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Dye-Binding 'Stoichiometry' and Selectivity of Cresol Red with Various Proteins

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ABSTRACT

Dye-binding stoichiometry and selectivity of the reactions of several standard proteins and protein matrices with the phthalein dye cresol red (CR) are presented. Spectrophotometric measurements show that the interactions are selective; amino acids, peptides, and albumin-type proteins do not react at all. The optimum analytical conditions of the reactive proteins—human gamma globulin (HGG), casein (C), milk protein (MP), soya bean (SBM), meat (MM), and fish meals (FM)—with CR are given. Dye-binding values obtained with soya bean meals are complemented by the sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns (SDS–PAGE) of the same samples, in order to show a qualitative picture of the molecular weight distribution of three untreated samples when comparing them to their respective denatured ones.

INTRODUCTION

The physicochemical characteristics and the analytical use of dye-binding reactions taking place between proteins and different naphthalenesulfonic

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acid dyes have been extensively examined (Rattee & Breuer, 1974). The characteristic variable reactivity of soya proteins with the phthalein dye, cresol red (CR), has been described earlier (Frölich, 1954*a*; 1954*b*; Olomucki & Bornstein, 1960). Previous experiments have shown that 'properly heat-denatured'† soya bean proteins bind significantly more CR than the 'under-heated'† samples, and 'over-heated'† proteins bind somewhat more than the 'properly heat-denatured' ones (Frölich, 1954*b*; Olomucki & Bornstein, 1960). In the absence of a systematic study, no reasonable interpretation can be found in the literature for the characterization of this CR-soya protein interaction. The practical use of the method in soya analysis has recently been described by Holmes (1987) as '... one of the most convenient tests for the protein quality... based on the absorption of the cresol red dye by the protein.'

We report here on the characteristics and optimum analytical conditions for CR–protein interactions by using spectrophotometric measurements and gel electrophoretic tests.

MATERIALS AND METHODS

Model materials

Amino acids, peptides (oxytocin, ribonuclease, and insulin), bovine albumin (BA), human albumin (HA), and human gamma globulin (HGG) were obtained from Sigma, St. Louis, Missouri, USA. Caseins (C) were obtained from Phylaxia, Budapest, Hungary (C_1), and from Serva, Feinbiochemica, FRG (C_2). Soya bean meals (SBM), numbered 1–3 of the same type (Varietas: NKS 1346, Glycine max.), were from different commercial sources. SBM_1 and SBM_2 samples originated from the USA and SBM_3 from Brazil. Sample 1 is fat free: samples 2–3 contain fat. Subscripts show the treatment of the samples. Samples $SBM_{1/1}$ and $SBM_{1/2}$ were acidified to $pH \simeq 2$ and $pH \simeq 1{\cdot}8$, respectively, with a mixture of phosphoric and hydrochloric acids (molar ratio 2:1), and neutralized immediately with sodium and calcium carbonate. $SBM_{2/1}$ and $SBM_{3/2}$ were acidified to $pH \simeq 2$ with a 2:1 mixture of phosphoric and hydrochloric acids without neutralization. $SBM_{3/1}$ was heated to 100°C for 5 min by applying a microwave heat-treatment. The soybean samples have been treated in the laboratory of the Hajduság Agrarian Industrial Association, Nádudvar, Hungary; milk protein (MP) was the product of Milk Industrial Répcelak,

† The terms 'properly heat-denatured', 'under-heated' and 'over-heated' proteins were proposed by Frölich (1954*b*).

Hungary. Meat and bone meals (MM) were obtained from the Feed-Protein Manufacturer Co. Solt (MM_1) and Debrecen (MM_2), both in Hungary. Fish meals (FM) were commercially imported samples from the FRG (FM_1), and Sweden (FM_2), of unknown origin.

Reagents
All reagents used were of highest analytical purity, obtained from Reanal (Hungary).

Apparatus
An electric shaker (Labor MIM, Hungary), accomodating 24 flasks, and a spectrophotometer (Spectromom 361, MOM, Hungary) were used.

Procedures

Spectrophotometric measurement
The CR–protein dye-binding reactions were carried out according to Frölich (1954*b*), with the following exceptions: the optimum amount of sample, i.e. those dye–protein ratios which ensure 100% excess of dye after quantitative reaction (no dye-binding), and the shortest reaction time necessary for the quantitative reactions were used (for details see the data of Table 1). The original Frölich reagent (Frölich, 1954*b*) contained one part of alcoholic CR solution (0·2%) and nine parts of 0·1 M hydrochloric acid.

Samples, ground to pass through a 60-mesh sieve, were weighed and placed in a 100-cm^3 glass-stoppered flask. Glass beads and 10 cm^3 Frölich reagent were also added and the flask was shaken for optimum shaking time. (Optimum shaking times needed for dye-binding are as follows: SBM, 20 min; MP, 10 min; MM and FM, 40 min; C, 40 min; and HGG, 10 min.) After that time, i.e. after quantitative dye-binding, the solutions were filtered through glass filter paper. Then 1 ml of the filtrate was diluted with 25 ml of 0·02 M sodium hydroxide. The optical densities of the diluted filtrates were measured in a 1 cm cuvette at a wavelength of 570 nm against distilled water.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
Measurements were performed in a discontinuous Trisborate buffer system according to Neville (1971). Slab gels were stained with coomassie brilliant blue R-250. For quantitative measurement an LKB 2202 Laser densitometer was used, connected to an LKB 2220 computing integrator. A low molecular weight calibration kit (LMWCK) (Pharmacia AB, Uppsala, Sweden) was used for standards. The molecular weight range was 14 400–94 000.

TABLE 1
Dye-Binding Selectivity and Stoichiometry of Various Proteins with CR as a Function of the Reaction Time

Sample	Optimum amount (mg)	Reaction time (min) 5	10	20	40	60	120	Mean[a]	SE	SE %	Ew_{av} ($\times 10^4$)
		Bound CR (mg/g protein)									
BA	100	—	—	—	—	—	—				
HA	100	—	—	—	—	—	—				
C_1	50	(17·60)	(19·40)	21·98	22·50	22·50	22·13	22·28	0·26	1·18	1·72
C_2	50	(20·80)	(22·53)	(25·53)	24·97	24·87	24·77	24·87	0·10	0·40	1·54
HGG	100	(11·61)	12·25	12·30	12·08	—	12·28	12·23	0·10	0·82	3·13
SBM_1	100	(19·32)	19·87	19·68	19·78	—	19·68	19·75	0·09	0·46	1·94
MP	50	(16·50)	16·80	16·76	16·54	—	16·60	16·68	0·12	0·74	2·26
MM_1	50	(33·40)	36·96	34·55	34·95	35·30	35·23	34·80	0·58	1·69	1·10
MM_2	50	(33·45)	(33·47)	35·44	36·52	36·16	36·31	36·11	0·47	1·30	1·06
FM_1	50	(31·18)	(33·22)	34·26	33·97	34·46	34·46	34·29	0·23	0·67	1·12
FM_2	50	(28·56)	(30·00)	(32·48)	33·28	33·00	33·06	33·11	0·15	0·45	1·16

[a] Data in parentheses not included, — not yet quantitative values.
Note: The optimum values in all cases were obtained in two steps. At the first step, different amounts of all proteins investigated were reacted with the same amount (10 cm^3) of the Frölich reagent for 2 h, i.e. a substantially longer reaction time than needed for the quantitative dye-binding. At the second step: the optimum amount of the given protein (those amounts of proteins which consumed about 5 cm^3 of the Frölich reagent—100% excess—after 2 h reaction time) was reacted with 10 cm^3—Frölich reagent using shorter reaction times than 2 h (including 2 h as well): the optimum reaction time for the given protein is regarded as the shortest time necessary for the quantitative reaction.

RESULTS AND DISCUSSION

Data obtained proved that CR–protein reactions are fundamentally different from the dye-binding reactions performed previously with naphthalenesulfonic acid dyes (Udy, 1956; Hurrel *et al.*, 1979; Molnár-Perl *et al.*, 1985*b*; 1986*a*; Molnár-Perl & Pintér-Szakács, 1986).

Preliminary experiments have shown that the amount of dye bound depends exponentially on the amount of protein interacted (Figs 1 and 2). Later data draws attention to the fact that, for each kind of protein tested, an optimum CR–protein ratio has to be chosen. In order to obtain comparable results and better reproducibility in inter-laboratory studies, the same CR–protein ratio should be used.

Our results have shown that optimum analytical conditions can be achieved when the dye–protein ratio is 100% in excess of the bound dye after

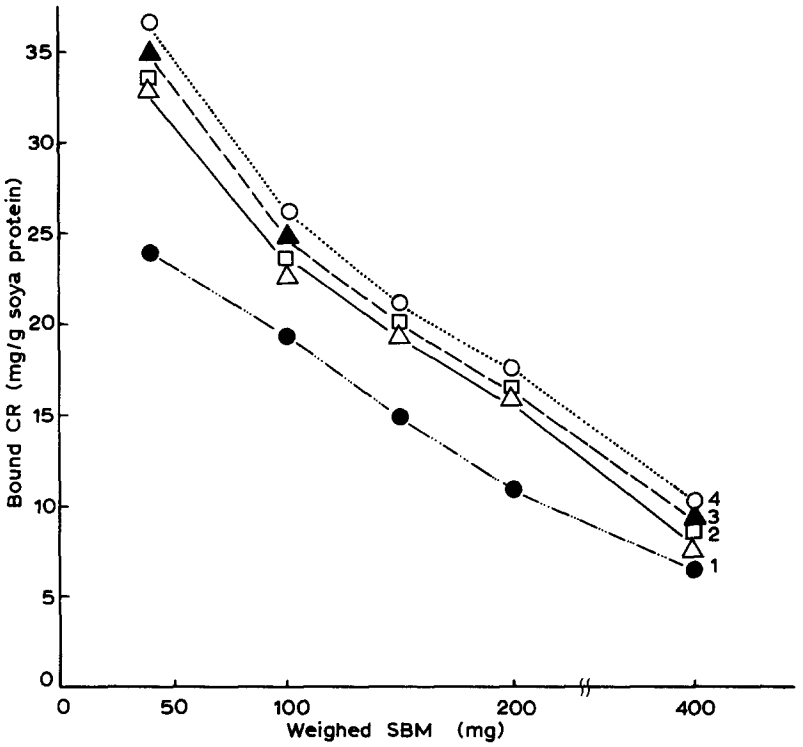

Fig. 1. CR-binding capacity of soya samples. (●) SBM_{1-3}; (△) $SBM_{1/1}$; (□) $SBM_{1/2}$; (▲) $SBM_{2/1}$ and $SBM_{3/2}$; (○) $SBM_{3/1}$.

quantitative reaction. The excess CR ensures a short time for quantitative reaction and an acceptable reproducibility (Table 1).

The optimum pH of the CR–protein reactions, in the range pH 1–6, was tested with the SBM_3 and $SBM_{3/1}$ samples (Fig. 3). Investigations were performed in parallel, ensuring the required pH, partly with different amounts of 0·1M HCl (Fig. 3, plot $SBM_{3/1}$), and partly with 0·2M HCl–0·2M KCl buffer (Fig. 3, plot SBM_3). Results show that bound CR was lower in the buffer system than in the HCl of the same pH (Fig. 3, plots SBM_3 and $SBM_{3/1}$). Later results suggest that this can probably be explained by the greater ionic strength of the buffer necessary to ensure the given pH. Thereafter, the Frölich reagent was used and the pH values checked with a pH meter. pH varied between 1·1–1·3 depending on the sample investigated.

Dye-binding tests of the model proteins investigated showed that the interactions are selective, i.e. amino acids, peptides (oxytocin, ribonuclease, and insulin), and albumin-type proteins do not react at all (Table 1), confirming the results of Hurrel and Carpenter (1975). In addition, the dye-binding reactivity values of a given protein matrix are independent of their

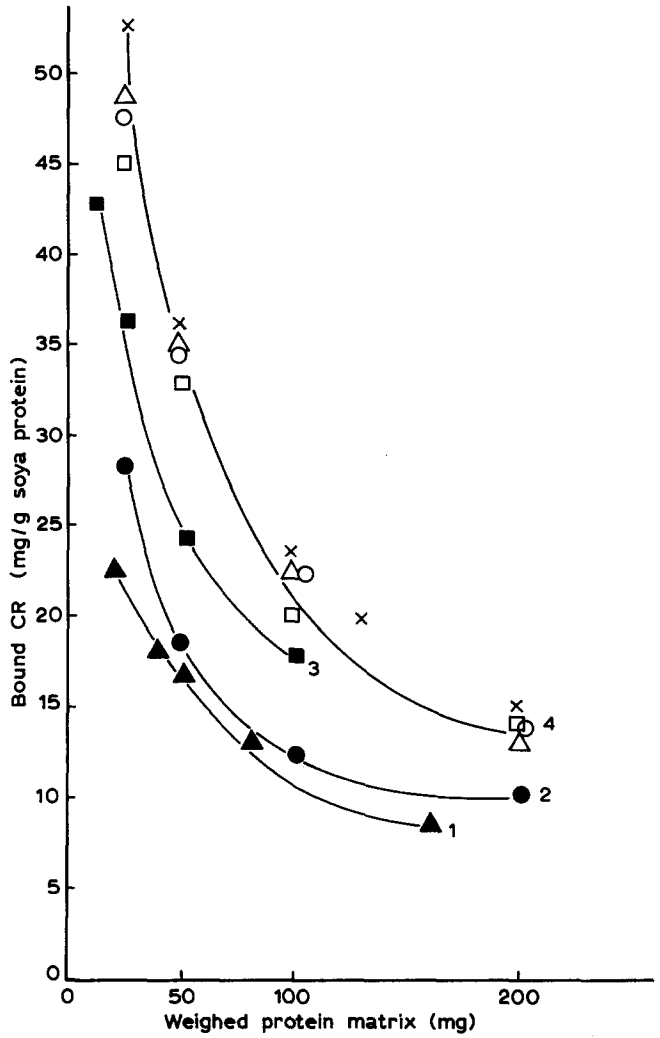

Fig. 2. CR binding capacity of different protein matrices. (▲) MP; (●) HGG; (■) C_2; (△) MM_1; (×) MM_2; (○) FM_1 and (□) FM_2.

origin and are the same within the experimental error of the method (see the dye-binding data of C_1, C_2, MM_1, MM_2, FM_1, and FM_2 Table 1), and of SBM untreated samples (SBM_1–SBM_3, Table 2, and Figs 4 and 5). As for the 'stoichiometry' of the dye-binding reactions of different proteins (Table 1), it has been shown that the molar ratios of the dye bound to the protein are characteristic of proteins, quantitative after 10–40 min, and do not change through the whole period of reaction time (120 min).

In order to confirm the nature of the dye-binding interactions, we assumed that free amino groups bind CR ('CR-reactive amino groups'), and calculated the average 'equivalent weight' (Ew_{av}) of the protein matrix tested

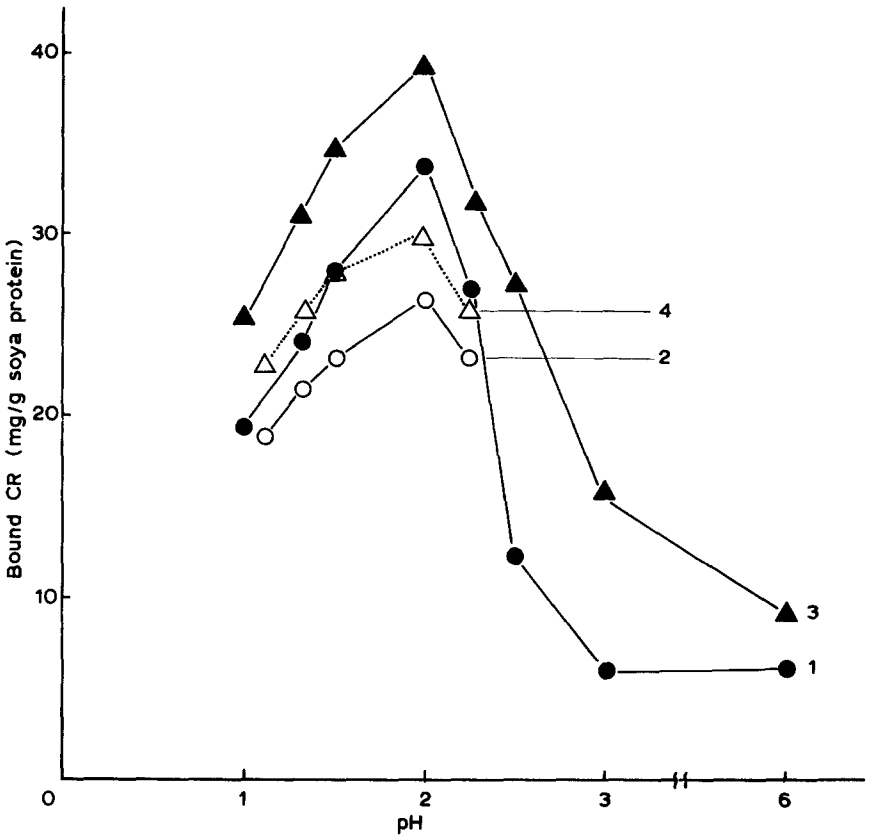

Fig. 3. The extent of CR binding capacity of soya samples as a function of pH. 1(●), 2 (○) = SBM_3; 3 (▲), 4 (△) = $SBM_{3/1}$; the pH values of tests presented by plots 1 and 3 have been adjusted with an additional amount of 0·2M hydrochloric acid (pH = 1), and with 0·02M sodium hydroxide (pH = 3); results given by plots 2 and 4 were carried out applying 0·2M HCl/ 0·2M KCl buffers. The amount of SBM samples were 100 mg; time of reaction: 30 min.

(Tables 1 and 2; calculated from the mean values of bound CR (mg/g protein) using a molecular weight of CR = 382·4). It should be emphasized that these Ew_{av} values are relative numbers, but excellent figures for evaluating, in part, the quality of the protein (Table 1) and, in part, the extent of soya's acidification and/or heat-treatment. Ew_{av} values provide evidence for a definite and reproducible type of dye-binding as follows:

(i) The single EW_{av} values of various proteins determined are in acceptable agreement with each other (Ew_{av} values of samples C_1 and C_2, MM_1 and MM_2, FM_1 and FM_2, in Table 1; and those of SBM_1–SBM_3 in Table 2).

(ii) The reproducibility of the interactions can be characterized by dye-binding data obtained with increasing reaction time.

TABLE 2
Dye-Binding Capacity of Soy Samples Processed by Different Methods

Sample	*Bound CR (mg/g protein)*		Ew_{av} ($\times 10^4$)
	*Measured**	*Calculated**	
SBM_1	19·75		1·93
$SBM_{1/1}$	22·87	3·1	1·67
$SBM_{1/2}$	24·00	4·3	1·59
SBM_2	19·24		1·94
$SBM_{2/1}$	25·23	5·8	1·51
SBM_3	19·57		1·95
$SBM_{3/1}$	25·89	6·3	1·48
$SBM_{3/2}$	24·63	5·1	1·55

* Differences obtained by subtracting the dye-binding values of the untreated samples ($SBM_1 - SBM_3$) from the values of the respective processed ones ($SBM_{1/1} - SBM_{3/2}$).

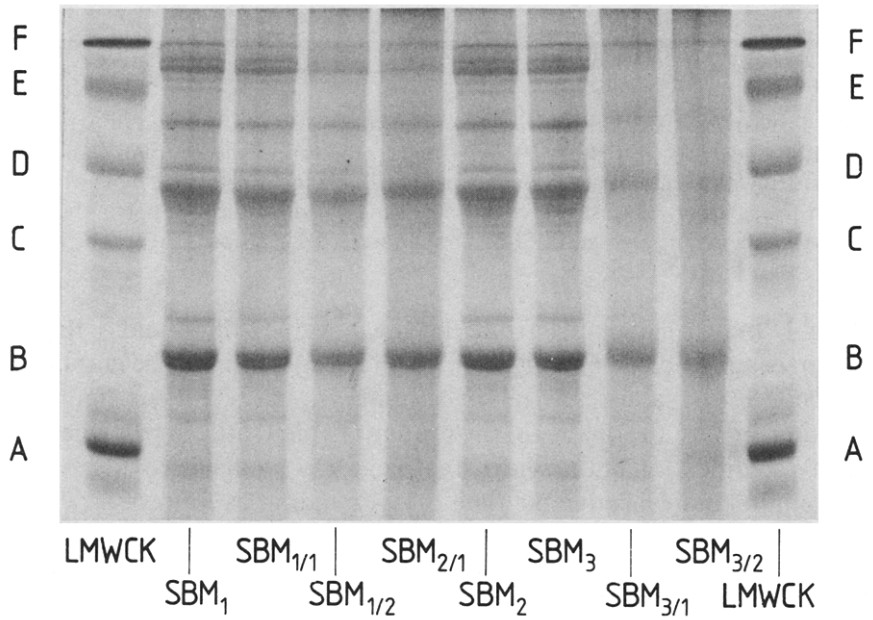

Fig. 4. SDS–PAGE electrophoretic pattern of SBM samples and LMWCK.

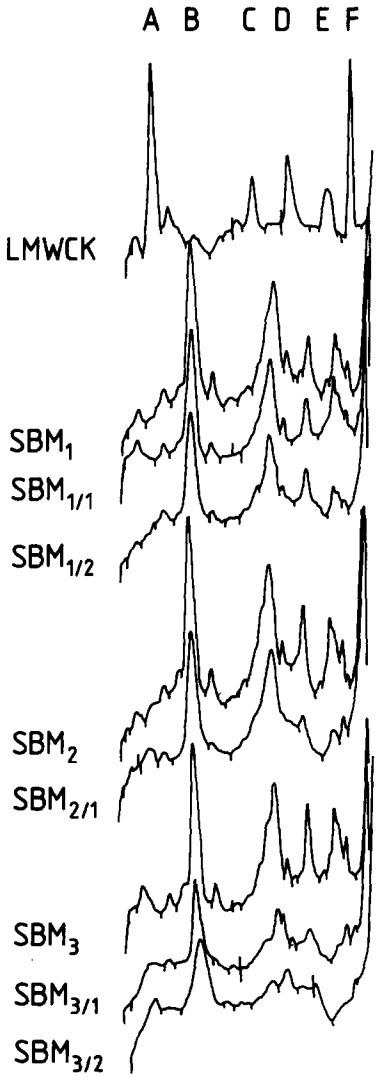

Fig. 5. Densitograms of the individual SBM samples and that of the LMWCK obtained by the LKB 2220 computing integrator. Peaks: A and C–F components of the LMWCK with the given molecular weights (Mw)—A: $1{\cdot}23 \times 10^4$, C: $2{\cdot}50 \times 10^4$, E: $6{\cdot}70 \times 10^4$, F: $9{\cdot}40 \times 10^4$. Mw of the main components, peaks B ($1{\cdot}8 \times 10^4$), present in all SBM samples, are calculated using the correlation of Rf/log Mw of the known proteins of LMWCK.

As for the increased dye-binding capacity of denaturated SBM samples (Table 2), it is clear that when subtracting the dye-binding values of the untreated samples (SBM_1–SBM_3) from the values of the respective processed ones ($SBM_{1/1}$–$SBM_{3/1}$), the differences obtained show reasonable relation with each other and with the strength of the denaturation method applied.

In addition to the latter results, demonstrating an increased number of 'CR-reactive amino groups' in the processed SBM samples, complementary evidence has been supplied by the SDS–PAGE method—using the same amounts of the samples (Fig. 4)—and by the qualitative comparison of the individual proteins upon evaluating the laser densitometer tracings (Fig. 5) of the electrophoretic patterns.

The SDS–PAGE patterns and their qualitative evaluation completed our dye-binding data. The SDS–PAGE patterns of the untreated SBM samples (SBM_1–SBM_3) and their laser scans (Fig. 5) are the same. From the SDS–PAGE patterns and the laser scans of the untreated samples (SBM_{1-3}) to their respective processed ones ($SBM_{1/1-3/2}$), it can be seen that, as a result of the various treatments, the amounts of the main components decrease (Figs 4 and 5). In comparison with the LMWCK standard these include component B, component D, and components E–F. At the same time, it can be seen that the SDS–PAGE patterns of the treated samples (Fig. 4, $SBM_{1/1-3/2}$) are uniformly darker. This is probably due to the fact that, under the conditions of various pretreatments, several compounds of different molecular weight are formed, increasing the number of 'CR-reactive amino groups'.

This study provides an improved testing method in protein chemistry (proving the dye-binding 'stoichiometry' and selectivity of cresol red with various proteins), as well as the optimum conditions in the analysis of CR-reactive proteins, particularly in soya bean quality control. The measurement of CR–protein interaction is a sensitive method of determining the extent of Maillard reaction browning in soya bean samples. Data obtained (Table 2) were in good agreement with other methods recently described (Molnár-Perl *et al.*, 1984; 1985*a*; 1986*b*).

This work, together with our earlier studies, is part of a comprehensive programme to develop new, improved, and faster analytical methods for monitoring Maillard reactions (Molnár-Perl *et al.*, 1984; 1985*a,b*; 1986*a,b*; Molnár-Perl & Pintér-Szakács, 1986).

In applying our methods for the measurement of available lysine (Molnár-Perl *et al.*, 1985*b*; 1986*a*), and the well known hydrolytic products of the deoxyfructosyl-lysine, i.e. pyridosine and furosine (Molnár-Perl *et al.*, 1986*b*), we used the large saccharide and protein-containing soya beans, excellent matrices for Maillard interactions.

In order to be able to evaluate the results in all of our investigations, the same soya bean samples (SBM_{1-3}) and the same denaturation procedures were used. (Since in this study only chosen samples and procedures were applied, their indications are not the same as in our earlier studies (Molnár-Perl *et al.*, 1984; 1985*a,b*; 1986*b*). Thus, it has been shown that the increased reactivity of the processed samples ($SBM_{1/1}$–$SBM_{3/2}$, Table 2) with CR are

in close agreement with the transformation of the active components of the Maillard reaction (i.e. the free ε-amino groups of lysine and the reducing saccharides) (Molnár-Perl & Pintér-Szakacs, 1986). The 'available lysine' content of various processed samples ($SBM_{1/1}$–$SBM_{3/2}$), as measured by our dye-binding procedure (Molnár-Perl *et al.*, 1985*b*; Molnár-Perl & Pintér-Szakács, 1986), as well as by their pyridosine and furosine contents (Molnár-Perl *et al.*, 1986*b*), is significantly decreased when comparing it to both the total lysine value of the same sample and the available lysine value of the corresponding untreated sample.

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