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Auteurs: drs H.L. Elenbaas
F.W. Janssen (KvW Zutphen)
W. Haasnoot

Projektleider: drs H.L. Elenbaas

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Working paper for Codex Committee on vegetable proteins (CXVP) on:

Quantitative Methods for Differentiation of Vegetable and Animal Proteins in foods III.

Prepared by H.L. ELENBAAS*, F.W. JANSSEN** and W. HAASNOOT*

INTRODUCTION

This paper is a follow-up of those of November 1981(1) and September 1983 (2) respectively.

In this paper some analytical methods which are being currently investigated or used to assay meat products on the presence of vegetable proteins are discussed with emphasis on recent developments. Identification of meat (and milk) proteins is another topic and in our view of minor interest to CXVP, although the investigations on this subject are of interest in respect of the analytical methods employed. There is an increasing world-wide interest in a sound and reliable method of analysis for vegetable proteins. In this context, note should be taken of collaborative studies carried out in the past two years, especially in Europe. Probably due to the economic recession, work has been limited to studies on soya proteins for the repressive chemical control of foods, rather than to fundamental research to establish procedure and parameters applicable to all kinds of vegetable proteins.

Use of immunoassays has been favoured in the inter-laboratory tests, in spite of two severe problems: solubilisation of denaturated proteins and loss of binding sites on the antigen by heat processing. Nevertheless the immunological methods appear to give good performance in routine analysis.

Methods reviewed in this paper are classified into 3 groups: Immunoassay, Electrophoresis and Other Methods. Each group will be discussed in detail.

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* State Institute for Quality Control of Agricultural Products, P.O. Box 230, 6708 PD Wageningen, the Netherlands

** Food Inspection Service, P.O. Box 9012, 7200 GN Zutphen, the Netherlands

METHODOLOGY

I Immunological methods

Several immunological methods are available. They all depend on the specific binding of an immunoglobulin to the antigenic sites of a protein. This binding takes place only under approximate physiological conditions.

Two major problems limit the applicability of these methods:

1. Heat denatured proteins are as a rule very difficult to dissolve in physiological buffers, and
2. By heat processing the binding sites on the antigen (the epitopes) are progressively lost.

Although these problems can be remedied to a certain extent by choice of extractants, they limit the applicability of techniques such as immunodiffusion, counter-electrophoresis, rocket electrophoresis and immuno-electrophoresis, which are based on precipitation of antigen/antibody complexes.

Kaltwasser et al. (3) describe a counter-electrophoretic system in which the samples are dissolved in buffers with reducing agents (0,1 M barbiturate, pH 8.6 with dithioerythritol DTE or mercaptoethanol M.E.). Although they claim that by this method they are able to detect a textured vegetable (soya)protein in a heated frankfurter type sausage, it is questionable whether this claim will hold good for many types of textured proteins. In our experience some soya preparations are very difficult to detect, even in the unheated state.

Very small amounts of antigenic proteins can be detected with assays which do not rely on the formation of an antigen/antibody precipitate but in which the binding is made visible by some amplification system. Examples are Radioimmunoassay (RIA), and Enzyme Immuno Assay (EIA) etc. It is anticipated that many of these systems which are currently used in clinical chemistry will find their way into analytical chemistry of foods.

Ring and Sacher (4) describe a method to determine soya in heated meat products by indirect haemagglutination (which could be considered as one of the oldest amplifying systems). The method is a modification of that published by Herrmann (5).

The modification consists of the inclusion of 0.7% M.E. and 1% urea in the extraction buffer.

However the method is quite complicated and requires considerable experience in evaluating the agglutination pattern.
One of the most salient advantages of the more sensitive assays is the possibility of extracting proteins under denaturing conditions with e.g. 8 M urea, guanidine-HCl, sodium dodecylsulfate (SDS), mercaptoethanol (ME), dithioerythritol (DTE) etc. and dilute to physiological conditions.
This fact, and not the type of amplifying system is the most important feature of the ELISA method as published by Hitchcock in 1981 (6). In the second working paper (2) it was mentioned that two collaborative trials (MAFF and Euvepro) have been carried out. The results have since been published respectively in the Journal of the Association of Public Analysts (7) and in an Euvepro report (8). Although results of both trials were encouraging more work has to be done before these methods can be considered as "quantitative". The testing of other antisera (against heat denatured soya proteins - currently being investigated at Unilever Research Lab, Colworth House G.B. (Thomas, priv. comm.) by the Food Safety and Inspection Service of the USDA (Ellis priv. comm.) and by Kaltwasser (3) in the FRG). The development of other extraction procedures is also necessary (the use of approxim. 2 liter organic solvent to prepare one sample may be considered prohibitive).

In addition it has to be established the extent to which the antisera are specific. Brehmer (9, 10) gives a fair warning that commercial antisera against soya proteins give a high incidence of false negative results especially with heated products.
In a simple test such as immunodiffusion it is possible, by observing the precipitation lines, to distinguish between identity and non-identity (false positive reaction).
ELISA does not offer such an option. It is therefore of great importance to ensure the specificity of the antisera. For this reason the inclusion of more "negative" samples in the collaborative trials is considered to be essential.
Griffiths et al. (11) recommend for non-specialised laboratories an ELISA procedure with commercially available immunoreagents. In his opinion these reagents are suitable for routine use in measuring levels of soya protein in raw and processed meat mixtures.

The response of commercial soya ingredients relative to an arbitrary standard soya proteins isolate varied much and was as a rule less than 100%. However when a standard soya protein was added to beefburgers, there was a good agreement between the observed and calculated levels in heat-set samples. Sterilized samples gave a decreased but linear response.

The results of an investigation by Barnett and Howden (12) on a rocket immuno-electrophoretic method for the detection of heat-treated peanut protein also suggest that the use of a (monospecific) antiserum to a heat-resistant (glyco)protein may extend the usefulness of immunological techniques.

Of interest are the two articles of Guenther et al. (13, 14). They describe an interlaboratory test of the immunological identification of proteins in chocolate and in baker's and confectioner's goods respectively. The proteins investigated were the vegetable proteins from soya bean, wheat, peanut, corn, almond and coco-nut as well as animal protein: casein and chicken meat protein. Using antisera with high titer made according to Baudner's prescription (which are also commercially available), the proteins involved could be identified in heat-treated products even with Ouchterlony double-diffusion plate technique. With counterimmunoelectrophoresis carry-over proteins were detected.

An advantage of the immunological methods over electrophoresis is that the immunoglobulins are able to bind to precipitated or dispersed protein particles which are not able to penetrate into the gel matrix.

Kurth and Rodgers (15) describe the covalent coupling of non-meat proteins to myosin by transglutaminase. Such coupling could result in electrophoretic patterns which are not interpretable whilst - provided the binding is outside the epitope - immunological response might be retained to some extent.

II Electrophoretic methods

Electrophoresis is probably the most frequently used analytical tool of the analyst working in this field.

There are some drawbacks when quantitative work is required.

One of these is again the solubilizing power of the buffers used. Common additives as urea, SDS, ME, or DTE are necessary to dissolve heated proteins. When one of the most potent solubilizing additives: SDS is used, the whole analytical system has to be based on an SDS reagent...

Using SDS of the two electrophoretic separation parameters charge and size, (differences in) charge (are) is getting lost with an inherent loss of the capability to optimize the separation of fused bands.

The variability of binding of dye (Coomassie Blue R250) from the staining solution by the separated protein fractions is a more serious problem. Dye binding is a complex phenomenon in which several types of non-covalent bonds are involved. Proteins that have been heated in complex formulations as such as batters may to some extent loose their ability to bind dye. This is a serious problem when quantitative analysis is required. Even, within the categories soya protein (isolates, concentrates and texturates) correlation between optical density (O.D) of the stained protein bands and the protein content of the product is poor. Consequently the conversion factors obtained are highly inaccurate.

A comprehensive literature on staining systems other than non-Coomassie BBR exists, e.g. silver staining methods (sensitivity 10-100 * CBBR) - for a review see 16,17. These methods are even more alchemistic in nature and we do not expect that a better correlation O.D/protein-content will be obtained with these staining systems.

The Euvepro collaborative trial (8) included also a test of an electrophoretic system (Armstrong, 18). Results were in general comparable to those of the ELISA method. The method as prescribed is however very laborious and uses very inconvenient gel thickness.

Several refinements which are already common practice in biochemistry have been adopted by food analysts.

Heinert et al. (19) state that by using a gradient gel (5-12% T) the resolution of the protein bands could be improved. They were able to separate one of the soya bands from an otherwise fused poultry protein band.

Ring et al. (20) claim that, when using guanidine-HCl instead of 8 M urea more protein dissolved and the separation improved.

In our opinion, a modern SDS system would consist of a vertical slab gel system with a thickness of 1-2 mm, in a gradient mode. It would contain urea in the gel. The sample buffer would be based on 8 M urea or guanidine-HCl, DTE or ME.
A very interesting development, which perhaps has good prospects - especially in qualitative work - is the transfer of the proteins separated by any electrophoretic method to a nitrocellulose sheet by the so called blotting procedure (Towbin *et al*. 21). By such a method the protein fractions which are otherwise buried inside the polyacrylamide matrix become accessible and can be detected and identified by specific immunoprobes. However this transfer is not very quantitative especially with proteins of high molecular size.
The method could perhaps be used to check whether in the ELISA method false positive results were obtained.

III Other methods

1. Non-chemical analysis: microscopy and histology

A collaborative study of a quantitative microscopic procedure, based on the stereological technique of Flint and Meech, designed and conducted in the U.K. in 1980/1981 has been briefly reported in 1984 by Crimes *et al*. (7). The participants analysed uncooked homogenized sausage-type meat products containing hydrated soya flour and/or hydrated textured soya. The results of the trial were unsatisfactory, the histological method was limited to a qualitative screening assay and needs skilled operators.
An interlaboratory test in the USA (22) in which histology was the method under consideration demonstrated that at least 3% soya flour could be measured. The identification of the flour was based on the histologically recognizable layers of the soya seed. This method also needs expert skill and will not be valid for the identification of soya isolate.

2. Chemical analysis

a. Methods based on the determination of protein fragments.

The results of the two Dutch studies on the differentiation of proteins by pyrolysis spectrometry combined with multivariate data analysis (23) and by computerized interpretation of the amino acid pattern (24, 25, 26), show that these methods are satisfactory for extended meat products when the respective pyrolysis and amino acid pattern of the composing materials are actually known.
However both methods are neither simple nor directly applicable assay techniques. The former method needs a mass spectrometer and both required computer techniques.
Pyrolysis spectrometry has the advantage of allowing direct analysis of blends of meat and soya protein without prior chemical treatment. The method still has to be tested on industrially produced blends.
A similar technique, direct probe mass spectrometry, for the diffentiation of meat species had been under investigation by Puckey and Jones (27).
With the method based on amino acid composition amounts of soya isolate and/or wheat gluten and/or casein can be determined quantitatively together with the amount of lean meat, collagen and/or liver in meat products, provided the type of ingredients is well-known. The history of heat processing of the product has little or no influence on the results (24). Inclusion of the N^{t}-methyl-histidine (3-methylhistidine) content (as a measure for the lean meat content) in the multivariate analysis of the protein raw materials of meat products improves the analytical results (25).
Analysis of the protein components in meat products produced in industry gave negative results (25, 26). The results were influenced to a different extent mainly by variations in the amino acid composition of the meat raw materials and the presence of organs other than liver.

Since suitable columns for High Performance Liquid Chromatography (HPLC) have become available, this technique is being used increasingly as a technique for the separation of proteins and peptides.

With columns specially designed for protein and peptide analysis, separation is possible according to size, charge, hydrophobicity and differences in isoelectric points (chromatofocusing).
The application of HPLC to establish the origin of final products or to quantify the addition of vegetable proteins has so far been limited to the determination of a specific soya peptide in enzymatically hydrolysed meat mixtures with soya protein (28, 28a).
The lack of more applications can be ascribed in part to the novelty of this technique but also to the limitations of the equipment with respect to the use of detergents and high salt concentrations.
Recently these limitations have been removed by the introduction of a more robust equipment (Fast Protein/Polypeptide/Polynucleotide Liquid Chromatography (FPLC)-system) in which the contact between liquid and metal parts has been avoided, so that detergents and high salt concentrations can be used without damaging the equipment (29).
This system has his limitation in that the working pressure may not exeed 4 MPa, special designed columns with low back-pressure overcome this problem.
Further research is recommended on the possible application of these systems and of HPLC with respect both to the quantification of vegetable proteins and to the establishment of the origin of final products.

b. Indirect methods

To our opinion indirect methods based on the determination of carbohydrates, sterol or particular metals from the soya bean protein are not satisfactory. In the Netherlands much hope was placed on the phytate method as a routine method in spite of the limitations; phytate being only a associated constituent of the protein.
In our studies (not yet published) we found differences in conversion factors of phytic-phosphate to soya protein from flour, concentrate and isolate, due to differences in N-conversion factors (30) and in the ratio of phytic-P/protein. Phytate to protein ratio's and phosphorus content of whole soya extracts and of various soyabean protein fraction are determined by Brooks and Moore (31) and by Honig et al. (32).

Kloczko and Rutowski (33) found that the phytic-P content of beef blends made with soya protein increased with increasing heat treatment and amount of added inorganic phosphate. Since the iron and zinc chelating properties of phytate are disadvantageous from a nutritional point of view, efforts will be directed to its removal (either by breeding or by technological means). Frequent recalibration of conversion factors would than be necessary. Such considerations force us to abandon the phytate method for regular use.
Quantifying soya protein in extended meat products by the determination of the artificial marker titanium dioxide, as had been practised in the USA, would appear the most reliable procedure on the analytical point of view. However addition of a foreign chemical substance solely for enforcement purposes meets with objections. Obligation to carry a tracer for certain food ingredients and not for others has a discriminatory effect. Quite properly the USDA has been rescinded in the USA Federal Register of 9 May 1984 (Bates, Euvepro, priv. comm.) the practice of TiO_2 tracing.

IV Meat proteins

As mentioned in the Introduction the differentiation of meat proteins is a parallel topic and corresponds with the identification of meat species, a specialism which is receiving widespread attention.
It is important to pursue the developments in meat species identification, because this subject and the differentiation of vegetable proteins have two problems in common: the solubility of heated proteins and the production and checking of antisera in immunoassays.
In this context we wish to draw attention to the CEC Workshop on "Biochemical Identification of Meat Species" organized in November 1984. Nearly all the experts present, among which were three participants from non-EEC countries (Kenya, Australia and USA) have presented their work and insights (34). Also six statements have been made about the methods used and about future co-operation for agreeing a tentative official method.

CONCLUSIONS

There are two major fields of application:

1. Quality control by producers of meats and sausages.
2. Repressive control analysis by official analysts.

Whereas in the first case the analyst will have access to information about the type of vegetable protein actually used, the formulation of the meat product and the retorting process, in the second case the control analyst will be deprived of such information.

The former is able to work with specially developed conversion factors to cope with variations in ingredients, recipes and in processing. The problems facing the latter are considered to be the most onerous. If analytical control of composite products is to be effective it is of the greatest importance that methods are developed to aid the determination of vegetable protein irrespective of processing conditions of the vegetable product as well as the final food product.

To achieve this goal all efforts should be directed towards the development of methods for some key ingredients e.g. soya protein. The experiences thus acquired will make it less difficult to convert the analytical method to use with other vegetable proteins e.g. rapeseed protein, peanut protein, gluten etc.

Immunological as well as electrophoretic methods offer both good starting points for the design of such quantitative methods. They offer complementary information on the products under study.

With the ELISA method efforts should be directed to the development of better antisera and to more realistic sample pre-treatment.

Efforts to improve the SDS-electrophoretic method should be directed towards the development of other buffer systems with a still greater potential to dissolve the heat treated proteins and to the development of methods to combat staining inhibition.

None of the other methods produces reliable results. The indirect methods, which indeed are simple tests compared with the analysis of protein fragments, do not only depend on the species, type and processing of the vegetable product, but are also affected by the various ingredients and additives used in a composite foodstuff.

The methods based on the determination of protein fragments with multivariate data analysis are suitable for simple meat blends, but they are complex. They need expert skill and the equipment is expensive.

These methods however are able to supply at once much more information about the composition of the product than the univariate methods. Surveying the literature, the most promising approach in methodology appears to lie in immunology, and may be in the near future in liquid chromatography (FPLC and HPLC).

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LAST MINUTE NOTE

Two new articles of interest were published just after finishing the paper.

- Kaiser and Krause (35) give a review of the analysis of proteins in foods by means of electrophoretic and chromatographic methods. Investigations on fish, meat and derived products, non-meat proteins in meat products, milk, cheese, cereals and products made of cereals, oilseed proteins, legumes, fruits and vegetables described in literature are presented.
- Bauer and Stachelberger (36) describe a counter-immunoelectrophoretic (CIE) method on agarose gels for the detection of non-meat proteins in meat products. They are able to detect in heated meat products (Bruehwurst-type sausages) 0.1% wheat gluten, 0.02% powderd whey, 0.01% soya protein, 0.004% dried whole egg and 0.001% Na-caseinate. For detecting wheat gluten the sample is extracted with barbital buffer (pH 8.2) containing 7M urea. The extract is mixed with a SDS-solution and the whole is saturated by incubation with normal rabbit serum.

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