SPECIES IDENTIFICATION IN HEAT PROCESSED MEAT PRODUCTS.

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INTRODUCTION

Various tests for detection of raw meats, based on species-specific antigen-antibody interaction, are now commercially available in kit form. These include adaptations of the Ouchterlony immunodiffusion principle, as in the dry-disc 'Domino 5' system (Jones et al., 1986a; available from Immunodiagnostic Systems Ltd.), and immunometric (capture) versions of enzyme-linked immunosorbent assays (ELISA), such as the Biokits assay and 'Checkmeat' (Patterson et al, 1984). The first type provides a convenient, inexpensive test which can be operated with minimal resources and is suitable for screening most raw meat materials in all situations. The more expensive ELISA tests require greater expertise and care in operation but have the advantage of speed and higher sensitivity (down to 0.5%).

These procedures are all based upon detection of the native blood proteins such as albumin and/or immunoglobulin. Since blood proteins are present in all animal tissues, it is not possible to distinguish offals from muscle nor, because of the variability in the amount of residual blood in carcass meat, can an accurate measure of species meat content be gained by this procedure, as shown for beef by Griffiths and Billington (1984).

Detection of pig meat is of particular interest because of its rejection by Moslem peoples. Research elsewhere has revealed other more unusual biochemical properties of pig muscle that can be used as indicators for its presence in raw and heated products. The rare muscle-specific peptide balanine (beta-alanyl-3-methylhistidine), which amongst the common meat species is virtually unique to the pig, can be detected by HPLC (Carnegie et al., 1983). Also a rare fatty acid, 11,14-eicosadienoic acid (C 20:2) has been found in pork and lard (Saeed et al, 1986) using gas chromatographic analysis of the methyl esters; in this case, a detection limit of 1% pork in beef and mutton was achieved. Although these methods may be suitable for quality control in special circumstances, considerable investment in equipment and expertise is required.

Several independent versions of the ELISA tests have now been reported which can identify pig meat in heated meats and meat products. The capture ELISA of Berger et al. (1988) has the ability to detect very low levels of heated pig meat extract in beef, chicken, or horse muscle extract. It is based on the detection of a highly soluble, heat-resistant component of fresh, unheated pork, which had been isolated in very pure form and used successfully as the immunogen. However, estimation of the apparent content of pig meat was not considered. A simpler indirect assay, described by Kang'ethe and Gathuma (1987) detected similar soluble "thermostable" antigen analytes derived from saline extracts of the meat. This approach was effective for the species identification of autoclaved, boiled or raw meats and, in the examples quoted, was capable of detecting ca. 3% of specified species in a mixture of meats.

The competitive-indirect ELISA for pig meat evolved by Manz (1985) showed many similarities to the original soy protein ELISA work of Hitchcock et al. (1981). As in the former, this assay was applied to heated meat products after they had been fully solubilised with 8M urea and mercaptoethanol. This work identified an α_1-globulin as the likely heat stable antigenic analyte. Quantitative estimation of pig meat content was attempted, but the level of responses to pig offal materials

was not given. Also, the full method was quite cumbersome and considered inappropriate for use by the majority of meat quality laboratories.

This paper describes the development of a practical protocol for sample preparation, and two ELISA procedures for detection of heated lean meats, particularly pig meat, in a wide variety of meat materials and products. They are intended for use in meat industry quality control laboratories, public analyst laboratories, and by others acquainted with immunoassay techniques.

MATERIALS AND METHODS

As the procedure is under commercial development at present, a detailed account of the experimental methodology cannot be given; but an outline follows.

Preparation of antigen and antiserum

Trimmed lean species meats (pig, beef, sheep, horse, chicken, turkey) from various leg and shoulder muscles, were individually cut into small pieces and comminuted finely. Thermostable muscle components were prepared by a special autoclaving and extraction procedure, and used as antigens to produce the anti-species muscle antisera. Sheep and goats were used as the principal host animals, and each received one primary injection of immunogen containing Freunds Complete Adjuvent at four intramuscular sites in the shoulder and hindquarters. Five booster injections, in Incomplete Freunds, were given at 4-6 week intervals over six months. Blood was withdrawn from the jugular vein at intervals after the booster injections and at slaughter. After clotting, expressed serum was centrifuged and freeze-dried for long-term storage, or stored in liquid form stabilized with sodium azide. Each batch of antiserum was screened for specificity of response to the relevant antigen by an ELISA procedure (see below) and its specificity improved as required by use of a tailored "blocking" solution (Jones and Patterson, 1986b) to nullify the heterologous cross-reactions.

Preparation of sample extracts

Meat materials from different species types, including raw species offals, raw lean mixtures of known composition and a wide range of manufactured, heat processed products, were prepared for testing by comminution, extraction and autoclaving at 121°C for a range of times, depending on the cooked state of the starting material. After reducing the autoclave pressure and cooling bottles, the warm extracts were homogenised and filtered through Whatman No. 113 paper.

Samples with a high fat content produced a surface layer of molten fat in the hot extract which was removed by suction before filtration; also, if an extract could not be filtered effectively (eg, products formulated with fillers or extenders), then approximately 20 ml of the warm liquid was centrifuged at 4-5000 rpm for 15 min and the supernatant filtered. If excessive frothing occurred during homogenisation, 2-3 drops of octan-2-ol (capryl alcohol, BDH) were added.

ELISA techniques

ELISAs of the indirect type, in which the wells of the microtitre plate are coated with antigen (muscle/sample extract), were used 1) to screen the antisera for specificity to pig protein, 2) to characterise the nature of this response ie whether exclusive to muscle proteins or also to components of blood, offals, collagen, fat, etc, and 3) for quantitation and estimation of lean meat content. Although basically of the same ELISA format, there were procedural differences between the three systems, to bias the assays towards either maximum sensitivity or broadest range for quantitation; for example, the latter was achieved by introduction of a 'competitive' step early in the procedure.

Horse radish peroxidase conjugated to anti-goat IgG was used as coupled

second antibody and detector enzyme; hydrogen peroxide + ABTS (Sigma A1888) in substrate buffer was added and, after incubation, the optical density (OD) of the solutions in the wells recorded at 405 mm by an automatic plate reader. Colour development was stopped by addition of 2% solution of sodium fluoride.

RESULTS

Antiserum specificity.

Initially, significant cross-reactions of the anti-pig antiserum was observed with the muscle extracts of the other common meat species. However, these were suppressed effectively by the blocking procedure, as shown by the results in Table 1. The Table shows differences greater than 1.0 OD units between the high colour response to pig muscle extract and the low colour response to beef, sheep, goat, chicken, horse and rabbit extracts. However a smaller difference of 0.77 OD units was recorded between pig and venison, indicating a higher level of cross-reaction to that species. This cross reactivity was also reduced for later tests by the same principle.

Table 2 shows that various pure pig organs including heart muscle all gave significantly lower OD values than samples of pure pig skeletal muscle. In the same assay, OD results for pork/beef mixtures with an increasing substitution of pork lean were related to the percentage of pork by a highly significant ($p<0.001$) linear regression, yielding comparative "lean meat percentages" for each tissue response. Apparent equivalences of 20-30% pork lean were given by heart and liver; kidney, spleen and lungs gave 10-15%; and brain gave the lowest response (5.4%). Gelatin (derived from pig skin) gave a high response equivalent to 30% of pig lean. However, the highest OD values for non-muscular tissue were produced by two extracts of pig fat: 0.82 and 0.53 respectively, equating to 50 and 31% of pork lean. Thus although the simple indirect assay clearly detected less 'active' pig antigen in the heat treated organs and fat than in skeletal muscle (and tongue), it did not provide an unequivocal distinction between these offals and pork/beef mixtures containing less than 50% pork meat. However, there was no response either to skeletal muscle, tongue, heart or any of the offals of bovine, ovine or equine origin.

Further tests on model lean meat mixtures by indirect ELISA demonstrated a highly significant linear correlation ($r = 0.97$) between corrected OD values and increasing percentages of pork in beef, lamb and chicken meats. Fig. 1 shows the combined results of two assay runs on model mixtures (extracted and analysed in duplicate) containing pork from 3 to 99%, mixtures without pork, and the pure species meats (pig, beef, sheep and chicken). In all the model mixtures tested, OD readings for 5% of pork lean in any other species were significantly different from the corrected OD readings of the other pure meats or the meat mixtures without pork at the 99% probability level, with a least significant difference of 0.08 OD units. Maximum responses were obtained from mixtures containing greater than 90% pig meat. Analysis of eight samples of whole muscle from different sites within the pig carcass gave a 5% coefficient of variation about the mean OD value of 1.63; the comparative result for the tongue was 1.40.

Responses from other heat-treated meat product materials not containing pigmeat were minimal. Corrected OD values were <0.05 for extracts of soy protein, milk powder and a beef sausage mixture containing a high proportion of rusk.

Quantitative estimation of lean content

The competitive form of the assay was designed to give optimum sensitivity to pork lean content between 5 and 100%, and to accommodate variation in the composition of sample extracts due to the presence of other non-meat components.

Table 1. ELISA response of the 'blocked' anti-pig antibody reagent to heat-treated skeletal muscle extracts of the common meat species.

Species	OD values at 405 nm: Mean value of 4 wells	Standard Deviation	Control blank value (2 wells)	Difference from pig
Pig	1.34	0.04	0.04	-
Beef	0.05	0.01	0.04	1.29
Horse	0.08	0.01	0.05	1.26
Chicken	0.17	0.02	0.04	1.17
Sheep	0.08	0.01	0.05	1.26
Goat	0.06	0.01	0.03	1.28
Venison	0.57[a]	0.02	0.04	0.77
Rabbit	0.14	0.01	0.04	1.20

a) Cross-reactivity was later reduced by solution of 5% of venison extract to the reagent.

FIGURE 1

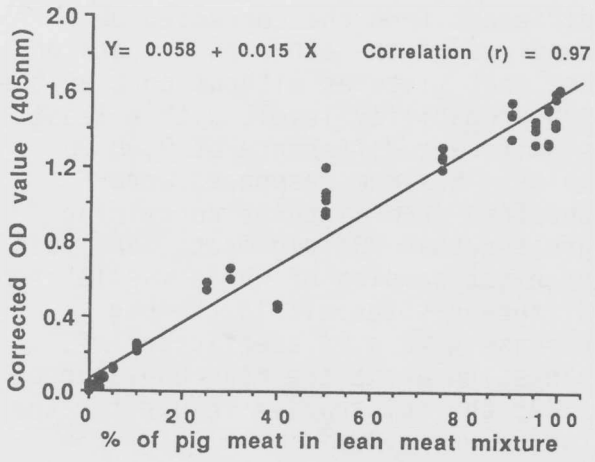

Figure 1. Response of indirect ELISA to heated pig meat in lean meat mixtures with beef, lamb or chicken.

FIGURE 2

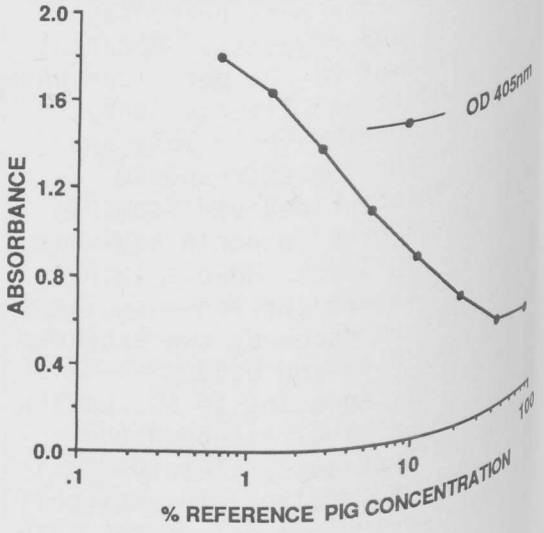

Figure 2. Typical absorbance curve for the competitive ELISA.

Table 2. Response of the anti-pig reagent to heat-treated species offals and to a range of pork and beef mixtures.

Carcass component	OD values at 405 nm mean of three values corrected values			
	Pig	Beef	Sheep	Horse
i) Typical muscle	1.57	0.01	Zero	Zero
Tongue	1.40	0.03	nt	nt
Heart	0.48 (28.6%)[a]	0.02	0.01	0.06
Liver	0.37 (21.8%)	0.02	0.02	Zero
Lungs	0.22 (12.4%)	0.02	0.03	nt
Kidney	0.25 (14.2%)	Zero	0.02	nt
Brain	0.11 (5.4%)	0.01	nt	0.03
Spleen	0.25 (14.2%)	0.02	nt	nt
Gelatin	0.49 (29.0%)			
fat (subcutaneous)	0.82 (50%)			
(mesenteric)	0.53 (31%)		nt = not tested	
ii) Model Pork/Beef mixtures:		Linear regression constants: y=mx + c		
5% pork in beef	0.10 OD		m = 0.016	
10% "	0.15		c = 0.023	
25% "	0.41		r = 0.994	
50% "	0.86			
75% "	1.37			
95% "	1.45			
iii) Skimmed milk powder and soya protein gave OD values <0.10.				

a) Percent values refer to the equivalent percentage of pork in beef estimated from the linear response of six raw pork/beef mixtures (ii), the responses of other species pure muscle meats and offals extracted with equivalent heat treatment and analysed during the same assay for comparison.

Predictions of pork lean content were derived from the data of standard dose/response curves as follows: dilutions of pure, 1:10 diluted, cooked pork meat extract (from representative muscles) gave increasing OD values through seven doubling dilutions to the limit of sensitivity at 0.63% of the original concentration. This standard curve was plotted for each plate within an assay, as shown by the examples of Figure 2 and Table 3. The assay response was linear between 30 and 2.5% concentrations, but outside these limits it tended to a curve and plateau. Thus evaluations of high pork lean content (>75%) were less accurate because small variations in the low OD values of individual wells resulted in large differences of estimated pork lean on the logarithmic scale; also minor variations in background colour had a

more significant effect.

Most sample extracts were diluted three- and six-fold for the assay, to provide two independent parallel points within the optimum sensitivity range. This was done to compensate for potential variation arising from the different extract matrices derived from the range of meats and meat products. The average pork lean content was then calculated via appropriate multiplication factors. In this way, the majority of heated meat mixtures could be analysed for apparent pork lean content with reasonable accuracy, although samples with a high pork lean content and individual muscle extracts required higher dilution, ie. 5- and 10-fold.

The major pig offals, fatty tissues and gelatin gave only small reductions in OD from the average MAB value (Table 3). Only pig brain extract at the 1:3 dilution returned a measurable value for apparent pig lean content (4.5%). Other "negative" results were recorded for extracts of beef, sheep and chicken offals. A slight reduction in OD was noted between the 1:6 and 1:3 dilution in each case; however this was attributed to the difference in solution matrix and concentration change of the extracted components. The same effect was also observed in extracts of meat products not containing pig meat and the soy protein and milk powder, but all gave a negative result for lean content in relation to the standard pig response. Thus in contrast to the foregoing indirect procedure, this quantitative ELISA for heated pig meat is virtually specific for the presence of **muscle** antigens. This is because, in this form, the assay was not sufficiently sensitive to detect the relatively low concentrations of heat-stable antigen present in the tissues of the main offals or fat. Black Pudding, a meat product rich in pig blood and fat, also gave a negative result.

Figure 3 compares the ELISA results for pork lean content in a range of carefully formulated lean meat mixtures of low (pork) fat content. For these quantitative tests, several batches of minced pig lean from different sources were mixed with lean meats of beef, sheep or chicken. Points on the graph represent the estimate of single extracts, from the average results of four wells, two at 1:3 dilution and two at 1:6. Overall, there was a significant linear relationship between the estimated content of pig meat and the original formulation (correlation coefficient $r=0.97$ on 52 points, $p<0.001$).

CONCLUSION

These ELISA procedures for the identification and determination of heated pig meat are simple and rapid to perform and require only a thorough autoclaving extraction procedure to prepare all samples for analysis, whether initially raw or heated. Since the antigen is present in the exudate of cooked pig meats, all exudate must be incorporated fully into each meat sample before extraction, otherwise a reduction in the quantitative response for pork will be obtained.

In both assays, the range of sensitivity permits detection of 5% of pork mixed with other lean meats; whilst the indirect assay can provide identification of pig protein in a selection of the offals, its response to the blood components was very low. On the other hand, the competitive assay was apparently specific for the muscle-meat content. However, since a significant variation in the response to individual pig muscles was obtained, the evaluation of the "lean meat content" by this ELISA cannot, unfortunately, be considered as truly absolute.

Table 3. Results of a competitive ELISA for heat-treated pig meat and offals. Corrected OD values at 405 nm from two wells.

	Authentic pork lean							
Standard dilutions, %	80	40	20	10	5	2.5	1.25	0.63
OD:	0.28	0.37	0.56	0.80	1.04	1.35	1.61	1.77

MAB[a] OD: 2.28 (±0.12)

Corrected OD values (and equivalent pig lean content) of Pig Offals

MAB[a] OD: 2.28 (±0.12)

Extract Dilution	Heart	Liver	Lungs	Kidney	Spleen	Brain
1:3						
1:6	2.07(-)	2.13(-)	2.16(-)	1.81(-)	1.78(-)	1.55(4.5)[b]
	2.12(-)	2.18(-)	2.23(-)	1.96(-)	2.00(-)	1.83(-)

	Fat (c)	Fat (d)	Gelatin(e)
1:3			
1:6	1.88(-)	2.05(-)	2.19(-)
	2.05(-)	1.98(-)	2.30(-)

a) Maximum antibody binding value: mean (± sd) of 4 wells
b) Lean content corrected for dilution factor
c) Mesenteric fat
d) Subcutaneous fat
e) Gelatin derived from pig skin

FIGURE 3

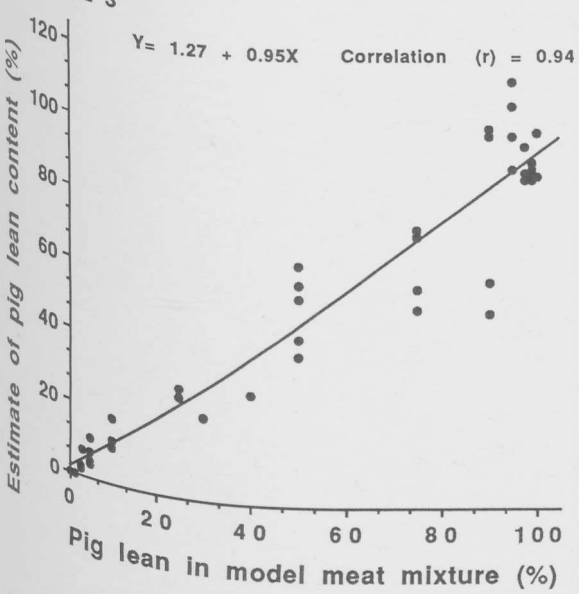

Figure 3. Pig lean meat estimation in model meat mixtures.

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