Journal of Chromatography, 597 (1992) 315–322
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 033

Immunoenzymatic assay of dyes currently used in affinity chromatography for protein purification

P. Santambien, P. Girot, I. Hulak and E. Boschetti*

SEPRACOR Bioprocessing Division, 92390 Villeneuve La Garenne (France)

ABSTRACT

Cibacron Blue F3-GA, Basilen Blue E3-G and Procion Red HE-3B are dyes currently used in affinity purification, and are commonly determined by spectrophotometry with limited sensitivity. An assay method is described based on a specific immunochemical recognition of the dyes amplified by a final enzymatic reaction. The sensitivity is close to 1 ng/ml of dye and the method is applicable any time that sensitive and accurate results are necessary. This method has actually been applied with success to the determination of trace amounts of dyes in the presence of affinant protein. The method was also applied to the demonstration of dye leaching from affinity sorbents when treated under acidic and/or alkaline conditions.

INTRODUCTION

The use of reactive dyes in pseudo-affinity chromatography is a frequent choice in the separation of biological substances [1,2]. This choice can be explained by the wide variety of dye molecules associated with their interactions with biological macromolecules that sometimes allow such a high specificity that the protein of interest can be purified from a complex mixture in a single step [3]. To this important advantage must be added their ready availability at low cost and their chemical stability. Owing to these features, one can envisage the specificity of the dye ligands being exploited on an industrial scale [4].

However, one of the drawbacks frequently mentioned in the use of immobilized dyes in protein purification is leakage, with the risk of finding traces of dyes in the final biological preparation. The classical spectrophotometric methods for determining dyes are considered to be very low sensitivity for this application (*ca.* 1 μg/ml).

In this paper, we describe a sensitive approach to the quantification of dyes based on an immunochemical recognition. This method has been applied to the determination of the leaching mechanism of dye sorbents and to the determination of trace amounts of dyes in the presence of proteins. The investigation was focused on three major dyes used in affinity chromatography: Cibacron Blue F3-GA [5], Basilen Blue E-3G and Procion Red HE-3B [6]. The first two have similar structures, except for the position of one sulphonic group on an aromatic ring (see Fig. 3), and very similar behaviour as affinity ligands. They are both used to synthetize "Blue" dye affinity sorbents.

EXPERIMENTAL

Abbreviations

CB = Cibacron Blue F3-GA; BB = Basilen Blue E-3G; PR = Procion Red HE-3B; ELISA = enzyme-linked immunosorbent assay; KLH = keyhole limpet haemocyanin; BHb = bovine haemoglobin; HSA = human serum albumin; IgG = immunoglobulin G; PBS = phosphate-buffered saline; PBST = phosphate-buffered saline containing 0.02% Tween 20; PBST/BHb = phosphate-buffered saline containing 0.02% Tween 20 and 0.5% bovine haemoglobin; PNPP = *p*-nitrophenyl phosphate disodium salt. Cibacron Blue F3-GA and Procion Red HE-3B are trade names of Ciba Geigy and ICI, respectively.

0021-9673/92/$05.00

Chemicals

Reactive dyes Cibacron Blue F3-GA, Basilen Blue E-3G and Procion Red HE-3B (also called Reactive Red 120), KLH, HSA, BHb, goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase and *p*-nitrophenyl phosphate disodium salt (PNPP) were purchased from Sigma (St. Louis, MO, USA). Chemical derivatives of dyes were a generous gift from Vilmax (Buenos Aires, Argentina). Chromatographic sorbents were obtained from the following suppliers: Blue Sepharose, Pharmacia-LKB Biotechnology (Uppsala, Sweden); Red Agarose, Sigma; and Blue Trisacryl, Red Trisacryl and Trisacryl GF05, IBF (Villeneuve La Garenne, France).

NUNC Immuno MAXISORP 96-well microtitre plates were obtained from Poly Labo (Strasbourg, France). Tween 20 was supplied by Aldrich (Strasbourg, France). Plasminogen was a gift from Dr. O. Bertrand (INSERM U 76, Paris, France).

Dye coupling on proteins

Dyes were conjugated to KLH for the immunization of rabbits and to BHb for the coating of microtitre plates.

A 50-mg amount of protein (KLH or BHb) was dissolved in 12 ml of 0.01 *M* carbonate buffer (pH 10.5) and then 50 mg of reactive dye CB or PR were added. The mixture was shaken overnight at room temperature and desalted to remove buffer salts and excess of dye through a column of Trisacryl GF05 (10 cm × 2.5 cm I.D.). The protein–dye complex was then lyophilized and stored at 4°C.

Spectrophotometric analysis indicated that 10–15 mol of dye were coupled per mole of protein.

Rabbit immunization

Two rabbits per KLH–dye conjugate were immunized according to a classical protocol in the presence of Freud adjuvant [7]. Antisera were obtained after coagulation of rabbit blood and centrifugation to separate clotted material. Antiserum was then used without any other operation (except dilution; see below) for the immunochemical determination of dyes.

ELISA protocol by competitive inhibition

Microtitre plates were first coated with BHb–dye conjugates according to a classical method [8]. Briefly, 100 μl per well of BHb–dye conjugate at 100 μg/ml in 100 m*M* carbonate buffer (pH 10.5) were incubated for 2 h at 37°C. After washing the microwell surface was saturated with 200 μl of free BHb (3% in PBST) overnight at 4°C before extensive washing with PBST for keeping or for direct use in the ELISA test.

A 50-μl volume of dye standard solution or sample to be assayed was introduced into each precoated well, followed immediately by the addition of 100 μl of PBST/BHb diluted antiserum (the optimum dilution was determined to be 1:2000). The mixture was incubated for 1 h at room temperature; each well was then rinsed three times with 200 μl of PBST-BHb and 100 μl of goat anti-rabbit IgG antibodies coupled to alkaline phosphatase (previously diluted to 1:500 in PBST/BHb) were added. Incubation for 60 min was necessary to complete the antigen–antibody interaction. After elimination of anti-IgG phosphatase alkaline conjugate in excess by washing each well three times with PBST (200 μl per well), enzymatic reaction leading to the formation of the *p*-nitrophenol chromophore was performed by addition of 200 μl per well of phosphatase substrate (PNPP) at 1 mg/ml in 0.5 *M* carbonate buffer (pH 10.6) containing 1 m*M* $MgCl_2$.

Finally, the absorbance was mesured at 405 nm on an automatic microtiter plate reader. All assays were done in triplicate.

Determination of trace amounts of dyes in the presence of specific proteins

The major practical interest in the use of an ELISA-based test to determine trace amounts of dyes is its applicability in the field of quality control for the measurement of dye contaminants in biological preparations containing proteins with affinity for the explored dye.

In this context, trials have been effected by adding, first, BB in HSA solutions (HSA is widely known for its strong interaction with CB or BB [3,9], and second, PR in plasminogen solutions (plasminogen has been purified on immobilized PR [10]). In another experiment, BB and PR were mixed at different concentrations, added to a complex protein mixture and then assayed using both dye antibodies separately.

In the first instance, two series of experiments were performed by mixing into the protein solu-

tions different amounts of dyes at concentrations between 1.2 and 5000 ng/ml. HSA and plasminogen concentrations were in the range 1–50000 ng/ml. All results were compared with those for standard solutions of dyes without proteins.

In the second instance, different concentrations of BB and PR mixtures (containing between 10 and 500 ng/ml for each dye) were added to a sweet whey solution containing mainly α-lactalbumin, β-lactoglobulin and γ-globulins, previously diluted in PBST-BHb to 1:200. The final protein concentration was 0.71 mg/ml in each sample. The results were analysed in comparison first with controls (dyes alone) and second with values obtained with the same dye mixtures without any addition of proteins.

Assay of leached dyes from dye affinity sorbents

One of the major applications of a sensitive dye assay is to check the leaching level of a dye chromatographic sorbent and its progressive elimination by washing. This generally occurs when sorbents are submitted to drastic sequences of regeneration and clean-up with acidic or alkaline solutions.

In this respect, 2 ml of Blue and Red sorbents were incubated with 2 ml of buffers at different pH values between 0.3 and 14 for 24 h at room temperature. The chromatographic sorbents tested were Blue Sepharose and Red Agarose (based on cross-linked agarose) and Blue and Red Trisacryl (based on a synthetic cross-linked polymer). After incubation, each supernatant was collected by centrifugation, neutralized and assayed. The buffers used were hydrochloric acid for pH 0.3–1.0, 0.5 *M* glycine–HCl for pH 2.5, 0.5 *M* acetate buffers for pH 3.5–5,0; 0.5 *M* citrate–phosphate buffers for pH 3.5–7.0, 0.5 *M* carbonate buffers for pH 9–11 and 0.5 *M* sodium hydroxide for pH 13.7.

RESULTS AND DISCUSSION

As shown in Fig. 1, it appeared that the most appropriate anti-dye antiserum dilution for the ELISA test was *ca.* 1:2000. Such a dilution corresponds for each dye concentration studied (from 62.5 ng/ml to 1 μg/ml) to the middle of the linear portion of the dose–response curves.

Similar results were obtained with anti BB and anti-PR antisera. They were therefore used at the same dilution (1:2000) for the development of the dye detection ELISA system.

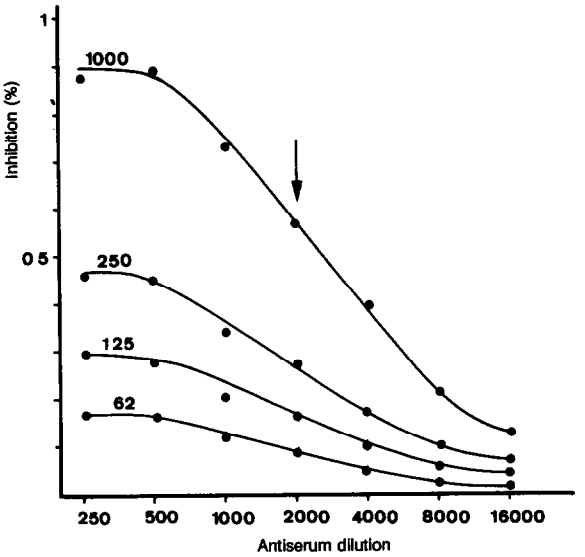

Fig. 1. Dilution curves of anti-CB antibodies at different dye concentrations. The arrow indicates the optimum zone of antiserum dilution (50% of the maximum absorbance) for each concentration of dye. Numbers on the curves indicate dye concentrations in ng/ml.

The typical competitive inhibition curves shown in Fig. 2 indicate that the 50% inhibition point was

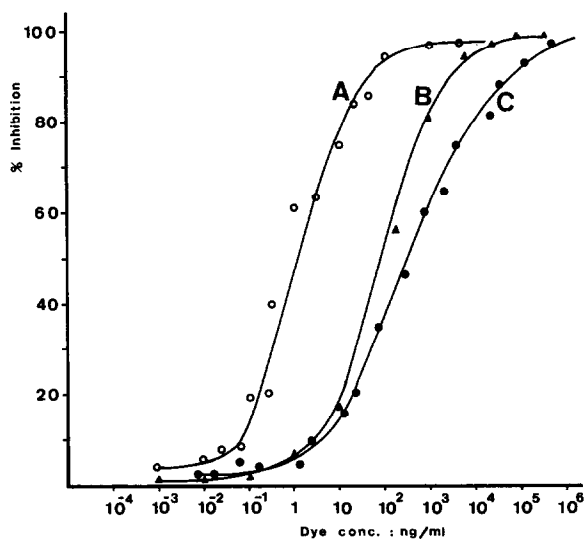

Fig. 2. Calibration graphs for determination of dyes using the competitive inhibition ELISA method: (A) Cibacron Blue F3-GA; (B) Basilen Blue E-3G; (C) Procion Red HE-3B.

reached with a concentration of about 1 ng/ml for CB, 150 ng/ml for BB and 500 ng/ml for PR. The sensitivity for these dyes was calculated to be *ca.* 0.1 ng/ml for CB, 1 ng/ml for BB and 10 ng/ml for PR. These levels of sensitivity are substantially higher (between 10^{3-} and 10^4-fold) than those obtained using spectrophotometric analysis where the threshold is close to 1 μg/ml. However, these values were valid only for native dyes.

To determine the specificity of the immunoenzymatic assay, several dye derivatives were synthesized and tested (Fig. 3). Derivatization were effected by the introduction of chemical substituents through the mobile chlorine of the triazine ring. Cross-reactions were then investigated with these derivatives.

When determining dose–response curves of dye-derivatives (Fig. 4), different results were obtained. The numerous trials indicated that the anti-CB antibodies recognized all chemical derivatives of CB. Conversely, they did not show any positive reaction with PR and PR derivatives. The 50% inhibition point and the level of sensitivity were modulated by the chemical nature of the substituent, however. This general behaviour was also verified in the case of anti-BB and anti-PR antibodies. In fact, anti-Red and anti-Blues antibodies (anti-Basilen and anti-Cibacron) did not cross-react with blue dyes (CB and BB) or red dyes, respectively, even at high concentrations. This absence of cross-immunochemical reaction demonstrated that the triazine ring which is a common part of the three molecules does not

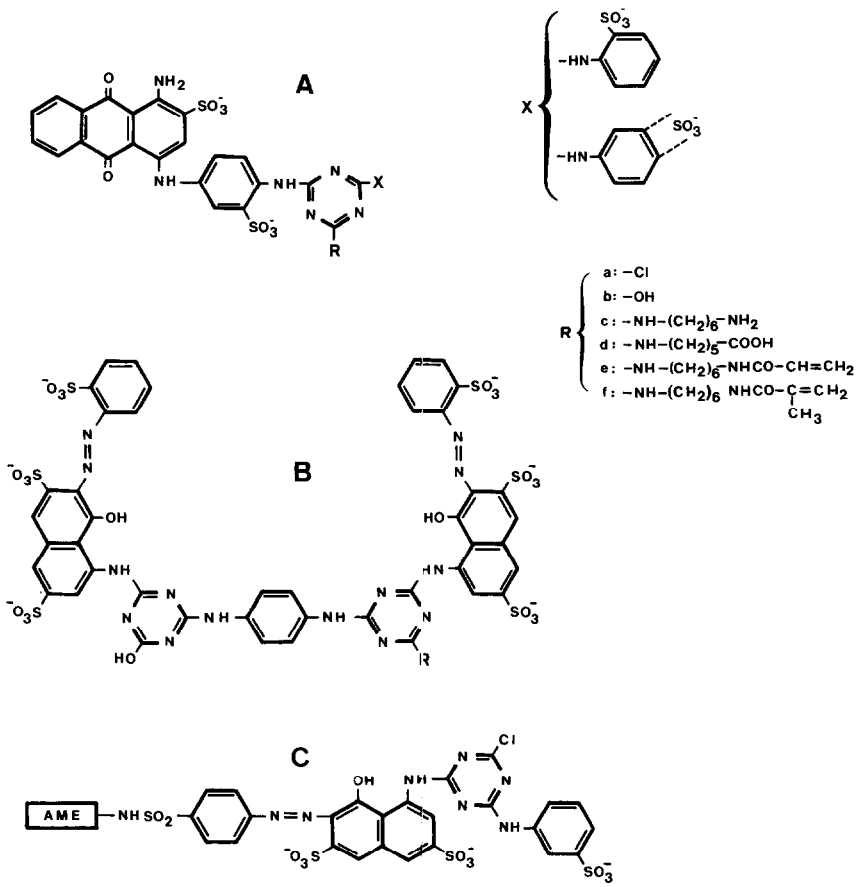

Fig. 3. Structures of CB, BB (A) and PR (B) and their derivatives. R is a chlorine in the case of native dyes (a) and is replaced by substituents b, c, d, e and f to give the corresponding derivatives. ME 29708 (C) is a special structure with some common parts compared with PR.

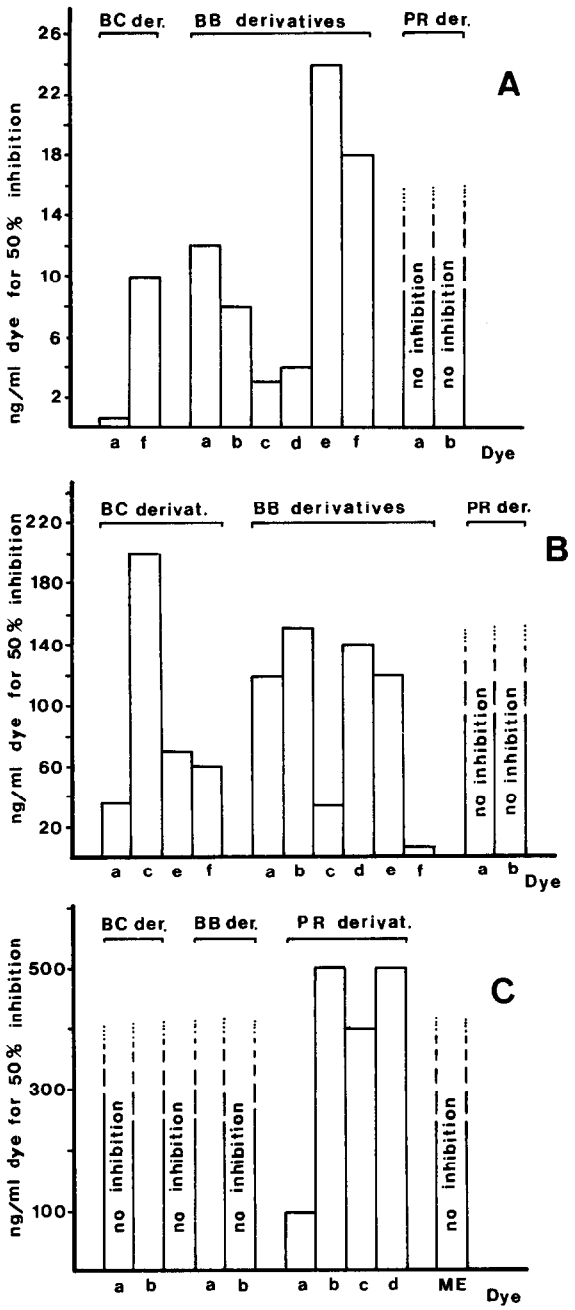

Fig. 4. Concentration of dye giving 50% immunochemical reaction inhibition with anti-CB (A), anti-BB (B) and anti-PR (C) antibodies; a–f refere to the structures shown in fig. 3.

play a fundamental role in the recognition mechanism.

It should be noted, for example, that a large difference in the test sensitivity exists when using anti-BB antibodies with a "c"-dye derivative (5 ng/ml for 50% inhibition) and with an "e" derivative (24 ng/ml for 50% inhibition). This is also true with anti-BC and PR antibodies (see Fig. 4).

Anti-PR antibodies were also tested against a dye (ME 29708; see Fig. 3C) which contains in addition to the triazine ring another important commun structure with PR (the naphthyl ring with OH and sulphonic groups linked via a diazonium salt). Even with these chemical similarities, the interaction between PR antibodies and the dye was negative, at least in the range of concentrations studied (5 pg/ml–10 μg/ml).

Results from the determination of trace amounts of dyes in the presence of proteins are shown in Tables I and II. HSA in the case of BB and plasminogen for PR did not significantly modify the results of the assays in the concentration range studied. This is an important feature when one knows that HSA and plasminogen have a strong affinity for BB (or CB) and PR from which they have been purified by affinity chromatography [3,4,10]. The presence of such proteins, however, was not able to alter the recognition of the dyes by the antibodies under the described conditions. These results are of importance and suggest that this kind of ELISA test could be applied to the determination of trace amounts of dyes in biochemical preparations when dye leaching from a chromatographic sorbent occurs even at very low level.

Table II shows the results of the determination of BB and PR simultaneously present either in a buffer (PBST/Hb) or in a prediluted (1:200 in the same buffer) complex biological liquid (sweet whey) containing numerous proteins, the major ones being α-lactalbumin, β-lactoglobulin and immunoglobulins. This experiment was designed to verify that biological substances which could be purified from complex protein mixtures by sequential affinity chromatography on different immobilized dyes could be tested for the presence of trace amounts of dye(s) only in the final purification step. In fact, as previously mentioned, dye affinity chromatography is particularly adapted to the one-step purification of biological substances from complex protein solutions such as serum or plasma. However, the absence of trace amounts of dyes in the final product could be a critical point, particularly when the pro-

TABLE I

DYES IN THE PRESENCE OF INTERACTING PROTEINS

Protein	Protein concentration (ng/ml)	Inhibition of immunochemical reaction (%)				
		CB[c] concentration (ng/ml)				
		1.22	19.5	78.1	313	5000
HSA[a]	0 (control	2.5	8.5	18.6	37.0	78.2
	1	2.77	8.33	16.11	33.47	75.28
	10	0	6.14	18.05	36.39	74.17
	100	0	9.17	22.22	33.47	73.33
	1000	4.86	11.39	18.47	31.25	71.53
	10 000	7.64	12.5	15.00	30.97	76.97
		PR[d] concentration (ng/ml)				
		1.22	19.5	78.1	313	5000
PL[b]	0 (control)	4.94	17.45	27.89	39.79	84.82
	1	3.91	13.58	25.15	39.10	93.26
	10	2.94	12.66	22.80	36.03	93.55
	100	0	12.37	23.36	37.47	93.51
	1000	0	11.87	20.86	35.83	91.61
	10 000	0	14.93	22.48	31.94	92.35
	50 000	5.77	17.33	25.08	38.15	92.35

[a] Human serum albumin.
[b] Plasminogen.
[c] Cibacron Blue F3-GA.
[d] Procion Red HE-3B.

tein is intended for *in vivo* diagnostic or therapeutic use.

Taking in account the standard deviation due to the experimentation itself, no significant difference appeared between the samples containing only mixed dyes and those containing in addition a large excess of proteins (protein/dye ratio *ca.* 1400). The presence of proteins at high concentrations seems to

TABLE II

DETERMINATION OF BASILEN BLUE E-3G (BB) AND PROCION RED HE-3B (PR) SIMULTANEOUSLY PRESENT IN SWEET WHEY

Dyes concentration (ng/ml)		Inhibition (%)					
		Controls		Mixed dyes alone		Mixed dyes with proteins of sweet whey (710 μg/ml)	
BB	PR	BB	PR	BB	PR	BB	PR
500	10	42.0	14.0	51.8	15.7	58.6	18.9
350	150	38.0	37.0	51.2	42.5	57.6	44.8
250	250	35.0	40.0	40.8	43.8	42.9	48.6
150	350	31.0	43.0	34.4	48.4	40.4	49.2
10	500	13.0	45.0	16.0	53.6	7.8	52.0

TABLE III

DETERMINATION OF DYE LEACHING FROM SORBENTS TREATED AT DIFFERENT pH VALUES FOR 24 h AT ROOM TEMPERATURE

pH	Dye released per ml of sorbent (μg)			
	BS[a]	RA[b]	BT[c]	RT[d]
0.3	n.d.[e]	>350	n.d.	3.4
1.0	3.13	n.d.	0.14	n.d.
2.5	n.d.	1.68	n.d.	0
3.5	0.35	4.48	0.07	0.72
5.0	0.84	0	0.28	0.10
7.0	0.91	0	0	0.48
9.0	0	n.d.	0.14	n.d.
9.5	n.d.	0.05	n.d.	2.56
11.0	0.09	1.36	0.28	4.00
13.7	n.d.	68.00	n.d.	57.60
14.0	2.00	n.d.	10.00	n.d.

[a] Blue Sepharose.
[b] Red Agarose.
[c] Blue Trisacryl.
[d] Red Trisacryl.
[e] n.d. = Not determined.

cause no interference with the immunoassay, confirming the reliability of the method.

Another important application of the described assay is in the determination of the leaching level of dye sorbents.

Generally, leaching occurs when the sorbents are submitted to strongly acidic or alkaline regenerations between runs. The treatment of two different kinds of sorbents (natural and synthetic) substituted by dyes at different pH values indicated (Table III) that the dyes were released at acidic and alkaline pH in agarose-based sorbents. At low pH (0.5 *M* HCl), leakage is important with values of *ca.* 100 μg/ml of gel for Blue Sepharose and 375 μg/ml of gel for Red Agarose.

An increase in pH leads to a diminution of leakage (very low values at pH 5–11), but at an extremely alkaline pH (*e.g.*, pH 14), small amounts of leached dyes were found, with values of about 5 μg/ml of sorbent for Blue Sepharose and 50 μg/ml for Red Agarose.

With dyes immobilized on Trisacryl Plus, we did not find any traces of leached dyes at acidic pH; trace amounts of leached dyes were found at strong alkaline pH only, 10 μg/ml for Blue Trisacryl and 60 μg/ml for Red Trisacryl.

These leakages, however, never affected the sorbent performance or sorption capacity. The differences in the behaviour of these sorbents when treated at various pH values could be explained by the chemical nature of the linkage between the dye and the polymeric matrix. Dyes are bound on the agarose moiety directly on a hydroxyl group reacting with the mobile chlorine of the triazine ring; these linkages are actually sensitive to extreme pH values. On the Trisacryl, the dyes are chemically immobilized by means of a spacer arm attached to the polymer through an amido bond, and on the dye side via an alkylamine linkage, with a certain level of sensitivity at alkaline pH only.

Whatever the sensitivity of the chemical bond and the behaviour of the matrices themselves, any dye leakage should be accurately quantified. Before starting a separation cycle, in fact, it could be very important to ensure that all traces of dye have been totally washed out. For this purpose, the described immunoenzymatic assay seems very suitable.

CONCLUSIONS

The use of dye antibodies allowed their utility and efficiency in the quantitative assay of trace amounts of dye to be demonstrated using an immunoenzymatic method based on competitive inhibition principle. The sensitivity of this approach is much higher than those obtained by spectrophotometric measurement and the method should be applicable to the determination of trace amounts of dyes resulting from leaching mechanisms during affinity purification processes. Apart from its high sensitivity and specificity, the method is applicable in the presence of proteins having a great affinity for the dye itself. This is of importance because the major risk of finding contamination of dyes in biological preparations is actually due to ligand leakage from dye affinity columns, where the protein of interest is first adsorbed and then eluted.

The absence of cross-reactions between dye antibodies and dyes of a different nature makes the existence of false-positive results improbable.

Partial sorbent hydrolysis with subsequent dye release, which might occur during intensive regeneration procedures, can be followed using the devel-

opment immunoenzymatic assay. This is useful to ensure perfect column washing before its use in a separation cycle.

Having demonstrated the applicability of an ELISA method to provide evidence for leaching of material from a chromatographic packing, it could be envisaged that it could be extended and perhaps generalized to most leaching problems in downstream processing, any time that specific antibodies can be prepared.

ACKNOWLEDGEMENTS

This work was partially supported by a grant from the French Ministère de la Recherche et de la Technologie as part of a EUREKA project (EU-384). We express gratitude to Dr. Mazza of Vilmax (Argentina) for the preparation of some dyes and dye derivatives and to Professor M. Monsigny and Dr. O. Bertrand for their valuable help with the technical development of this work.

REFERENCES

1 T. Atkinson, P. H. Hammond, R. D. Hartwell, P. Hughes, M. D. Scaven, R. F. Sherwood, D. A. P. Small, C. J. Bruton, M. J. Harvey and C. R. Lowe, *Biochem. Soc. Trans.*, 9 (1981) 290.
2 R. K. Scopes, *Anal. Biochem.*, 165 (1987) 235.
3 J. Travis and R. Pannel, *Clin. Chim. Acta*, 49 (1973) 49.
4 M. Allary, J. Saint-Blancard, E. Boschetti and P. Girot, *Bioseparation*, 2 (1991) 167.
5 E. Giannazza and Ph. Arnaud, *Biochem. J.*, 203 (1982) 637.
6 D. H. Watson, M. J. Harvey and P. D. G. Dean, *Biochem. J.*, 173 (1978) 591.
7 R. U. Lemieux, D. A. Baker, W. M. Weinstein and C. M. Switzer, *Biochemistry*, 20 (1981) 199.
8 M. M. Palcic, R. M. Ratcliffe, L. R. Lamontage, A. M. Good, G. Alton and O. Hindsgaul, *Carbohydr. Res.*, 195 (1989) 361.
9 R. J. Leatherbarrow and P. D. G. Dean, *Biochem. J.*, 189 (1980) 27.
10 N. Harris and P. Byefield, *FEBS Lett.*, 103 (1979) 162.