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Determination of Soya Protein in Meat Products by a Commercial Enzyme Immunoassay Procedure: Collaborative Trial

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The results of a collaborative trial of enzyme immunoassay kits supplied by Biokits Ltd for the determination of soya protein in meat products are reported. Fourteen laboratories analysed uncooked sausages, uncooked beefburgers, uncooked pâtés, and cooked pâtés containing different known levels of soya flour, isolate, concentrate and/or texturate. Blanks, duplicates and standard controls were included in a total of nineteen samples; accuracy and precision were estimated by statistical analysis. Overall recoveries were 80–100 per cent. across the range of levels of the different soya ingredients studied. Multiple analysis of one sample indicated an 11 per cent. overall coefficient of variation. Reproducibility between different laboratories was better than that of an immunoassay procedure previously tested but unavailable in kit format. The current kit was easy to use: all the specialised materials (antiserum, enzyme conjugate, substrates, pre-sensitised microwells, soya standards, etc.) were included and none of the participants reported any significant problems in following the protocol provided. No prior knowledge about the type of soya ingredient nor the composition of the meat product is necessary. Within the limitations of this study it may be concluded that, using a single arbitrary soya standard as a reference, the levels of soya protein in a raw or pasteurised meat product of entirely unknown composition may be reliably estimated with repeatability and reproducibility up to 1 and 2 g/100 g respectively, with a recovery of 80–100 per cent. of added soya protein; these characteristics are significantly better than found in previous trials.

The determination of non-meat proteins in meat products of mixed composition has always presented an analytical problem. With the advent of immunoassay procedures, the specific identification and quantitation of proteins in complex mixtures has become technically feasible. Of the non-meat protein additives soya protein has always been considered to be the most significant component requiring analysis in meat products. For these reasons the Ministry of Agriculture, Fisheries and Food sponsored a collaborative trial in 1983 employing an enzyme-linked immunosorbent assay (ELISA) procedure[1].

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The results of that trial, together with those of a stereological procedure to estimate soya material, were published in 1984[2] with the observation that the inherent weakness of the ELISA procedure was associated with differences in responses from different soya flours or textured concentrates to the same ELISA procedure and soya standard. Reported results, therefore, required to be corrected back to the standard concentrate using response factors which had been determined for the soya materials used in the trial. In unknown samples the soya material (and hence the response factor) would not be known. In addition participants in the trial commented adversely on the quantity of solvents and labour time required to prepare each acetone powder for Kjeldahl analysis and solubilisation for the ELISA procedure. However, it was concluded that the ELISA method gave considerably better results than those reported for the stereological method based on non-immunochemical staining and microscopy[2]. A later collaborative trial comparing SDS-polyacrylamide gel electrophoresis and the ELISA method[3] concluded that while both these methods were useful both required further refinements to make them generally acceptable for control purposes.

Since 1983 an independent group has worked on the development of an improved enzyme immunoassay for the quantitation of soya protein in meat products[4]. The method was developed to permit rapid aqueous extraction of meat samples into a liquid form suitable for analysis. The enzyme immunoassay reagents relied on pre-prepared components in a ready-to-use kit form employing a specially developed antiserum to renatured soya protein isolate. The same renatured soya protein isolate was used to prepare stable liquid standards and this combination of antiserum and standard, together with a rapid renaturation procedure for extracted samples, resulted in a decreased variation in response from different soya preparations. The analysis, including sample preparations, could be completed within the working day with the actual enzyme immunoassay taking less than 60 minutes to complete.

Because of these improvements to the procedures tested in 1983 and the present ready availability of the soya protein enzyme immunoassay kit, the performance of the procedure was evaluated by collaborative trial.

Organisation of the trial

LABORATORIES

Thirteen laboratories agreed to participate in the collaborative trial, all of which returned their results.

METHOD, APPARATUS AND REAGENTS TO BE USED

All the laboratories were required to follow the given protocol as outlined in Appendix I. The enzyme immunoassay kits (Biokits Limited) and some of the other reagents to be used, were provided centrally to all the analysts.

SAMPLES

Participants each received 19 trial samples, the compositions of which are given in Table I. They were asked to analyse them in two phases, i.e. the initial

TABLE I

COMPOSITION OF SAMPLES USED IN THE COLLABORATIVE TRIAL OF THE "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE FOR THE DETERMINATION OF SOYA PROTEIN IN MEAT SAMPLES

Component	Content in final product *per cent. m/m*								
Recipe type	Sausage			Beefburger			Pâté		
Recipe code	S1	S2	S5	B2	B3	B4	P1	P3	P4
Sample code uncooked	32	12/51[a]	30/52	27/60	69/95	61/87		15/71	
cooked							20	33/65	28/72
Beef forequarter (70VL)	22·5	22·5	22·5						
Beef flank (60VL)	5·8	5·8	5·8						
Pork belly (60VL)	5·86	5·86	5·86				45·3	42·3	41·3
Pork back fat	12·85	12·85	12·85						
Pork rinds (90VL)	5	5	5				8	8	8
Rusk	15·2	13·2	13·22						
Seasoning[b]	2·5	2·5	2·5						
Beef forequarter (95VL)				70	60	70			
Beef clod fat				10	10	10			
Seasoning[c]				2	2	2			
Pigs liver (98VL)							26·7	26·7	26·7
Onions (rehydrated)							5	5	5
Salt							2	2	2
Sodium tripolyphosphate							0·3	0·3	0·3
Sodium nitrite							0·02	0·02	0·02
Garlic powder							0·3	0·3	0·3
Sodium caseinate							—	1	—
Water	30·29	30·29	30·27	16	18	15	12·4	12·4	12·4
Soya product:	None						None		
Isolate[d]		2	0	0	0	0		2	2
Concentrate[e]		0	2	2	0	0		0	2
Grits[f]		0	0	0	10	0		0	0
Texturate[g]		0	0	0	0	3		0	0
Total soya product	0	2	2	2	10	3	0	2	4
Total soya protein	0	1·74	1·18	1·3	4·9	1·98	0	1·74	2·92
Total meat content	52·01	52·01	52·01	80	70	80	80	77	76

[a] Sample 12/51 also formed the pre-trial sample.
[b] Seasoning; No. E 255/256 (Lucas Ingredients Ltd).
[c] Seasoning; "Stars and Stripes" burger seasoning (Lucas Ingredients Ltd).
[d] Soya isolate; PP500E (McAuley Edwards Ltd).
[e] Soya concentrate; Arcon A (British Arkady Ltd).
[f] Soya grits; defatted soya grits No. 20 (Lucas Ingredients Ltd).
[g] Textured soya; Danprotex-B TSP concentrate (Interfood Ltd).

pre-trial sample (which was to be analysed eight times) followed by a single analysis of each of the 18 remaining samples as the main part of the trial. The results of the analysis of the pre-trial sample were to be approved by the organisers of the trial before the participants were permitted to attempt the second phase of the trial.

The samples were prepared by the British Food Manufacturing Industries Research Association (BFMIRA), using normal commercial procedures. They followed examples of typical recipes for uncooked U.K. sausages and burgers,

and cooked and uncooked pâtés[5]. The pre-trial sample was the uncooked sausage mix which also formed sample 12.

FAMILIARISATION WITH THE BIOKITS METHOD

As the method to be used was new to many of the participants in the trial it was decided by the organisers that all efforts should be made to ensure that they became familiar with the procedures and principles involved before attempting the analysis of the trial samples proper. To this end a full day's "Workshop" was held which all participants in the trial attended and at which instructions on the trial and opportunity to use the apparatus involved in the trial were given. In addition the analysts were given a "pre-trial" experimental sample to analyse before undertaking analysis of the trial samples proper.

Results

The results reported by the analysts are given in Tables II to XI, and are summarized in Table XII. It should be noted that none of the results was corrected for any response variations of soya components used relative to the standard.

TABLE II

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

PRE-TRIAL SAMPLE: UNCOOKED SAUSAGE (CODE NUMBER 12)

Laboratory	Soya protein (replicates) *g/100 g*								Mean *x*	Coefficient of variation *CV*
1	1·58	1·61	1·84	2·10	1·84	1·82	1·78	2·22	1·85	11·8
2[e]	1·36	1·49	1·35	1·40	1·58	1·47	1·56	1·56	1·47	6·4
3[e]	—	—	—	—	—	—	—	—	—	—
4	1·47	1·52	1·46	1·65	1·52	1·42	1·66	1·68	1·55	6·56
5	1·49	1·45	1·56	1·89	1·65	1·62	1·76	1·85	1·66	9·76
6	1·49	1·53	1·46	1·63	1·75	1·53	1·58	1·55	1·56	5·82
7	1·31	1·35	1·28	1·36	1·35	1·52	1·36	1·38	1·36	5·0
8	1·38	1·49	1·59	1·77	1·91	1·82	1·53	1·59	1·63	12·27
9	1·46	1·71	1·02	1·63	1·62	1·51	1·35	1·20	1·44	16·4
10	1·89	1·99	1·76	1·90	1·83	2·10	1·92	1·81	1·90	5·67
11	1·42	1·32	1·22	1·29	1·81	1·51	1·42	1·23	1·40	13·8
12	1·71	1·53	1·59	1·81	1·63	1·87	1·99	1·76	1·74	8·6
13	1·50	1·65	1·67	1·63	1·82	1·65	1·48	1·32	1·59	9·1
14	1·45	1·84	1·93	1·87	1·72	2·03	2·01	1·67	1·82	10·7
Overall mean (x)									1·61	
Overall coefficient of variation										10·84
Actual total soya protein content (g/100 g)[f]						1·74				

For key see Table XIII.

TABLE III

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

CONTROL (BLANK) SAMPLES: COOKED PATÉ CONTROL (20)/UNCOOKED SAUSAGE CONTROL (32)

	Soya protein *g/100 g*	
Laboratory	Sample code number 20 Pâté	Sample code number 32 Sausage
1	not detected	not detected
2[e]	0	0
3[e]	0	0
4	—	—
5	Nil	Nil
6	<0·20	<0·20
7	0	0
8	0	0
9	<0·68	<0·68
10	—	—
11	<0·69	<0·69
12	not detected	not detected
13	<0·70	<0·70
14	<1·00	<1·00
Mean (x)[a]	—	—
Repeatability (r)[a]	—	—
Reproducibility (R)[a]	—	—
Actual total soya protein content (g/100 g)[f]	0	0

For key see Table XIII.

Statistical analysis of the results

The results obtained were statistically analysed according to the procedures outlined by the British Standards Institution[6]. Significant differences between the pairs of individual results were identified using Cochran's Test and the extremes of magnitude of pairs of results were identified by Dixon's Test. Outlying results are marked in Tables III to XI. The value of the means, repeatabilities and reproducibilities were calculated from the remaining results and these are also given in these Tables. No extensive statistical analysis was applied to the results from the pre-trial sample.

Discussion

GENERAL COMMENTS

The consistency of response to different soya ingredients in different products depends on the properties of the antibodies used as well as the detailed procedure followed. The method tested here differed from those used in previous collaborative trials, as a readily available convenient commercial kit (including pre-sensitised immunoassay plates, optimised antisera developed for

TABLE IV

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 69/95: UNCOOKED BURGER

Laboratory	Soya protein *g/100 g*	
	Sample code number 69	Sample code number 95
1	4·82	4·27
2[e]	3·82	3·40
3[e]	3·38	3·36
4	4·01	4·44
5	4·06	4·36
6	3·82	4·03
7	3·37	4·15
8	4·19	4·92
9	2·47	2·43
10	4·40	4·83
11	4·88	4·92
12	4·80	4·35
13	5·86	4·69
14	3·00	3·21
Mean (x)	4·08	
Repeatability (r)	1·03	
Reproducibility (R)	2·28	
Actual total soya protein content (g/100 g)[f]	4·90	

For key, see Table XIII.

TABLE V

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 61/87: UNCOOKED BURGER

Laboratory	Soya protein *g/100 g*	
	Sample code number 61	Sample code number 87
1	1·55	1·56
2[e]	0·86	1·62
3[e]	0·66	1·21
4	1·85	1·40
5	1·51	1·64
6	1·89	1·62
7	1·18	1·44
8	3·47	4·89
9	2·68	1·26
10	2·38	2·47
11	1·81	1·86
12	2·35	1·57
13	1·74	1·94
14	1·65	2·22
Mean (x)	1·69	
Repeatability (r)	1·14	
Reproducibility (R)	1·35	
Actual total soya protein content (g/100 g)[f]	1·98	

For key, see Table XIII.

TABLE VI

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 27/60: UNCOOKED BURGER

	Soya protein *g/100 g*	
Laboratory	Sample code number 27	Sample code number 60
1	1·26	1·13
2[e]	1·24	0·87
3[e]	1·20	0·82
4	1·34	1·23
5	0·99	1·03
6	1·09	1·12
7	1·04	0·92
8	1·24	2·22
9	1·19	0·90
10	1·61	1·27
11	1·10	1·17
12	0·84	1·09
13	1·25	1·21
14	1·68	0·94
Mean (x)	1·18	
Repeatability (r)	0·78	
Reproducibility (R)	0·81	
Actual total soya protein content (g/100 g)[f]	1·30	

For key, see Table XIII.

TABLE VII

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 33/65: COOKED PÂTÉ

	Soya protein *g/100 g*	
Laboratory	Sample code number 33	Sample code number 65
1	2·57[b]	1·53[b]
2[e]	1·54	1·39
3[e]	1·38	1·01
4	1·72	1·41
5	1·25	1·33
6	1·38	1·52
7	1·43	1·46
8	0·61[b]	3·69[b]
9	1·48	1·08
10	1·94	2·03
11	1·98	1·87
12	1·48	1·34
13	1·65	2·04
14	1·59	1·28
Mean (x)	1·52	
Repeatability (r)	0·49	
Reproducibility (R)	0·81	
Actual total soya protein content (g/100 g)[f]	1·74	

For key, see Table XIII.

TABLE VIII

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 28/72: COOKED PÂTÉ

Laboratory	Soya protein *g/100 g*	
	Sample code number 28	Sample Code Number 72
1	2·70	2·67
2[e]	2·56	2·30
3[e]	2·05	2·13
4	2·74	2·43
5	2·24	2·41
6	2·37	1·97
7	2·24	2·44
8	2·86[b]	5·04[b]
9	2·39	2·33
10	2·74	3·17
11	3·01	3·77
12	1·70	2·94
13	2·55	2·99
14	1·84	2·27
Mean (x)	2·50	
Repeatability (r)	0·97	
Reproducibility (R)	1·27	
Actual total soya protein content (g/100 g)[f]	2·92	

For key, see Table XIII.

TABLE IX

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 15/71: UNCOOKED PÂTÉ

Laboratory	Soya protein *g/100 g*	
	Sample code number 15	Sample code number 71
1	1·98	1·50
2[e]	1·59	1·42
3[e]	1·58	1·20
4	1·71	1·36
5	1·20	1·07
6	1·08	1·10
7	1·10	1·45
8	0·41[c]	0·24[c]
9	1·45	1·18
10	1·94	2·03
11	1·76	1·47
12	0·91	1·41
13	1·78	1·77
14	1·25	1·32
Mean (x)	1·45	
Repeatability (r)	0·58	
Reproducibility (R)	0·88	
Actual total soya protein content (g/100 g)[f]	1·74	

For key, see Table XIII.

TABLE X

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 30/52: UNCOOKED SAUSAGE

	Soya protein *g/100 g*	
Laboratory	Sample code number 30	Sample code number 52
1	1·10	1·41
2[e]	1·41	1·08
3[e]	1·17	0·80
4	1·42	0·94
5	1·23	1·14
6	1·05	1·15
7	1·10	0·88
8	2·93[b]	0·64[b]
9	1·06	0·96
10	1·57	1·37
11	1·19	1·15
12	1·11	1·21
13	1·44	1·41
14	1·06	1·67
Mean (x)	1·20	
Repeatability (r)	0·57	
Reproducibility (R)	0·60	
Actual total soya protein content (g/100 g)[f]	1·18	

For key, see Table XIII.

TABLE XI

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SAMPLE 12/51: UNCOOKED SAUSAGE

	Soya protein *g/100 g*	
Laboratory	Sample code number 12	Sample code number 51
1	1·48	1·51
2[e]	1·44	1·40
3[e]	1·36	1·06
4	1·98	1·53
5	1·43	1·51
6	1·15	1·50
7	1·48	1·60
8	1·53	1·93
9	1·95	1·34
10	2·06	1·88
11	1·38	1·85
12	1·79	1·65
13	1·69	2·10
14	1·56	1·98
Mean (x)	1·61	
Repeatability (r)	0·67	
Reproducibility (R)	0·77	
Actual total soya protein content (g/100 g)[f]	1·74	

For key, see Table XIII.

TABLE XII

COLLABORATIVE TRIAL: DETERMINATION OF SOYA PROTEIN IN MEAT PRODUCTS USING "BIOKITS" ENZYME IMMUNOASSAY PROCEDURE

SUMMARY OF RESULTS

Sample code	Sample type	Soya product(s) added[f]	Amount of soya product added *g/100 g*	Protein content of soya product *g/100 g*	Soya protein content in sample *g/100 g*	Total soya protein content in sample *g/100 g*	Observed soya protein content *g/100 g*	Observed repeat-ability r	Observed repro-ducibility R
12/51	Uncooked sausage	Soya isolate direct (PP500E)	2	87	1·74	1·74	1·61	0·67	0·77
15/71	Uncooked pâté + caseinate	Soya isolate direct (PP500E)	2	87	1·74	1·74	1·45	0·58	0·88
20	Cooked pâté	None (control)	—	—	—	—	—	—	—
27/60	Uncooked burger	Soya concentrate direct (Solve 70)	2	65	1·30	1·30	1·18	0·78	0·81
28/72	Cooked pâté	Isolate (PP500E)	2	87	1·74	2·92	2·50	0·97	1·27
		Concentrate (Arcon A)	2	59	1·18				
30/52	Uncooked sausage	Soya concentrate direct (Arcon A)	2	59	1·18	1·18	1·20	0·57	0·60
32	Uncooked sausage	None (Control)	—	—	—	—	—	—	—
33/65	Cooked pâté + caseinate	Soya isolate direct (PP500E)	2	87	1·74	1·74	1·52	0·49	0·81
61/87	Uncooked burger	Textured soya (Danprotex-B)	3	66	1·98	1·98	1·69	1·14	1·35
69/95	Uncooked burger	Soya grits (Lucas Defatted No. 20)	10	49	4·90	4·90	4·08	1·03	2·28
Pre-trial sample	Uncooked sausage	As 12/51 above	2	87	1·74	1·74	1·61	—	—

For Key, see Table XIII.

TABLE XIII

KEY TO TABLES II–XII

The reported results were examined for evidence of individual systematic error ($P \leq 0{\cdot}05$) using Cochran's and Dixon's tests progressively, by procedures described in BSI "Precision of Test Methods", BS 5497; Part 1; London, 1979, as used for the subsequent calculations of mean (x), repeatability (r), and reproducibility (R).

(a) No statisticaly analysis performed on these results.
(b) Results rejected by Cochran's Test, $P \leq 0{\cdot}05$. Values not used in calculation of x, r and R.
(c) Results rejected by Dixon's Test, $P \leq 0{\cdot}05$. Values not used in calculation of x, r and R.
(d) Standard deviation calculated from all individual results but not including those rejected by Cochran's or Dixon's tests.
(e) Laboratory 2 reported two independent series of results for second phase of trial—coded as Laboratory 2 and 3.
(f) For sample composition, see Tables I and XII.

the purpose, liquid ready-to-use standards) as well as the modified and simplified protocol were used. These changes have resulted in an overall improvement in accuracy and precision; this improvement is illustrated in Table XIV, which compares the results given in Tables II to XII and those from the previous collaborative trials. In order to facilitate comparison of different samples, the observed mean level and the repeatability/reproducibility are expressed as percentages of their associated means. These results are consistent with the report of a more limited evaluation of the same protocol carried out at a single laboratory[5].

The pre-trial sample was analysed eight times at all thirteen laboratories to which it was sent (Table II). The mean observed levels were between 1·36 and 1·90 g/100 g, while the coefficients of variation were between 5·0 and 16·4 per cent. Because the authors considered these ranges to be satisfactory, all the laboratories were permitted to continue with the trial samples proper. The overall coefficient (10·8 per cent.) is acceptable when compared with suggested interlaboratory norms[7] (5–10 per cent. within assays; 7–15 per cent. between assays) for ELISA.

ACCURACY

The mean observed level of soya protein after 104 separate determinations of the pre-trial sample was 1·61 g/100 g, somewhat lower than that calculated from the recipe (corresponding to 92·5 per cent. recovery), and this underestimation was fairly consistently repeated when the trial samples proper were analysed (Table XIV). However, this observed level depends on the response of the standard soya protein relative to the soya protein actually present in the sample; since the recoveries varied from 101·7 to 83·3 per cent. over a range of soya ingredients and sample types, the standard chosen minimised the chance of overestimation rather than maximised accuracy. For instance, the use of another standard (or of a constant correction factor) could have produced apparent recoveries centred on 100 per cent. (e.g. 90–110 per cent.).

This trial and the two previous collaborative trials[2,3] the results of which are summarised in Table XIV are all based on an ELISA procedure published in 1981[1], but certain differences must be emphasised before meaningful comparisons between them can be made. The MAFF protocol[2] stipulated an

TABLE XIV

COMPARISON OF RESULTS OF PRESENT COLLABORATIVE TRIAL WITH THOSE OBTAINED FROM PREVIOUS TRIALS

Sample type	Accuracy parameters			Precision parameters			
	Soya protein content						
	Actual *g/100 g*	Observed *g/100 g*	Recovery % *100x*			*100r*	*100R*
	c	x	c	r	R	x	x
BIOKITS[a]							
Sausage with isolate	1·74	1·61	92·5	0·67	0·77	41·6	47·8
Uncooked pâté with isolate	1·74	1·45	83·3	0·58	0·88	40·0	60·7
Burger with concentrate	1·30	1·18	90·8	0·78	0·81	66·1	68·6
Cooked pâté with isolate + concentrate	2·92	2·50	85·6	0·97	1·27	38·8	50·8
Sausage with concentrate	1·18	1·20	101·7	0·57	0·60	47·5	50·0
Cooked pâté with isolate	1·74	1·52	87·4	0·49	0·81	28·2	53·3
Burger with texturate	1·98	1·69	85·4	1·14	1·35	67·5	79·9
Burger with grits	4·90	4·08	83·3	1·03	2·28	25·2	55·9
EUVEPRO[b]							
Sausage meat product (cooked at 80°) with:							
Isolate	1·50	1·85	123·3	1·10	2·01	59·5	108·6
Isolate	2·35	2·64	112·3	0·92	1·20	34·8	45·5
Flour	1·61	1·71	106·2	1·03	1·53	60·2	89·5
Concentrate	1·96	2·17	110·7	0·88	1·90	40·6	87·6
Texturate	2·81	3·96	140·9	1·41	2·39	35·6	60·4
Texturate	2·81	4·30	153·0	1·18	3·72	27·4	86·6
MAFF[c]							
Sausage with flour	1·16	0·96	82·8	0·86	0·93	89·6	96·9
Sausage with flour	2·26	1 93	85·4	0·49	1·26	25·4	65·3
Sausage with flour	4·32	3·88	89·8	2·19	2·62	56·4	67·5
Sausage with texturate	1·87	2·06	110·2	1·10	1·73	53·4	87·0
Sausage with texturate	3·64	4·43	121·7	2·63	3·51	59·4	79·2
Sausage with texturate	6·95	7·19	103·5	4·52	5·15	62·9	71·6

[a] This trial.
[b] Olsman, W. J., Dobbelaere, S., and Hitchcock, C. H. S., *J. Sci. Fd. Agric.*, 1985, **36**, 499–507.
[c] Crimes, A. A., Hitchcock, C. H. S., and Ward, R., *J. Assoc. Publ. Analysts*, 1984, **22**, 59–78.

unoptimised antiserum which was commercially available; it involved sensitisation of ELISA plates with a standard soya protein concentrate (Unico-75). Since the soya flour and texturate ingredients of the MAFF samples responded to the ELISA at 118 and 120 per cent. respectively, the MAFF results were corrected. The dependence of the observed response on the soya type was identified as one of the inherent weaknesses of the method at the time. The EUVEPRO protocol[3] was identical to the MAFF protocol, but a wider range of soya ingredient types was analysed, and no corrections were made. In both these trials, recoveries of non-textured soya protein from various meat products (processed at less than 80 °C) ranged from 82·8 to 123·3 per cent. (Table XIV). Although this range was considered satisfactory, it did not include samples containing textured soya protein (the manufacture of which involves extrusion

at temperatures greater than 100 °C). These samples required the application of an empirical correction before a satisfactory recovery is obtained. The accuracy of the method for the determination of textured soya protein when incorporated as an unknown ingredient in meat products (and in samples after sterilisation at 121 °C) was therefore regarded with concern. In the current study, the commercial kit supplied by Biokits was tested. The protocol had been significantly modified and simplified; in particular, the antiserum provided had been specially developed by Biokits for the purpose; the preparation of an acetone powder and of sensitised plates was unnecessary; the results were obtained using the standard soya provided and calculated without correction for response factor differences. The data reported here demonstrate that the recoveries were significantly better than those obtained in previous trials[2,3], indicating that better accuracy has been achieved over the wide range of samples analysed, including the one containing the texturate.

PRECISION

The precision of the method should not depend on the sample matrix or the soya ingredient in it; examination of Table XIV shows little correlation between the precision parameters and the sample type. The percentage coefficients of reproducibility (measuring precision between laboratories) varied from 47 to 80 per cent. Corresponding ranges obtained in previous trials were 45–109 per cent. and 65–97 per cent. The ranges for repeatability in this trial are 25–68 per cent. whereas 28–61 per cent. and 26–90 per cent. were obtained in the previous trials. There is a slight indication that the presence of texturate adversely affects precision; this may be explained by the difficulty with which strongly heated soya protein dissolves even in denaturing solutions. The precision of the current test is better than in previous trials, since although repeatability does not seem to have changed, the reproducibility coefficient has generally decreased. This is probably due to the provision of standardised sensitised plates subject to factory quality control as well as to the simplified procedure which would be expected to have a greater impact on the "between" rather than the "within" laboratory variances.

SENSITIVITY

The lowest detectable level of soya protein is about 0·7 g of soya protein per 100 g sample; by doubling the meat sample weight to 24 g the kit would enable detection down to 0·35 g/100 g, but this protocol was not used in the collaborative trial. Samples containing no soya protein always gave a blank reading which in this assay is at or around the absorbance value of the maximum binding microwell. Participants reported the control (blank) samples in a variety of ways which ranged from "nil" to "less than 1·00 g/100 g" (Table III).

Conclusion

Within the limitations of this trial, it may be concluded that, using a single arbitrary soya standard as a reference, the method tested permits the estimation of the levels of soya protein in a raw or pasteurised meat product of entirely

unknown composition containing an unknown soya ingredient (whether grits, flour, isolate, concentrate or texturate) with a recovery of 80–100 per cent.; the method has repeatability and reproducibility values, when expressed as percentage of the mean, in the order of 45 and 60 per cent. respectively. This accuracy and precision implies that, at a typical laboratory, a single observation at a level of 2·0 g of soya protein per 100 g sample is subject to a standard deviation of 0·22 g/100 g (Table II); the true figure therefore probably lies between 1·5 and 2·5 g/100 g (95 per cent. confidence interval). Because of the uncertain recovery, one such observation therefore should be interpreted as 1·5–3·1 g/100 g. For four observations about a mean of 2·0 g/100 g, the corresponding range would be 1·7–2·8 g/100 g. Nevertheless it is recommended that each laboratory check its own data using standard samples in order to estimate its own confidence limits.

None of the participants had any significant problems in performing the analyses. The test of this first enzyme immunoassay kit for the quantitative determination of a food ingredient has defined useful analytical parameters which encourage the belief that immunoassays will become increasingly useful to food analysts in the future. Further testing of this kit with a wider variety of commercial soya ingredients in different meat products (including those canned at 121 °C) is ideally required; nevertheless the way lies open for the application of ELISA to other food analytes[8] including proteins (meat, milk, egg, wheat, etc.) and trace components (mycotoxins, enterotoxins, anabolic agents, antibiotics, antinutrients, vitamins, pesticides, etc.).

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Appendix I: Analysis Protocol

1. Samples for analysis

Nineteen 100 g vacuum packed frozen coded samples were supplied to each participant and stored at −18 °C until use. The samples were analysed in accordance with the "Biokits" enzyme immunoassay procedure as follows:

The pre-sample was sampled twice (i.e. 2 × 12 g independently homogenised) and duplicate extractions of each analysed as replicates in one two-strip assay such that eight data points were achieved.

The remaining eighteen formal evaluation samples were randomly divided by each participant into two groups of nine samples, each group being analysed in a two-strip assay on a separate date. Each coded sample was homogenised and extracted once only providing one data point per sample.

2. Biokits enzyme immunoassay procedure

PRINCIPLE OF THE ASSAY

The BIOKITS SOYA PROTEIN ASSAY is an indirect competitive enzyme immunoassay. The meat samples are homogenised and then extracted (solubilised) in a urea-dithiothreitol buffer at 100 °C followed by rapid renaturation in a cystine-containing diluent. The assay is performed in plastic microwells which have been pre-coated with a purified preparation of soya protein. In the initial competititon reaction, a fixed amount of the diluted extract of the meat sample is added into the soya protein-coated microwell along with a fixed volume of specific rabbit anti-soya protein. With increased concentrations of soya protein in the diluted extract, the amount of rabbit anti-soya protein binding to the soya protein attached to the well, will *decrease*. After allowing this reaction to proceed, the unbound material is removed by aspiration and washing.

The amount of rabbit anti-soya protein remaining bound to the soya protein-coated well is determined by reacting a fixed amount of peroxidase-conjugated swine anti-rabbit globulin. After incubation, the excess conjugate is removed by aspiration and washing, and the bound peroxidase activity determined by adding a fixed amount of 2 : 2′-azino-di-(3-ethyl benzthiazoline sulphonic acid (ABTS)* substrate which develops a green colouration in the presence of peroxidase. The colour development is inversely proportional to the original soya protein concentration in the diluted extract. The concentration of soya protein in the meat can be determined by reading off a calibration curve derived from standards of known soya protein concentration.

* ABTS® is a registered trademark of Boehringer Mannheim GmbH.

KIT COMPONENTS

The following components are provided in each kit:

A. Five vials, each containing 3 ml of one of the following pre-diluted SOYA PROTEIN STANDARDS: 3·5; 7; 15; 35; 70 μg/ml soya protein, respectively in phosphate buffered saline of pH 7·2, with 0·1 per cent. Bovine Serum Albumin (BSA) and 0·01 per cent. thiomersal as a preservative.

B. One vial containing 0·5 g of SOYA PROTEIN CONTROL. This comprises a soya protein isolate powder with no other additives. The powder has been calibrated for soya protein content by Kjeldahl nitrogen analysis using 6·25 as the conversion factor. The soya protein value is stated on the vial label.

C. One vial containing 6 ml of pre-diluted RABBIT ANTI-SOYA PROTEIN in phosphate buffered saline of pH 7·2 with 0·1 per cent. of BSA and 0·01 per cent. of thiomersal as preservative.

D. One vial containing 11·5 ml PEROXIDASE CONJUGATE (peroxidase-conjugated swine anti-rabbit globulin) in phosphate buffered saline of pH 7·2 with 0·1 per cent. of BSA, 0·01 per cent. of thiomersal and stabiliser.

E. One vial containing 0·55 ml of ABTS CONCENTRATE (15 mg/ml of 2:2′-azino-di-(3-ethylbenzthiazoline sulphonic acid) in distilled water).

F. One vial of PEROXIDE-CITRATE BUFFER containing 12·0 ml of a solution of 2·3 per cent. w/v of citric acid monohydrate and 0·015 per cent. of hydrogen peroxide (pH 4·0).

G. One bottle of WASH SOLUTION CONCENTRATE containing 100 ml of a ten-fold concentrate of Tris buffered saline with 5 per cent. of Tween 80 and 0·01 per cent. of thiomersal as preservative. The pH of the concentrate is approximately 7·9.

H. One vial of DILUENT CONCENTRATE containing 20 ml of a five-fold concentrate of phosphate buffered saline with 0·5 per cent. of BSA and 0·05 per cent. of thiomersal as preservative. The pH of the concentrate is approximately 6·9.

I. One vial of STOP SOLUTION containing 6 ml of 1·5 per cent. w/v of sodium fluoride in distilled water.
CAUTION: sodium fluoride is toxic—avoid ingestion or contact with skin or eyes.

J. One SOYA PROTEIN SENSITISED MICROWELL MODULE comprising six double column strips of microwells (96 assay wells) held in a plastic frame and packed in a foil laminate pouch with desiccant bag; each strip has 2 columns of 8 microwells; the interior of each microwell has been coated with a pre-determined amount of soya protein and dried.

K. One package insert.

L. Three sheets of pre-labelled graph paper.

M. One example work sheet and assay layout guide.

MATERIALS REQUIRED BUT NOT PROVIDED IN KIT

For sample preparation

Reagents:

1. Urea ("Analar").
2. Dithiothreitol (DTT, Cleland's Reagent).
3. Tris(hydroxymethyl)methylamine ("Analar").
4. L-cystine.
5. Sodium chloride ("Analar").
6. Sodium hydroxide solution 1·0 M.
7. Hydrochloric acid 1·0 M.

Equipment:

1. Waring Blendor, with 37–110 ml capacity bowl (obtainable from Gallenkamp, Belton Road West, Loughborough, Leicestershire LE11 0TR).
2. Ultra Turrax Homogeniser, Model TP18/10, with 18N shaft, speed control and stand (obtainable from Scientific Supplies Co. Ltd, Scientific House, Vine Hill, London EC1 5EB).
3. Borosilicate conical flasks, 50 ml, with 19/26 neck size, and 19/26 stoppers.
4. Whatman filter papers, 18·5 cm. diameter, Grade No. 1.
5. Water or steam bath, 100 °C.
6. Water bath, 50 °C.

Miscellaneous glassware required includes measuring cylinders, volumetric flasks, and pipettes.

For enzyme immunoassay

1. Precision micropipette capable of delivering 50 µl (e.g. Gilson Pipetman P200, available from Anachem, Anachem House, 20 Charles Street, Luton, Bedfordshire LU2 0EB).

2. Precision repeating dispenser capable of delivering 50 and 100 µl (e.g. Labsystems Finnpipette Stepette 10–1000 µl, with 800 µl syringe: steptip 2, and 3200 µl syringe: steptip 3, available from Jencons (Scientific) Ltd, Cherrycourt Way Industrial Estate, Leighton Buzzard, Bedfordshire LU7 8UA).

3. Orbital plate shaker with mixing speed of 1200–1400 r.p.m. (e.g. Flow Titertek Plate Shaker, fixed speed, available from Flow Laboratories Limited, Woodcock Hill, Harefield Road, Rickmansworth, Herts WD3 1PQ).

4. Microwell washer, (e.g. NUNC Immuno Wash 8, available from Gibco Limited, P.O. Box 35, Washington Road, Abbotsinch Industrial Estate, Paisley PA3 4EP, Scotland).

5. Microwell plate reader, fitted with 414 nm interference filter. (e.g. Uniskan I or Uniskan II available from Labsystems (U.K.) Limited, 12 Redford Way, Uxbridge, Middlesex.)

BioKits — **Soya Protein Assay**

QUANTITATION OF SOYA PROTEIN IN MEAT PRODUCTS

Date: 11/7/85 Operator: CCH

	SOLUTION TESTED	Absorbance (414nm) 1	2	Mean	Soya Protein Concentration Read from Calibration Curve (μg/ml) (S.P.)	Weight of Meat Sample (grams) W1	Weight of .05M Tris-HCl Buffer (grams) W2	Weight of Homogenate Added to Extraction Flask (grams) W3	Weight of Soya Protein Control Added to Extraction Flask (mg) W4	Weight of Meat Extracted (mg) $\frac{W1}{W1+W2} \times W3 \times 1000$ W5	SAMPLE $\frac{(S.P.)}{W5} \times 100$ % SOYA PROTEIN IN MEAT SAMPLE	CONTROL $\frac{(S.P.)}{W4 \times \text{PROTEIN VALUE}} \times 100$ % RECOVERY OF SOYA PROTEIN CONTROL
	3.5μg/ml SOYA PROTEIN STANDARD	1·139	1·184	1·162								
	7.0μg/ml SOYA PROTEIN STANDARD	·864	·809	·837								
	15μg/ml SOYA PROTEIN STANDARD	·646	·580	·563								
	35μg/ml SOYA PROTEIN STANDARD	·335	·329	·332								
	70μg/ml SOYA PROTEIN STANDARD	·206	·192	·199								
C	SOYA PROTEIN CONTROL PROTEIN VALUE: ·83	·349	·368	·359	31·5				40·6			93·5
M	MAXIMUM BINDING	1·963										
T 1	ECONOMY SAUSAGE	·744	·694	·719	9·6	12·47	47·96	2·47		509.7	1·9	
2												
3												
4												
5												
6												
7												
8												
9												

Fig. 1. Example worksheet with data and results.

PREPARATION AND EXTRACTION OF TEST SAMPLES AND CONTROL

Preparation of extraction reagents (sufficient for 32 assay wells)

1. Stock 0.25 M Tris–HCl pH 8·6 (200 ml)
   1.1 Weigh out exactly 6·06 g of Tris (hydroxymethyl) methylamine "Tris" into a 250 ml beaker.
   1.2 Add approximately 150 ml of distilled water and mix until all the Tris is in solution.
   1.3 Adjust the pH to 8·6 by adding 1·0 M HCl (approximately 11–13 ml).
   1.4 Quantitatively transfer to 200 ml volumetric flask and make up to exactly 200 ml with distilled water.

2. 0·05 M Tris–HCl Buffer pH 8·6 (600 ml)
   2.1 Dilute stock 0·25 M Tris–HCl pH 8·6 1 : 4 by adding 120 ml of stock to 480 ml of distilled water.

3. Urea-DTT Extraction Buffer (100 ml)
   3.1 Weigh out 80·0 g of urea into a 250-ml conical flask.
   3.2 Add 20 ml of stock 0·25 M Tris–HCl solution and 20 ml of distilled water.
   3.3 Heat gently over a Bunsen flame with constant mixing until all the urea has dissolved.
   3.4 Add 0·29 g of DTT to the hot urea solution and mix by swirling until dissolved.
   3.5 Transfer the flask to a 100 °C water bath and maintain at this temperature.

4. Renaturation Solution (1000 ml)
   4.1 Weigh out 1·8 g of L-cystine in a 50-ml beaker. Add 20·0 ml of 1·0 M NaOH solution using a volumetric pipette. Allow the L-cystine to dissolve completely by gentle mixing.
   4.2 Weigh out 3·5 g of NaCl and dissolve in 900 ml of distilled water in a 1-litre beaker.
   4.3 With constant mixing, slowly add the 20 ml of L-cystine solution to the NaCl solution; complete the transfer by rinsing out the residual L-cystine solution with distilled water.
   4.4 With constant mixing slowly add 8 ml of 1·0 M HCl to the L-cystine–NaCl solution while monitoring the pH.
   4.5 Finally adjust to pH 9·0 by adding approximately a further 1·5–3 ml of 1·0 M HCl; make up the total volume to 1·0 litre with distilled water.

Preparation of meat slurry (for soya protein content of 1 to 10 per cent.)

1. Weigh accurately about 12·0 g of the meat sample into the blender container. Record the weight as W1 on the work sheet illustrated in Figure 1.

2. Weigh accurately about 48·0 g of 0·05 M Tris–HCl Buffer (pH 8·6) into the blender container. Record the weight as W2 on the work sheet.

3. Blend the meat sample until a fairly smooth homogeneous mixture is obtained.

4. Transfer as much as possible of the slurry to a 100 ml beaker taking care not to leave any significantly large pieces of tissue in the blender bowl.

5. Using the Ultra-Turrax tissue homogeniser, complete the sample homogenisation. The final mixture should be a smooth easily pipettable homogenate (using pipettes with 1-mm tip diameter).

6. Weigh accurately about 2·5 g of each meat homogenate into separate 50-ml stoppered conical flasks. (This may be done by placing the flask directly on the balance pan and transferring the homogenate using a pasteur pipette. Before pipetting, ensure that the homogenate is thoroughly mixed and has not been allowed to settle out.) Record the weight as W3 on the work sheet.

7. Place each flask in a 50 °C water bath; this pre-warms the flask and meat homogenate before the addition of the urea-DTT extraction buffer.

Preparation of soya protein control

1. Weigh accurately about 40 mg of the SOYA PROTEIN CONTROL into a 50-ml stoppered conical flask. Record the weight as W4 on the work sheet.

2. Add 2·5 ml of 0·05 M Tris–HCl buffer (pH 8·6) to the flask; gently mix the powder into suspension by swirling.

3. Place the flask in a 50 °C water bath to pre-warm as for meat samples.

Extraction of meat slurry and soya protein control

1. Using a graduated pipette, add 7·5 ml of the urea-DTT extraction buffer at 100 °C to each of the meat samples flasks and to the soya protein control flask in the 50 °C water bath.

Note: Leave the pipette standing in the flask of urea-DTT extraction buffer to prevent cooling and crystallisation of urea in the pipette tip.

2. Stopper each flask and mix thoroughly by gentle swirling to achieve a uniform suspension. Immediately transfer all the flasks to a 100 °C water bath.

3. Incubate the flasks at 100 °C for 60 minutes with occasional mixing.

4. Remove all the flasks from the 100 °C water bath and place them in the 50 °C water bath to prevent the urea-DTT extraction buffer from crystallising out.

RENATURATION OF EXTRACTED SAMPLES AND CONTROL

1. With constant mixing slowly add 20 ml of Renaturation Solution at 50 °C to each flask in the 50 °C water bath. Mix the contents of each flask thoroughly by swirling and replace the flasks in the 50 °C water bath.

2. Remove each flask in turn from the 50 °C water bath and quantitatively transfer its contents to a 100 ml volumetric flask using three 10 ml volumes of Renaturation Solution at 50 °C. Mix thoroughly by swirling and allow to cool to room temperature. Make up the volume to exactly 100·0 ml with Renaturation Solution at room temperature.

3. Mix thoroughly by repeated inversion and immediately filter the contents of each flask through a pleated or folded 18·5-cm diameter Whatman No. 1 filter paper.
Collect only the first 10 ml of filtrate.

SHELF LIFE

Extraction reagents and extracted samples

The Extraction Reagents should be prepared on the day of use and not stored for longer than the working day. The extracted samples (filtrates) may be stored for up to 48 h at 2 to 6 °C prior to assay.

Kit reagents

The reagents and the SOYA PROTEIN SENSITISED MICROWELL MODULE of the BIOKITS SOYA PROTEIN ASSAY KIT should be stored at 2 to 6 °C. The shelf life of the kit and components is indicated by the expiry data on the respective labels. Once the kit reagents have been opened they should be used within two weeks.

PREPARATION OF KIT MATERIALS

A. *Soya protein standards*

SOYA PROTEIN STANDARDS are supplied pre-diluted in buffer. No preparation is necessary other than mixing contents thoroughly by repeated inversion. (Do not shake)

B. *Soya protein control*

The SOYA PROTEIN CONTROL is supplied as a powder which requires to be extracted in a similar fashion to the meat samples (see page 23).

C. *Rabbit anti-soya protein*

RABBIT ANTI-SOYA PROTEIN is supplied pre-diluted in buffer. No preparation is necessary other than mixing contents thoroughly by repeated inversion. (Do not shake)

D. *Peroxidase conjugate*

PEROXIDASE CONJUGATE is supplied pre-diluted in buffer. No preparation is necessary other than mixing contents thoroughly by repeated inversion. (Do not shake)

E/F. *ABTS concentrate and peroxide-citrate buffer*

ABTS CONCENTRATE is supplied as a 25-fold concentrate and requires to be diluted 1:24 in PEROXIDE-CITRATE BUFFER to prepare the Working ABTS Solution.

N.B. Dilutions of ABTS CONCENTRATE should be freshly prepared and used within 4 h of preparation.

Mix contents of each separate vial by inversion; dilute the ABTS CONCENTRATE in the PEROXIDE-CITRATE BUFFER according to either of the following protocols:

e.g. for 96 assay wells: add 0·5 ml ABTS CONCENTRATE to the 12·0 ml of buffer contained in the PEROXIDE-CITRATE BUFFER vial, stopper the vial and mix well by repeated inversion.

—for any other (smaller) number of assay wells, dilute ABTS CONCENTRATE in the ratio 1 : 24 with PEROXIDE-CITRATE BUFFER, e.g. for groups of 32 assay wells pipette 3·6 ml of PEROXIDE-CITRATE BUFFER into a clean container and add to this 0·15 ml of ABTS CONCENTRATE, mix well.

N.B. Take care not to cross-contaminate the ABTS CONCENTRATE and PEROXIDE-CITRATE BUFFER in their respective vials.

G. *Wash solution concentrate*

WASH SOLUTION CONCENTRATE is supplied as a 10-fold concentrate and requires dilution 1 : 9 in distilled or deionised water to prepare the Working Wash Solution.

e.g. for 96 assay wells; use the total contents of the WASH SOLUTION CONCENTRATE vial (100 ml) by rinsing out and making up to 1·0 litre in a volumetric flask with distilled or deionised water.

—for any other (smaller) number of assay wells, dilute the WASH SOLUTION CONCENTRATE 1 : 9 with distilled or deionised water, e.g. for groups of 32 assay wells add 30 ml of WASH SOLUTION CONCENTRATE to 270 ml of distilled or deionised water. The pH of the Working Wash Solution should be in the range 7·7–7·9.

H. *Diluent concentrate*

DILUENT CONCENTRATE is supplied as a 5-fold concentrate and requires to be diluted 1 : 4 in distilled or deionised water to prepare the Working Assay Diluent Solution.

N.B. This *diluted* reagent is used for the final 1 : 9 dilution of the sample extract and the SOYA PROTEIN CONTROL extract.

e.g. for 96 assay wells; use the total contents of the DILUENT CONCENTRATE vial (20·0 ml) by rinsing out and making up to 100 ml in a volumetric flask with distilled or deionised water.

—for any other (smaller) number of assay wells dilute the DILUENT CONCENTRATE 1 : 4 with distilled or deionised water, e.g. for groups of 32 assay wells add 6·5 ml to 26 ml of distilled or deionised water. The pH of the Working Assay Diluent Solution should be in the range 7·1–7·3.

I. *Stop solution*

No preparation is necessary other than mixing contents thoroughly by repeated inversion.

CAUTION: STOP SOLUTION contains 1·5 per cent. of sodium fluoride, which is toxic. If contact with skin is made, wash affected area immediately with copious amounts of water.

J. *Soya protein sensitised microwell module*

Open the foil laminate pouch with the label side uppermost by cutting along the inside margin of the crimp seal with a sharp pair of scissors. Remove the module keeping the wells open side uppermost; if only a small number of assays are to be run (e.g. 32 assay wells) then remove two strips of wells and fit into a spare frame; replace the remaining frame and strips in the foil laminate pouch taking care that the desiccant bag lies underneath the module; reseal the pouch with adhesive tape.

DETAILED ENZYME IMMUNOASSAY PROCEDURE

The BIOKITS SOYA PROTEIN ASSAY KIT can be divided into three 32 assay well groups (2 strip assay); two 48 assay well groups (3 strip assay) or the entire plate of 96 assay wells (6 strip assay) may be used. Example assay layouts are shown in Figure 2.

It is recommended when first familiarising oneself with the kit that 32 assay well groups are selected. All reaction wells, except the Blank and Maximum Binding wells, should be run in duplicate and the mean absorbance value of each pair of wells calculated. The results may be recorded on the 32 assay well work sheet provided. Of the 32 assay wells, 10 are used for the standards (five duplicates) two are used for the control (one duplicate) and 1 well each for Substrate Blank and Maximum Binding respectively. This leaves 18 wells for test samples (nine duplicates).

Note: With a pencil, number the columns 1–4 on the lower frosted edge of the strip; this preserves the identity of the strips should they become detached from the frame.

Note: When an assay has been started, all steps should be completed without interruption.

1. Allow all reagents and the SOYA PROTEIN SENSITISED MICROWELL MODULE to reach room temperature before starting the assay.

2. Prepare the meat samples, the SOYA PROTEIN CONTROL and the necessary kit materials.

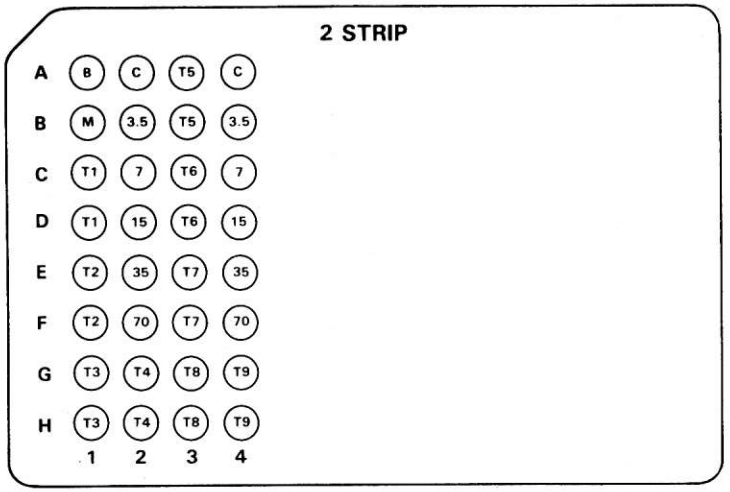
Immediately prior to assay dilute the sample and control filtrates 1 : 9 by adding 0·90 ml of Working Assay Diluent Solution to 0·10 ml of filtrate.

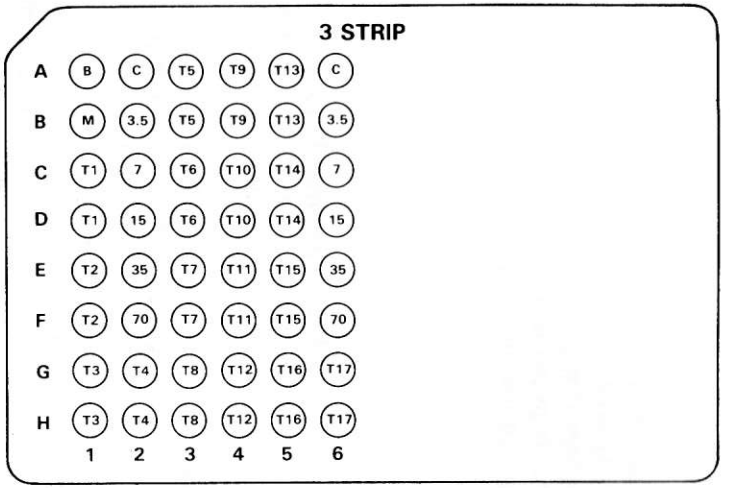
3. Using the precision micropipette, place 50 µl of each SOYA PROTEIN STANDARD, the prepared SOYA PROTEIN CONTROL and each of the prepared samples, into duplicate microwells.

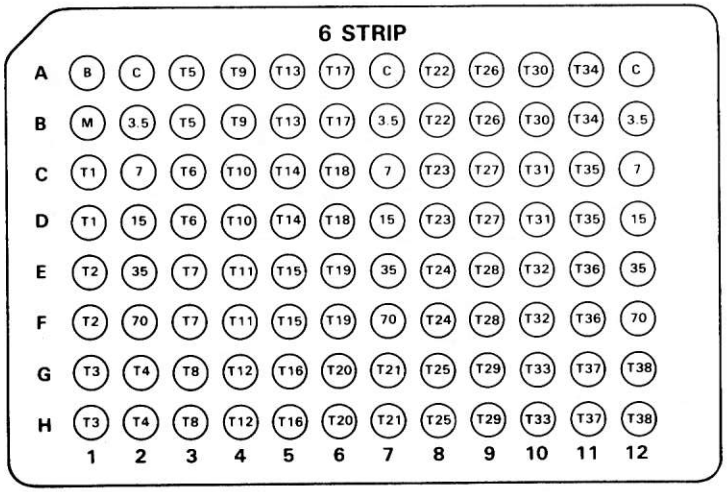
Note: Use a separate disposable tip for each pipetting step to avoid cross-contamination.

N.B.: Ensure that the addition of standards, control and samples follows the layout on the template provided.

4. Using the precision micropipette, place 50 µl of Working Assay Diluent Solution into the Maximum Binding well. The Substrate Blank well should be left empty.







EXAMPLE ASSAY LAYOUTS

B	Substrate Blank
M	Maximum Binding
3.5-70	Soya Protein Standards
C	Soya Protein Control
T1-T38	Test Sample

Fig. 2. Example assay layouts (template).

5. Using the repeating dispenser, with the steptip 2 syringe, add 50 µl of the RABBIT ANTI-SOYA PROTEIN to each well, except the Substrate Blank well. The repeating dispenser unit with steptip 2 syringe in place should be used to dispense a column of eight wells before refilling the syringe.

Caution: Do not allow tip of pipette to contact the material already in the microwells. Work in an orderly sequence starting at well "M" in column 1 and completing the addition to column 1 before refilling the dispenser and filling out column 2 starting at position A2. Repeat this procedure with the other columns.

N.B. Once this step has been started it must be completed without interruption.

6. Place the MICROWELL MODULE on the orbital plate shaker and mix for 10 minutes.

7. At the end of the incubation period, aspirate the material from all the wells of column 1 using the Microwell washer. Then fill all the wells of column 1 with Working Wash Solution. Complete this sequence for each successive column of wells (all the column wells are now filled with wash solution). Return the Microwell washer to column 1 and repeat the whole aspiration/fill sequence a further four times across all the columns (each column will have received a total of five aspirations and five fills). Finally, use the Microwell washer to aspirate each column of wells and then tap the plate upside down on several layers of absorbent tissue to remove residual droplets of wash solution.

Note: When inverting the plate be sure to squeeze the plastic frame at the centre of the long edges to prevent the strips from falling out of the frame.

8. Using the repeating dispenser, with the steptip 3 syringe, add 100 µl PEROXIDASE CONJUGATE to each microwell, except the Substrate Blank. The repeating dispenser unit with steptip 3 syringe in place should be used to dispense two columns of eight wells before refilling the syringe. Use the same orderly sequence described in section 5 above.

N.B. Once this step has been started, it should be completed without interruption.

9. Place the MICROWELL MODULE on the orbital plate shaker and mix for 10 minutes.

10. At the end of the incubation period, repeat the washing sequence described in Step 7.

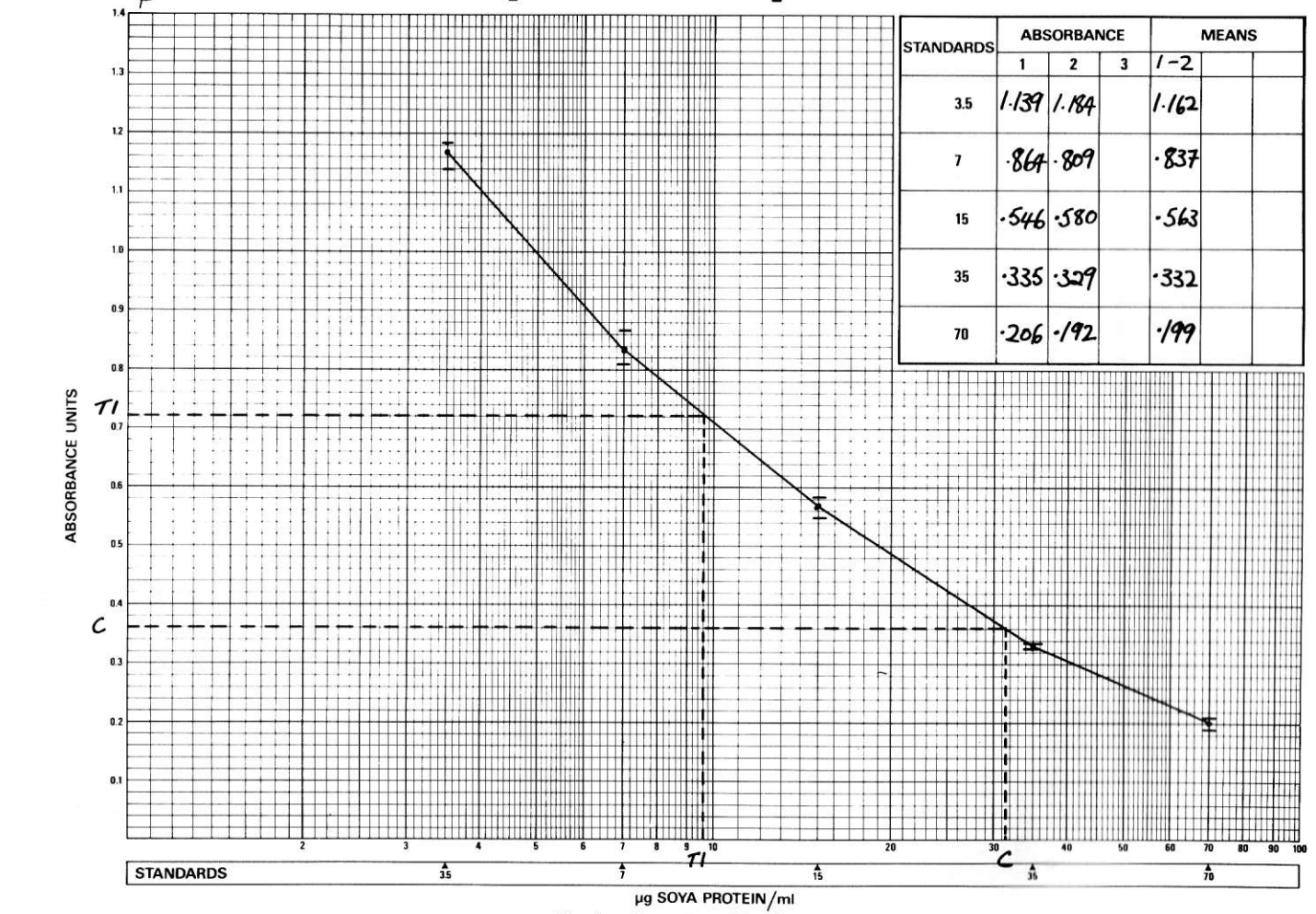
11. Using the repeating dispenser with a clean steptip 3 syringe, add 100 µl of the Working ABTS Solution to each well.

Refill the syringe after dispensing two columns of eight wells. Use the same orderly sequence described in section 5 above.

N.B. Once started, this step should be completed without interruption.

12. Place the MICROWELL MODULE on the orbital plate shaker and mix for 10 minutes.

13. At the end of the incubation period, add 50 µl of STOP SOLUTION to each well, using the repeating dispenser with a clean steptip 2 syringe.



STANDARDS	ABSORBANCE 1	2	3	MEANS 1-2		
3.5	1.139	1.184		1.162		
7	.864	.809		.837		
15	.546	.580		.563		
35	.335	.329		.332		
70	.206	.192		.199		

Fig. 3. Example calibration curve.

N.B. Add the STOP SOLUTION to each column of eight wells in the same orderly sequence as that used to add the Working ABTS Solution.

14. Mix for 10 seconds on the orbital plate shaker to distribute the STOP SOLUTION uniformly.

15. Using a microplate reader fitted with a 414 nm filter, set the reader to zero on the Substrate Blank well (co-ordinates A1). Then measure the absorbance of each of the assay wells starting at well "M" in column 1; complete the reading of column 1 before moving to the top of column 2. Repeat this process until all wells have been measured. All readings should be completed within 90 minutes of adding the STOP SOLUTION. Record the results on the 32 assay well worksheet provided and calculate the mean absorbance values.

Results: Calculation of the value of the unknown

The unknown values for soya protein concentration in meat samples are determined from a calibration curve. To construct the calibration curve, use the pre-labelled graph paper supplied. Plot the mean absorbance value for each of the five SOYA PROTEIN STANDARDS and draw straight lines to join each pair of neighbouring points. To determine the soya protein levels of the meat samples and the SOYA PROTEIN CONTROL, take the mean absorbance value from each duplicate and interpolate the corresponding soya protein concentration from the calibration curve.

Figure 1 illustrates the data and results of a typical BIOKITS SOYA PROTEIN ASSAY. Figure 3 shows the calibration curve and the interpolation of soya protein concentrations from the mean absorbance values, for the SOYA PROTEIN CONTROL and for a meat sample containing soya protein.

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The Reduction of Nitrate in a Swimming Pool Water

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The production of ammonia in a swimming pool water, with consequent high values for combined residual chlorine was found to be caused by the growth of nitrate-reducing bacteria within the filter media.

Two years ago, a somewhat unusual problem was encountered in a newly commissioned swimming pool. Shortly before the really serious trouble developed, a number of teething troubles were experienced, firstly difficulty in controlling the automatic sodium hypochlorite dosing equipment, and secondly, scale formation on parts of the pool.

The first problem was found to be due to an unusually high aluminium content in the water that interfered with the electrode of the automatic chlorine dosing apparatus. Concentrations of aluminium of the order of 0·3 mg per litre were found instead of the normal figure of less than 0·01 mg per litre. This was due to the use of an excessive amount of alum and to the high pH of the water. The Langelier index of the water was found to be +0·9, and the pH was sometimes above 8·0. When the pH was lowered, the scaling problem disappeared.

The main problem was that the ammoniacal content of the pool water increased, and, more importantly, the combined residual chlorine also increased, sometimes to very high levels. One of the specifications for this pool was that the combined residual chlorine should be less than 1·0 mg per litre. However, levels as high as 6·0 mg per litre were recorded, and the high level fluctuated although the pool had not then been opened to the public.

Some of the first analyses of the pool water showed levels of nitrate nitrogen below that of the mains water, but it was thought that this might be due to variations in the composition of the supply. This proved to be wrong. When the high levels of combined residual chlorine were found, the nitrate nitrogen of the mains water was 8·23 mg per litre, but this had dropped to 6·30 mg per litre in the pool.

Since the pool had been little used by the public, the high combined residual chlorine could not be due to urine residues, and must, therefore, have been dichloramine. The absence of any significant amount of urine was confirmed by the low potassium levels. All the evidence pointed to the production of ammonia within the system, and it was concluded that this was occurring within the filters. Accordingly, it was decided that samples of the sands used to charge the three filters should be examined.

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The fine grade of sand used was found to contain a substantial proportion of silt; the free ammonia present, however, amounted to only 30 mg per kilogram, which would not account for the continuous production of ammonia in the pool water. Other supplies of this type of filter sand, moreover, contained only approximately one twentieth of this amount of free ammonia.

A portion of the fine sand was incubated with mains water for 3 days at 30 °C and at the end of this time, the nitrate nitrogen content of the water had dropped from 8·2 mg per litre to 4·5 mg per litre, a corresponding quantity of ammonia having been produced. In the second test, the fine sand was washed with sterile water and then gently ignited. When this treated sand was incubated with mains water for 3 days at 30 °C, no reduction of the nitrate nitrogen occurred, ruling out the possibility of the sand reducing nitrate by a catalytic type of action.

In the course of a full bacteriological examination of the pool water, the presence of Pseudomonas organisms was established, and it was realised that these were among the soil organisms capable of reducing nitrates. Accordingly, the colonies from the culture plate were dispersed in sterile Ringer's solution, and this suspension used to inoculate 200 ml of mains water. The inoculated water was incubated for 2 days at 30 °C at the end of which time it was found that almost the whole of the nitrate nitrogen had been reduced to ammonia.

Subsequently, bacteriological examination of samples of water leaving the swimming pool filters showed all to contain Pseudomonas organisms, and when one of the filters was opened, a strong smell of ammonia was detected. These tests had established that the nitrate content of the pool water was being partially reduced to ammonia in the filters, and it was apparent that the silt present in the fine sand had provided a source of carbon and had acted as a nucleus for bacterial growth. The complete identity of the organisms was not established, but clearly they were heterotrophic denitrifying bacteria.

The filter problem was finally cleared up after the Thames Water Authority substantially increased the air scour operation prior to back washing. The air scour stage, designed to expand the sand bed and to separate and clean sand grains by abrasion, played a vital part in breaking up the clumps of bacterial slime within the sand. With the more vigorous treatment of the filters, the reduction of nitrate to ammonia ceased.