Food Chemistry 37 (1990) 201–219

Detection of Pork in Processed Meat: Experimental Comparison of Methodology

Wajih N. Sawaya,[a] T. Saeed,[a] M. Mameesh,[b] E. El-Rayes,[a] A. Husain,[a] S. Ali[a] & H. Abdul Rahman[a]

[a] Kuwait Institute for Scientific Research, PO Box 24885, 13109 Safat, Kuwait
[b] Biochemistry Department, Kuwait University, PO Box 5969, 13060 Safat, Kuwait

(Received 5 May 1989; revised version received and accepted 28 September 1989)

ABSTRACT

Four different methods were compared to detect pork in processed meats. These included: analysis of meat fat by high performance liquid chromatography (HPLC) for triglycerides (TGs) and by gas chromatography (GC) for fatty acids; and analysis of meat proteins by enzyme-linked immunosorbent assay (ELISA), and of ophidine dipeptides by HPLC.

Low levels of pork in processed meats can be detected by either fat or protein analysis; fat analysis can also be used for all food products that contain pork fat. TG analysis is more reliable than fatty acid analysis using C20:2 as a marker; however, the GC method is simpler, faster and requires less sample preparation. Both TG and GC methods can detect levels as low as 2% pork in processed meat.

In the ELISA technique, crude preparations of sheep-antipig antiserum can detect low levels (2%) of pork in beef or mutton samples heated at 70, 100 and 120°C. The analysis of ophidine dipeptides can also detect low levels (2–5%) of pork in heated/processed meats; however, this method was not tested for differences in sex, breed, diet and muscle type.

INTRODUCTION

The detection of pork in various food products, particularly in cooked or processed meat, has been an important subject of study in many countries, especially where religious laws prohibit the consumption of pork products.

Food Chemistry 0308-8146/90/$03·50

Most of the research work reported on this subject has concentrated on the analysis of pork fat and the identification of pork proteins.

Pork fat is unique in its peculiar fatty acid distribution and triglyceride (TG) composition. Since the fatty acid 11,14 eicosadienoic acid (C20:2) was reported to be present in pork fat and absent in other commonly consumed meats and fats (Saeed *et al.*, 1986), its presence in meat products was used as an indicator for the presence of pork. However, the presence of C20:2 was recently reported in some beef and mutton samples (Firestone, 1988). Rugraff and Karleskind (1983) separated saturated TGs and obtained 2-monoglycerides using pancreatic lipase, and used the ratio of palmitic acid in the saturated TGs and the 2-monoglycerides to detect pork in meat. This unique composition of intact pork TGs was utilized for the detection of pork in processed meats (Sawaya & Saeed, 1988). In contrast to other animal fats, pork TGs are mostly esterified by saturated fatty acids at the C-2 position. For example, about 80% of total palmitic acid content is esterified at the C-2 position in pork (Bradford *et al.*, 1965), but only 15–27% in beef, lamb and deer. According to the number of saturated (S) or unsaturated (U) fatty acids in the TG molecules, TGs are classified into four types: S_3, S_2U, SU_2 and U_3. Two types, S_2U and SU_2 can exist in two isometric forms, giving SUS and SSU, and UUS and USU respectively. Chacko and Perkins (1965) reported that pork fat contains 38% SSU, 41% USU, 1% SUS and 7% UUS. In other animal fats (Bradford *et al.*, 1965), the TG composition was distinctly different: 9–14% SSU, 13–38% SUS and 26–38% UUS. The characteristic composition of fat TGs and the use of high performance liquid chromatography (HPLC) in the last ten years for separation and identification of the TGs of natural fats and oils (Plattner *et al.*, 1977; Herslof *et al.*, 1979; Herslof, 1981; Plattner, 1981) have been useful in detecting pork in meat products.

Several methods have been used for speciation of fresh meat, including immunodiffusion (Swakt & Wilks, 1982; Doberstein & Greuel, 1982; Shaw *et al.*, 1983), electrophoresis (Slattery & Sinclair, 1983; Glesson *et al.*, 1983) and enzyme-linked immunosorbent assay (ELISA) (Kang'ethe & Patterson, 1982; Whittaker *et al.*, 1983; Jones & Patterson, 1986). Speciation of cooked meat is more difficult because the duration and temperature of heating affect the protein structure and species-specific antigenic determinants (Murakimi *et al.*, 1983; Katsube & Imaizumi, 1968); however, reports in the literature indicate the presence of thermostable antigens in animal tissues. Hayden (1981), utilized adrenal heat-stable antigens for species identification of cooked sausage (core temperature, 71°C), using agar gel immunodiffusion. King (1984), analyzed the thermostable enzyme adenylate kinase (EC 2.7.4.3) in pork, renaturated with 6M urea or 6M guanidine hydrochloride, using enzyme staining of isoelectrofocusing gels. Kang'ethe *et al.*

(1986) and Kang'ethe and Gathuma (1987) used thermostable muscle antigens (TMA) for speciation of meat products sold in Kenya's retail markets and of autoclaved meat samples by immunodiffusion and enzyme immunoassay (EIA), respectively. However, these antigens are present in low concentrations and are not highly monospecific.

Analysis of histidine dipeptides (β-alanyl-L-histidine, carnosine; β-alanyl-L-methylhistidine, anserine; β-alanyl-L-3-methylhistidine, balenine) has been used for meat speciation (Carnegie *et al.*, 1982). Tinbergen and Slump (1976) showed that chicken could be identified in luncheon meats by analysis of anserine and carnosine. Carnegie *et al.* (1982) showed that the anserine–balenine ratio is useful in comparing tinned hams. Olsman and Slump (1981) reviewed the problem and concluded that the histidine dipeptides could be useful for the identification of meat species used in meat products. Carnegie *et al.* (1984) used this method to estimate pig content in different processed meats; however, the use of peptide ratios for species identification is limited by large variations among different muscles in the same animal (Crush, 1970; Tanaki *et al.*, 1977) and by small differences between closely related species.

Although several methods have been reported for detection of pork fat or pork meat in meat products, there is still a need for reliable sensitive and simple methods to detect low percentages of pork in heat-processed meat. This investigation was undertaken to explore such methods and to evaluate them by analysis of mixed meats, fats and heated meat products of known composition.

MATERIALS AND METHODS

Collection of samples

Authentic samples of pork from animals raised under controlled conditions were obtained from the Danish Meat Research Institute (Rosklide, Denmark). Samples of beef and mutton were obtained from the local market.

Production of antispecies/antisera

Pork meat extract (antigen) was prepared from representative samples of lean pork. The meat sample was homogenized with 5% saline (1:1 v/v) and autoclaved at 120°C for 30 min. The homogenate was cooled, allowed to settle and then filtered (Whatman filter paper No. 42). Two sheep were immunized by injecting 2 ml of pork antigen (autoclaved and filtered meat extract) in Freund's complete adjuvant (3 ml of meat extract to 5 ml Freund's complete adjuvant) intramuscularly and subcutaneously at multiple sites.

Booster doses were given every other week using the antigen in Freund's incomplete adjuvant. The animals were bled first after 4 weeks and thereafter every 2 weeks. The collected blood was centrifuged at 1500*g* and the serum was then stored in 2–3 ml portions at −20°C.

Sampling

Laboratory prepared samples (standards) were made from either pure fat or lean meat (i.e. without visible fat). Single-species meat samples were minced separately, combined in different proportions and mixed thoroughly before processing. Commercially prepared meat samples in tinned cans (size U4, 300 × 207, weight approximately 270 g/tin), containing pure pork luncheon meat, pure beef sausage or a mixture of 0, 1 and 5% pork/beef sausage mix were processed by the Institute of Food Research, Bristol Laboratory, and the Campden Food Preservation Research Association (Chipping Campden, Gloucestershire, UK). The retort processing met a Fo 16 value, to give reasonable protection against thermophilic spoilage (total process time was 80 min). These samples were used as representative of commercially canned meat (unknowns) in testing different methods of determining the content percentage of pork.

Sample preparation

HPLC analysis of TGs

One to two gram samples were extracted with 15 ml chloroform/methanol 1:2 v/v. The combined filtrate was evaporated to dryness under vacuum by a rotary evaporator and stored under nitrogen in a deep freeze. TGs were separated from other lipids by column chromatography on acid-washed florisil (Carrol, 1976), using a chromatographic column (1·5 × 20 cm) packed with 30 g of acid-treated florisil in hexane as slurry. The flow rate was adjusted to 1 ml/min. The sample (100 mg) was introduced as a hexane solution at the top of the column and was washed with 20 ml hexane followed by 20 ml 5% diethyl ether in hexane. The TGs were then eluted with 20 ml 15% ether in hexane.

For ozonolysis, 10 mg TGs were dissolved in 5 ml hexane in a centrifuge tube. The tube was cooled in a mixture of dry ice/acetone, and ozone from a microozonizer was allowed to pass through the solution at 200 ml/min for 15 min, until the iodine/starch indicator solution changed colour. The tube was then flushed with nitrogen to purge unreacted ozone. The ozonide was cleaved to the aldehyde by adding 2–3 mg Lindle catalyst and stirring the solution under hydrogen for about 30 min.

For the formation of derivatives, the solvent was evaporated from the

aldehyde solution and 10 mg *p*-nitrobenzyloxamine hydrochloride (PNBA) and 100 μl pyridine were added. The tube was capped and heated for 1 h at 50°C. When the reaction was complete, the pyridine was evaporated and the sample dissolved in methylene chloride and washed twice with water (2 × 3 ml).

GLC analysis of fatty acid

A 1–2 g sample was extracted with 20 ml chloroform/methanol (2:1) in an ultrasonic bath for 20 min. The dissolved fat was filtered and the filtrate evaporated to dryness. The extracted fat was dissolved in 1 ml hexane, and 250 μl 0·1 N methanolic KOH was added to effect transesterification. The vial was shaken for 10 min and the layers were allowed to separate. An aliquot (1 μl) from the hexane layer was injected into a gas chromatograph.

Histidine dipeptides

Pure pork, beef and sheep and different mixtures of pork/beef (P/B) and pork/sheep (P/S) were made from ground muscle tissues. Histidine dipeptides were extracted from 3 g meat samples with 30 ml 0·9% saline and 120 ml 8% 5-sulfosalicylic acid. The mixture was homogenized using a tissue homogenizer for 2 min and the homogenates were centrifuged at 9000*g* at room temperature for 1 h. The supernatant fractions containing the dipeptides were filtered through a Millipore pre-filter (type AW) and filter (type GS, 0·22 μm diameter pore).

Enzyme-linked immunosorbent assays

Samples of pure pork, beef, sheep and different mixtures of P/B and P/S were prepared in the laboratory by co-homogenization in an equal volume of 1:1 saline (0·85 g/100 ml H_2O) of the finely mixed pure meat samples which had been autoclaved at 120°C for 30 min, followed by centrifuging at 47 000*g* for 30 min at 4°C. The supernatant was filtered through Whatman No. 42 filter paper and stored frozen in 3 ml aliquots. Various extraction conditions of the commercially-canned meat samples, were tested to obtain the highest recovery of pork muscle antigens. The following procedure was found to give optimal results, and was thus used for extraction. A 10 g sample of meat was homogenized in 90 ml hot (90–95°C) saline (3%) and transferred to a heat-resistant glass bottle, which was sealed and then autoclaved for 30 min at 121°C; after cooling, the meat mixture in the bottle was rehomogenized, centrifuged at 10 000*g* for 15 min and the supernatant filtered through Whatman filter No. 3.

Analytical procedure

TGs analysis

HPLC analysis of the TGs was carried out using a Shimadzu LC 4A HPLC instrument equipped with gradient capability and a variable UV detector.

The S_2U fraction was analyzed using a silica C_{18} (octadecylsilica) reversed phase column, 25 cm long and 5 mm in diameter, packed with 5 μm particles. Data were acquired and processed using a Shimadzu CR-3A with floppy disk drive and monitor. Derived TGs of different pork samples were analyzed under isocratic conditions using acetonitrile/methylene chloride (90:10) as the mobile phase. All other samples were run using a gradient elution. The gradient program was as follows: 5% CH_2Cl_2 in acetonitrile for the first 10 min after which the gradient was programmed to 27% CH_2Cl_2 for 5 min and this composition held for 25 min. The flow rate for the mobile phase was 1·5 ml/min. The wavelength used for the UV detector was 254 nm.

Fatty acid analysis

Fatty acids were analyzed using a gas chromatograph equipped with an inlet splitter for the capillary column and a flame ionization detector as reported by Saeed *et al.* (1986) with some modification. The FFAP capillary column was replaced with an OV-225 (25 cm × 0·25 mm) column since the FFAP stationary phase is generally less stable than OV-225 and bleeds quite rapidly, resulting in loss of column efficiency unless compensated for by a longer column (50 m). A hexane layer (1 μl) was injected into the column with a split ratio of 1:30. Nitrogen carrier gas flow through the column was adjusted to 2 ml/min. Column temperature, initially 140°C, was programmed to reach 210°C at 2°C/min. The final temperature was held for 20 min. Injection port temperature was kept at 250°C and the detector at 260°C. Fatty acids were identified by comparing their retention times with those of the standard. Quantification was based on area normalization.

Histidine dipeptide analysis

The dipeptides, in 5 μl of extract, were separated on a Whatman Partisil-10-SCX column with a lithium formate buffer containing 0·2M lithium hydroxide titrated to pH 2·9 with formic acid (Carnegie *et al.*, 1984). The column was kept at 40°C at a flow rate of 0·7 ml/min from a Shimadzu LC-6A pump. The eluate from the column was mixed with *o*-phthalaldehyde (OPA) reagent (Nakamura *et al.*, 1979) delivered at a rate of 1·2 ml/min with another Shimadzu LC-6A pump. OPA reagent was prepared by dissolving 49·5 g of boric acid in 900 ml of distilled and deionized water, and titrating to pH 11·4 with 50% sodium hydroxide solution. To this were added 3·0 ml

30% Brij 35 (trade name for a commercially available series of polyoxyethylene alcohols), 2·0 ml mercaptoethanol and 10 ml methanol containing 500 mg OPA. Deionized distilled water was added to make 1 litre. After thoroughly purging with nitrogen, the OPA reagent remained stable for at least 2 weeks at room temperature in the dark. The OPA reagent was mixed with the column eluate as a post-column derivation reagent in a mixing coil. The derivatives thus formed were detected by a Shimadzu RF-530 HPLC fluorescence detector. The excitation wavelength was 340 nm and emission wavelength was set at 450 nm. The detector output was recorded and processed using a Shimadzu integrator (Model CR-3A). Quantitation was done using an external standard solution containing 312·5 ppm each of carnosine, anserine and balenine (the balenine was a gift from Dr J. Wolff, National Institute of Health, Bethesda, MD, USA).

Competitive ELISA

Sheep antipig antisera (300 μl) was pipetted into 24 ml of phosphate buffer saline plus 0·2% Tween 20 (PBST) containing 2 ml each of beef, sheep and horse meat extracts (heated at 120°C for 30 min and extracted with 5% saline). The mixture was shaken gently for 1 h at 20–25°C (room temperature). Optimal blocking conditions were established by preliminary checkerboard format microtitration assays according to Jones and Patterson (1986). The effects of heterologous treatment and antibody dilution were evaluated by mixing aliquots of the sheep-antipig antisera, serially diluted in PBST, with equal volumes of the three heterologous meat extracts (250 μg, 310 μg and 1 mg/ml from each of beef, sheep and horse) in the same diluent and incubating as above before application to the wells of the ELISA plate precoated with the various meat extracts.

Antigen, antibody and conjugate working dilutions for each antigen were determined on a checkerboard titration. Titration curves of antigen versus antibody concentrations were performed to determine optimal conditions for the ELISA method, including pH, incubation time for substrate enzyme reaction, substrate selection and concentration and IgG optimal dilutions.

The competitive ELISA method was used to detect different P/B or P/S mixtures. Pig muscle extract (180 μl), raw and/or heated at different temperatures and diluted 1:100 with PBS (phosphate buffer saline, 0·15M, pH 7·2), was added to an ELISA microplate (96 high-binding and flat-bottom well plates, Nunc, Denmark) and incubated at 45°C for 40 min (antigen coating). The plate was then washed three to four times with PBST and dried by tapping on clean paper towels. Sheep-antipig antiserum was diluted with blocking buffer (1:100) and incubated for 1 h at 4°C. Serial dilutions (1:1, 1:5, 1:10, 1:25, 1:50, 1:100) of meat extracts from different

species were added in duplicate to each well (90 μl). Then 90 μl of sheep-antipig antiserum in blocking buffer, was added to each well. The plate was shaken for 20 min (Dynatech, Microplate Shaker), washed three to four times with PBST and dried by tapping on clean paper towels. Diluted goat immunoglobulins (1:1000 in PBST), HRP-conjugated (horseradish peroxidase-conjugated, Dakopatt, Denmark), was added to all wells except one blank well. The plate was shaken for 20 min, washed three to four times with distilled water and dried by tapping on clean paper towels.

Azino-bis solution (ABTS) (azino-bis-di, 3-ethyl benzyl thiazoline sulphuric acid diammonium solution) was prepared by adding 2 ml ABTS stock solution (15 mg ABTS/ml) to 50 μl 6% H_2O_2 in 25 ml citrate phosphate buffer 0·15 M, pH 5·0 and 180 μl was added to each well. The plate was shaken for 20 min until the colour was clear green. The absorbance was measured in each microwell by a microELISA plate reader (MR-70 Dynatech) at 405 nm. The reaction was stopped by adding 150 μl 2·5% NaF to all wells. Low colour intensity indicated positive response and high colour density, negative response.

RESULTS AND DISCUSSION

Four different methods for detecting pork in beef were evaluated for accuracy and precision: HPLC analysis of fat TGs, HPLC analysis of histidine dipeptides, fatty acid (C20:2) analysis and ELISA. Results of laboratory prepared samples (standards) were compared with results from commercially manufactured samples containing canned beef sausage, pork luncheon meat and different mixtures of P/B. These samples contained different proportions of P/B and were labelled as follows: A = pure beef (12·1% fat), A1 = 0·5% pork (12·0% fat), A5 = 1% pork (12·3% fat), A10 = 10% pork (10·7% fat), A30 = 5% pork (10·7% fat) and A20 pork luncheon meat (17·8% fat).

HPLC analysis of fat TGs (S_2U fraction)

HPLC analysis was performed on laboratory-prepared samples of lean pork, beef and P/B mixtures (0, 1, 3, 5, 10, 30 and 50% pork) and commercially canned P/B samples (0, 0·5, 1·0, 5·0, 10 and 100% pork). A typical HPLC chromatogram (sample A30) is shown in Fig. 1. The SSU/SUS ratio was determined for beef, sheep, pork and P/B and P/S mixtures (Saeed *et al.*, 1989). Since pork contains mostly the SSU isomer, but no significant amounts of SUS, any addition of pork to pure beef should result in an increased SSU/SUS ratio. Moreover, the change in the SSU/SUS ratio of

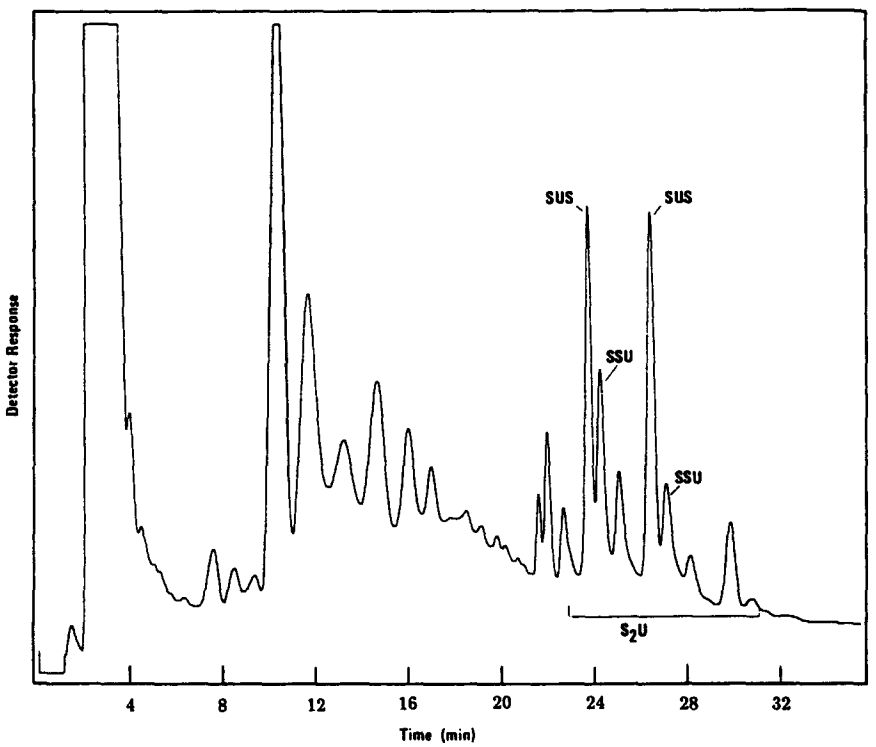

Fig. 1. HPLC analysis of sample A30 triglycerides.

different carbon equivalents (CE), where CE is the total carbon numbers in the TG molecule (i.e. 39, 41, 43 and 45), will vary with the differing proportions of SSU of these carbons in pork TGs. The SSU/SUS ratio of CE 43 would change more significantly than that of the CE 41 when pork is added to beef. The SSU/SUS ratio for CE 45 would not change much since the SSU of this carbon equivalent is present in pork in small amounts. The ratio of total CE 41 and CE 43 for SSU/SUS isomers would also reflect addition of pork.

In the analysis of fat TGs in the commercially prepared samples (unknowns), the SSU/SUS ratios for CE 41, CE 43, and the total of CE 41 + CE 43 (Table 1) matched those of P/B, and hence were identified as pork in beef. All ratios were increased by increasing the amounts of pork in the mixtures. This was observed even with small additions of pork indicating that as low as 2% of pork was detectable by this method. When the SSU/SUS ratios for different CEs were plotted against percentages of P/B (Fig. 2), and regression analysis was performed, the general form of the equation was linear of the first degree: $y = ax + b$. The correlation coefficient (R) was 0·99 for all CEs (mean of five replicate analyses) indicating a high positive correlation between SSU/SUS ratio and percentage of P/B. When

TABLE 1
SSU/SUS Ratios of Unknown Samples

Sample no.	*Pork meat (%)*	*CE41*	*CE43*	*Total*[a]	*Estimated pork fat (%)*
A	0	0·70	0·38	0·40	<1
A1	0·5	0·71	0·42	0·46	≃1
A5	1	0·75	0·44	0·54	3–4
A10	10	0·76	0·61	0·66	7–8
A20	100	ND	ND	ND	100
A30	5	0·75	0·46	0·56	4
Lean beef	0	0·68	0·38	0·43	—
Lean sheep	0	0·85	0·65	0·70	—

[a] Total = SSU (CE41 + CE43)/SUS (CE41 + CE43).
ND, not determined since SUS was too low.

the SSU/SUS ratios of the fat TGs in commercially canned meat (Table 1) were statistically compared with those of the laboratory prepared samples using the *t*-test, there was no significant difference at the $P > 0{\cdot}05$ level, indicating that the SSU/SUS ratio of all CEs of the TGs can be used to detect low levels of pork in canned meat mixtures, with the highest value being that of CE 43 followed by CE 43 + 41 and CE 41.

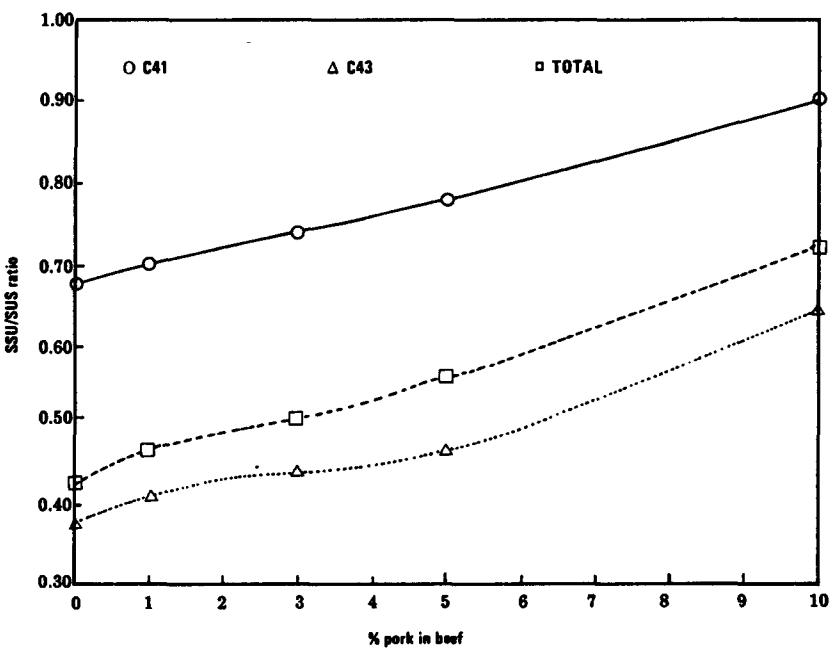

Fig. 2. SSU/SUS ratios of pork/beef mixtures.

The HPLC analysis of the TGs can thus be utilized for samples containing meat and fat. Since fat is not significantly affected during processing, the method applies to fresh as well as processed meats. The method permits the detection of 2% pork in beef and 3% pork in mutton, and has been investigated for different variables such as age, sex, muscle type and diet. The major disadvantage is the lengthy and tedious sample preparation, a batch of six samples requiring at least 3 days. The method is not applicable to fats that have been chemically modified, e.g. hydrogenated fats. The reproducibility of HPLC data could be poor if contamination of the sample during the lengthy preparation is not carefully avoided.

HPLC analysis of histidine dipeptides

HPLC analyses of histidine dipeptides extracted from autoclaved mixtures of laboratory-prepared lean P/B (1–50% pork) and of commercially-canned meat samples containing lean beef (35%), pork (58%) and P/B (35%) mixtures (0, 0·5, 1, 5, 10 and 100% pork) were performed (Fig. 3), and the ratios calculated of carnosine–anserine (C/A) and balenine–anserine (B/A) Tables 2 and 3). Since beef and pork have similar values for carnosine and

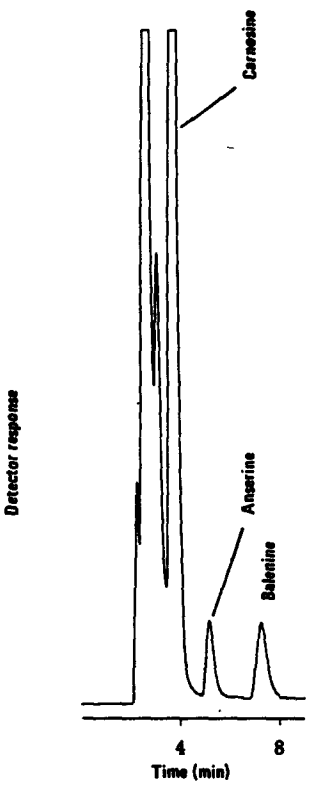

Fig. 3. HPLC chromatogram of sample A20, histidine dipeptides.

TABLE 2
Dipeptide Ratios in Laboratory-Prepared (Standard) Pork/Beef Mixtures

Pork/beef (%)	*C/A*	*B/A*
0	5·13	0·031
1	5·10	0·046
2	5·19	0·068
5	5·20	0·135
10	5·16	0·267
30	5·26	0·828
50	5·24	1·520
100	5·24	4·000

anserine, the C/A ratio was of no value in detecting the addition of pork to beef. Both pork and beef are relatively high in carnosine and low in anserine, which is the major dipeptide in sheep. Balenine, however, is uniquely high in pork. These differences are reflected in the C/A and B/A ratios for different meats. The B/A ratio for pork is high compared with other commonly consumed meats and can be used as an indicator for pork. The C/A ratio, although not useful for detecting pork, could indicate the presence of meats for which this value is low, e.g. sheep, chicken, kangaroo and rabbit (Carnegie *et al.*, 1982). However, there is a linear relationship between B/A ratio and percentage of pork in the laboratory-prepared samples (Table 2), and the addition of only 1% pork resulted in a significant increase in B/A ratio. Regression analysis indicated a high correlation ($R = >0{\cdot}99$) between B/A value and percentage of pork.

The B/A ratios of the commercially canned samples A, A1, A5, A10, A20 and A30 (Table 3), were statistically compared with those of the laboratory-prepared samples (Table 2) using the *t*-test. There was a significant difference between the two at the 1% and 5% levels. This difference could be due to muscle source or age of the animals (Carnegie *et al.*, 1982). Other factors that may affect the concentration of dipeptides in meat samples are breed, sex and diet. The results obtained with standard samples prepared in the laboratory indicate that histidine dipeptides could be used to detect low levels of pork in processed meats. However, the reliability and scope of this method depends on determining the effect of the sources of variability on the concentration of histidine dipeptides in beef, sheep and pork.

HPLC analysis of histidine dipeptides for pork detection is a relatively simple process. Sample preparation is simple and rapid, taking an average of 90 min. HPLC analysis is also fast (10 min). A detection level of 5% pork in beef or sheep is attainable. After normalization of the sources of variation,

TABLE 3
Dipeptides Ratios in Commercial Meat Samples

Sample	*Actual pork (%)*	*C/A*	*B/A*	*Estimated pork lean (%)*
A	0	6·66	0·021	<1%
A1	0·5	5·71	ND	0
A5	1	5·38	0·028	1
A10	10	5·57	0·073	Approx. 3
A20	100	15·62	0·770	Approx. 30
A30	5	4·54	0·066	Approx. 2·5

ND, not determined since B was too low.

the detection limit may be further reduced. The method, however, requires a complicated setup for HPLC analysis (post-column derivation).

Fatty acid analysis

A typical gas chromatogram of the fatty acid methyl esters of one of the commercially canned samples (A10) is shown in Fig. 4, and the fatty acid composition of the six samples presented in Table 4. Eicosadienoic acid was detected in five of the samples, indicating the presence of pork. It was previously reported by Saeed *et al.* (1986), that this method could not be used to determine low levels of pork mixed with other meats since the C20:2 content in pork meat is variable. However, since the method was reported to detect as little as 1% pork in beef and mutton mixtures, the presence of C20:2 is a positive indicator of > 1% pork in the sample. Unknown samples were analyzed for C20:2, and the results compared with those obtained by Saeed *et al* (1986).

C20:2 was not detected in sample A (Table 4), indicating a lard content of less than 1%, which is the detection limit for this method. Sample A1 showed little C20:2, indicating that the amount of pork present was near the detection limit. The exact amount of pork cannot be reliably determined from the value of C20:2 alone since C20:2 content can vary considerably in pure pork (Hubbard & Pocklington, 1968). The composition of the rest of the fatty acids was similar to that of sample A. Samples A5 and A10 contained slightly more C20:2 than sample A and were estimated to contain 2–5% pork fat. Sample A20 was significantly different from the other samples. First, it contained more C20:2 than that reported by Saeed *et al.* (1986) for pure pork. Secondly, its overall fatty acid composition was similar to that of pure pork fat. Thus, sample A20 was estimated to contain at least 80% pork fat. Sample A30 was similar to samples A1, A5 and A10, except

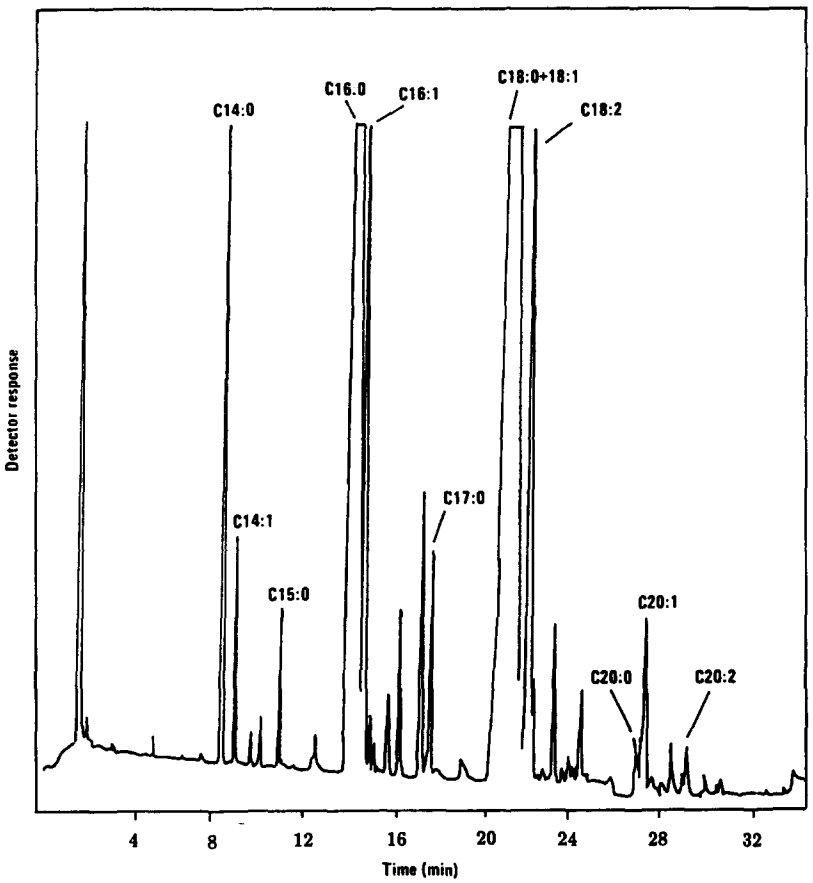

Fig. 4. Gas chromatogram of fatty acid (methyl esters) of sample A10.

that it contained higher amounts of C20:2. The pork fat content of this sample was estimated at about 10%.

In general, the gas chromatographic method using eicosadienoic acid is rapid. Sample preparation after modification is simple and can be completed within 1·5 h since chromatographic analysis takes about 40 min and the total time required for fatty acid analysis is about 2 h. The method has the advantages of being straightforward, since it uses the presence or absence of C20:2, and sensitive, detecting as little as 1% pork content. Since the method is based on fat analysis, it is applicable to fresh and processed meat and other fat-containing samples.

The method was found to detect as little as 1% pork in model beef and mutton mixtures; the presence of C20:2 could be considered as a positive indicator of greater than 1% pork fat in the sample. However, the method cannot accurately determine pork concentration since the eicosadienoic acid content in pure pork is highly variable (Hubbard & Pocklington, 1968).

TABLE 4
Fatty Acid Composition of Commercial Meat Samples

Fatty acid	*A*	*A1*	*A5*	*A10*	*A20*	*A30*
C14:0	3·45	2·17	3·20	2·98	1·19	2·89
C14:1	0·67	0·30	0·76	0·63	ND	0·64
C15:0	0·71	0·58	0·70	0·56	ND	0·56
C16:0	24·80	24·67	26·10	25·20	19·80	25·29
C16:1	2·85	2·53	3·29	3·70	1·90	3·72
C17:0	1·94	1·35	1·18	1·20	0·45	0·95
C18:0 + C18:1	51·30	57·32	51·30	54·55	50·50	54·00
C18:2	5·54	2·88	3·99	5·60	17·37	4·65
C19:0	0·23	1·10	1·17	0·67	0·24	0·57
C20:0	ND	0·60	0·28	0·20	0·15	0·20
C20:1	0·47	0·78	0·40	0·72	1·28	0·69
C20:2	ND	0·06	0·15	0·16	0·97	0·22
Estimated pork fat	<1%	1%	2–4%	2–5%	100%	10%

ND, not detected, i.e. less than 0·1%.

Moreover, Firestone (1988) recently reported the presence of C20:2 in the tissues of animals other than pig, casting doubt on its reliability as the sole indicator of low levels of pork in processed meat.

Enzyme-linked immunosorbent assays (ELISA)

Low levels of P/B (2, 3 and 4%), that were not available commercially were prepared by mixing commercially canned pure pork luncheon meat and beef sausage, either by mixing appropriate volumes of pork and beef extracts (v/v), or by mixing the two pure meats in defined proportions by weight (w/w) and then extracting the mixture. When competitive ELISA tests were performed on different v/v samples, there was a difference of 0·522 OD (DOD) between pure beef ($OD = 0{\cdot}824 \pm 0{\cdot}097$) and 2% P/B ($OD = 0{\cdot}309 \pm 0{\cdot}0348$). However, for 1% P/B, the DOD was 0·41 which is large enough to detect the presence of pork. Similar results were obtained with different percentages of P/B prepared on a wt/wt basis, where a 0·54 OD difference was obtained between pure beef and 2% P/B. The OD difference was only 0·25 at the 1% level, indicating that it is more difficult to detect less than 2% pork on a w/w basis. The different detection limits of samples prepared by v/v and w/w methods, could be due to the high fat content of the meat mixture (50%), which is usually removed by centrifuging before the v/v samples are prepared for analysis. The detection limit in both types of samples would be close if normalized according to fat content.

To check if ELISA results for commercially canned samples agreed with those obtained from laboratory-prepared P/B standards, statistical analysis using the *t*-test was performed on the DOD values of both. The results showed no significant difference at the 1 and 5% levels between the experimental (unknown samples) and the standard DOD values at all percentages of P/B tested (1–10%), indicating that the sheep-antipig antiserum produced against autocalved whole-pig muscle extracts can be used to detect low percentages of P/B in unknown canned/processed meat mixtures (Fig. 5). It was also demonstrated that the same antiserum can be used to detect low percentages (2%) of pork in laboratory-prepared meat mixtures that have been heated at 70 and 100°C for 30 min (Sawaya *et al.*, 1990).

These results confirm the presence of heat-stable antigens in animal tissues, particularly muscle (Hayden, 1981; Kang'ethe *et al.*, 1985, 1986; Kang'ethe & Gathuma, 1987). The successful use of unpurified extracts of meat or meat mixtures as antigens for the production of antisera is reported herein for the first time, but was previously suggested by Patterson and Spencer (1985). The proposed method eliminates complex purification procedures and has the ability to discriminate between close species. Moreover, the use of a complementary species for the required antisera, e.g. antipig antisera being raised in sheep, is more effective than using rabbits, as previously reported by Patterson and Spencer (1985), and by Jones in 1986

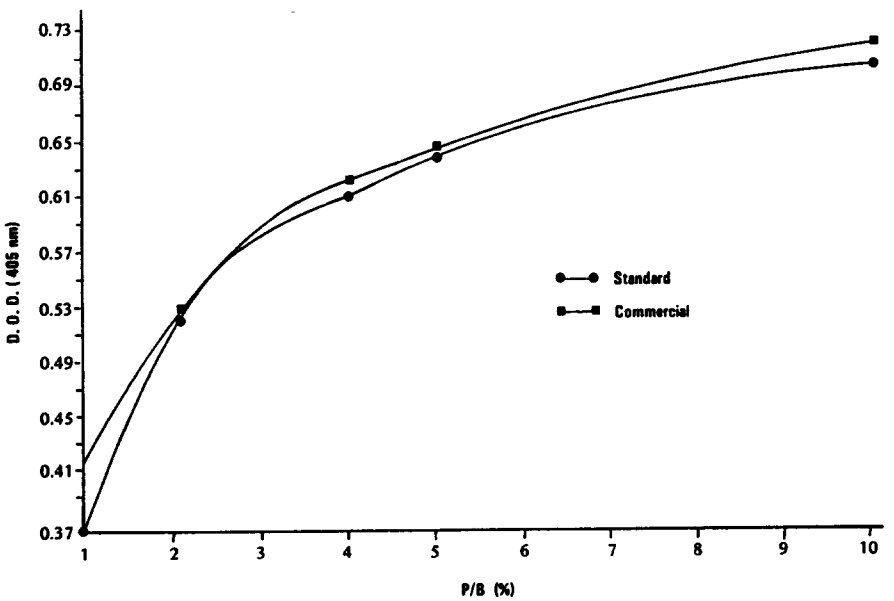

Fig. 5. Standard versus commercial DOD up to 10% P/B.

(private communication). In fact, rabbit anti-pig antiserum produced to the same antigen used in sheep (muscle extract autoclaved at 120°C for 30 min) showed high cross-reactivity with the heterologous species and could not be used to detect low percentages of P/B or P/S even after treatment with a blocking buffer. The advantages of ELISA are the simple preparation procedure, the short time required to obtain results and the low cost of analysis. The disadvantage of the immunoassay method is that it can only detect pork proteins and not pork fat. Moreover, immunochemical methods are subject to biological variability requiring frequent standardization.

In summary, the results of testing a limited number of commercially-prepared canned meat samples indicate that pork can be detected in processed meats by fat as well as protein-based detection methods. The fat method is more universal, i.e. it can be used for the detection of both meat or fat. HPLC analysis of TGs is more reliable than GC fatty acid analysis using C20:2 as a marker, particularly since C20:2 was recently reported in animal tissue other than pig (Firestone, 1988); also the variation in C20:2 content between different muscles is large.

If the sample contains meat, then either protein-based method is preferable to either fat-based method. However, the immunoassay method is easier and cheaper to use than the determination of ophidine dipeptides, and requires less sample preparation and overall time. The ophidine dipeptide method, although fairly reliable, has yet to be tested for different variables such as breed, muscle type, sex and diet.

In conclusion, we recommend the immunoassay method for the detection of pork in processed meats and the ophidine dipeptide method as a complementary alternative. If the sample contains only fat, then we recommend the C20:2 method for preliminary screening and the HPLC analysis of the TGs for confirmation.

ACKNOWLEDGEMENTS

This project was partially funded by the Kuwait Foundation for the Advancement of Sciences (KFAS), whose financial and administrative support is gratefully acknowledged.

The authors thank Mrs S. Jones (AFRC Institute, Bristol, England) for her advice and guidance, particularly her recommendation on antigen extraction: Mr B. Yasin (University of Kuwait, Animal Resources Center) for maintaining the rabbits; and Dr M. A. Razzaque (KISR) for donating the sheep for production of antibodies; and Dr M. Khan (KISR) for statistical and computer analysis of the results.

REFERENCES

Bradford, R. A., Luddy, F. E., Herb, S. F., Magidman, P. & Esimeuschneider, R. W. (1965). Glyceride distribution in adipose and liver glycerides of animals. *J. Am. Oil Chem. Soc.*, **42**, 446.

Carnegie, P. R., Hee, K. P. & Bell, A. W. (1982). Ophidine (β-alanyl-L-3-methylhistidine), Balenine and other histidine dipeptides in pig muscles and tinned hams. *J. Sci. Food Agric.*, **33**, 795.

Carnegie, P. R., Collins, M. G. & Ilic, M. Z. (1984). Use of histidine dipeptides to estimate the proportion of pig meat in processed meats. *Meat Sci.*, **10**, 145.

Chacko, G. K. & Perkins, E. G. (1965). Anatomical variation in fatty acids composition and triglyceride distribution in animal depot fats. *J. Am. Oil Chem. Soc.*, **42**, 1121.

Carroll, K. K. (1976). *Lipid Chromatography Analysis, Vol. 1*, ed. G. V. Marinet. Marcel Dekker, New York, p. 176.

Crush, K. G. (1970). Carnosine and related substances in animal tissues. *Comp. Biochem. Physiol.*, **34**, 3.

Doberstein, K. H. & Greuel, E. (1982). The preparation of specific antisera for the identification of meat of African antelope species in the agar gel precipitation test. *Fleischwirtschaft*, **62**, 1011.

Firestone, D. (1988). Fats and oils. *J. Assoc. Off. Anal. Chem.*, **71**(1), 76.

Glesson, L. J., Slattery, W. J. & Sinclair, A. J. (1983). Species identification of meat samples—An unusual case. *Aust. Vet. J.*, **60**, 127.

Hayden, A. R. (1981). Use of antisera to heat stable antigens of adrenals for species identification in thoroughly cooked beef sausages. *J. Food Sci.*, **46**, 1810.

Herslof, B. J. (1981). HPLC of triglycerides using UV detection. *J. High Resol. Chromatography and Chromatographic Communi.*, **4**, 471.

Herslof, B. J., Padlaha, O., Toregard, B. & Karshamns, A. B. (1979). HPLC of triglycerides. *J. Am. Oil Chem. Soc.*, **56**, 864.

Hubbard, A. W. & Pocklington, W. D. (1968). Distribution of fatty acids in lipids as an aid to the identification of animal tisuses. *J. Sci. Food Agric.*, **19**, 571.

Jones, S. & Patterson, R. L. S. (1986). A modified indirect ELISA procedure for raw meat speciation using crude anti-species antisera and stabilized immuno-reagents. *J. Sci. Food Agric.*, **37**, 767.

Kang'ethe, E. K. & Gathuma, J. M. (1987). Species identification of autoclaved meat samples using antisera to thermostable muscle antigens in an enzyme immunoassay. *Meat Sci.*, **19**(4), 265.

Kang'ethe, E. K., Jones, S. J. & Patterson, R. L. S. (1982). Identification of the species origin of fresh meat using an enzyme-linked immunosorbent assay procedure. *Meat Sci.*, **7**, 229.

Kang'ethe, E. K., Lindqvist, K. J. & Gathuma, K. I. (1985). Immunological reactions of thermostable muscle antigens and their possible use in speciation of cooked and fresh meat. In *Proceedings of CBC Workshop on Biochemical Identification of Meat Species, Brussels, 27–8 November 1984*, ed. R. L. S. Patterson. Elsevier Applied Science Publishers, London, pp. 129–44.

Kang'ethe, E. K., Gathuma, J. M. & Lindqvist, K. J. (1986). Identification of the species of origin of fresh, cooked and canned meat and meat products using antisera to thermostable muscle antigens by Ouchterlony's double diffusion test. *J. Sci. Food Agric.*, **37**, 157.

Katsube, Y. & Imaizumi, K. (1968). Serological identification of animal meats. *Jap. J. Vet. Sci.*, **30**, 218.

King, N. L. (1984). Species identification of cooked meats by enzyme-staining of isoelectric focusing gels. *Meat Sci.*, **11**, 59.

Murakimi, H., Umaki, F., Makumura, T. & Huruta, M. (1983). Application of microelectrophoresis and micro-gel diffusion technique in differentiation of household meat. *J. Food Hygi. Soc.*, **10**, 434.

Nakamura, H., Zimmerman, C. L. & Pisano, J. J. (1979). Analysis of histidine containing dipeptides, polyamines and related amino acids by HPLC: Application to Guinea pig brain. *Anal. Biochem.*, **93**, 423.

Olsman, W. J. & Slump, P. (1981). *Developments in Meat Science*, ed. R. A. Lawrie. Applied Science Publishers, London, p. 195.

Patterson, R. M. & Spencer, T. L. (1985). Differentiation of raw meat from phylogenic related species by enzyme-linked immunosorbent assay. *Meat Sci.*, **15**, 119.

Plattner, R. D. (1981). High performance liquid chromatography of triglyceride controlling selectivity with reverse phase columns. *J. Am. Oil Chem. Soc.*, **58**, 638.

Plattner, R. D., Spencer, G. F. & Kleiman, R. (1977). Triglyceride separation by reverse phase high performance liquid chromatography. *J. Am. Oil Chem. Soc.*, **54**, 511.

Rugraff, L. & Karleskind, A. (1983). Analysis of animal fat mixtures. Application in checking that tallows and subsidiarily raw or cooked meat products and derivatives are free from pork fat. *Revue Francaise des Corps Gras*, **30**(9), 323 (in French).

Saeed, T., Abu-Dagga, F. & Abdul Rahman, H. (1986). Detection of pork and lard as adulterants in beef and mutton mixtures. *J. Assoc. Off. Anal. Chem.*, **69**, 999.

Saeed, T., Ali, S., Abdul Rahman, H. & Sawaya, W. N. (1989). Detection of pork and lard as adulterants in processed meat: Liquid chromatographic analysis of triglycerides. *J. Assoc. Off. Anal. Chem.*, 77(6), 921.

Sawaya, W. N. & Saeed, T. (1988). Detection of Pork in Processed Meat: Phase II. Report No. KISR2669, Kuwait Institute for Scientific Research, Kuwait.

Sawaya, W. N. Mameesh, M. S., El-Rayes, E., Husain, A. & Dashti, B. (1990). Detection of pork in processed meat by an enzyme-linked immunosorbent assay using antiswine antisera. *J. Food Sci.*, (in press).

Shaw, F. D., Deane, E. M. & Cooper, D. W. (1983). An immunodiffusion method for the identification of the species of origin of meat samples. *Aust. Vet. J.*, **60**, 25.

Slattery, W. J. & Sinclair, A. J. (1983). Differentiation of meat according to species by the electrophoretic separation of muscle lactate dehydrogenase and esterase isoenzymes and isoelectric focusing of soluble muscle proteins. *Aust. Vet. J.*, **60**, 47.

Swakt, K. S. & Wilks, C. R. (1982). An immunodiffusion method for the identification of the species of origin of meat samples. *Aust. Vet. J.*, **59**, 21.

Tanaki, N., Nakamura, M., Harada, M., Kimura, K., Kawano, H. & Hama, T. (1977). Anserine and carnosine contents in muscular tissue of rat and rabbit. *J. Nutri. Sci. Vitaminol.*, **23**, 319.

Tinbergen, B. J. & Slump, P. (1976). The detection of chicken meat in meat products by means of the anserine/carnosine ratio. *Z. Lebensm. Unters.-Forsch.*, **161**, 7.

Whittaker, R. G., Spencer, T. L. & Copland, J. W. (1983). An enzyme linked immunosorbant assay for species identification of raw meat. *J. Sci. Food Agric.*, **34**, 1143.