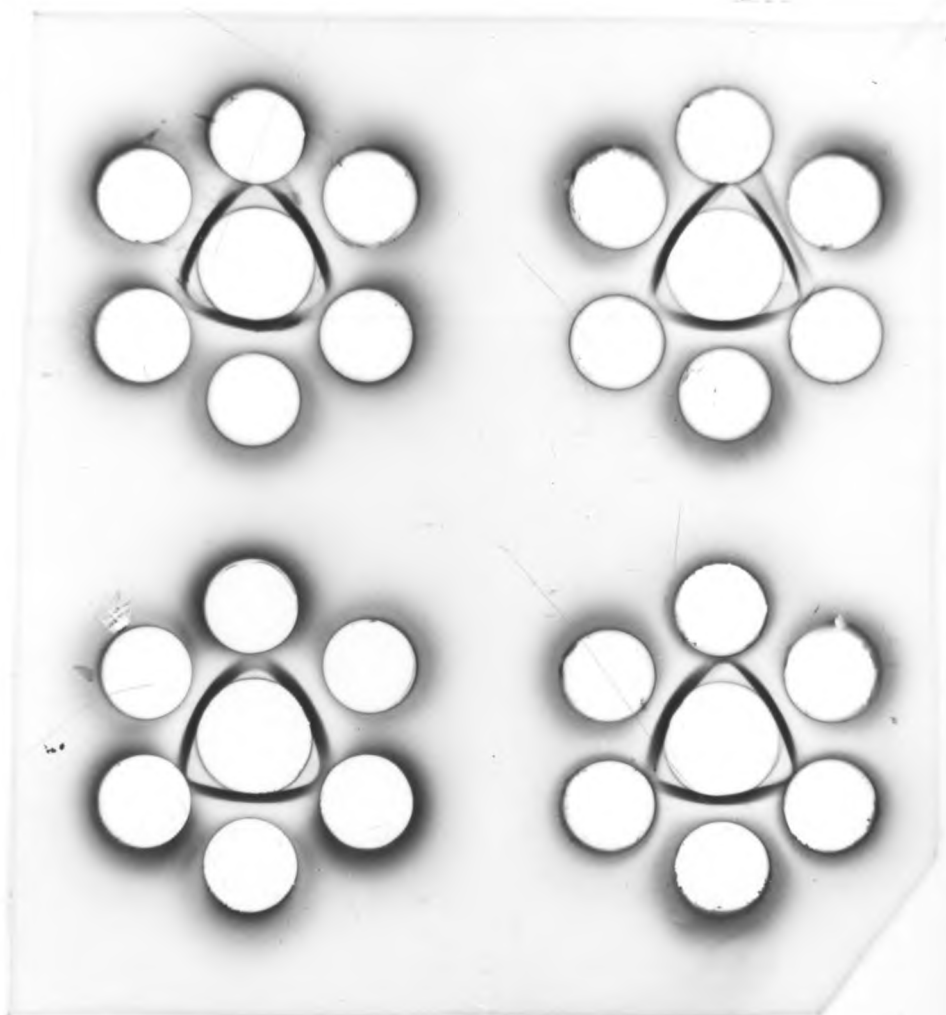


Plate 5. Shows the antigenic properties of purified anti-HSA. Wells a, c and e in the 4 tests contain horse meat extract while b, d and f in 1, 2, 3 and 4 contain beef, sheep, venison or veal extracts respectively.

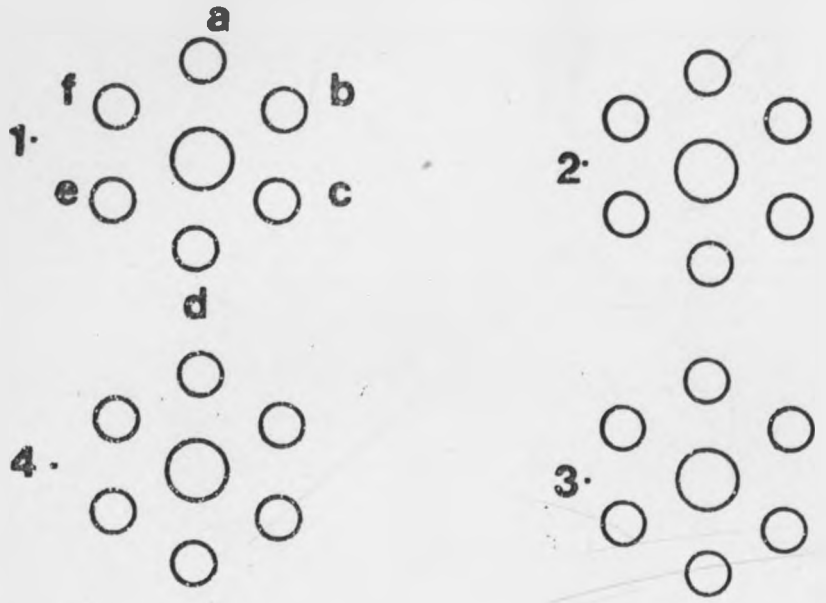
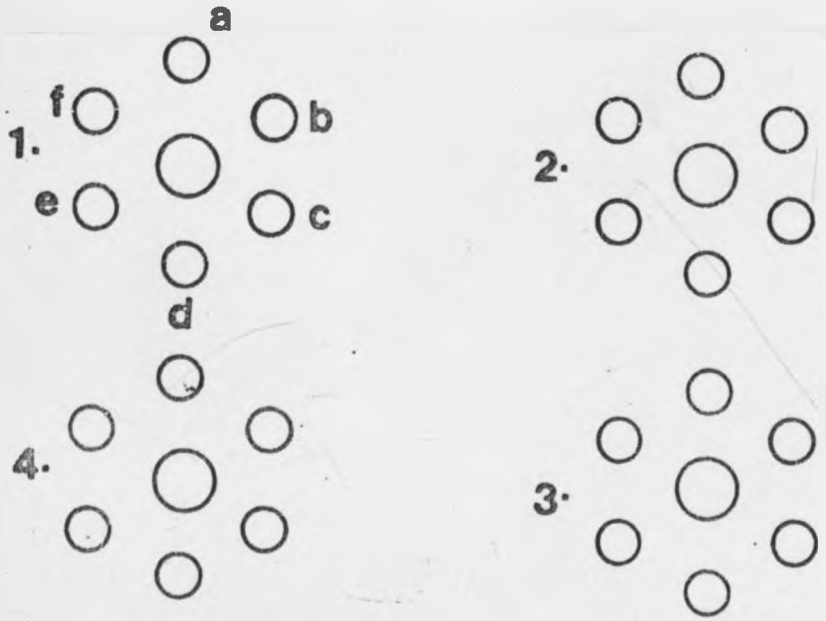
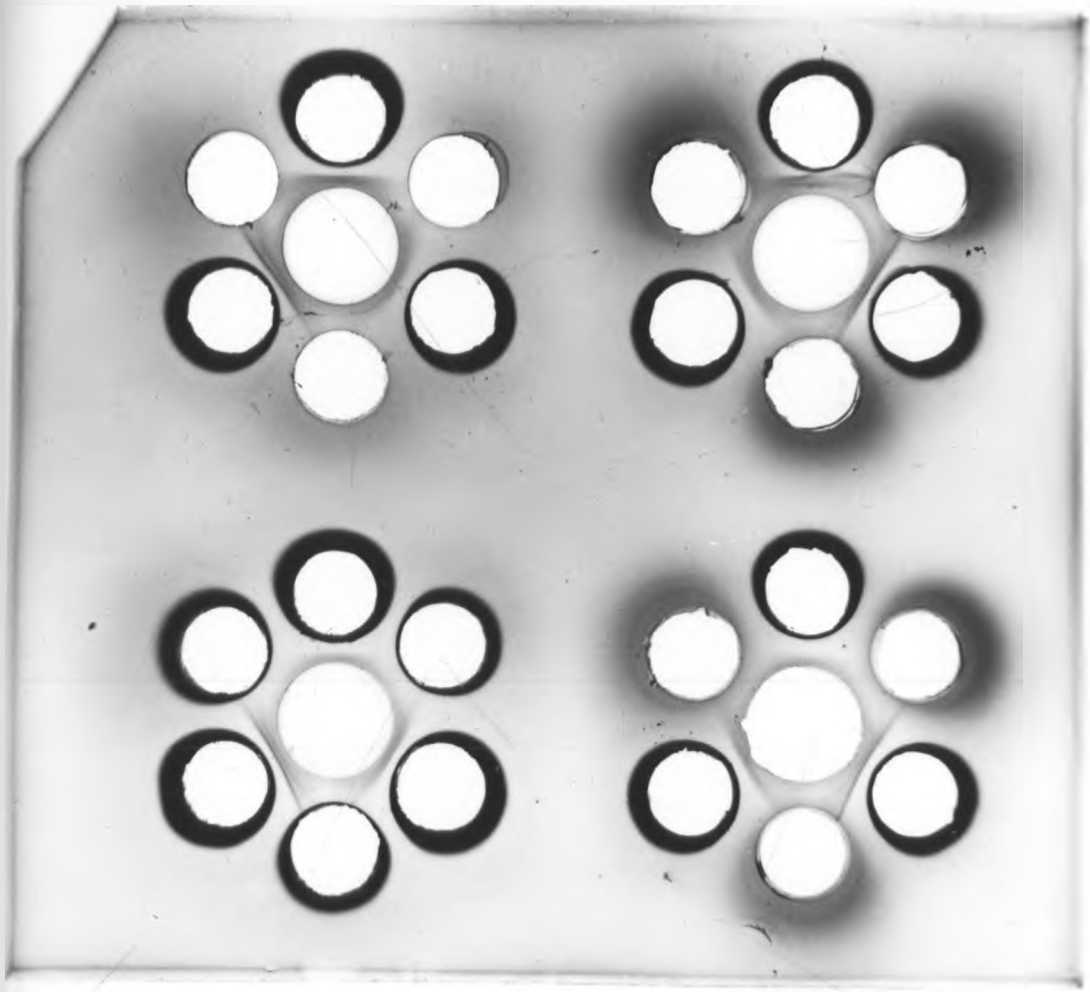
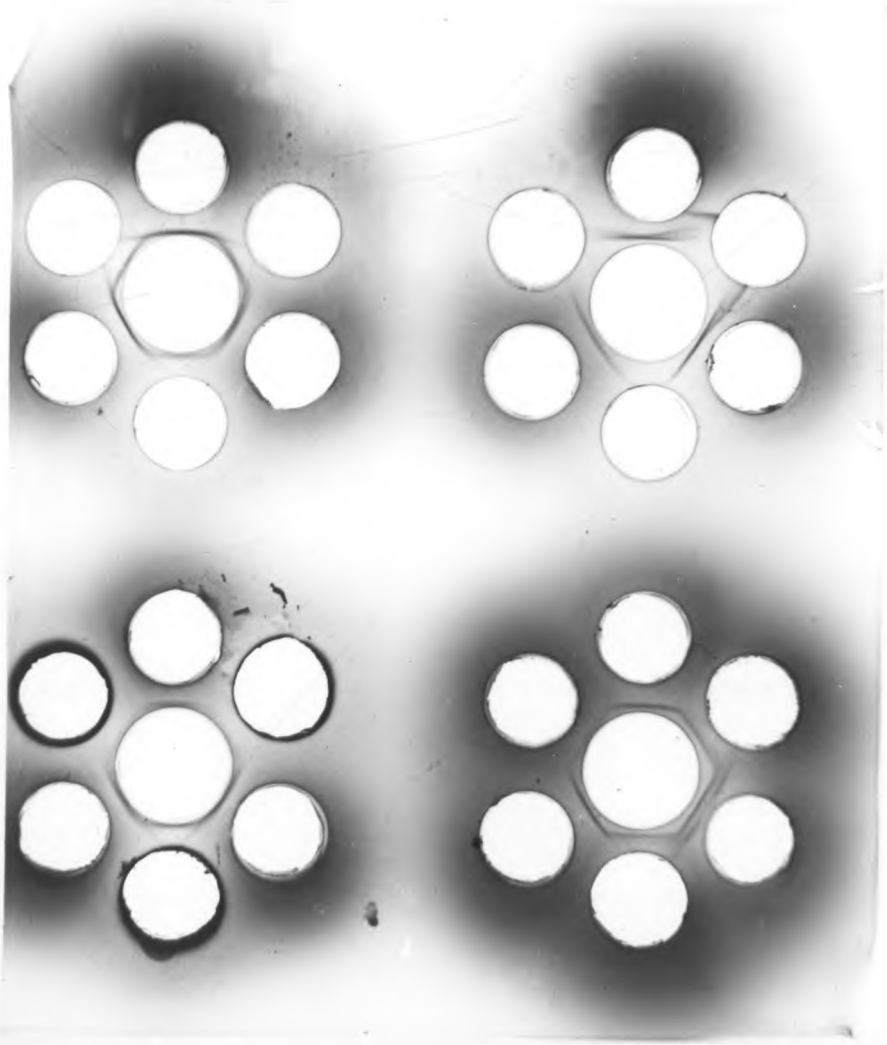


Plate 6. Shows antigenic relationship of purified anti-SSA. Wells a, c and e contain SSA (1 mg/ml) while b, d and f in 1, 2, 3 and 4 contain beef, horse, venison or veal extracts respectively.







Optimisation of antigen and antiserum working dilutions

The results revealed that against their homologous meat extract antigens, anti-BSA, -SSA and -HSA specific fractions required dilution to 1:200, 1:400 and 1:400 respectively in PBS-Tween. These dilutions lay on the straight portions of the curves where sensitivity was high (see Appendix Tables 4-6). The antigen dilution range varied between 1:64 to 1:16,384 for beef and horse meat extracts and 1:512 to 1:32,764 for sheep meat extract, employing the already determined antiserum dilution. The antigen dilutions representing the regions of minimum interference from other extracted proteins are shown (Appendix Figs. 9-11).

Qualitative identification of the species of origin of meat using specific antisera and ELISA

Beef was effectively discriminated from sheep, horse and veal using anti-BSA specific fraction (Fig. 3). This differentiation was highly significant ($P \leq 0.001$) (Table 1) over the range of antigen dilutions used.

Horse meat was similarly differentiated from beef and sheep meat using anti-HSA specific fraction (Fig. 4). The overall results were highly significant ($P \leq 0.001$) (Table 1) over the range of antigen dilutions employed.

Sheep meat was differentiated from horse, venison and beef (Fig. 5) at differing levels of significance but not from goat (Table 1) over the range of antigen dilutions used.

Table 1. Discrimination between meats from different animal species by use of species specific antisera.

Antiserum (specific fraction)	Meat extract	Degrees of freedom	Student's t-value
Anti-BSA	Beef vs horse	7	14.20 ***
	" " sheep	7	11.01 ***
	" " veal	7	15.68 ***
Anti-HSA	Horse vs beef	7	21.65 ***
	" " sheep	7	23.78 ***
Anti-SSA	Sheep vs beef	6	2.71 *
	" " horse	6	7.35 ***
	" " goat	6	2.15 NS
	" " venison	6	4.18 **

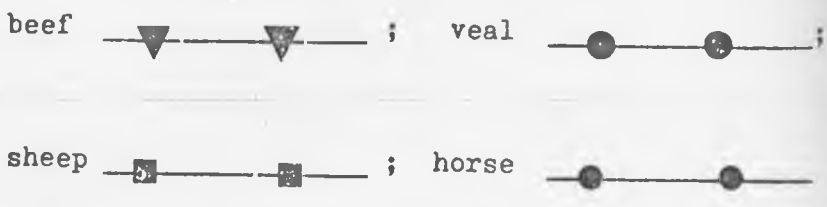
* Significant at $P \leq 0.05$

** " " $P \leq 0.01$

*** " " $P \leq 0.001$

NS Not significant at $P \leq 0.05$

Fig. 3. Identification of beef using anti-BSA specific fraction in the ELISA test.



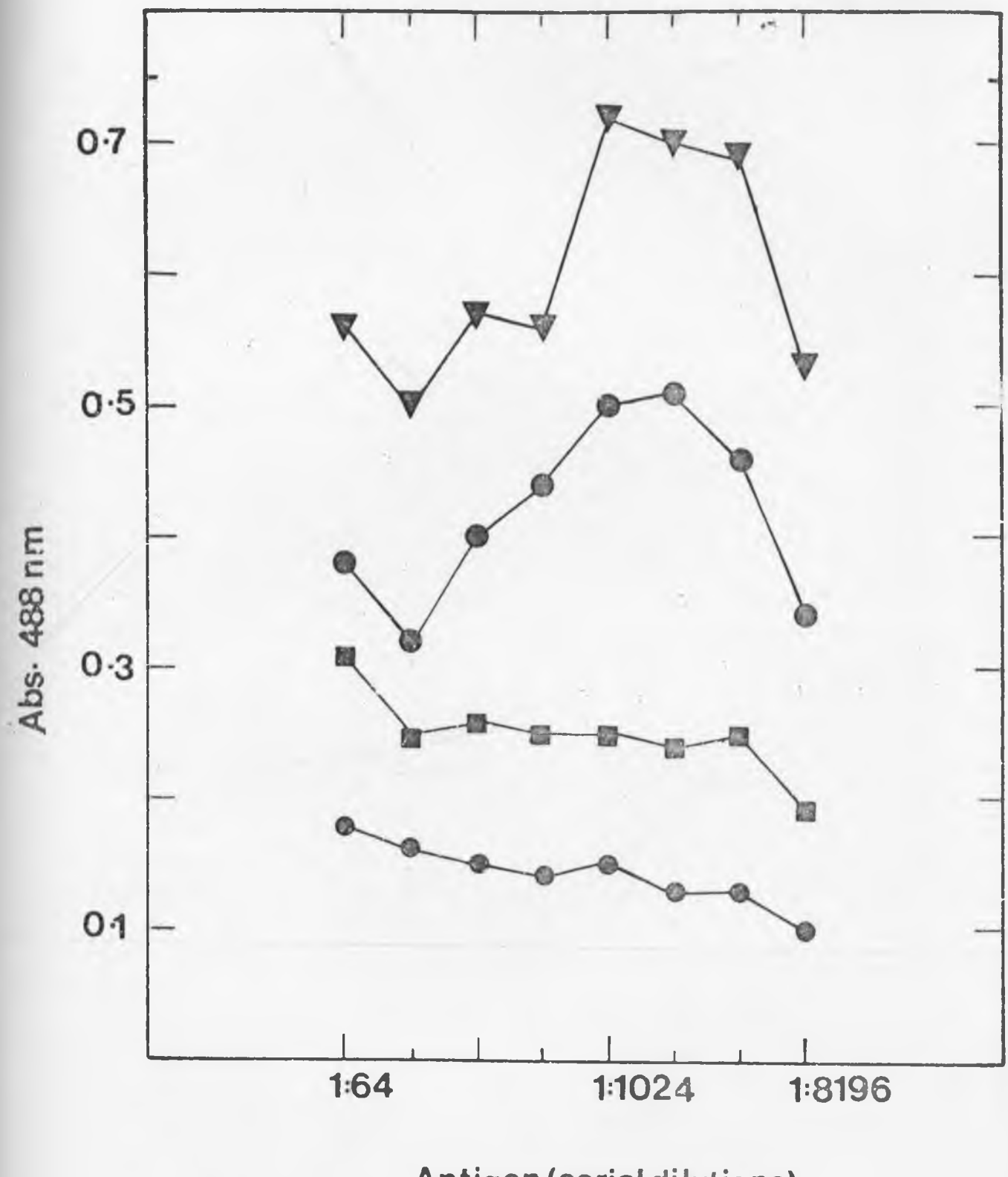
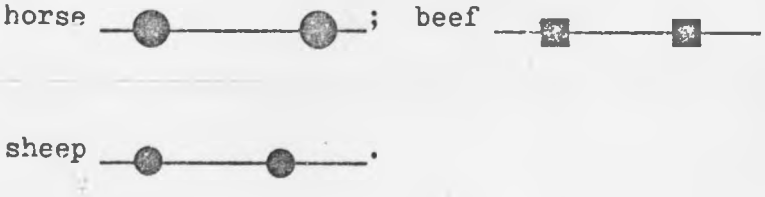

0·7
0·5
0·3
0·1
Abs· 488 nm
1:64
1:1024
1:8196
Antigen (serial dilutions)·

Fig. 4. Identification of horse meat using anti-HSA specific antibody fraction in the ELISA test.



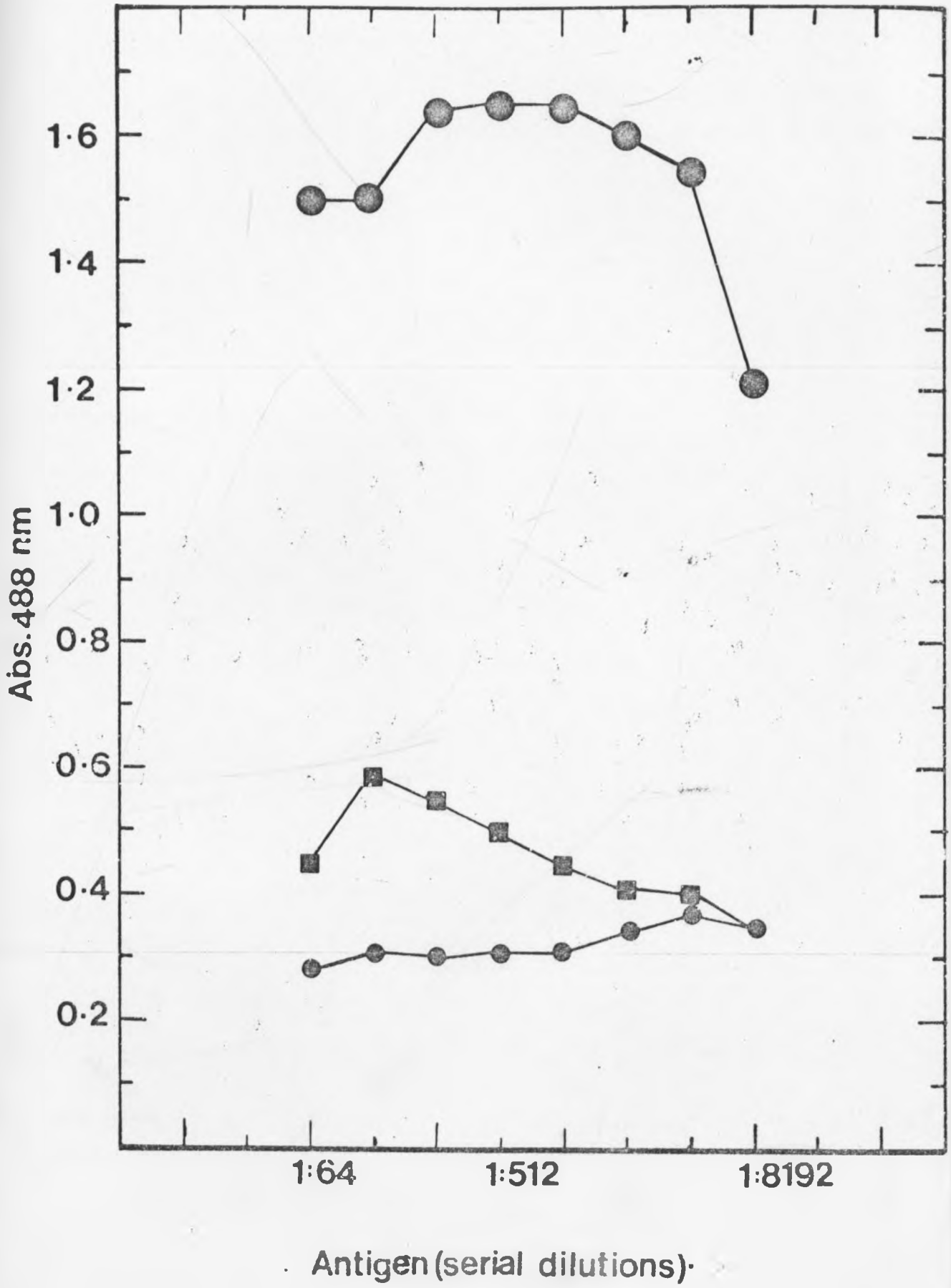
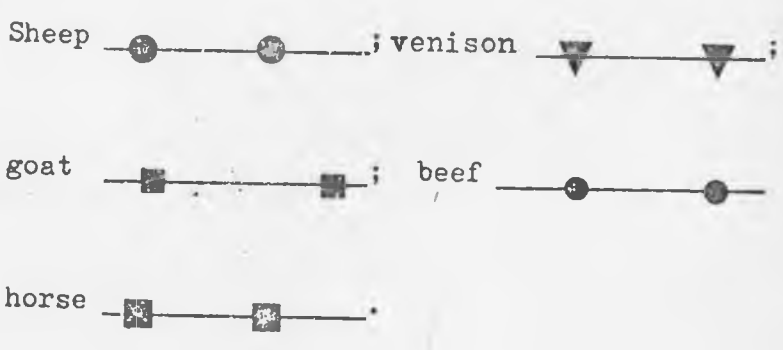

1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Abs.488 nm
1:64
1:512
1:8192
Antigen (serial dilutions)·

Fig. 5. Identification of sheep meat using anti-SSA specific fraction in the ELISA test.



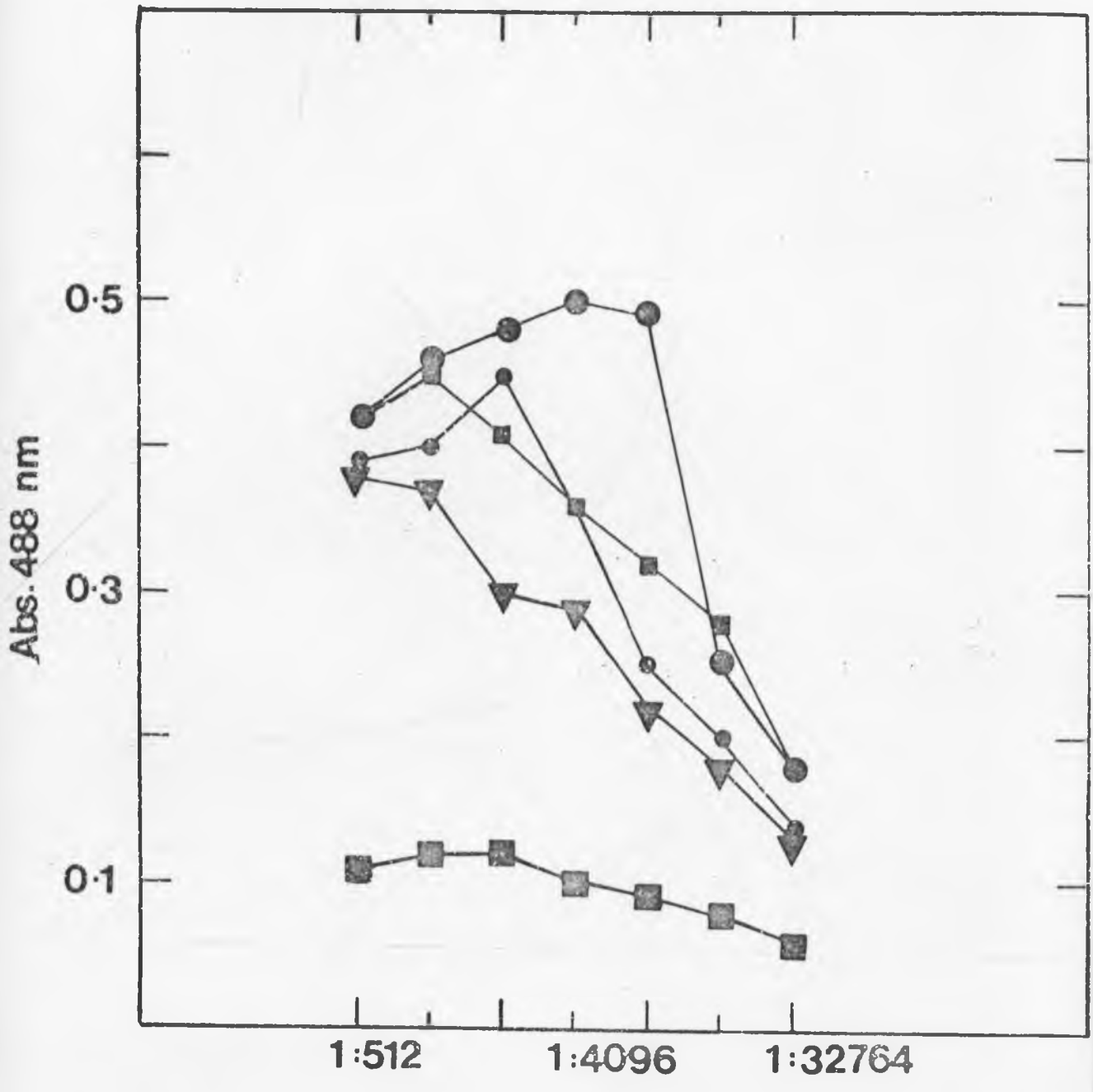

0·5
0·3
0·1
Abs. 488 nm
1:512
1:4096
1:32764
Antigen(serial dilutions)·

Table 3. Estimated percentage of horse meat in beef mince in samples of known composition.

Known % of horse meat in beef	Absorbance value at 488 nm	% estimated from constructed curve
50	0.90	12.05
20	0.72	7.26
10	0.57	4.05
5	0.46	1.92
2	0.29	-1.07

Curves constructed using absorbance values for known concentrations of horse meat in beef mince are not applicable as reference standards for subsequent analyses.

Table 2. Similarity of absorbance curves. Analysis of variance data for displacement or parallelism.

Source	SS	DF	MS	F-value	Significance
Displacement	0.01	4	0.003	0.97	NS
Parallelism	0.001	2	0.0007	0.23	NS
Within curves	0.09	30	0.003		

SS = Sum of squares.

MS = Mean sum of squares.

DF = Degrees of freedom.

NS = Not significant at $P \leq 0.05$.

$$Y = A + B_e^{-kx} \quad \text{(i)}$$

$$X = \frac{-1}{K} \log_e \frac{Y-A}{B} \quad \text{(ii)}$$

Although this method appears to be a plausible way of estimating the percentage of horse meat in beef mince, the results (Table 3) clearly indicate that quantitative data cannot be obtained by this technique. It was apparent from the overall lower absorbance values obtained for these latter mixtures that although they were consistent within the set, they were quite different from the previous samples.

Results of the Competitive ELISA test showed that inhibition of the anti-HSA specific fraction by BSA was negligible. The results of samples used to test the reliability of the Competitive ELISA method showed that the HSA content of 40% horse meat in beef mince was 0.23 µg while pure horse meat extract contained 0.63 ± 0.16 µg/ml HSA (95% confidence interval). The HSA content of samples containing 0.5, 5 and 10% horse meat in beef could not be estimated accurately because the absorbance values lay outside the sensitive range of the curve. Maximum sensitivity was found to lie between 0.35 and 0.15 (absorbance value) representing a range of HSA concentration from 0.04 to beyond 40 µg/ml respectively (Fig. 8).

Qualitative and quantitative detection of horse meat in beef mince

Small differences in the absorbance values were recorded between pure beef extract and extracts of beef mince containing 0.2, 0.5, 2.0 and 5% horse meat, but the differences were statistically non-significant at $P = 0.05$. However, similar percentages of horse meat in beef synthesised by mixing appropriate aliquots of pure beef and pure horse meat extracts were differentiated significantly down to 0.5% ($P \leq 0.05$) (Fig. 6). Mixtures of 10 to 80% horse meat in beef mince were significantly differentiated from pure beef. Similarly the test differentiated up to 50% horse meat in beef mince from pure horse meat. The maximum concentration tested (80%) was not significantly differentiated from pure horse meat.

Further analysis of the absorbance values from the curves in Fig. 6 was carried out in the expectation of improving the quantification of the horse meat content of beef mince. Analysis of variance (Table 2) of the plots of percentage horse against absorbance for the three antigen dilutions 1:256, 1:1024 and 1:4096 revealed that neither parallelism nor displacement was significant, thereby permitting representation of the individual curves by a common curve (Fig. 7). To test the reliability of this technique in quantitative work, new extracts of known percentages of horse meat in beef (2, 5, 10, 20 and 50%) were assayed over the whole range of antigen dilutions. The mean values for each concentration at five dilutions of antigen were used to estimate the percentage of horse meat in beef from the previous common fitted curve, or calculated using equation (ii) derived from equation (i) when the absorbance values were less than 0.45.

Fig. 6. Detection of horse meat in beef mince using anti-HSA specific fraction in the ELISA test.

(a) 100% horse; (b) 45%; (c) 25%; (d) 15%; (e) 5% and (f) 0.5% horse meat in beef mince respectively and (g) 100% beef.

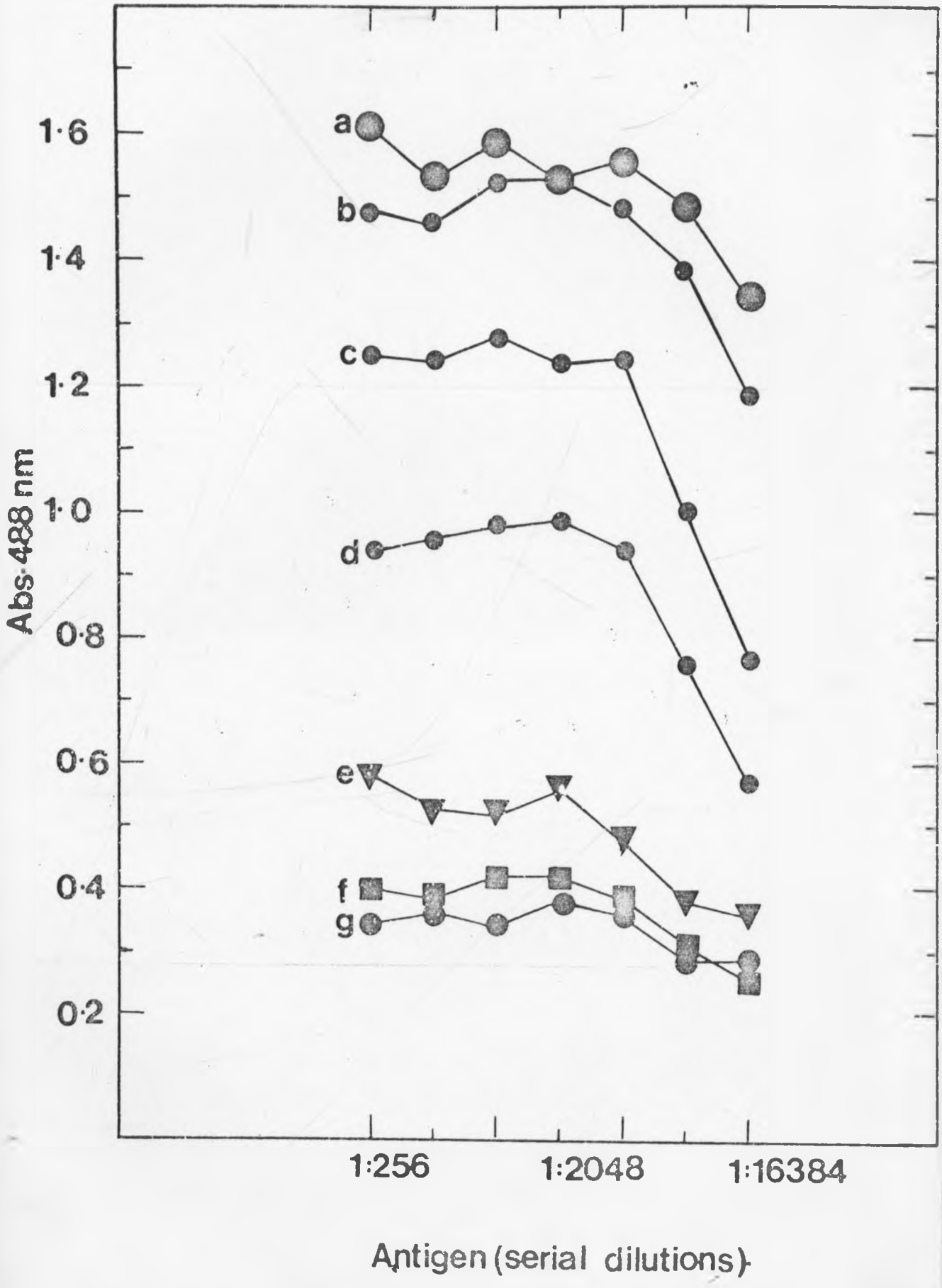

1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Abs 488 nm
a
b
c
d
e
f
g
1:256
1:2048
1:16384
Antigen (serial dilutions)

Fig. 7. A common curve (antigen dilutions 1:256 to 1:4096) for quantification of the percentage horse meat in beef mince.

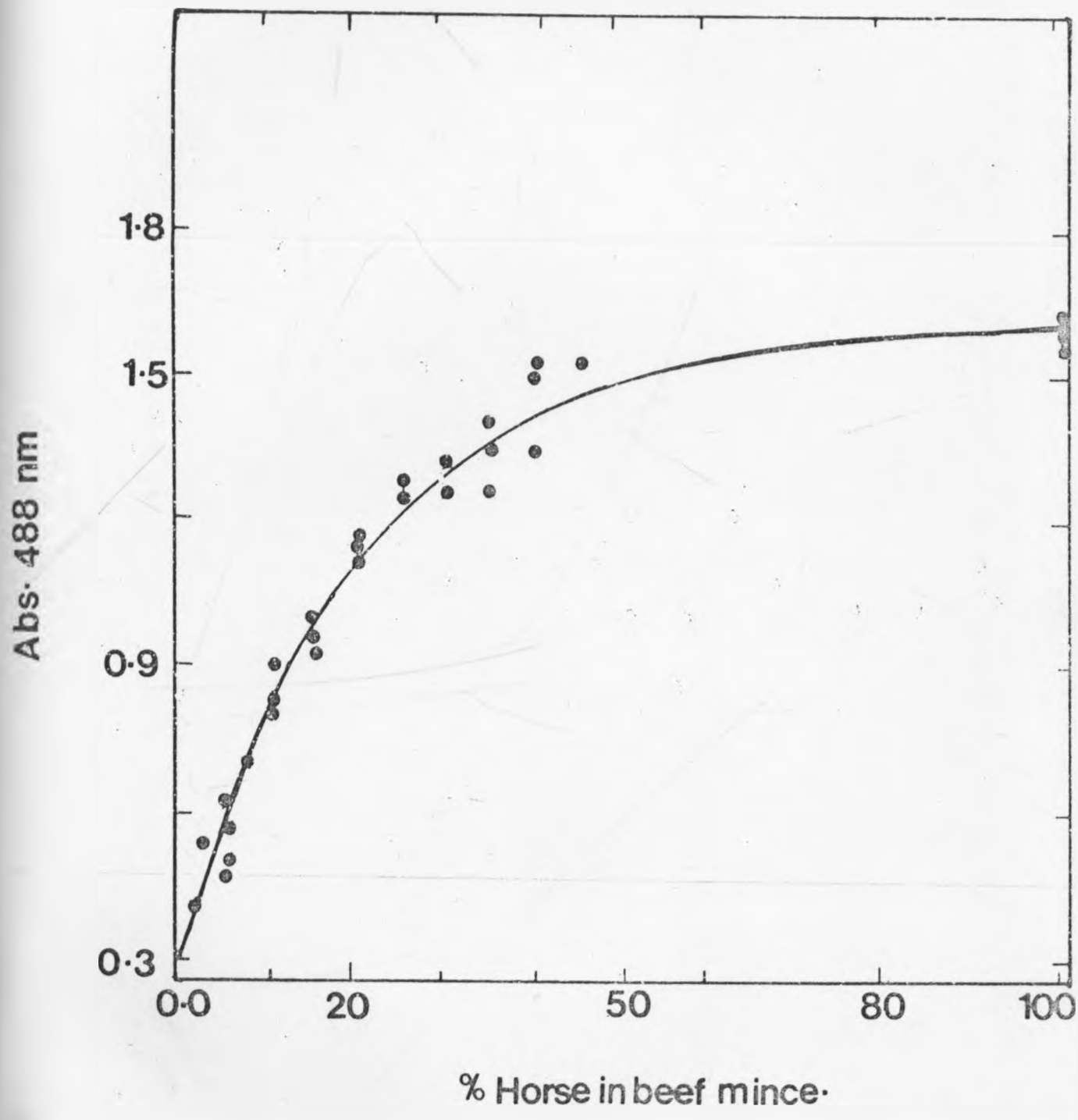
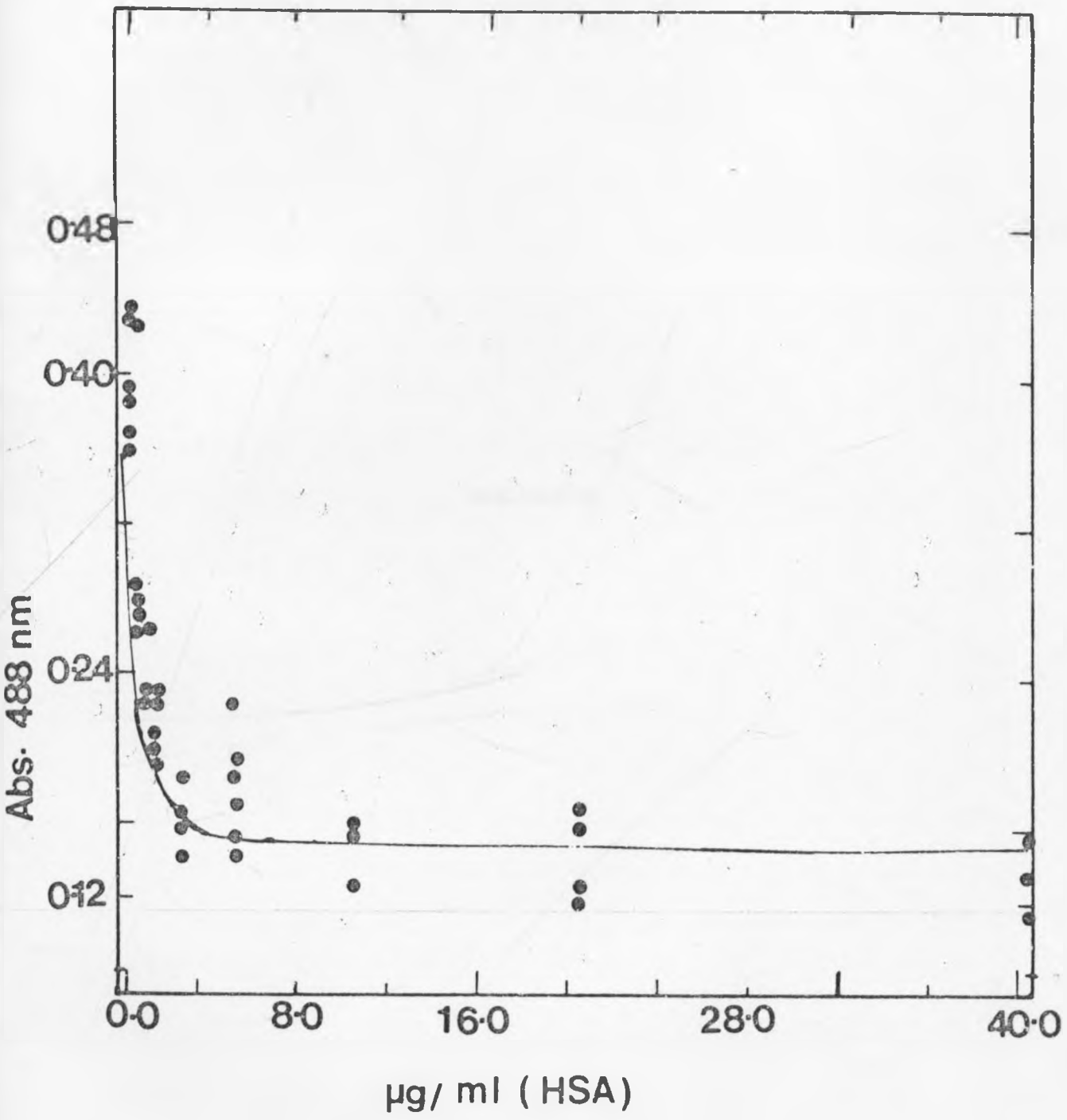

1·8
1·5
0·9
0·3
Abs· 488 nm
0·0
20
50
80
100
% Horse in beef mince·

Fig. 8. Standard curve for estimating the amount of HSA (µg/ml) using the competitive antigen modification of the indirect ELISA test.


0·48
0·40
0·24
0·12
Abs. 488 nm
0·0
8·0
16·0
28·0
40·0
µg/ ml (HSA)

DISCUSSION

The results show the importance of using a monospecific antiserum in serological identification of animal proteins whether in precipitin reactions or in the more sensitive ELISA test. The precipitin tests showed clearly that the principal antigens in each of the meat extracts detected by the antisera shared common antigenic determinants with the serum albumins. This is in agreement with the findings of Haydem (1978) and Kamiyama et al (1978(a)). In addition the meat extracts were found to contain other antigens detected by the antisera. The possibility of muscle components in extracts reacting with antisera to serum albumin was suggested by Katsube et al (1966). No attempts have been made to characterise these second antigenic determinants.

Beef was effectively discriminated from sheep meat using anti-SSA specific fraction by the ELISA technique but not in the precipitin tests, demonstrating the improved sensitivity of the ELISA method. However, the anti-SSA specific fraction failed to identify beef at the same high level of significance ($P \leq 0.001$) as beef was differentiated from sheep meat by the anti-BSA specific fraction. Benjamin et al (1971) showed that BSA and SSA were closely similar in their amino acid composition and peptide maps. These authors further reported that the cross reactivity between anti-BSA serum and heterologous albumins was due to related rather than identical antigenic determinants. From the specificity of the antisera, it seems probable that the antigenic determinants on BSA related to SSA are fewer than those on SSA related to BSA. If this is true then it may explain why the purification of anti-SSA serum through columns linked with BSA and then HSA still resulted in some antibodies reacting with the beef extract whilst this was not observed with the purified anti-BSA fraction.

Detection of the species of origin of various meats was successfully carried out using ELISA. The failure of anti-SSA specific fraction to discriminate sheep from goat is due most probably to the fact that purification through a column linked with goat serum albumin was not performed. From the relatively low level of significance at which goat was differentiated from sheep meat (P = 0.06), it is likely that irrefutable identification could be achieved after such a purification step.

The ELISA method detected horse meat in beef mince over a wide range of concentrations (10-50%). This sensitivity is better than that reported by Katsube et al (1968) for the commonly used agar gel diffusion precipitin test in which 25% adulterant was the minimum detectable. The range could be extended downwards to 0.5% but only by mixing appropriate aliquots of pure beef and pure horse meat extracts (Fig. 6). The reason for the poor sensitivity in the mixed meat extracts appears to be due to the poor efficiency of extraction of horse serum albumin from the normal mixed meats rather than to a fault in the ELISA method itself which was capable of detecting 0.5% of horse meat in the mixed pure extracts. Similarly Kamiyama et al (1978(b)) were able to detect 0.2% of pigmeat in beef using synthetic mixtures; however, when the ratio of pigmeat was greater than 5%, the passive haemagglutination inhibition test employed could not differentiate it from an extract of pure pigmeat. Thus the ELISA technique can be employed over a wider range of adulterant ratios and is more sensitive than both the passive haemagglutination inhibition test and the precipitin test.

It is clear that accurate quantification of the amount of horse meat in beef over the full range of concentrations has not been achieved using indirect ELISA. This may have been due to the many steps in the ELISA technique that are critical and require strict standardisation to guarantee success in the assay. Of these, the amount of antigen that adheres to the solid phase is the most crucial.

Antigens have been shown to attach to plastic polymer surfaces mainly by weak physical forces (Van Oss et al, 1966). The nature of these forces is such that there is considerable doubt about the stability of the antigen phase during the assay. Antigen leakage from polystyrene and nylon solid phases of the order of 30 and 60% respectively has been reported (Lehtonen et al, 1980). These authors recommend covalent coupling of the antigen to the solid phase to reduce the variability observed between and within plates attributed to such losses. However, attempts to achieve a uniform attachment of antigen to the solid phase by pre-activating plates with 1% glutaraldehyde was unsuccessful. It resulted in high background readings and the practice was eventually abandoned (Duance, (1981) personal communication).

A second major cause of variation may have been due to different amounts of residual blood left in the various muscles after slaughter, affecting the concentration of serum albumin. This variation introduced by the vagaries of the slaughtering process probably resulted in non-standardised quantities of antigen adhering to the solid phase. This in turn would be reflected in differing absorbance values depending upon the amount of serum albumin present, irrespective of the actual percentage of adulterant meat added, as is apparent in the results in Table 3. This not only explains why quantification was not possible but also indicates the unsuitability of serum albumin as an antigen in quantitative work. Another antigen, for example myoglobin, may be more appropriate because its content in the muscle is not subject to the efficiency of exsanguination during slaughter.

Conclusions

This project was undertaken to try to develop an enzyme-linked immunosorbent assay as a technique for identifying the species of origin of different raw meats. The ELISA method described in this study was found to be highly sensitive and can be adopted for qualitative detection of the species origin of raw meats. Quantitative detection of adulteration of beef with horse meat was not possible with indirect ELISA but may be achieved by use of competitive antigen modification of indirect ELISA test, provided an antigen is used whose content in the muscle is not subject to gross variation as found with the serum albumin investigated here.

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I would like to thank the Commonwealth Commission of United Kingdom for the scholarship and the University of Nairobi for granting study leave.

Finally, I would like to express my deep-felt gratitude to my wife, Loise and daughter, Grace, for their love and encouragement during this period of separation, without which I would not now be in the position to submit this dissertation.

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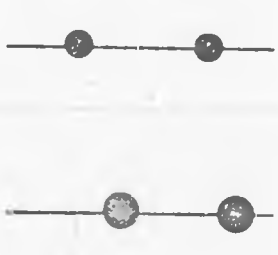
APPENDIX

Fig. 9. Optimisation of antigen working dilution (beef meat extract) using anti-BSA specific fraction (1.200) in PBS-Tween.



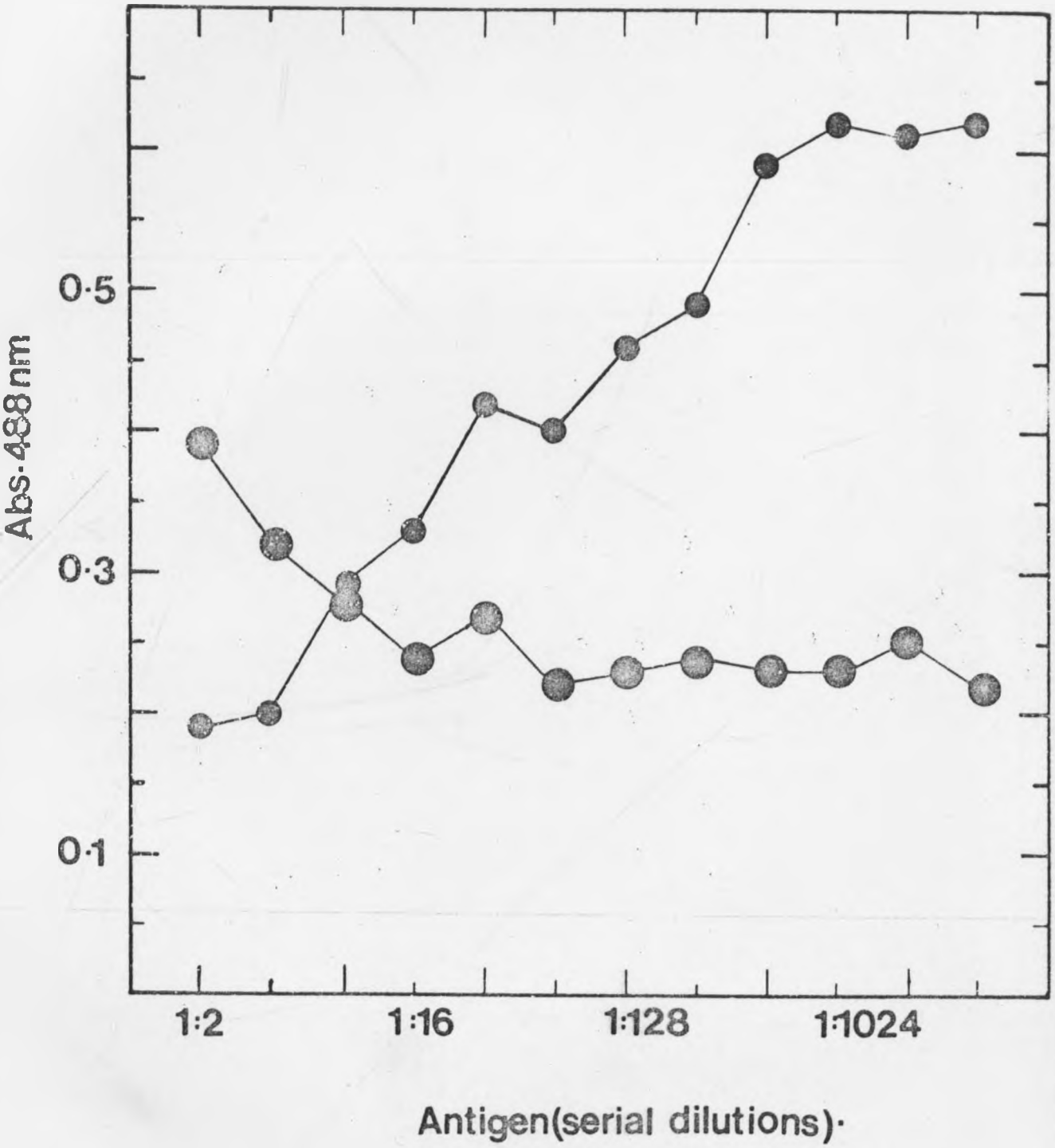
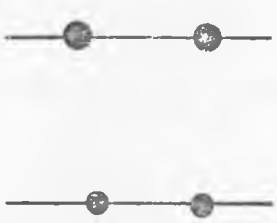

Abs.488nm
0·5
0·3
0·1
1:2
1:16
1:128
1:1024
Antigen(serial dilutions)·

Fig. 10. Optimisation of antigen working dilution (horse meat extract) using anti-HSA specific fraction (1:400) in PBS-Tween.



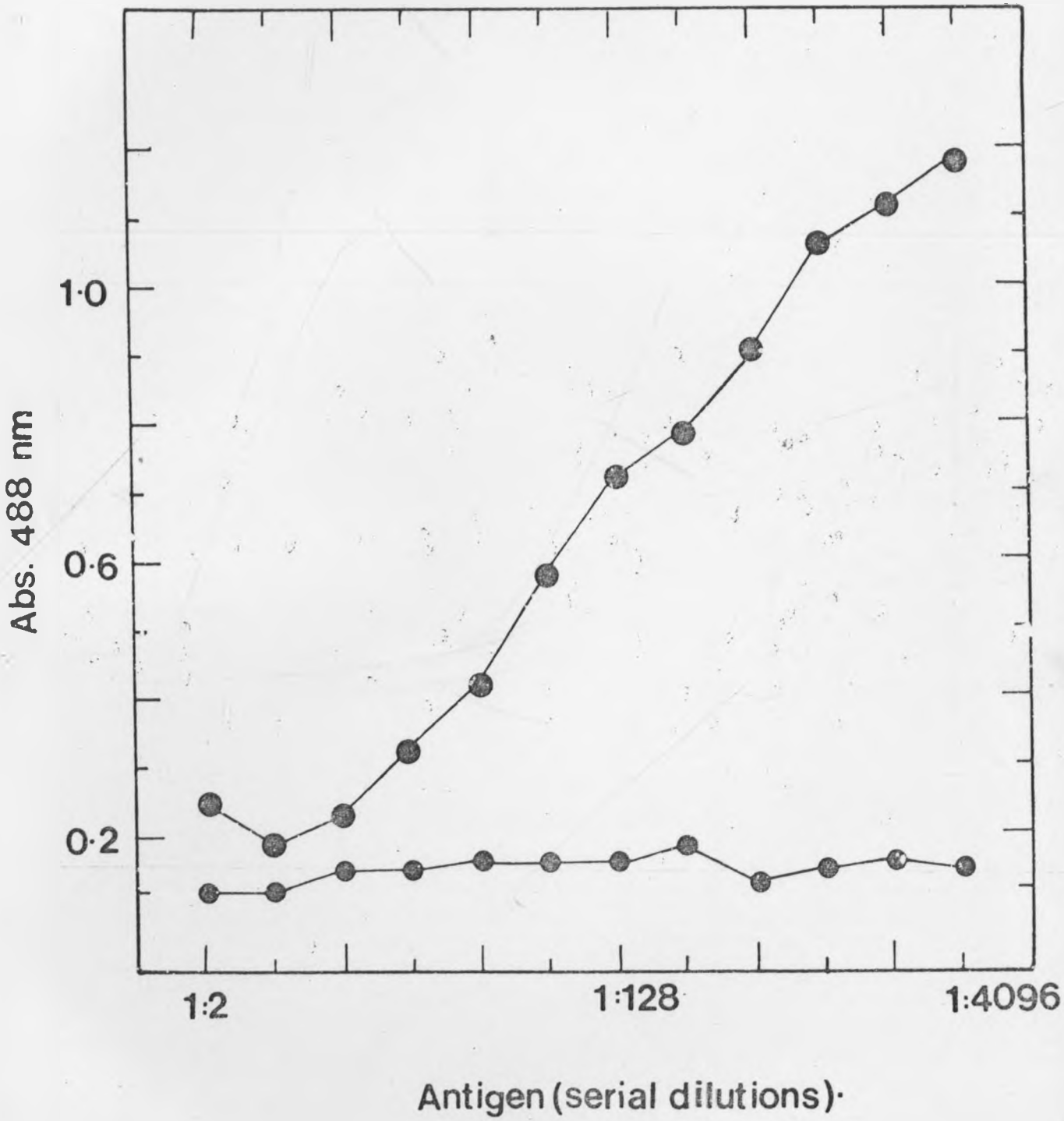
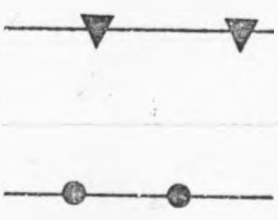
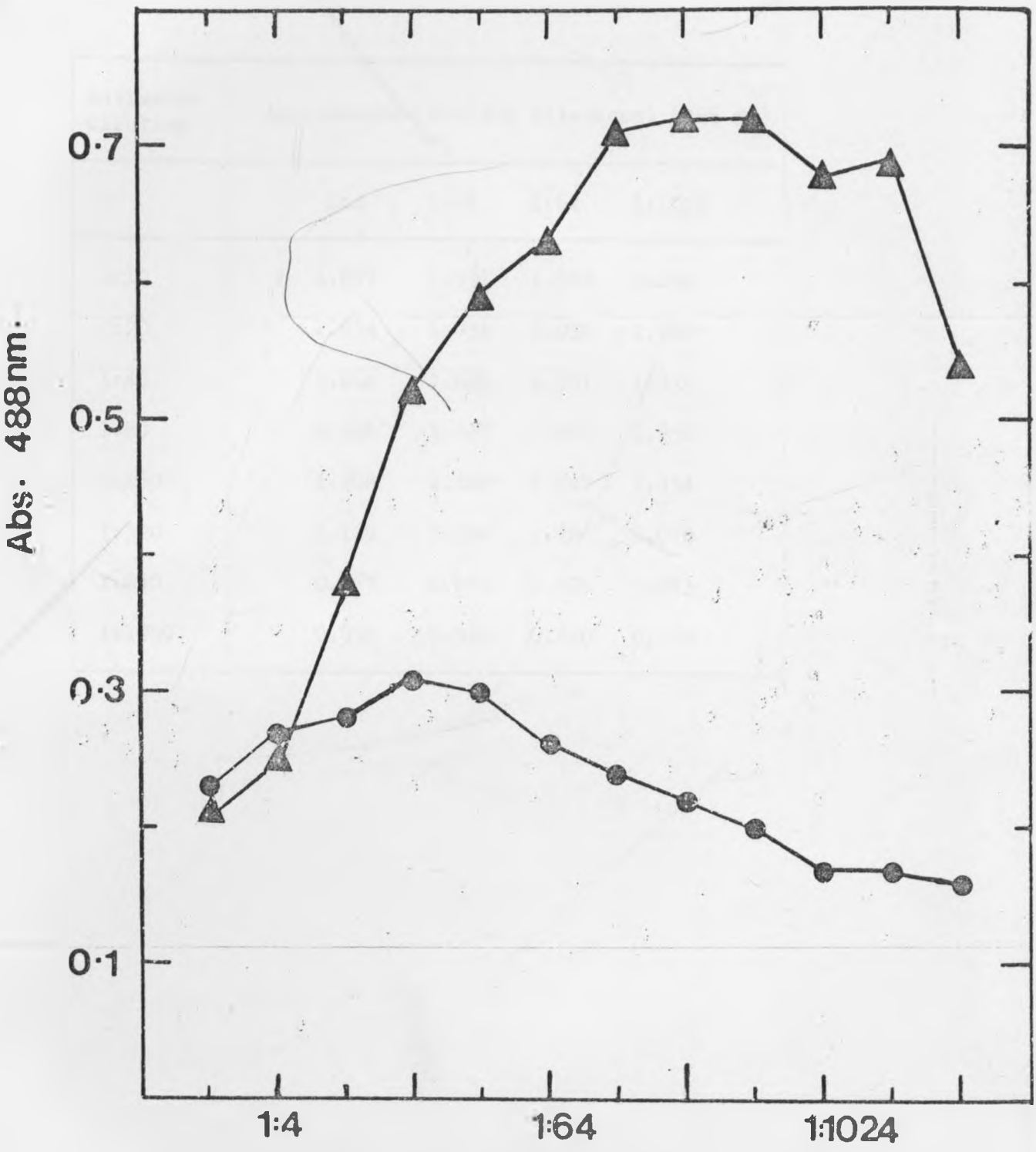

1·0
0·6
0·2
Abs. 488 nm
1:2
1:128
1:4096
Antigen (serial dilutions)·

Fig. 11. Optimisation of antigen (sheep meat extract) working dilution using anti-SSA specific fraction (1:400) in PBS-Tween.




0·7
0·5
0·3
0·1
Abs. 488nm.
1:4
1:64
1:1024
Antigen (serial dilutions).

Appendix Table 4. Optimisation of antiserum dilution (anti-SSA specific fraction).

Antiserum dilution	Absorbances (Antigen dilutions) (488 nm)			
	1:4	1:16	1:64	1:1024
1:10	1.897	1.931	1.968	2.010
1:20	1.834	1.931	2.010	1.968
1:40	1.864	1.864	1.931	1.779
1:80	1.688	1.667	1.806	1.630
1:160	1.408	1.408	1.547	1.454
1:320	1.159	1.096	1.286	1.070
1:640	0.867	0.831	0.920	0.873
1:1280	0.596	0.549	0.690	0.524

Appendix Table 5. Optimisation of antiserum (anti-BSA specific fraction) dilution.

Antiserum dilution	Absorbances (Antigen dilutions) (488 nm)			
	1:4	1:16	1:64	1:1024
1:10	1.688	1.731	1.579	1.667
1:20	1.579	1.595	1.533	1.595
1:40	1.197	1.454	1.376	1.408
1:80	1.112	1.184	1.232	1.217
1:160	0.876	1.135	0.883	0.876
1:320	0.600	0.755	0.607	0.619
1:640	0.582	0.554	0.495	0.536
1:1280	0.367	0.417	0.398	0.323

Appendix Table 6. Optimisation of antiserum (anti-HSA fraction)(dilution.

Antiserum dilution	Absorbances (Antigen dilutions) (488 nm)			
	1:4	1:16	1:64	1:1024
1:10	1.709	1.896	1.931	1.896
1:20	1.667	1.834	1.834	1.864
1:40	1.755	1.864	1.1806	1.834
1:80	1.667	1.864	1.612	1.834
1:160	1.579	1.688	1.896	1.779
1:320	1.478	1.595	1.648	1.547
1:640	1.294	1.491	1.387	1.519
1:1280	1.080	1.177	1.107	1.254