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Anjali A. Bhide[a]; Rajesh M. Patel[b]; Jyeshtharaj B. Joshi[c]; Vishwas G. Pangarkar[c]
[a] ALFA LAVAL INDIA LTD., PUNE, MAHARASHTRA, INDIA [b] Arati Drugs Ltd. M.I.D.C., Tarapur Dist., Thane Maharashtra, India [c] DEPARTMENT OF CHEMICAL TECHNOLOGY, UNIVERSITY OF BOMBAY, BOMBAY, INDIA

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Affinity Partitioning of Enzymes Using Unbound Triazine Dyes in PEG/Phosphate System

ANJALI A. BHIDE
ALFA LAVAL INDIA LTD.
DAPODI, PUNE 411012 MAHARASHTRA, INDIA

RAJESH M. PATEL
ARATI DRUGS LTD.
M.I.D.C.
TARAPUR, DIST. THANE MAHARASHTRA, INDIA

JYESHTHARAJ B. JOSHI and VISHWAS G. PANGARKAR*
DEPARTMENT OF CHEMICAL TECHNOLOGY
UNIVERSITY OF BOMBAY
MATUNGA, BOMBAY 400019, INDIA

ABSTRACT

The partitioning of alcohol dehydrogenase glucose-6-phosphate dehydrogenase and hexokinase from pressed bakers' yeast was studied in polyethylene glycol (PEG)/potassium phosphate aqueous two-phase system using unbound reactive triazine dyes as affinity ligands. The various parameters investigated were ligand type and concentration, pH of the system, phase composition of the system, and molecular weight of PEG. It was found that the best ligands gave an increase in partition coefficient of 2 to 3 times over that in the absence of the ligand.

INTRODUCTION

Aqueous two-phase systems (ATPS) are finding increasing use in the recovery of proteins (1). Proteins and enzymes of high purity are required in clinical and diagnostic research and in food applications. For this, a method based on selective partitioning is desired. Plain ATPS have the

* To whom correspondence should be addressed.

disadvantage of nonselective partitioning of enzymes. In order to overcome this drawback, plain ATPS can be modified by attaching biospecific affinity ligands to one of the aqueous phases (2). This approach is termed *affinity partitioning*.

Free ligands, when used in polymer–polymer ATPS, such as polyethylene glycol (PEG)/dextran, partition in both the phases. Therefore, it is desired to bind ligands covalently to one of the phase-forming polymers. Thus, in most of the earlier work dealing with affinity partitioning, affinity ligands (AL) were covalently bound to PEG in the PEG/dextran system (3, 4). Recently, Wang et al. (5) suggested that free ligands can be used in PEG/salt systems for the separation of enzymes. These PEG/salt systems have some advantages including low cost and ease of handling. Apart from the work of Wang et al. (5), practically no information on the extraction of proteins by affinity partitioning in PEG/salt system is available in the literature. Therefore, it was thought desirable to further explore this approach for a few selected enzymes of the dehydrogenase and kinase classes.

In the present work, various affinity ligands have been screened in terms of their partitioning ability for alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PDH), and hexokinase (HK). A systematic study of the effects of various parameters, such as pH of the system, phase composition, molecular weight of PEG, and concentration of ligands, on partition coefficients of the selected enzymes has been carried out.

MATERIALS

Pressed bakers' yeast was used as the source of the enzymes. PEG and potassium phosphate were used for the formation of two aqueous phases in water. PEG (6000) and potassium phosphate were obtained from Loba Chemie Pvt. Ltd. (Bombay, India). PEG (4000) was purchased from S. d. Fine Chem. Ltd. (Bombay, India).

All substrates and coenzymes used in the enzyme assays, were purchased from S.R.L. Chemicals (Bombay, India). Reactive triazine dyes were kindly gifted by ATIC Industries (Balsar, India), and Cibacron Blue F-3G-A was procured from Ciba-Geigy (UK). All the chemicals used in the present work were of analytical grade.

EXPERIMENTAL TECHNIQUES

The aqueous two-phase system was prepared by employing the equilibrium phase compositions given by Albertsson (6) (Table 1). The phases were separated into a PEG-rich top phase and a salt-rich bottom phase.

TABLE 1
Phase Compositions of the System (w/w %) Polyethylene Glycol (4000)/Potassium Phosphate (K_2HPO_4 + KH_2PO_4) (6)[a]

Tie line	PEG (%)	K_2HPO_4 (%)	KH_2PO_4 (%)	Water (%)
1	9.55	7.36	3.68	79.44
2	9.42	7.58	3.99	79.20
3	9.19	7.98	3.99	78.84

[a] System pH = 7. 28°C.

A known quantity of pressed bakers' yeast was added to a known volume of the PEG phase. The homogeneous suspension was subjected to acoustic cavitation by using a horn-type ultrasonic generator (22.7 kHz frequency, 4 μm amplitude, 240 W power) for the release of intracellular enzymes. The ultrasonicated yeast suspension was centrifuged (1000 rpm for 20 minutes) to separate the released enzymes from the cell debris. A measured volume of this PEG solution was equilibrated with a known volume of the phosphate phase. The initial and final activities of the enzymes in the PEG phase were assayed spectrophotometrically (7, 8).

The partition coefficient was defined as the ratio of activity of the enzyme in the top phase to that in the bottom phase (1, 6). The partition coefficients for the selected enzymes were determined in the presence as well as absence of ligands.

In order to obtain suitable conditions for extraction of enzymes and proteins by liquid–liquid partitioning, various parameters, such as pH of the system, phase composition, concentration of ligand, and molecular weight of PEG, were investigated.

RESULTS AND DISCUSSION

The partitioning of ligands was studied initially in plain PEG and salt systems. Since the ligands are not bound to PEG in the present case, it was desirable to study the partitioning of ligands in the PEG/salt system. A PEG phase containing a known concentration of ligand was equilibrated with a salt phase for this purpose.

It was observed that the K values for various ligands were very high (>100), implying that the ligands partitioned preferentially in the polymer phase. This may be due to interactions between ligand and PEG. The ligands contain monochloro- or dichlorotriazine functional groups (Fig. 1). Monochloro- and dichlorotriazines have high affinity for hydroxyl

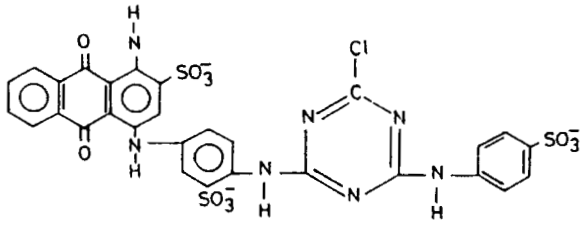

FIG. 1 Structural formula of Cibacron Blue F-3G-A.

functions, particularly in open chains or polymeric structures like cellulose. In the present case the hydroxyl group of PEG shows affinity toward the ligands and gets physically bound to them. This is predominant at lower temperatures whereas at higher temperatures a covalent bond, which is much more stable, is formed (9).

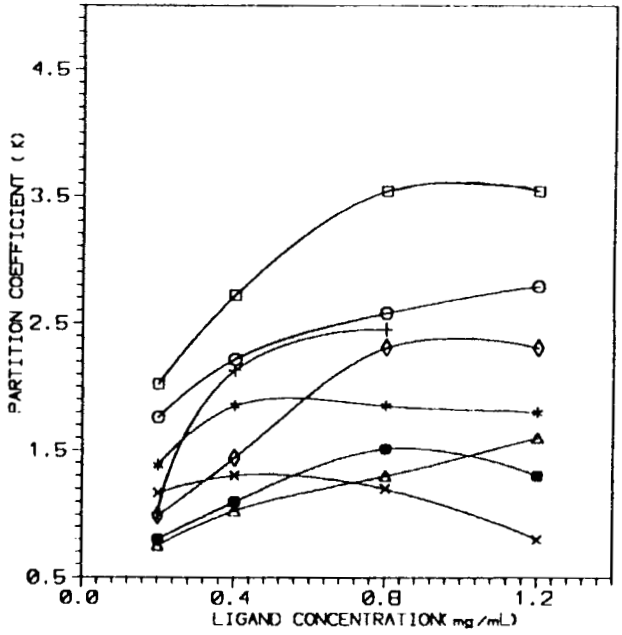

FIG. 2 Effect of ligand concentration on partitioning of ADH. System pH = 7. Tie line 2 phase composition. (○) K_{CB}, (□) K_{PB}, (◇) K_{PT}, (+) K_{PG}, (×) K_{PY}, (*) K_{PR8}, (△) K_{PR7}, (●) K_{PR5}.

Effect of Ligand Type and Concentration

In order to obtain a suitable type of ligand for partitioning of an enzyme, various types of ligands of known concentration were added to the phase system. Further, the concentrations of ligands were varied to observe the effect of ligand concentration on partitioning of the selected enzymes. It can be observed from Figs. 2, 3, and 4 that the partition coefficients increase up to a certain ligand concentration, after which there is negligible change in K. Table 2 gives the partition coefficients of the selected enzymes in the absence of ligand. The effectiveness of a ligand for partitioning a given enzyme can be quantified in the form of an enhancement factor, ϕ, defined as the ratio of partition coefficient in the presence of ligand to that in the absence of ligand. The highest ϕ values for ADH, HK, and G6PDH were obtained with Procion Blue, Procion Green, and Procion Navy Blue dyes, respectively. With Procion Yellow, a decrease in K with increasing concentration of dye was observed. A possible explanation for the constancy in K at relatively high ligand concentrations is as follows.

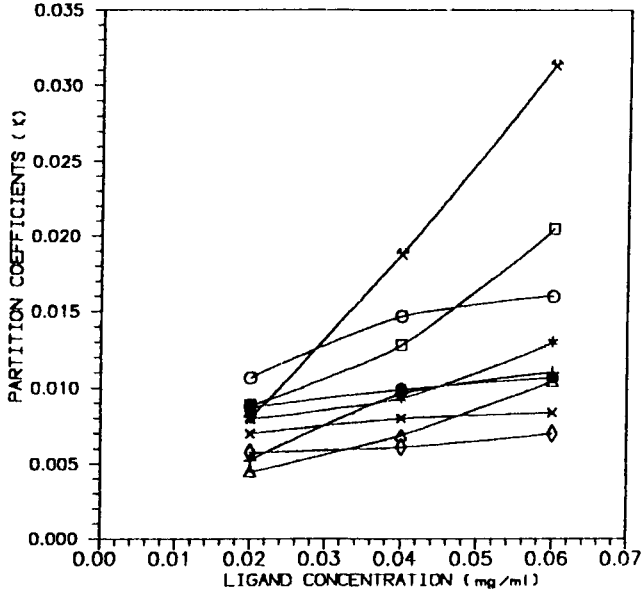

FIG. 3 Effect of ligand concentration on partitioning of G6PDH. System pH = 7. Tie line 2 phase composition. (○) K_{CB}, (□) K_{PB}, (◇) K_{PT}, (+) K_{PG}, (×) K_{PY}, (*) K_{PR8}, (△) K_{PR7}, (●) K_{PR5}, (×) K_{PNB}.

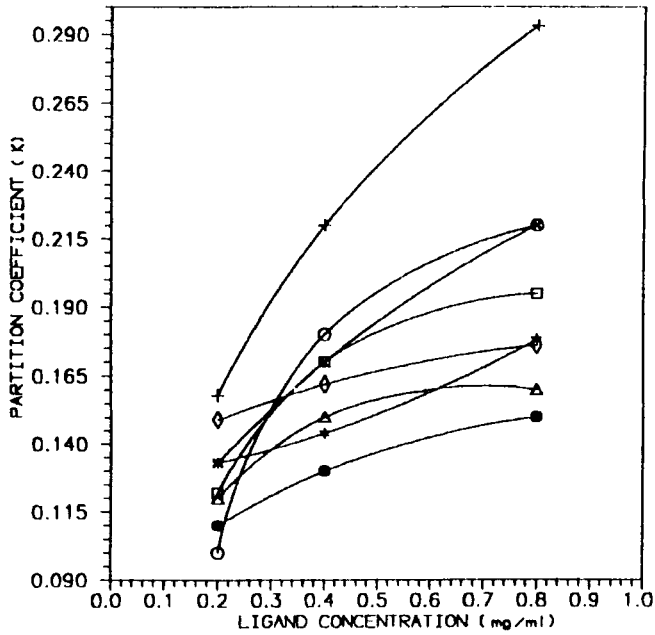

FIG. 4 Effect of ligand concentration on partitioning of HK. System pH = 7. Tie line 2 phase composition. (○) K_{CB}, (□) K_{PB}, (◇) K_{PT}, (+) K_{PG}, (×) K_{PY}, (*) K_{PR8}, (△) K_{PR7}, (●) K_{PR5}.

It is known that there are specific interactions between the enzyme and dye. These interactions are relatively weak but nevertheless must have a stoichiometry. Thus, for a given enzyme–dye pair, the binding can be represented by

$$\text{Enzyme} + Z\ \text{dye} > \text{enzyme–dye complex}$$

TABLE 2
Partition Coefficients of the Enzymes without Ligands[a]

No.	Enzymes selected	K_w
1	Alcohol dehydrogenase	1.08
2	Glucose-6-phosphate dehydrogenase	0.00447
3	Hexokinase	0.105

[a] System pH = 7. 28°C. Phase composition: tie line 2. 9.42% PEG (4000) + 7.58% K_2HPO_4 + 3.99% KH_2PO_4 + 79.2% water.

Initially, when the ligand concentration is low, it is possible that the dye may be the limiting component in the reaction equation. At this stage, increasing the dye concentration makes more sites available for enzyme binding, and K increases rapidly. However, when the dye concentration reaches the stoichiometric quantity for a given enzyme concentration, a further increase in dye concentration converts the enzyme into the limiting or stoichiometrically deficient component. Beyond this point, the dye concentration would obviously have no effect on K.

Basically, protein molecules contain polar and nonpolar groups on their surface. The polar groups act as catalytic sites and nonpolar groups act as hydrophobic sites on the protein surface. The increase in K with increasing ligand concentration can be attributed to hydrophobic interactions between the enzyme and ligand (1, 6). This behavior was observed for all the dyes except Procion Yellow.

Effect of Phase Composition on Partitioning

The phase compositions were varied to determine their effect on partitioning. It can be observed from Tables 3, 4, and 5 that K increases from tie line 1 phase composition (Table 3) to tie line 2 phase composition and subsequently decreases from tie line 2 to tie line 3 phase composition.

It is likely that the increase in K may be due to decreased solubility of proteins with an increase in salt concentration. This may lead to salting out of proteins (1).

Effect of pH on Partitioning

The partitioning of all enzymes was studied in the basic range in which the enzymes are active (8). It can be observed from Fig. 5 that ADH, when partitioned at acidic pH, exhibits no activity, while it shows appreciable

TABLE 3
Effect of Phase Composition on Partitioning of Alcohol Dehydrogenase[a]

Tie line	$K_{\text{without dye}}$	K_{PB}	K_{CB}	Enhancement factor ϕ_{PB}	Enhancement factor ϕ_{CB}
1	1.01	2.36	2.3	2.33	2.27
2	1.08	3.54	2.58	3.27	2.38
3	0.589	0.937	0.833	1.59	1.41

[a] System pH = 7. Concentration of ligand: 0.8 mg/mL. 28°C.

TABLE 4
Effect of Phase Composition on Partitioning of Glucose-6-phosphate Dehydrogenase[a]

Tie line	K_w	K_{PB}	K_{CB}	K_{PNB}	ϕ_{PB}	ϕ_{CB}	ϕ_{PNB}
2	0.00447	0.0128	0.01469	0.0188	2.86	3.29	4.20
3	0.00195	0.0063	0.00653	0.00671	3.25	3.35	3.44

[a] System pH = 7.25. Concentration of ligand: 0.04 mg/mL. 28°C.

TABLE 5
Effect of Phase Composition on Partitioning of Hexokinase[a]

Tie line	$K_{without\ dye}$	K_{PG}	ϕ_{PG}
1	0.095	0.25	2.63
2	0.105	0.293	2.79
3	0.046	0.097	2.10

[a] System pH = 7. Concentration of ligand: 0.8 mg/mL. 28°C.

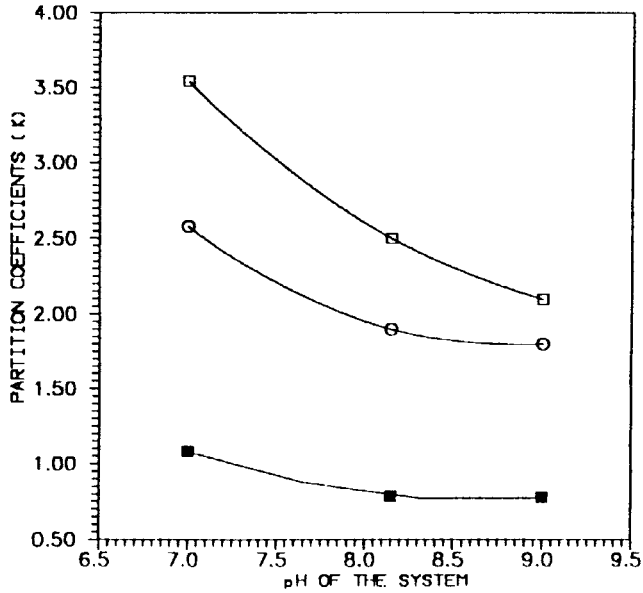

FIG. 5 Effect of pH on partitioning of alcohol dehydrogenase. Ligand concentration = 0.8 mg/mL. Tie line 2 phase composition. (■) K_w, (□) K_{PB}, (○) K_{CB}.

TABLE 6
Effect of PEG Molecular Weight on Partitioning of Alcohol Dehydrogenase[a]

Molecular weight	$K_{without\ dye}$	K_{PB}	K_{CB}	Enhancement factor ϕ_{PB}	Enhancement factor ϕ_{CB}
4000	1.08	3.54	2.58	3.27	2.38
6000	0.42	0.70	0.55	1.658	1.315

[a] System pH = 7. Concentration of ligand: 0.8 mg/mL. 28°C. Phase composition: tie line 2. 9.42% PEG + 7.58% K_2HPO_4 + 3.99% KH_2PO_4 + 79.2% water.

TABLE 7
Effect of PEG Molecular Weight on Partitioning of Glucose-6-phosphate Dehydrogenase[a]

Molecular weight (PEG)	K_w	K_{CB}	K_{PNB}	K_{PB}	ϕ_{CB}	ϕ_{PNB}	ϕ_{PB}
4000	0.00447	0.01469	0.01881	0.01281	3.28	4.20	2.86
6000	0.00248	0.00399	0.00498	0.00408	1.60	2.01	1.64

[a] System pH = 7.25. Concentration of ligand: 0.04 mg/mL. 28°C. Phase composition: tie line 2. 9.42% PEG + 7.58% K_2HPO_4 + 3.99% KH_2PO_4 + 79.2% water.

activity at pH 7. A subsequent decrease in activity is observed at pH 8 and 9. The explanation for this behavior is as follows.

The proteins carry a positive charge at low pH values and a negative charge at high pH values. The isoelectric point for ADH and G-6-PDH are 5.4 and 4.5 (5). Therefore, the protein carries a negative charge at pH 7 and higher. An increase in pH may induce conformational changes in protein structure, due to which K decreases at pH 9.

Effect of Molecular Weight of PEG on Partitioning

The effect of the molecular weight of PEG was studied under standardized system conditions of pH 7, optimum ligand concentration, and tie line 2 phase composition. It can be observed from Tables 6, 7 (see Fig.

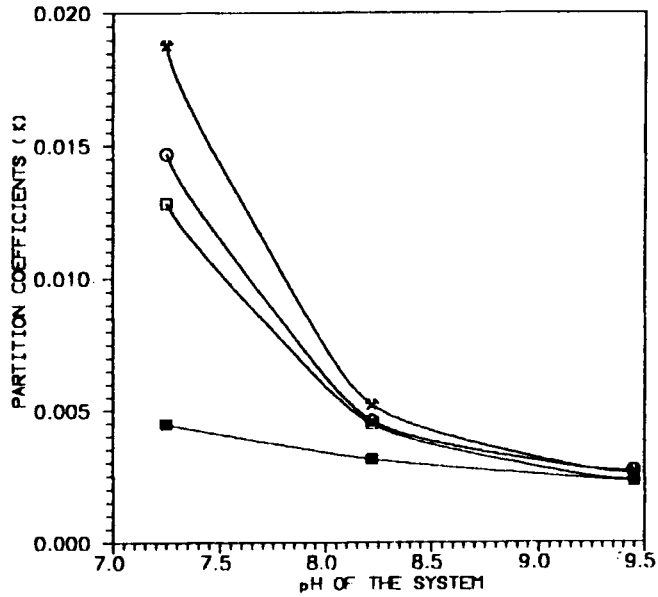

FIG. 6 Effect of pH on partitioning of glucose-6-phosphate dehydrogenase. Ligand concentration = 0.04 mg/mL. Tie line 2 phase composition. (■) K_w, (□) K_{PB}, (○) K_{CB}, (×) K_{PNB}.

6), and 8 (see Fig. 7) that there is an increase in K with a decrease in the molecular weight of PEG. This is probably due to the excluded volume effect of PEG (5). Thus, it is observed that PEG with a lower molecular weight promotes the partitioning of enzymes.

TABLE 8
Effect of PEG Molecular Weight on Partitioning of Hexokinase[a]

Molecular weight (PEG)	$K_{\text{without dye}}$	K_{PG}	Enhancement Factor, ϕ_{PG}
4000	0.105	0.293	2.79
6000	0.039	0.087	2.23

[a] System pH = 7. Concentration of ligand: 0.8 mg/mL. 28°C. Phase composition: tie line 2. 9.42% PEG + 7.58% K_2HPO_4 + 3.99% KH_2PO_4 + 79.2% water.

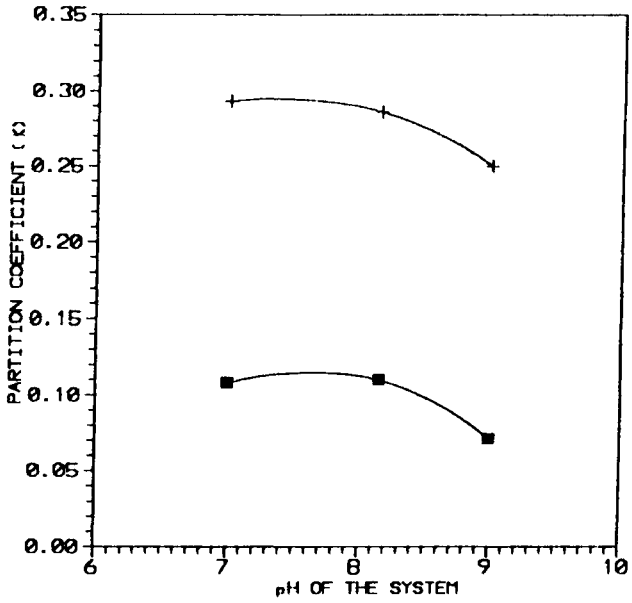

FIG. 7 Effect of pH on partitioning of hexokinase. Ligand concentration = 0.8 mg/mL. Tie line 2 phase composition. (□) K_w, (+) K_{PG}.

CONCLUSIONS

Aqueous two-phase systems can be employed with advantage for the extraction of biologically active proteins from a biomass. It was found that the free reactive triazine dyes not bound to PEG preferentially partitioned in the PEG phase in the PEG/phosphate system. The effect of various parameters such as types and concentrations of affinity ligands, pH of the system, molecular weight of PEG, and phase composition of the system on partitioning of ADH, G6PDH and HK was determined. It was observed that the affinity ligands gave an enhancement of two to three times in partition coefficient over that in the absence of the ligand.

NOMENCLATURE

ADH	alcohol dehydrogenase
G6PDH	glucose-6-phosphate dehydrogenase

HK	hexokinase
K	partition coefficient
K_w	partition coefficient without ligand
K_{CB}	partition coefficient with Cibacron Blue F-3G-A
K_{PB}	partition coefficient with Procion Blue M-R
K_{PT}	partition coefficient with Procion Turquiose M-GN-4
K_{PG}	partition coefficient with Procion Green HE-4BD
K_{PY}	partition coefficient with Procion Yellow M-3R
K_{PR8}	partition coefficient with Procion Red HE-8B
K_{PR7}	partition coefficient with Procion Red HE-7B
K_{PR5}	partition coefficient with Procion Red HE-5B
K_{PNB}	partition coefficient with Procion Navy Blue HE-R
ϕ	enhancement factor
ϕ_{PB}	enhancement factor with Procion Blue M-R
ϕ_{CB}	enhancement factor with Cibacron Blue F-3G-A
ϕ_{PNB}	enhancement factor with Procion Navy Blue HE-R
ϕ_{PG}	enhancement factor with Procion Green HE-4 BD

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