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Wendy J. Taylor^{a b} & J. Leighton Jones^{a b}

^a Campden Food and Drink Research Association, Chipping Campden, Gloucestershire, GL556LD, UK

^b Sera-Lab Ltd, Crawley Down, Sussex, RH104FF, UK

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An Immunoassay for Verifying the Identity of Canned Sardines

WENDY J. TAYLOR AND J. LEIGHTON JONES

Campden Food and Drink Research Association, Chipping Campden,
Gloucestershire, GL55 6LD, UK

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*An immunoassay has been developed which facilitates discrimination between canned whole sardine (*Sardina pilchardus* Walbaum) and other fish species (Pacific pilchard, mackerel, herring, sild and anchovy) that may be substituted for it in the canned product. The non-competitive indirect ELISA utilizes an antiserum raised against a crude water-soluble extract of canned sardine. Appropriate antiserum specificity was ensured by using extracts of those fish with which cross-reactivity was undesirable, to achieve post-production 'blocking' of unwanted cross reactions. This assay will be of value in upholding EC and UK law on the composition and labelling requirements of canned sardine products and provides a basis for the development of a rapid test format.*

Keywords: Adulteration, authenticity, ELISA, fish, food labelling.

INTRODUCTION

The composition and labelling of foods, within the UK, is subject to standards and regulations set down in UK and EC legislation. Countries without appropriate legislation follow guidelines of the FAO/WHO Codex Alimentarius Commission to facilitate international trade. In relation to canned sardines, EC Council Regulation No. 2136/89 (1989) states that preserved sardines "must be prepared exclusively from Fish of the Species *Sardina pilchardus* Walbaum". The UK Food Labelling Regulations (Statutory Instrument, 1984) also specify, in Statutory Instrument 1035, that fish labelled as sardine must be of the species *Sardina pilchardus* Walbaum. In contrast, many countries outside the European Community adopt the FAO/WHO Codex Worldwide Standard (1982) for canned sardines and sardine-type products, which allows other types of fish (e.g. pilchards, sprats, herring, brisling, anchovies and sardinella) to be labelled as sardines. A test method to verify the identity of fish of the species *Sardina pilchardus* is therefore required to uphold both EC and UK legislation.

Conventional methods of determining the species of origin of fish meats include electrophoresis and isoelectric focusing, where the protein patterns of the unknown fish sample are compared with those obtained for known reference samples (Lundstrom, 1979). Although electrophoretic methods have proved highly effective in many

Correspondence to J. Leighton Jones. Present address of Wendy J. Taylor: Sera-Lab Ltd, Crawley Down, Sussex, RH10 4FF, UK.

instances, they require sophisticated laboratory equipment and the use of highly skilled personnel. Moreover, for canned fish, much of the protein present has been denatured by the heat treatments administered during canning, so that it is difficult to achieve distinctive electrophoretic patterns even after relatively harsh treatment of extracts with cyanogen bromide and 6 M-urea to resolubilize the denatured fish proteins (Mackie & Taylor, 1972). Immunoassays offer an obvious approach to the development of rapid, technically undemanding methods for the identification of the species of origin of fish products, as evidenced by the commercial success of ELISA and dipstick tests for meat species testing (Goodwin, 1992). With regard to canned sardine, an immunoassay which detects proteins specific to *Sardina pilchardus* could prove ideal for discriminating between fish meat from this species and that from those of other fish species that could be substituted for it in the canned product.

Initially, protein purification investigations were undertaken to identify and isolate heat stable proteins (or protein complexes generated during the heating process) unique to canned *Sardina pilchardus*, for use as immunogens (Sidwell, 1992). However, no unique protein could be isolated in sufficient quantities for this purpose (Taylor *et al.*, 1992). An antiserum was therefore raised against a crude extract of authentic canned sardine, and the specificity of this antiserum adjusted post-production using the simple antibody/antigen 'blocking' technique described by Jones & Patterson (1986). This report describes the production of an anti-sardine antiserum, the successful post-production modification of its specificity and the development of an indirect non-competitive ELISA for verifying the identity of canned sardine amongst a range of possible canned substitutes.

MATERIALS AND METHODS

Antiserum Production

Protein extract of authentic canned *Sardina pilchardus*, for use as immunogen, was prepared by homogenizing the sardine (skin and bones removed) with two parts (volume) distilled water. After centrifugation at $13\,000 \times g$ and $20\,000 \times g$ (each for 20 min) the supernatant was filtered through Whatman No. 1 filter paper (BDH Chemicals, Poole, Dorset), dialysed against three changes of distilled water (approximately 10 times extract volume) for 24 h at 4°C in Spectra/Por1 (Pierce and Warriner, Chester) dialysis membrane (molecular weight cut-off 6000–8000) and freeze-dried. This lyophilized extract was then used for the production of a polyclonal antiserum. This antiserum was produced in rabbits using standard procedures by Beeches Biotech Ltd (Taunton, Somerset). The antiserum was provided as a 5 mg/ml preparation of the IgG fraction obtained using Protein A–Sepharose B columns, and was diluted in 10 mM-phosphate-buffered saline, pH 7.4 (PBS) for use in the 'blocking' and ELISA procedures.

'Blocking' Unwanted Cross-reactions of the Crude Antiserum

To 'block' unwanted cross-reactions, the antiserum was mixed with an extract of canned fish of one or two of the appropriate fish species as follows. Equal volumes of a 1:1000 dilution of the anti-sardine antiserum and the water-soluble extract of canned fish (2 mg/ml) of a single fish species or a mixture from two fish species (4 mg/ml for each fish species, which when mixed together in equal volumes is equivalent to 2 mg/ml for each species) were mixed in either microtitre plate wells (Dynatech, Billingham, Sussex) or suitable small containers, e.g. Bijoux (AgriYork 400 Ltd, Selby, Yorkshire) or Eppendorf microcentrifuge tubes (BDH Chemicals) and incubated for 16 h at 4°C. The relative immunoreactivity of the 'blocked' antiserum was assessed in

a non-competitive ELISA alongside the immunoreactivity of crude 'unblocked' antiserum as a control.

Non-competitive Indirect ELISA

A Dynatech 96-well polystyrene microtitre plate was coated with the water soluble extracts of canned sardine (*Sardina pilchardus*), sild (*Clupea harengus* or *Sprattus sprattus*), herring (*Clupea harengus*), mackerel (*Scomber* spp.), Pacific pilchard (*Sardinops sagax caerulea* or *S. s. sagax* or *S. s. melanostica*) and anchovy (*Engraulis* spp.). These extracts were prepared as described above. Fish extract (50 μ l) at a protein concentration of 0.1 mg/ml in a carbonate/bicarbonate buffer, pH 9.6 (1.58 g sodium carbonate and 2.92 g of sodium hydrogen carbonate dissolved in 1 l of distilled water) was incubated in each well for 1 h at 37°C. Plates were then washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBSt) using a Flow Titertek HandiWash 110 (ICN Flow, High Wycombe, Bucks). Fifty microlitres of 'blocked' or crude antiserum (diluted 1:2000 at the point of use) was incubated in each well for 2 h at 37°C. Plates were washed as above prior to the addition of 50 μ l of alkaline phosphatase-labelled goat anti-rabbit IgG (diluted in PBS as recommended by the suppliers—Sigma Chemical Company, Poole, Dorset). After a further 1 h incubation at 37°C the plates were washed five times as above, and finally 50 μ l of substrate (1 mg/ml *p*-nitrophenyl phosphate [Sigma] in 1 M-diethanolamine buffer, pH 9.8, containing 0.5 M-magnesium chloride) was added to each well and the plate incubated for 30–60 min at 37°C. The optical density (OD) of each well at 405 nm was determined and recorded using a Dynatech MR600 plate reader.

The percentage relative immunoreactivity (RI%) of the antiserum with extract of each fish species was calculated by taking the mean OD exhibited by the antiserum in wells coated with sardine extract as 100%, and calculating the mean OD obtained in wells coated with other fish species as a percentage of this.

RESULTS AND DISCUSSION

The crude anti-sardine antiserum was used to develop the indirect non-competitive ELISA. This antiserum showed high (and unwanted) relative immunoreactivities with mackerel, Pacific pilchard, sild and herring—over 70% in each case—though not with anchovy (Figure 1a). This broad specificity of the antiserum was not surprising as earlier isoelectric focusing work revealed apparently identical protein patterns for a wide range of canned fish meat extracts (Sidwell, 1992). These earlier results implied that the majority of proteins were common to many of the canned fish meat extracts examined.

In order to improve the specificity of the antiserum, extracts of those fish likely to be substituted for *Sardina pilchardus* were used to 'block' unwanted cross reactions of the crude anti-sardine antiserum. The fish extracts were added to the antiserum (diluted 1:1000 with PBS) either singly at 2 mg of the fish protein per ml, or in pairwise combinations again with 2 mg protein of each fish species per ml. Resulting immunoreactivities are shown in Figure 1 and Table 1.

Addition of a fish protein extract to the anti-sardine antiserum markedly reduced the immunoreactivity of the antiserum with that fish extract in the non-competitive ELISA. For example, addition of herring extract to the antiserum reduced the immunoreactivity of the serum with herring from 80% to 1% (Figure 1a and 1e). Broadly similar reductions in immunoreactivities were observed following the addition to the antiserum of extracts of mackerel (72–0%—Figure 1a and 1b), Pacific pilchard (112–3%—Figure 1a and 1c) and sild (104–19%—Figure 1a and 1d). Addition of a single fish extract to the antiserum also considerably reduced the

TABLE 1. Immunoreactivities of anti-sardine antiserum 'blocked' with mixed fish species extracts in a non-competitive ELISA

Fish extract used to 'block'	% Relative immunoreactivity in ELISA					
	Sardine	Mackerel	Herring	Pacific pilchard	Anchovy	Sild
No extract (i.e. crude antiserum)	100.0	71.9	79.7	112.5	0.1	104.3
<i>Mixed fish species extract at 2 mg/ml each species</i>						
Mackerel and herring	100.0	0	1.4	38.3	0	19.4
Mackerel and pilchard	100.0	0	3.8	0	0	31.5
Mackerel and anchovy	100.0	5.1	50.6	20.9	0	59.2
Mackerel and sild	100.0	0	5.9	53.1	0	7.1
Herring and pilchard	100.0	9.7	0	4.1	0.9	18.2
Herring and anchovy	100.0	3.2	8.6	12.1	0	14.3
Herring and sild	100.0	2.6	0	40.0	0	5.6
Pilchard and anchovy	100.0	45.3	58.1	17.8	0	43.2
Pilchard and sild	100.0	5.1	0	0	0	5.7
Anchovy and sild	100.0	6.1	9.6	8.4	0	4.6

Relative immunoreactivities (RI%) for the 'unblocked' and 'blocked' antisera were determined in a non-competitive ELISA on wells coated with extracts of fish species as shown. In each case the OD obtained with sardine extract is taken as 100% and the ODs obtained with the other extracts expressed as a percentage of this.

immunoreactivity of the antiserum with the other fish species in some cases. For example, addition of herring extract reduced the immunoreactivity of the antiserum with mackerel from 72% to 6% (Figure 1a and 1e), and the addition of Pacific pilchard extract reduced the immunoreactivity of the antiserum with herring from 80% to 14% (Figure 1a and 1c).

Addition of mixed fish extracts had even greater effects on the immunoreactivity patterns of the anti-sardine antiserum with those extracts (Table 1). For example, addition of sild extract to the antiserum reduced immunoreactivity of the serum with sild from 104% to 19% (Figure 1a and 1d), but addition of sild and anchovy extracts together (at the same total concentration) further reduced this to 4.6% (Table 1). Again, the addition of extracts reduced the immunoreactivity of the anti-sardine antiserum with species other than those of the added extract: the most dramatic effect observed was with the combined addition of Pacific pilchard and sild extracts to the antiserum, which reduced to less than 6% the immunoreactivities of the anti-sardine antiserum not only with the Pacific pilchard and sild, but also with herring and mackerel (Table 1). Other particularly effective combinations for 'blocking' include herring and Pacific pilchard, and herring and anchovy, both of which reduced immunoreactivities with all fish extracts to less than 20% (Table 1).

Evidently the 'blocking' technique provides an effective way of improving the antiserum specificity. The stability of the improved specificity antiserum was investigated in a preliminary experiment. Storage of the 'blocked' antibody solution at 4°C and -20°C for up to 14 days did not affect 'blocking' efficiency. Figure 2 illustrates that even after this storage, typical reduced immunoreactivities were still observed. Moreover this procedure for improving antiserum specificity is relatively simple. In contrast, methods such as affinity adsorption and separation are often more complex, more expensive and do not necessarily yield better results. Prior to the above 'blocking' experiments affinity separation methods were investigated to improve the specificity of the antiserum (Taylor *et al.*, 1992). Both conventional affinity chromatography on Sepharose 4B columns, and immunomagnetic separations using magnetiza-

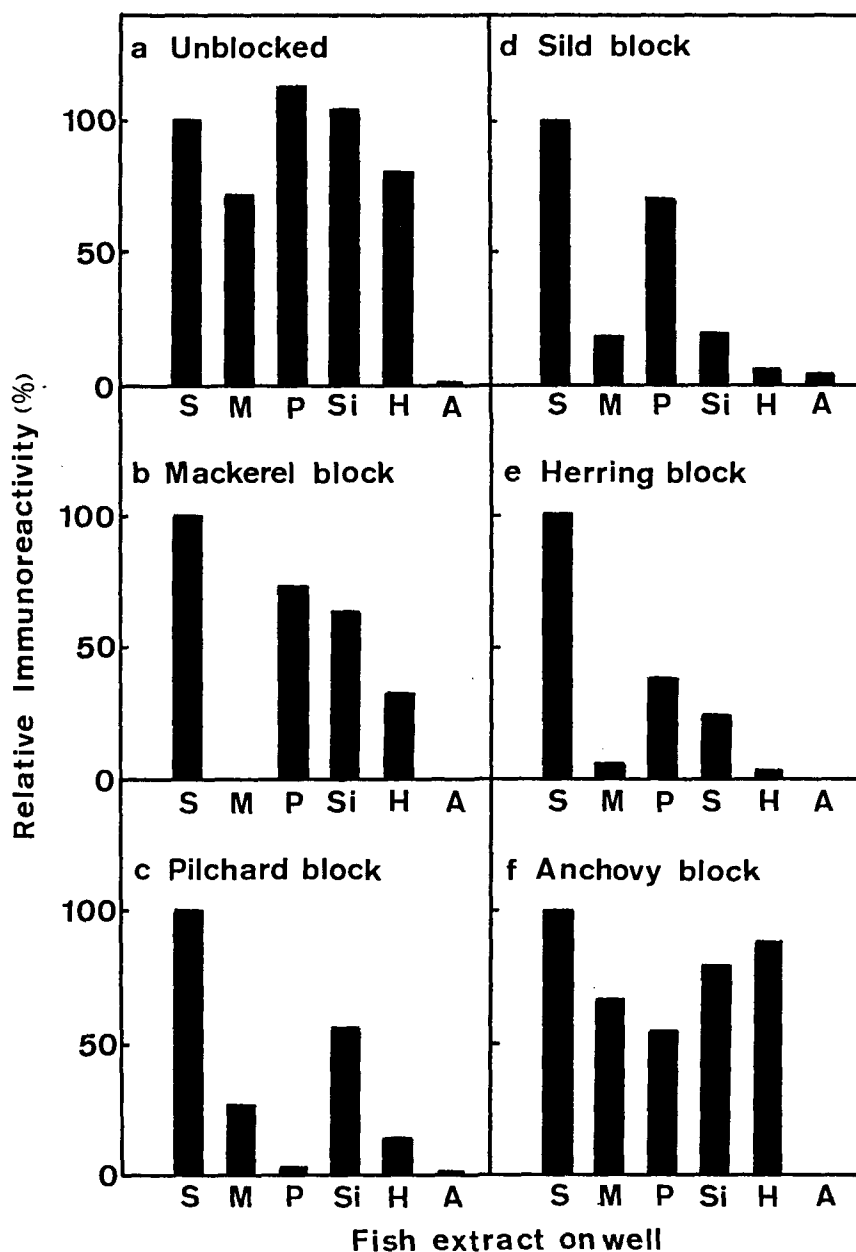


FIG. 1. Immunoreactivities of the anti-sardine antiserum 'blocked' with extracts of single fish species. The anti-sardine antiserum was used 'unblocked' (a) or 'blocked' with extract of each fish species in turn as indicated (b-f). The 'blocked' and 'unblocked' antisera were assessed in a non-competitive indirect ELISA on microtitre wells coated with extract of sardine (S), mackerel (M), Pacific pilchard (P), sild (Si), herring (H) and anchovy (A). Relative immunoreactivities were calculated by taking the OD obtained with sardine extract as 100% and expressing the ODs obtained for each of the other extracts as a percentage of this. As an example of signal intensity, the mean OD obtained with 'unblocked' antiserum on the sardine coated wells was 0.78.

ble particles as the solid phase, were investigated. However in this instance the antiserum 'blocking' technique proved not only to be the simplest, but also gave the greatest improvements in specificity (Taylor *et al.* 1992).

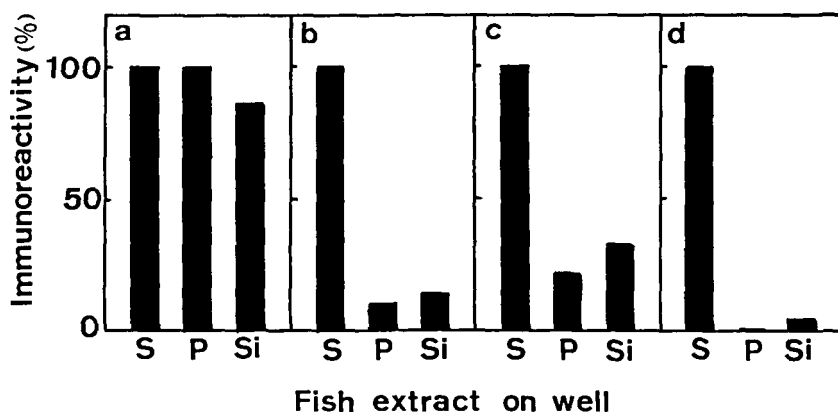


FIG. 2. Effect of storage on 'blocking' of anti-sardine antiserum with single fish extracts. The antiserum was used 'unblocked' (a), or 'blocked' with a combined extract of Pacific pilchard and sild and assessed immediately (b), or assessed after 14 days storage at 4°C (c) or -20°C (d). Assessment was by non-competitive indirect ELISA on microtitre wells coated with extract of sardine (S), Pacific pilchard (P) and sild (Si). Immunoreactivities were calculated by taking the OD obtained with sardine extract as 100% and expressing the ODs obtained for each of the other extracts as a percentage of this. As an example of signal intensity, the mean OD obtained with 'unblocked' antiserum on the sardine coated wells was 1.07.

Clearly, using this technique a series of immunoreagents (i.e. a single antiserum 'blocked' variously and appropriately with different antigens) have been obtained which could facilitate positive identification of intact canned sardines; of the species tested only sardine will give consistently positive signals with each of the blocked antisera. The approach would allow unequivocal identification of instances of substituted Pacific pilchard, mackerel, sild, herring and anchovy. However, a number of fish species are listed in the FAO/WHO Codex Worldwide Standard for canned sardines and sardine-type products (e.g. sardinella and Atlantic pilchard) and immunoreactivity data of the antiserum against these fish would need to be established before a test based on this assay could be widely implemented. Unfortunately no reliable source of these fish could be identified during this study, so that this aspect remains to be investigated.

Nevertheless, the data described clearly illustrates the potential of immunoassays for species testing of processed fish meats. Furthermore the commercial availability of simple dipstick tests for meat speciation testing, and the growing range of diagnostic kits for home use by non-trained personnel, demonstrates the applicability of immunoassays to on-site testing by food manufacturers, processors, retailers and enforcement bodies such as Trading Standards Officers and Customs and Excise.

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