A comparison of the dye-binding and fluorodinitrobenzene methods for determining reactive lysine in leaf-protein concentrates

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1. Twenty-eight leaf-protein concentrate (LPC) samples, subjected to different thermal treatments, were produced from five curd batches. For these samples, the fluorodinitrobenzene (FDNB)-reactive lysine values gave closer agreement with dye-binding lysine (DBL) than with the dye-binding capacity (DBC).

2. No relationship was established between the dye-bound-after-propionylation (DBAP) and the histidine+arginine value.

3. Comparison of dye-bound-protein values with those for tungstic-acid-precipitated nitrogen × 6·25 for the LPC samples showed the heat-damaged samples to lie below the regression line for the other samples.

4. Reactive-lysine values by dye-binding and by FDNB methods correlated well with total lysine, but the slopes of the regression line indicated closer agreement for values for samples not damaged by heat.

5. The correlation coefficients between DBC and total basic amino acids, DBC and histidine+arginine+DBL, and DBC and histidine+arginine+FDNB-reactive lysine were similar.

6. There was no correlation between the lightness of colour of the LPC samples and the availability of lysine.

In this study, samples of leaf-protein concentrate (LPC) were prepared using different curd-drying techniques and other heat treatments. These samples were used to investigate the effect of thermal treatment on the availability of lysine by two methods. LPC undergoes several processes in production (Pirie, 1971), but Byers (1971) found that damage to lysine occurred only during the heat-coagulation stage. It was shown previously (Walker, 1979) that even careful preparation of LPC results in samples in which some lysine ceases to be available (reactive by the dye-binding lysine (DBL) method). Using the protein efficiency ratio assay with rats (gain in body-weight per g protein), Shurpalekar *et al.* (1966) and Bickoff *et al.* (1975) found considerable losses of nutritional value on thermal drying of LPC curd as compared with freeze-drying. Henry (1964), who determined the biological value and true digestibility of LPC samples, found that hot-air drying in particular reduced the true digestibility of LPC, and Allison *et al.* (1973) showed, using a deamination method for available lysine, that these values correlated, significantly, with in vivo digestibility values for LPC samples.

The DBL method, described previously (Walker, 1979), is here compared with the direct measurement of fluorodinitrobenzene (FDNB)-reactive lysine (Carpenter, 1960), using the modification of Booth (1971). The FDNB-reactive lysine method is an established chemical method for detecting processing damage and has been correlated with the chick assay (Carpenter & Woodham, 1974), although it does not correlate so well with the rat assay. There is evidence that biological assay using chicks is not directly comparable to that using rats (Fisher, 1974), which are the laboratory animals of choice for comparison of human nutrient requirements. Ford (1964) and Henry & Ford (1965) found that the availability of lysine for various food materials (including an LPC sample), as measured by a microbiological procedure was more consistent with the rat assay than that measured by the FDNB reaction. Boctor & Harper (1968) compared the FDNB-reactive lysine value with the rat assay and showed that available lysine values for heat-treated foods varied greatly with the method of assay used. However, the number of samples that they investigated was limited,

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owing to the laborious nature of both the FDNB and rat assay methods, particularly the latter. For autoclaved egg albumin, the FDNB-reaction gave a value of 0·56 for lysine availability and rat growth assay a corresponding value of 0·20. Similar but smaller differences were obtained for autoclaved beef muscle. Boctor & Harper (1968) postulated that sugars bound to lysine interfere with the hydrolytic activity of digestive enzymes; thus FDNB-reactive lysine values may underestimate the extent of availability. If this is so, it may account for the reports of FDNB-reactive lysine being found in rat faeces (Hurrell *et al.* 1976). Therefore, correlation between the animal assay and the FDNB-reactive lysine method is not straightforward but, nevertheless, the FDNB method remains a good indicator of processing damage and there is information based on this method from many laboratories for many different foods. Jokinen & Reineccius (1976) examined several chemical methods for the determination of available lysine (excluding the DBL method) and found that the Booth (1971) modification of the Carpenter (1960) method gave comparable results in their laboratories to other values reported for the same food materials. In addition to these nutritional considerations, the disadvantages of the FDNB method are the period required for assay, the small number of samples that can be dealt with simultaneously and the considerable technical skill required. The DBL method offers advantages on these latter considerations.

In this paper reactive lysine values determined by the FDNB and DBL methods are also compared with total lysine determined by amino-acid analysis of acid hydrolysates. Experiments on the behaviour of the amino acids in the presence of quinones (Cranwell & Haworth, 1971) and humic acid (Perry & Adams, 1971) during acid hydrolysis show that an amino acid residue in which the amino group has become involved in bond formation is released only in part under the usual conditions of protein hydrolysis. As lysine in particular takes part in the reactions, it would be expected that total lysine values calculated from ion-exchange chromatography of acid hydrolysates would yield low values.

Darkening of the colour of LPC occurs during processing, and this is thought to be due to the reactions of the phenolic compounds of LPC (Bray & Humphries, 1978). The extent of darkening was measured to determine whether it could be used as an indicator of nutritional damage to LPC samples, when compared with the DBL and FDNB techniques.

Mossberg (1966) used dye-binding capacity (DBC) (with CI Acid Orange 10) as an indicator of processing damage, but Lea & Hannan (1950), who also used Acid Orange 10, noted that DBC did not indicate early Maillard damage. Similarly, Hurrell & Carpenter (1975) found, using CI Acid Orange 12 in comparison with the FDNB-reactive lysine, that DBC was insensitive to early Maillard damage, but was a good indicator of late Maillard damage. Both FDNB-reactive lysine and DBL were determined for some food samples by Hurrell & Carpenter (1976, 1978) and show close agreement. The DBL method used by these authors was similar to that described here and used the same dye-buffer reagent, but the samples were mixed with the dye for 10 min in a wet-milling process, using specially designed equipment.

EXPERIMENTAL

Preparation of LPC samples

The LPC samples studied are described in Table 1. The control samples were the same as those described previously (Walker, 1979). All samples were prepared during the 1975 and 1976 seasons from five batches of leaf juice by the method described previously (Walker, 1979). The juice extraction equipment used was a large-scale pulper and press (Davys & Pirie, 1960, 1965) for LPC batches nos. 1, 2 and 5 and a screw press (Bentalls 'Protessa' screw press; E. H. Bentalls and Co. Ltd, Maldon, Essex) for batches nos. 3 and 4. For each curd batch, a sample was dried overnight in a Vickers freeze-drier (Vickers-Armstrong

Table 1. *Details of leaf-protein concentrate (LPC) samples used*

Batch no.	Leaf source	Sample no.	Drying technique used
1	Lucerne (*Medicago sativa* L.)	3	FD
		12	RT
		13	50°, forced-air oven, overnight
		14	90°, forced-air oven, overnight
		15	90°, V
		16	FD, then heated 110°, 16 h at 100 g moisture/kg
		17	FD, then heated 110°, 24 h at 100 g moisture/kg
2	Lucerne	11	FD
		18	50°, forced-air oven, overnight
		19	90°, forced-air oven, overnight
		20	90°, V
		21	Evaporated under reduced pressure
3	Ryegrass (Italian) (*Lolium multiflorum* L. var. RVP)	1	FD
		4	RT
		5	50°, forced-air oven, overnight
		6	90°, forced-air oven, overnight
		7	90°, V
		8	FD, then heated 110°, 16 h at 100 g moisture/kg
		9	FD, then heated 110°, 24 h at 100 g moisture/kg
4	Ryegrass (Italian)	3	FD
		10	50°, forced-air oven, overnight
		11	90°, forced-air oven, overnight
		12	90°, V
5	Fescue (*Festuca arundinacea* L. var. Alta)	2	FD
		3	RT
		4	50°, forced-air oven, overnight
		5	90°, forced-air oven, overnight
		6	90°, V

FD, freeze-dried, overnight (see p. 456); RT, curd spread thinly and dried at room temperature over a period of 2 d; V, vacuum oven overnight.

(South Marston) Ltd, South Marston Works, Swindon), using a shelf heat of 30°. This was used as a control for other samples within the same batch which were dried or heated by different techniques. Samples were ground and stored as previously described (Walker, 1979).

Analysis of LPC samples

Dye-binding capacity (DBC), dye-bound-after-propionylation (DBAP), DBL, protein and amino acids (including total lysine) were measured and values calculated as described previously (Walker, 1979).

FDNB-reactive lysine. This was measured by the direct estimation of FDNB-reactive lysine as described by Booth (1971). As the molecular weight of the lysine residue had been used for the calculation of DBL and total lysine, this value was also used for the calculation of FDNB-reactive lysine, rather than the molecular weight of the amino acid. The FDNB-reactive lysine value was corrected for loss of FDNB-reactive lysine by using the correction factor 1·09 (Roach *et al.* 1967) for materials low in carbohydrate.

Colour. This was measured using a Hunter Colour Difference Meter which measures colour in terms of the *L*, *a*, *b* colour solid (*L*), lightness, where 0 is black and 100 is white; (−*a*), greenness; (+*b*), yellowness). Only lightness of colour is recorded here. The LPC powders were prepared for the instrument by pressing a sample between two Petri dishes

Table 2. *Protein (g/kg sample), dye-binding lysine (DBL) (mmol/kg protein) and fluorodinitrobenzene (FDNB)-reactive lysine (mmol/kg protein) of twenty-eight leaf-protein concentrate samples of five batches of leaf-protein curd subjected to different heat treatments*†

(Protein values uncorrected for moisture content; values in parentheses are percentages of freeze-dried batch control value; all values represent the mean of duplicate analyses)

Batch no.†	Leaf source	Sample no.	Protein (tungstic-acid-precipitated nitrogen × 6·25)	Dye-binding capacity‡	Dye-bound-after-propionylation§	DBL‖	FDNB-reactive lysine¶
1	Lucerne (*Medicago sativa* L.)	3	542	1211	728	483	402
		12	519	1208 (100)	778* (107)	430* (89)	325* (81)
		13	510	1190 (98)	712 (98)	478 (99)	308* (77)
		14	554	1120* (93)	699 (96)	421* (87)	261* (65)
		15	555	1125* (93)	708 (97)	417* (86)	315* (78)
		16	532	955* (79)	739 (102)	216* (45)	193* (48)
		17	561	997* (82)	740 (102)	257* (53)	208* (52)
2	Lucerne	11	553	1208	694	514	426
		18	494	1252* (104)	730* (105)	522 (102)	451 (106)
		19	548	1140* (94)	759* (109)	381* (74)	314* (74)
		20	529	1212 (100)	753* (109)	459* (89)	375* (88)
		21	487	1239 (103)	678 (98)	561* (109)	475* (112)
3	Ryegrass (Italian) (*Lolium multiflorum* L. var. RVP)	1	580	1205	694	511	385
		4	466	1203 (100)	762* (110)	441* (86)	391 (102)
		5	493	1223 (102)	739* (106)	484 (95)	351 (91)
		6	506	1172 (97)	672 (97)	500 (98)	357 (93)
		7	519	1137* (94)	666 (96)	471 (92)	367 (95)
		8	574	1000* (83)	710 (102)	290* (57)	210* (55)
		9	576	1068* (89)	728 (105)	340* (67)	255* (66)
4	Ryegrass (Italian)	3	545	1233	695	538	466
		10	536	1191* (97)	668 (96)	523 (97)	457 (98)
		11	546	1222 (99)	690 (99)	532 (99)	373* (80)
		12	563	1174* (95)	685 (99)	489* (91)	379* (81)
5	Fescue (*Festuca arundinacea* L. var. Alta)	2	613	1180	665	515	425
		3	625	1149 (97)	636 (96)	513 (100)	351* (83)
		4	587	1193 (101)	651 (98)	542 (105)	461 (108)
		5	642	1016* (86)	541* (81)	475 (92)	312* (73)
		6	633	1063* (90)	602* (90)	461* (90)	344* (81)
Standard error of replication (*n* 20)				20	18	25	19

* Significantly different from the value of the batch control ($P < 0{\cdot}05$).
† For details, see Table 1.
‡ For details, see p. 457.
§ For details, see p. 457.
‖ For details, see p. 457.
¶ By indirect measurement on FDNB-lysine (Booth, 1971).

to form a flat cake. The machine was calibrated against standard black and white tiles, and two determinations were made for each sample.

A preliminary plot was made of all paired data where relationships were being investigated. In all cases where a relationship clearly existed it was substantially linear over the range studied, and correlation coefficients were, therefore, determined.

RESULTS AND DISCUSSION

Table 2 shows results for protein, DBC, DBAP, DBL and FDNB-reactive lysine for the 28 LPC samples studied. Each observation is the mean of duplicate observations. An estimate of the standard error of replication of these observations, based on ten duplicate samples of each is also given in Table 2.

The mean value for the FDNB-reactive lysine was approximately 100 mmol/kg protein lower than the DBL value. The correlation coefficient (r) between FDNB-lysine and DBL values was 0·90 (n 28) and the regression equation was $y = -15{\cdot}5+0{\cdot}81x$, with y = FDNB-lysine (mmol/kg protein) and x = DBL (mmol/kg protein). The residual variance of the regression was 1300.

A regression analysis of the FDNB-reactive lysine and DBC values gave $r = 0{\cdot}83$ (n 28) and the regression equation $y = -571{\cdot}5+0{\cdot}80x$, where y = FDNB-reactive lysine (mmol/kg protein) and x = DBC (mmol/kg protein). The residual variance of the regression was 2000, which was not significantly different from the residual variance of the regression of DBL and FDNB-reactive lysine. However, the use of the latter regression equation allows more precision in the prediction of FDNB-reactive lysine, and the lower residual variance of this regression is in accordance with results reported by Hurrell & Carpenter (1978).

In Table 2 the DBC, DBAP and FDNB-reactive lysine values are also expressed as percentages of the corresponding value for the freeze-dried control for that batch. All batches showed some samples in which DBC was significantly decreased ($P < 0{\cdot}05$) when compared with the freeze-dried control. However, the decrease due to a particular treatment was not consistent for all LPC batches. Previously (Fig. 4; Walker, 1979) DBC was shown to correlate highly (r 0·98, n 20) with the protein content (tungstic-acid-precipitated N×6·25) for undamaged LPC samples. DBC results (in appropriate units) from Table 2 were compared with this regression line. Values for samples which had received the most severe heat treatment lie furthest below this regression line. This is to be expected, as DBC is the dye bound to the basic amino acids including lysine (Hurrell & Carpenter, 1975). This effect of thermal damage of a food sample on its DBC has been noted previously (Mossberg, 1966).

The DBAP values showed that there was little significant difference between samples dried by different drying techniques and their batch controls. In some instances (e.g. lucerne nos. 18, 19 and 20) there were significant increases which cannot be explained. There was no correlation between DBAP and arginine+histidine (r −0·18, n 28), which was unexpected, as in principle the histidine+arginine groups are all that remain for binding dye after reactive lysine has been blocked. This may imply that the DBAP value includes non-specific binding sites. The effects of the presence of food components other than proteins on the DBL values are not yet known.

The DBL values showed significant differences for many of the heat-treated LPC samples, when compared to their batch controls, and most of those which were significantly different from the batch control for the DBL value were also significantly different from the batch control for the FDNB-reactive lysine value. One LPC sample (lucerne no. 21) showed a significant increase in available lysine in relation to the batch control by both methods. This sample had been dried at room temperature by evaporation under reduced pressure, and was the only sample to be dried in this manner. Four of the heat-damaged samples

Table 3. *Basic amino acids, glutamic acid and aspartic acid (mmol/kg protein)* of twenty-eight leaf-protein concentrate samples subjected to different heat treatments†*

(All results are the mean of analyses of duplicate hydrolysates‡; values in parentheses are the percentages of the batch control value)

Batch no.	Leaf source†	Sample no.†	Aspartic acid	Glutamic acid	Lysine	Histidine	Arginine
1	Lucerne (*Medicago sativa* L.)	3	858	880	523	206	463
		12	889	936	497 (95)	200	411§
		13	892	864	483§ (92)	184	426
		14	857	829	448§ (86)	192	428
		15	865	876	461§ (88)	198	433
		16	868	906	371§ (71)	187	396§
		17	862	903	375§ (72)	191	410§
2	Lucerne	11	837	835	544	215	422
		18	829	862	553 (102)	209	451
		19	885§	887	497§ (91)	202	414
		20	909§	885	494§ (91)	193	418
		21	906§	851	541 (99)	193	420
3	Ryegrass (Italian) (*Lolium multiflora* L. var. RVP)	1	853	921	499	201	431
		4	801§	875	534§ (107)	195	447
		5	832	862	505 (101)	191	458
		6	833	881	481 (96)	187	445
		7	855	906	493 (99)	184	439
		8	824	960	397§ (80)	175	427
		9	816	902	435§ (87)	193	432
4	Ryegrass (Italian)	3	843	870	534	187	431
		10	843	912	540 (101)	187	438
		11	827	911	505§ (95)	185	432
		12	798	850	517 (97)	196	426
5	Fescue (*Festuca arundinacea* L. var. Alta)	2	818	898	517	179	435
		3	804	872	513 (99)	182	439
		4	797	865	529 (102)	187	444
		5	811	887	480§ (93)	192	440
		6	793	847	489 (95)	203	451
	Standard error of replication (*n* 20)		24	29	15	16	19

* Protein calculated from the amino acid residues recovered, including ammonia, and applying a correction factor for tryptophan (see p. 457.)
† For details, see Table 1.
‡ For details, see p. 457.
§ Variation from batch control significant ($P < 0.05$).

gave FDNB-reactive lysine values which were significantly different from the batch control, but they did not show a significant difference from the batch control for the DBL values.

DBL plotted *v.* total lysine from amino acid analysis shows a high correlation (r 0·91, n 28) as does a plot of FDNB-reactive lysine *v.* total lysine (r 0·93, n 28) (see Figs. 1 and 2 respectively). This type of relationship was to be expected from earlier reports (Cranwell & Haworth, 1971).

Table 3 shows the basic amino acids, glutamic acid and aspartic acid, which were determined by ion-exchange chromatography, together with an estimate of the standard error of replication of these observations based on ten duplicate samples of each. Aspartic acid and glutamic acid have been included because, under severe processing conditions, these amino acids have been shown to react with lysine to form isopeptides (Hurrell & Carpenter, 1975). Only for one LPC sample (ryegrass sample no. 4) was aspartic acid significantly

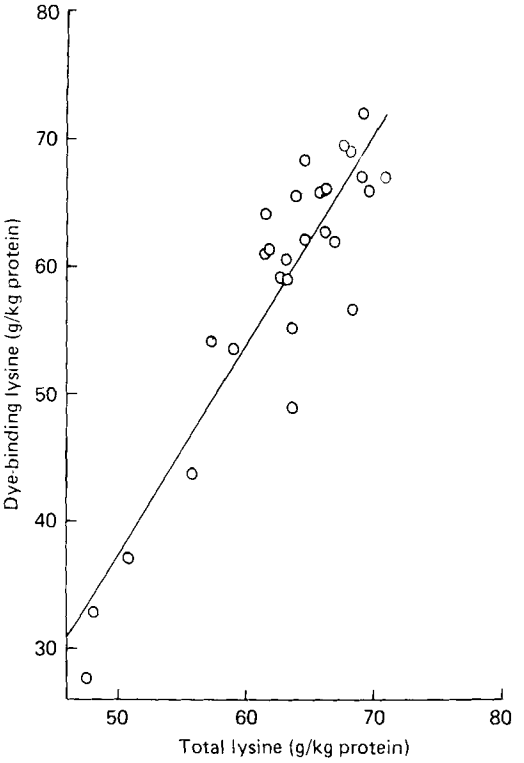

Fig. 1. The relationship between dye-binding lysine (g/kg protein (tungstic-acid-precipitated nitrogen × 6·25)) and total lysine (g/kg protein (for calculation, see p. 457)) by amino acid analysis of acid hydrolysates of twenty-eight leaf-protein concentrate samples subjected to different thermal treatments (for details of procedures and samples, see p. 456 and Table 1 respectively). $y = -45 + 1{\cdot}65x$.

lower than that of the batch control, and glutamic acid values showed no differences in comparison with the control. For three LPC samples (lucerne samples nos. 19, 20 and 21) the aspartic acid was significantly higher than for the batch control. No explanation for the latter phenomenon can be given. The over-all lack of change of these two amino acids would indicate that lysine is not involved in the formation of isopeptides with glutamic acid and aspartic acid to any pronounced extent during the processing of the LPC samples studied, even for those severely heat-damaged at 110° for 16 and 24 h (lucerne sample nos. 16 and 17 and ryegrass sample nos. 8 and 9). Table 3 also shows a significant decrease of total lysine when compared with batch controls for many of the heat-damaged LPC samples, which would be expected from results given in Figs. 1 and 2. Of the other two basic amino acids, only arginine showed a significant decrease on heating for some LPC samples in batch no. 1.

Hurrell & Carpenter (1975) showed that DBC was more closely correlated to histidine+arginine+FDNB-reactive lysine (HARL value) than to the total basic amino acids. DBC values for all LPC samples studied are here plotted *v.* (*a*) total basic amino acids (histidine, arginine and total lysine) (Fig. 3), (*b*) HARL value, calculated using the DBL value for reactive lysine (Fig. 4) and (*c*) HARL value, calculated using the FDNB-reactive lysine value (Fig. 5); *r* values were nearly identical (0·82, 0·81 and 0·82 respectively). Thus, for

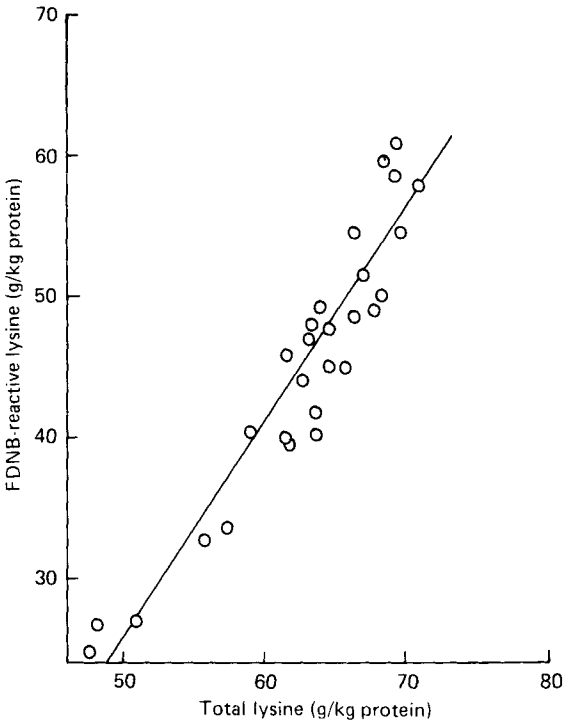

Fig. 2. The relationship between fluorodinitrobenzene (FDNB)-reactive lysine (g/kg protein (tungstic-acid-precipitated nitrogen $\times 6{\cdot}25$)) and total lysine (g/kg protein (for calculation, see p. 457)) by amino acid analysis of acid hydrolysates for twenty-eight leaf-protein concentrate samples subjected to different thermal treatments (for details of procedures and samples, see p. 456 and Table 1 respectively). $y = -51 + 1{\cdot}54x$.

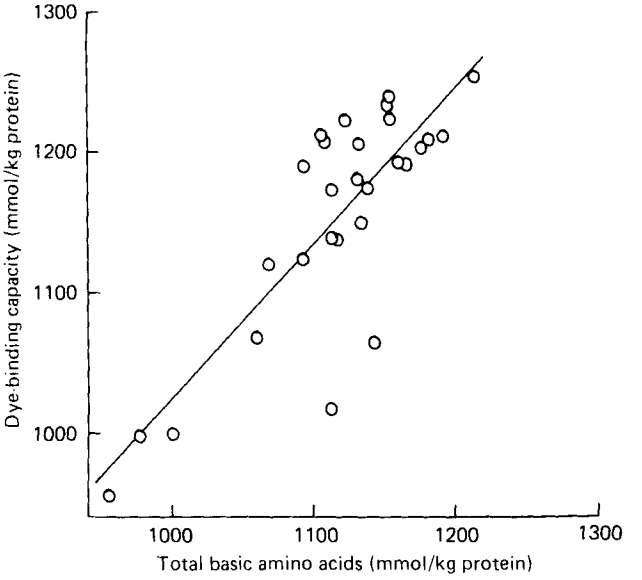

Fig. 3. The relationship between dye-binding capacity (mmol/kg protein (tungstic-acid-precipitated nitrogen $\times 6{\cdot}25$)) and total basic amino acids (mmol/kg protein (for calculation, see p. 457)) by amino acid analysis of acid hydrolysates of twenty-eight leaf-protein concentrate samples subjected to different thermal treatments (for details of procedures and samples, see p. 456 and Table 1 respectively). $y = -76 + 1{\cdot}10x$.

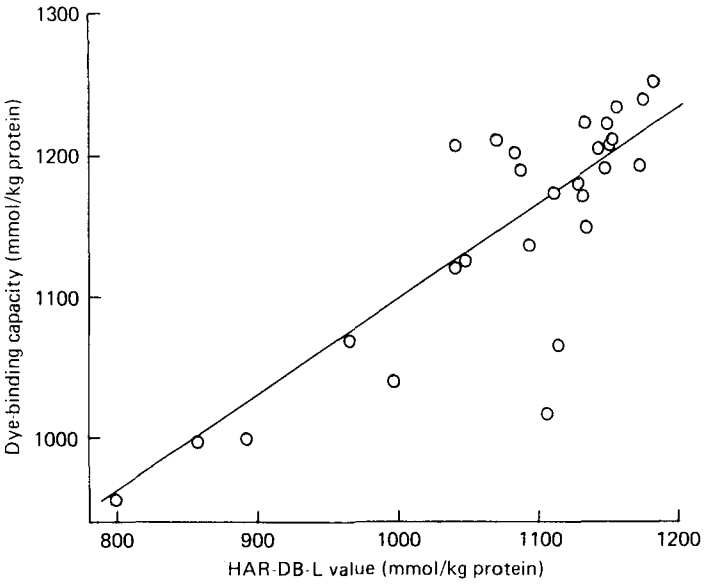

Fig. 4. The relation between dye-binding capacity (mmol/kg protein (tungstic-acid-precipitated nitrogen $\times 6{\cdot}25$)) and the histidine+arginine+dye-binding lysine (HAR-DB-L) value (mmol/kg protein) for twenty-eight leaf-protein concentrate samples subjected to different thermal treatments (for details of procedures, calculation of HAR-DB-L value and details of samples, see p. 456, p. 457 and Table 1 respectively). $y = 419+0{\cdot}68x$.

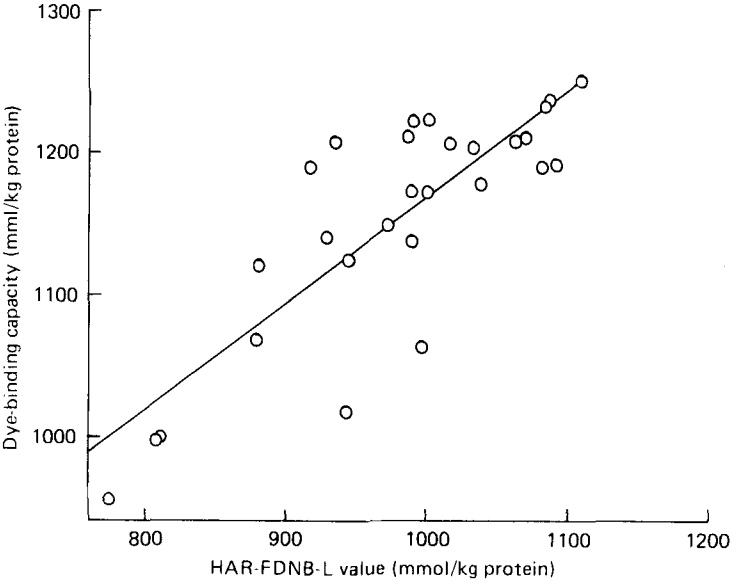

Fig. 5. The relation between dye-binding capacity (mmol/kg protein (tungstic-acid-precipitated nitrogen $\times 6{\cdot}25$)) and the histidine+arginine+fluorodinitrobenzene (FDNB)-reactive lysine (HAR-FDNB-L) value (mmol/kg protein) for twenty-eight leaf-protein concentrate samples subjected to different thermal treatments (for details of procedures, the calculation of HAR-FDNB-L value and details of samples, see p. 456, p. 457 and Table 1 respectively). $y = 417+0{\cdot}75x$.

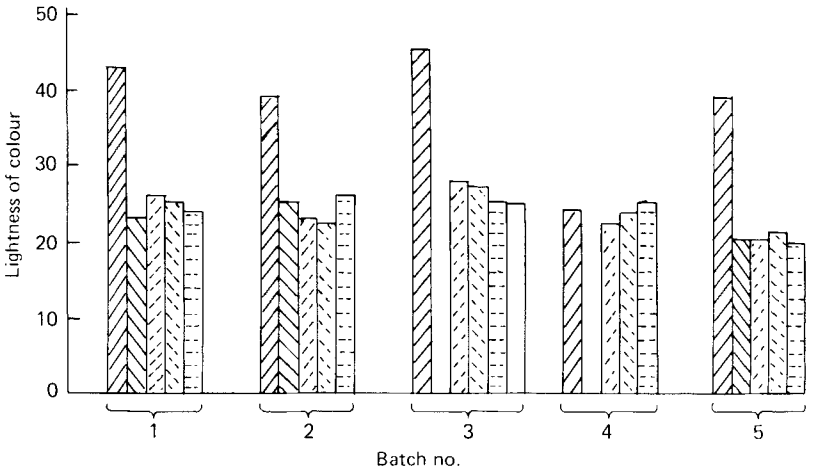

Fig. 6. Histogram to show lightness of colour (units of colour on *L*, *a*, *b* scale (0, black; 100, white); see p. 457), using the Hunter Color Difference Meter for twenty-four leaf-protein concentrate samples from five curd batches, subjected to different thermal treatments. ▨, Freeze-dried; ▧, dried at room temperature; □, dried by evaporation under reduced pressure; ▧, dried at 50°, overnight, forced-air oven; ▨, dried at 90°, overnight, forced-air oven; ▤, dried at 90°, overnight, vacuum oven. For details of samples, see Table 1.

the LPC samples studied the HARL value holds no advantage over total basic amino acids for comparison with DBC.

Fig. 6 indicates the lightness of colour of the different batches of LPC samples. There was no correlation between the lightness of colour and the nutritional value as assessed by available lysine determined by either the DBL or FDNB-reactive methods.

The DBL method described here provides a convenient, reproducible and simple method for the estimation of reactive lysine as an indicator of processing damage to LPC samples, so long as suitable experimental conditions are chosen. There appears to be no reason why it should not be applied as successfully to other food materials, provided that there is no interference from other food components. If this is so then the DBL method could be used as a quality control measurement during food processing. The simplicity of the methodology makes it very suitable for use in laboratories lacking sophisticated equipment.

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