idine hydrochloride plus MCE. To ensure that this was indeed the case, the fractions collected prior to the leading edge of the peaks (0–85 mL) were checked by SDS–PAGE. The electropherograms (not shown) had no detectable protein, confirming that large aggregates were not present. This supported our previous speculation that the aggregates were linked by disulfide interactions between proteins. The elution profiles (not shown) of unheated proteins and proteins from 70 °C gels solubilized with guanidine hydrochloride plus MCE were similar to those presented in Figure 5.

The SDS–PAGE electropherogram (Figure 5) also indicated that fibrinogen was at least partially broken into subunits since primarily α and β chains were observed in lane 2 and all three chains were observed in lane 5. The lack of well-defined subunit separation probably reflected lack of resolution by the gel filtration resin, although bonding between subunits cannot be dismissed.

Overall, the results indicated that noncovalent bonding alone or in combination with disulfide bonding accounted for the protein–protein interactions developed when myosin, fibrinogen, and myosin–fibrinogen gels were formed.

ACKNOWLEDGMENT

Paper No. 15169, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, MN.

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Received for review February 19, 1986. Revised manuscript received November 13, 1986. Accepted May 4, 1987.

Detection of Wheat Gluten, Whey Protein, Casein, Ovalbumin, and Soy Protein in Heated Meat Products by Electrophoresis, Blotting, and Immunoperoxidase Staining

Frederik W. Janssen,* Gerrit Voortman, and Johannes A. de Baaij

A method is reported by which it is possible to detect several nonmeat proteins in a heat-processed meat matrix. The proteins were extracted with a buffer containing sodium dodecyl sulfate, and aliquots of the extract were subjected to electrophoresis on a polyacrylamide gradient gel. The separated proteins were blotted on a nitrocellulose foil, and the nonmeat proteins were subsequently stained with a selective immunoperoxidase staining system. In model meat products, which had been heated up to 100 °C, detection of soy protein, whey protein, caseinate, egg albumin, and wheat gluten was possible down to the 0.1% level. The method provides a high level of information regarding the identity of the nonmeat protein under investigation. Screening the samples by a dot blot procedure proved to be an efficient way of sorting out samples that do not contain nonmeat proteins, thereby reducing labor costs.

Nonmeat proteins are added to meat products to enhance the emulgatory and water-binding capacity of meat proteins, especially in those cases where the emulgatory capacity of the meat proteins themselves is insufficient, as for example in low-meat-content formulations or in dietary products with a low-salt content.

Though a better product can thus be made, authorities in many countries are reluctant to give legal clearance for the use of these nonmeat proteins because, apart from the aforementioned aspect, they can also be used as meat extenders; i.e., part of the meat can be replaced by adding nonmeat protein and water.

Of the many nonmeat proteins currently in use, soy protein presumably ranks among the most frequently used ones. Of the many other proteins (wheat gluten, caseinate, whey protein, ovalbumin, peanut protein, rapeseed protein, cottonseed protein, sunflower protein) their use as meat extender has been documented (Hermansson, 1975; Hermansson and Akesson, 1975; Hand et al., 1981; Terrell et al., 1981; Wills and Kabirullah, 1981; Patana-Anake and Foegeding, 1985; Smith et al., 1973). Whether any of these proteins is actually (illegally) used remains obscure because adequate analytical methods to detect them in meat products are scarse, especially when the meat product has been heat preserved.

Of all electrophoretic methods, SDS electrophoresis is the method of choice (Lee et al., 1976; Armstrong et al., 1982; Heinert and Baumann, 1984) because even samples heated with a high-temperature/time record can be dissolved under the denaturing conditions required for this type of electrophoresis (by heating at 80–100 °C in a buffer containing ca. 2% sodium dodecyl sulfate). Quite frequently it is observed however that the electropherograms are either crowded with bands (products that have recieved

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0021-8561/87/1435-0563$01.50/0

only a mild heat treatment) or show up with only a continuous smear (liver sausages). In both cases it is extremely difficult to detect the presence of bands originating from added nonmeat proteins because these bands are always of minor intensity compared to bands originating from the meat proteins themselves.

Immunological methods like immunodiffusion and counterimmunoelectrophoresis (Kaltwasser et al., 1984; Menzel and Glatz, 1981; Ring and Sacher, 1984) have been extensively used to detect products of nonmeat origin in meats. They all share the same drawback: they fail when the meat product has been subjected to substantial heat treatment. The proteins are becoming more or less denatured then and are insoluble under the approximate physiological conditions needed to carry out the immunological assay.

Hitchcock et al. (1981) however succeeded quite elegantly to overcome this obstacle by using a very sensitive enzyme-linked immunosorbent assay to determine soy protein in a meat product. Though Hitchcock et al. (1981), Crimes et al. (1981), and Griffiths et al. (1984) claim the method to be quantitative, a round robin test initiated by the European Vegetable Protein Federation (Olsman et al., 1985) gave values that were too high when soy texturate had been added to a meat product. The method is applicable as a screening method but requires automated washing and reading equipment that may be economically feasible only when a high number of samples need to be assayed.

A more simple immunological method to detect soy protein in meat products was published by us (Janssen et al., 1985). In this method a dot immunoassay as described by Hawkes et al. (1982) was used.

A very promising quantitative dot blot procedure for determining soy protein in heat-processed meat products was recently published by Ravenstein and Driedonks (1986). They used especially developed antisera against the SDS-treated A1 fraction of glycinin.

Meanwhile, it is well recognized that gel techniques, for example immunodiffusion or immunoelectrophoresis, do provide more qualitative information than EIA or dot blot procedures (Gordon et al., 1984). There is a built-in identity check in the aforementioned methods as one may observe the confluency of the precipitation lines (immunodiffusion) or may compare the electrophoretic mobilities of sample constituents and reference proteins (immunoelectrophoresis).

When the results have to be used in courtroom testimony, we felt that there would be a need to confirm the results of an ELISA or dot blot procedure with a method providing more information about the identity of the nonmeat proteins. A method that included electrophoretic separation, blotting, and immunological detection with commercially available soya antiserum and retained a high level of information about the identity of the protein under investigation was designed by us in order to detect soy proteins in meat products (Janssen et al., 1986).

The fact that many more nonmeat proteins can be used as meat extenders prompted us to investigate whether it would be feasible to detect some of these proteins as well by this method.

MATERIALS AND METHODS

Materials. *Meat Products.* A meat product was prepared according to Table I. Soy protein isolate (Purina 500 E), caseinate (EM-spray bland), and whey protein (Lacprodan 80) were gifts from, respectively, Purina Protein Europe, Brussels, Belgium; DMV, Veghel, The Netherlands; and Danmark Protein A.S., Aarhus, Denmark. Wheat gluten was bought from Ruitenberg bv., Amersfoort, The Netherlands. Ovalbumin was made by us by separating the yolk and the white from commercially obtained hen's eggs. The white was lyophilized. Potato starch came from BDH, Poole, Great Brittain.

Table I. Recipe Model Meat Product (%)

meat/additive	I	II	blank	remark
beef (lean)	43.75	43.75	45.5	
pork (fat)	43.75	43.75	45.5	
curing salt	2	2	2	0.6% $NaNO_2$ in NaCl
potato starch	4	4	4	
ovalbumin	0.1			lyophilized
wheat gluten	0.1			
soy protein	0.1	0.1		isolate, Purina 500 E
caseinate		0.1		DMV spray bland
whey protein		0.1		Lacprodan 80
water	6.2	6.2	3	
total	100.0	100.0	100.0	

Preparation: Remove all sinews, dice and comminute. Weigh the required amount in a beaker, and mix with curing salt and potato starch. Divide into portions, and add the calculated amount of nonmeat proteins. Mix thoroughly. Weigh 5 g into a flat plastic pouch with area dimension of 8 × 8 cm. Remove air by gently rolling over the surface of the pouch with a pencil. Close the pouches by heat sealing. Immerse for 5 min in boiling water. Because the pouches are only 2–3 mm thick the complete meat analogue will reach 100 °C within a few seconds. Cool with running tap water. Store the pouches in a refrigerator.

Reagents. Sodium dodecyl sulfate (SDS), dithioerythritol (DTE), acrylamide, *N,N'*-methylenebisacrylamide (Bis), Coomassie Brilliant Blue R-250 (CBB R-250), and Tween-20 were purchased from Serva, Heidelberg, FRG. 4-Chloro-1-naphthol came from Fluka AG, Buchs, Switzerland. Nitrocellulose (NC) blotting paper (BA 83, 0.2-μm-diameter pore size) was obtained from Schleicher & Schuell, Dassel, FRG. Colloidal gold stain (Aurodye) was obtained from Janssen Life Sciences, Beerse, Belgium. All other chemicals were from Merck, Darmstadt, FRG.

Antisera. Rabbit soy antiserum (OTNG 04/05), rabbit gliadin antiserum (OTMZ 04/05), rabbit bovine casein antiserum (OTNF 04/05), rabbit whey antiserum (OTOS 04/05), rabbit chicken ovalbumin antiserum (OTNE 05/05), and goat rabbit IgG antiserum (GAR) (ORET 04/05) were obtained from Behringwerke AG, Marburg, FRG. Soluble peroxidase/antiperoxidase complex (rabbit PAP) came from Dako, Glostrup, Denmark, or Nordic, Tilburg, The Netherlands.

Methods. *SDS Electrophoresis.* Samples and standards were prepared by blending meat products (I, II, blank) or reference nonmeat proteins with a sample buffer, consisting of electrode buffer (0.38 M glycine, 50 mM Tris, pH 8.6) in which 1% SDS, 0.1% DTE, and 20% sucrose were dissolved.

The mixture was heated for 45 min in a boiling water bath, cooled to room temperature, and centrifuged. Electrophoresis was carried out on vertical polyacrylamide slabs with a pore gradient of 5.5–22.5% acrylamide and 3% Bis as a cross-linker.

A pH of 8.6 was used throughout the gel (gel buffer, 0.38 M Tris, adjusted to pH 8.6 with HCl). A BioRad electrophoresis apparatus, type Protean II, with gel dimensions 160 × 160 × 1.0 mm was used. Electrode buffer (see above) was used as lower (anode) buffer. Upper chamber (cathode) buffer contained in addition 0.2% SDS. Reference samples contained 0.2 or 0.0 4% nonmeat proteins (percent protein varying from 50 to 90). During electrophoresis a constant power of 30 W was applied for about 2 h.

Blotting. Electroblotting (Towbin et al., 1979) was carried out according to Burnette (1981) in a trans-blot apparatus (BioRad) equipped with a BioRad Model

Table II

procedure	solution	dilution	time, min
blocking of active sites	PBST[a]		50
primary antiserum	*b*	$^1/_{200}$ in PBST	60
wash[c]	PBST		10
linking antiserum	goat rabbit IgG antiserum[d]	$^1/_{100}$ in PBST	60
wash[c]	PBST		10
PAP	PAP complex[e]	$^1/_{200}$ in PBST	45
wash[c]	substrate buffer[f]		10
substrate	4-chloro-1-naphthol and H_2O_2[g]		5–10[h]
storage	*i*		

[a] Phosphate-buffered saline with Tween-20 (PBST) pH 7.0: 7.2 mM $Na_2HPO_4 \cdot 2H_2O$, 2.79 mM $NaH_2PO_4 \cdot 2H_2O$, 0.15 M NaCl, and 0.3% Tween 20 (v/v). This buffer serves to inactivate absorptive sites on the NC foil. The addition of preimmune serum, BSA, or gelatin proved to be unnecessary. [b] All primary antisera (soy, casein, whey protein, ovalbumin, wheat gluten) were of rabbit allotype. [c] All washings at least four changes. [d] It is essential that the GAR is applied at a relative high concentration, so that only one of the F(ab) moieties of the IgG binds to the primary antiserum and the other is still able to bind the PAP complex. [e] PAP complex (Sternberger et al., 1970) is a soluble complex of horseradish peroxidase (HRP) and antihorseradish peroxidase in a ratio 3:2. The anti-HRP has to be of rabbit allotype to ensure that it binds to goat rabbit IgG antiserum. [f] Substrate buffer: 0.01 M Tris adjusted to pH 7.6 with HCl. [g] Substrate: Dissolve 25 mg of 4-chloro-1-naphthol in 5 mL of ethanol. Mix with 45 mL of substrate buffer. Filter after 1 min and add 0.1 mL of hydrogen peroxide (3%) to the filtrate. [h] Color development takes place within a few minutes. [i] Wash out substrate, dry at ambient temperatures, and cover with plastic sealing tape of suitable dimensions.

250/2.5 power supply and a cryostat-controlled cooling coil.

The transfer buffer consisted of 12.5 mM Tris, 0.1 M glycine, and 10% ethanol (v/v). A constant voltage of 200 V was applied for 2 h with maximal cooling capacity (cryostat setting at 1 °C). After termination, the cassette was dismounted and the NC blot treated according to the immunoperoxidase staining protocol (Table II). The proteins left behind in the polyacrylamide gel were fixed with trichloroacetic acid and stained with CBB R-250 to check transfer efficiency.

Dot Blot. Samples were dissolved and heated and the suspensions centrifuged as described under SDS Electrophoresis, with the exception that no sucrose was added to the buffer. Drops of 5 or 1 μL were applied to a dry NC foil. The foil was subsequently stained according to the protocol described in Table II.

Staining of Blots and Dot Blots. Immunoperoxidase Staining. Immunoperoxidase staining was carried out according to Table II. *General Protein Staining.* Nonspecific colloidal gold staining was carried out by blocking the absorptive sites of the blot with blocking buffer (PBST) followed by overnight incubation with 0.15 mL of colloidal gold solution (Aurodye)/cm^2 of NC surface. Proteins stained magenta red.

RESULTS AND DISCUSSION

By blotting on a nitrocellulose foil the proteins, once buried within the narrow polyacrylamide matrix, become accessible to high specific probes like immunoglobulins or lectins.

When this method is applied to the problem of detecting nonmeat proteins in heat-processed meat products, a prerequisite is that the blotted protein and the immunoglobulin are still matched; i.e., damage to the molecular structure induced during the heat processing and the extraction of the meat proteins has to become repaired to a certain extent if the immunoglobulin has been raised against the antigen in its native state, especially if the epitopes are of the conformational type. Though it has been shown by Griffiths et al. (1984) that commercially available antisera against soy proteins can be used to detect soy protein in a heat-processed meat product by enzyme-linked immunoassay, if the meat product was extracted with 8 M urea, this will not necessarily hold for an extract made with an SDS-containing buffer or other assay systems.

It had already been established (Janssen et al., 1986) that the commercially available soy antiserum used in this study binds to all major (reduced and SDS-treated) soy protein fractions, α′-, α-, and β-conglycinin, the acidic and basic subunits of glycinin, and to epitopes of some minor fractions. These results were confirmed in this study, as can be seen in Figure 1.

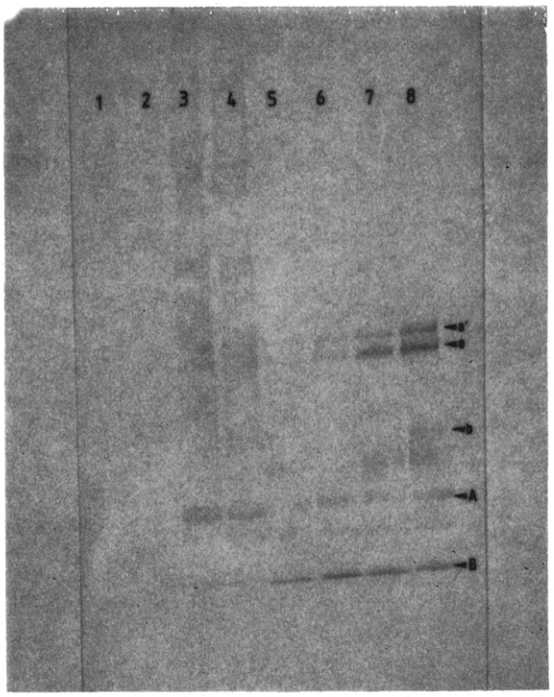

Figure 1. Immunological staining (soya protein antiserum). Key: (1) model meat, blank, unheated; (2) heated; (3) model meat II (containing 0.1% soya); (4) model meat I (containing 0.1% soya); (5) soya protein, 125 ng; (6) soya protein, 250 ng; (7) soya protein, 500 ng; (8) soya protein, 1000 ng; a = α-conglycinin; b = β-conglycinin; A = glycinin, acidic subunit; B = glycinin, basic subunit.

As shown by the results of the immunoperoxidase staining of the SDS blots (Figures 2–5), all other commercially available polyclonal antisera used in this study bind to epitopes of their antigenic parent substances as well.

When smearing of peroxidase-stained blots occurred, this problem could be remedied in all cases by diluting the sample extract.

From the same figures it can be concluded that it is possible to detect all types of nonmeat protein presently under investigation at the 0.1% level with the immunological staining system proposed. This was impossible when nonselective stains like colloidal gold stain (applied on a blot; Figure 6) or CBB R-250 stain (applied on the

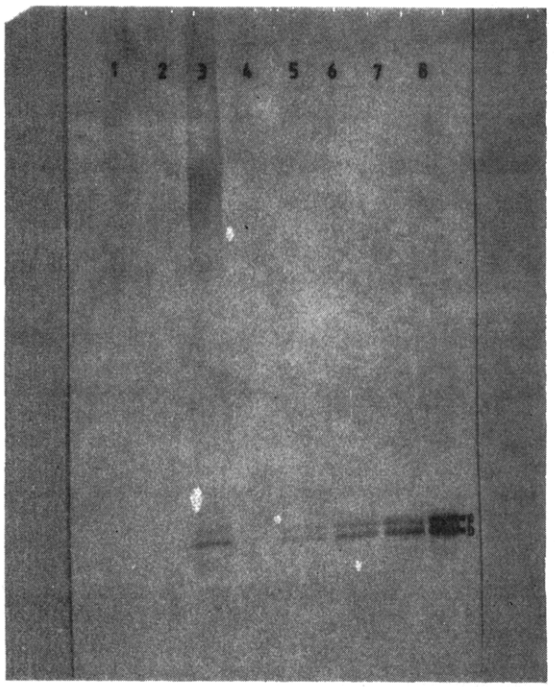

Figure 2. Immunological staining (casein antiserum). Key: (1) model meat, blank, unheated; (2) model meat, blank, heated; (3) model meat II (containing 0.1% caseinate); (4) model meat I (caseinate absent); (5) caseinate, 125 ng; (6) caseinate, 250 ng; (7) caseinate, 500 ng; (8) caseinate, 1000 ng; a = α-casein; b = β-casein.

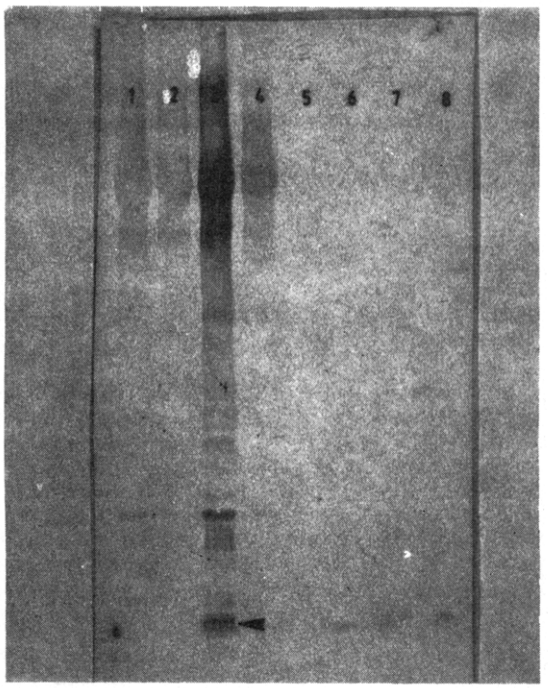

Figure 3. Immunological staining (whey protein antiserum). Key: (1) model meat, blank, unheated; (2) model meat, blank, heated; (3) model meat II (containing 0.1% whey protein); (4) model meat I (whey protein absent); (5) whey protein, 125 ng; (6) whey protein, 250 ng; (7) whey protein, 500 ng; (8) whey protein, 1000 ng. Arrow indicates lactoglobulin.

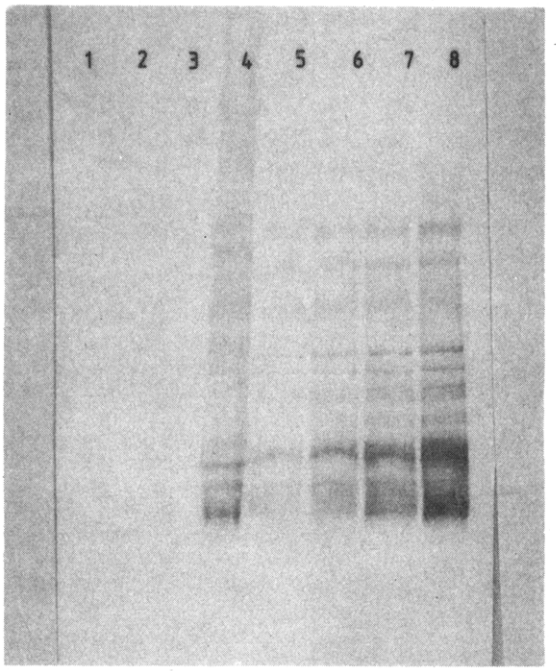

Figure 4. Immunological staining (gliadin antiserum). Key: (1) model meat, blank, unheated; (2) model meat, blank, heated; (3) model meat II (wheat gluten absent); (4) model meat I (containing 0.1% wheat gluten); (5) wheat gluten, 156 ng; (6) wheat gluten, 312 ng; (7) wheat gluten, 625 ng; (8) wheat gluten, 1250 ng.

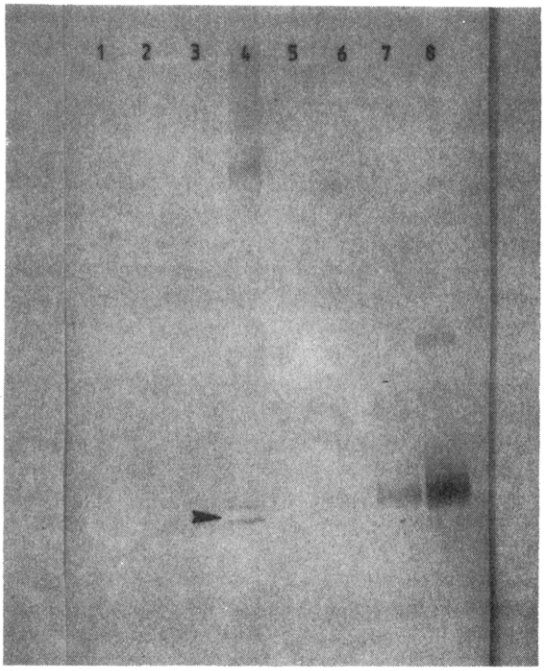

Figure 5. Immunological staining (chicken ovalbumin antiserum). Key: (1) model meat, blank, unheated; (2) model meat, blank, heated; (3) model meat II (chicken ovalbumin absent); (4) model meat I (containing 0.1% chicken ovalbumin); (5) chicken ovalbumin, 125 ng; (6) chicken ovalbumin, 250 ng; (7) chicken ovalbumin, 500 ng; (8) chicken ovalbumin, 1000 ng. Arrow indicates chicken ovalbumin.

gel itself; results not shown here) were used.

Furthermore, one can conclude from Figures 1–5 that the presence of other nonmeat proteins in the meat product in addition to the one to be detected does not impair specificity. Only in the case of anti-casein/anti-whey serum with casein/whey protein some aspecificity may occur, as can be seen in Figure 3, lane 3. But, with the method presented here one can easily distinguish between the presence of several nonmeat proteins.

Direct dot blotting of the samples on a nitrocellulose foil, omitting the electrophoretic separation step, proves a very efficient and low-cost way to sort out a number of samples with a negligible percent nonmeat protein, thereby reducing the number of samples that need to be analyzed by electrophoresis. By comparison of the intensity of the stained spots with reference dots only those samples that

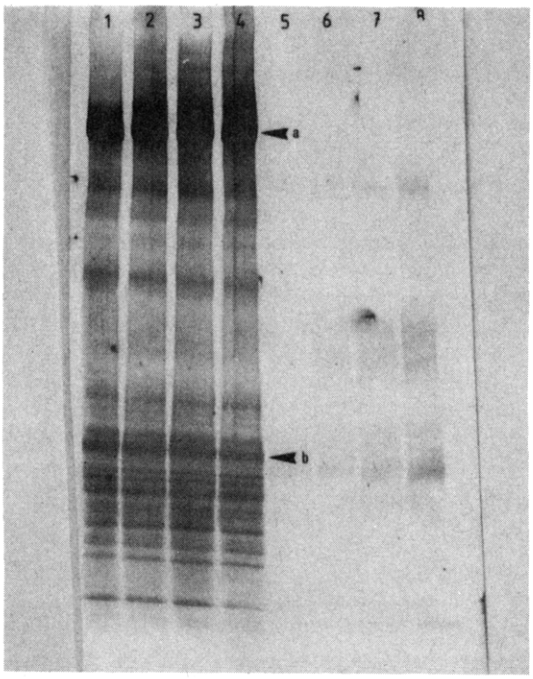

Figure 6. Aselective gold staining (AURODYE). Key: (1) model meat, blank, unheated; (2) model meat, blank, heated; (3) model meat II; (4) model meat I; (5) ovalbumin, 125 ng; (6) ovalbumin, 250 ng; (7) ovalbumin, 500 ng; (8) ovalbumin, 1000 ng; a = myosin; b = actin.

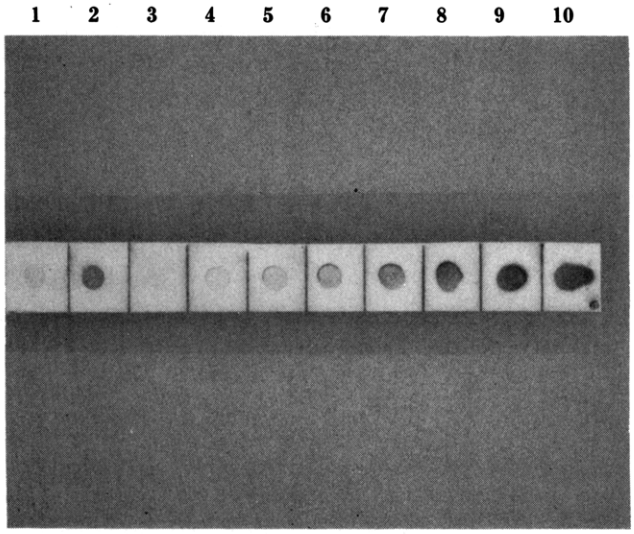

Figure 7. Immunological staining (gliadin antiserum), dot blot. Key (left to right): (1) model meat II (wheat gluten absent); (2) model meat I (containing 0.1% wheat gluten); (3) model meat blank; (4) wheat gluten, 156 ng; (5) wheat gluten, 312 ng; (6) wheat gluten, 625 ng; (7) wheat gluten, 1250 ng; (8) wheat gluten, 2500 ng; (9) wheat gluten, 5000 ng; (10) wheat gluten, 10 000 ng.

show up with a spot of an intensity exceeding that of a preset reference (e.g., 0.1% nonmeat protein) need to be confirmed with the complete electrophoretic procedure.

Results of a dot blotting experiment are shown in Figure 7. A series of wheat gluten reference dilutions and three meat model extracts were dotted. As can be seen, only meat product I, which contained 0.1% wheat gluten, shows a distinct spot. Dot blotting carried out with the other antisera gave comparable results, except that a cross-reactivity of casein antiserum toward whey protein and of whey protein antiserum toward casein was observed.

Cost effectiveness of the method may be improved by pooling of the specific antisera. The feasibility of this proceeding is dependent on the quality if the antisera, as all aspecifically binding IgG's of the individual antisera are present in the pool. Background may thus be significantly increased.

It is possible to estimate the percent nonmeat protein in the meat product by comparing the intensity of the spots with a series of references, either by the eye or by means of an optical scanning device. The accuracy of the determination is however affected by the variability of the antigenicity of the commercially used nonmeat proteins, which depends on type of nonmeat protein (meal, concentrate, isolate, texturate) and processing conditions. In addition some epitopes may get irreversibly damaged during the processing of the meat product itself. The heat processing applied to the meat products in this study is rather low. There are however indications that the method presented here works also when the meat product receives a more severe heat treatment (luncheon meat type products). Further research is needed to study the extent of epitope modification and its effect on detection limits, specificity, and quantitation.

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Received for review May 6, 1986. Accepted December 8, 1986.