*Journal of Chromatography*, 576 (1992) 71–77
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6254

# Single-step method for purification of human transferrin from a by-product of chromatographic fractionation of plasma

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(First received July 23rd, 1991; revised manuscript received December 19th, 1991)

ABSTRACT

A rapid, simple and convenient method is described for the isolation, on a pilot scale, of pure and functional human transferrin from an unexploited by-product of chromatographic fractionation of plasma. In a single chromatographic step on DEAE-Spherodex, 97% pure transferrin was obtained in 75% yield. A virus inactivation treatment was included in the preparative process in order to guarantee the safety of the final product, which could be used in culture media.

## INTRODUCTION

Human plasma transferrin is a monomeric glycoprotein of 678 amino acids, with a relative molecular mass of *ca.* 80 000 [1]. It can bind strongly to one or two ferric ions per molecule [2]. Some experiments have shown that transferrin could act *in vitro* as a growth factor, in addition to its role in iron transport, and it has been described as an essential component in defined culture media [3–6].

With the increasing need to eliminate fetal bovine serum in biotechnological industries, it is important to assess the utility and supply of serum-free media components. For several reasons, it seems preferable to use, in serum-free culture media, components of a human origin rather than components of an animal origin. Moreover, human transferrin used in culture media seems to be more effective than bovine transferrin [7].

Several methods for the separation of transferrin from human serum have been published [8–15], the majority of which involve several steps and laborious techniques, whose yields are often low. Furthermore, they are often impractical for large-scale work. Moreover, plasma or serum are not convenient sources for low-cost preparations of pure transferrin.

Some papers have been published [16–18] concerning the purification of transferrin, using by-products of plasma fractionation as starting materials. These low cost materials are certainly the most suitable sources for a large-scale purification of transferrin.

We have developed [19,20] a chromatographic technique for the purification of human albumin employing the Spherosil-Spherodex process. In its first chromatographic step on DEAE-Spherodex, the process gives rise to a by-product that contains essentially transferrin and immunoglobulin G (IgG). This paper describes the purification, on a pilot scale, of transferrin by an ion-exchange single step using as source material this by-product, which can be virally inactivated before the chromatographic step.

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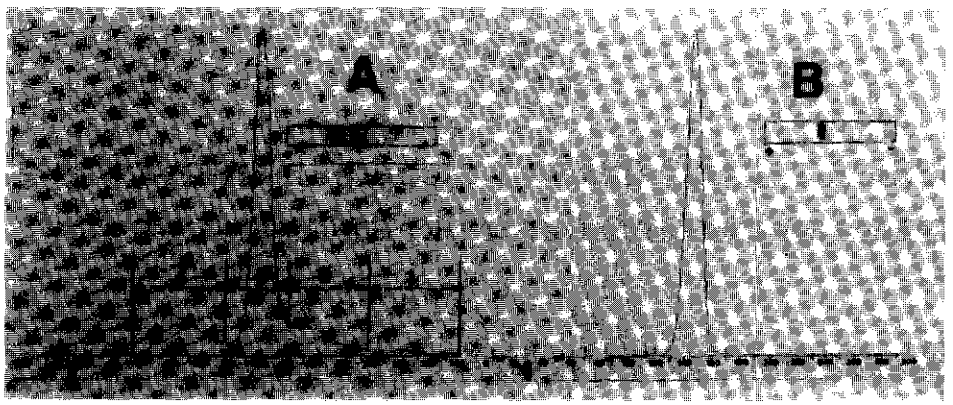


Fig. 1. Cellulose acetate electrophoretic analysis of the starting material D1 fraction (A) and of the transferrin fraction isolated by ion-exchange chromatography on DEAE-Spherodex (B). The migration zones of albumin, $\alpha_1$- + $\alpha_2$-globulins, $\beta$-globulins and $\gamma$-globulins are labelled 1, 2, 3 and 4, respectively.

## EXPERIMENTAL

### *Starting material*

Starting material was a fraction obtained from the albumin purification chromatographic process [19,20]. Briefly, Cohn supernatant II + III was dialysed against 0.01 *M* phosphate buffer (pH 5.2] and injected into a DEAE-Spherodex (Sepracor-IBF, Villeneuve la Garenne, France) column equilibrated in the same buffer. The breakthrough peak, labelled D1, which was not adsorbed on the ion exchanger, constituted the starting material for transferrin purification.

This fraction contained mainly $\beta$-globulins (70–80% of total proteins) as judged by cellulose acetate electrophoresis (Fig. 1A). Study of several D1 samples obtained from different industrial fractionations of Cohn supernatant II + III has shown that these fractions are very variable in composition (Table I). D1 fractions were obtained as a diluted protein solution containing 1–1.5 g of protein per litre.

### *Purification*

The D1 fraction was adjusted to pH 6.4 and 1.8 mS/cm by direct addition of concentrated solutions of NaOH and sodium phosphate. Then, the solution was ready to be injected into a DEAE-Spherodex column equilibrated in a 0.018 *M* phosphate buffer (pH 6.4). The breakthrough peak (immunoglobulin-rich fraction) was discarded. Transferrin was eluted by a 0.01 *M* phosphate, 0.055 *M* NaCl buffer (pH 5.6). The column was regenerated by three successive washings, with 1 *M* NaCl, 0.1 *M* HCl and 60% ethanol–0.5 *M* acetic acid. All the experiments carried out to determine the final optimal conditions of chromatography were performed on a 100-ml DEAE-Spherodex column.

On the pilot scale, a 95 cm × 14 cm I.D. column was used. Starting material was injected at 18 l/h (117 cm/h). The peak containing transfer-

TABLE I

PROTEIN COMPOSITION OF INDUSTRIAL D1 FRACTIONS

Fractions were obtained by DEAE-Spherodex fractionation of Cohn supernatant II + III, according to the method described in refs. 19 and 20. Dara were obtained by cellulose acetate electrophoresis.

| D1 samples | $\gamma$-Globulins (%) | $\beta$-Globulins (%) | $\alpha_1$- + $\alpha_2$-Globulins (%) | Albumin (%) |
|---|---|---|---|---|
| 1 | 10.7 | 81.1 | 3.4 | 4.8 |
| 2 | 14.7 | 69.0 | 15.0 | 1.3 |
| 3 | 18.5 | 74.0 | 6.7 | 0.6 |
| 4 | 9.4 | 72.0 | 3.5 | 15.3 |
| 5 | 21.1 | 75.0 | 3.9 | 0 |

rin was collected, dialysed against ultrafiltered water and freeze-dried.

Prior to chromatography, the starting material was virus-inactivated by treatment either with 0.3% (v/v) tri(*n*-butyl)phosphate (TNBP) and 1% (v/v) Tween 80 at 24°C for 6 h, or with 0.3% TNBP and 0.2% (w/v) sodium cholate at 30°C for 6 h, according to Edwards *et al.* [21].

*Analytical methods*

Protein assays were performed according to Bradford [22] using bovine serum albumin as standard. IgG and IgA assays were carried out by the method of Mancini *et al.* [23].

Cellulose acetate electrophoresis was carried out on a Helena apparatus (Saint Leu la Forêt, France). After migration, the absorbance of the revealed bands was read with a densitometer Profil 1-2 (Sebia, Issy les Moulineaux, France).

Purity was checked by immunoelectrophoresis [24] using horse antihuman whole serum and specific rabbit anti-IgG, anti-IgA, anti-IgM, anti-albumin and anti-transferrin antisera.

Fast protein liquid chromatography (FPLC) was carried out with the Pharmacia system using a Mono Q HR5/5 column. For this purpose a 20 m*M* triethanolamine buffer (pH 7.7) and a gradient from 0 to 1 *M* NaCl in the same buffer were used, respectively. Isoelectrofocusing and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out with the Pharmacia Phast System using Phast Gels IEF 3–9 and Phast Gel Gradient 8–25 respectively, following the manufacturer's instructions. Preparations of transferrin were compared with standard transferrins (Sigma, St. Louis, MO, USA).

The iron saturation coefficient was measured using the Bio Mérieux Fer-kit (Bio-Mérieux, Marcy l'Etoile, France). Iron saturation of transferrin was done according to Mazurier and Spik [25].

TNBP, Tween 80 and sodium cholate were assayed as described by Horowitz *et al.* [26].

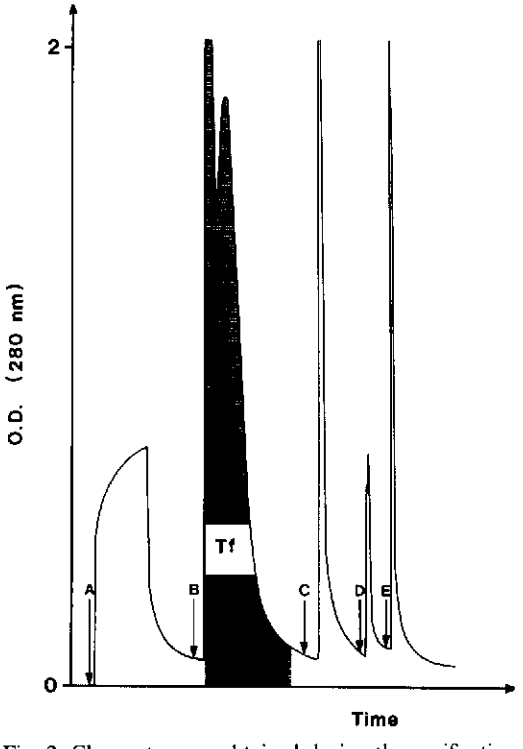


Fig. 2. Chromatogram obtained during the purification of human transferrin from industrial D1 fractions. D1 fractions equilibrated to pH 6.4 and 1.8 mS/cm with NaOH and sodium phosphate were injected (A) into a DEAE-Spherodex column equilibrated in the same buffer. Transferrin was eluted by a buffer containing 0.01 *M* sodium phosphate and 0.055 *M* NaCl (pH 5.6) (B). The column was regenerated by three washings: 1 *M* NaCl (C), 0.1 *M* HCl (D), and 60% ethanol–0.5 *M* acetic acid (E).

## RESULTS

*Transferrin purification*

Preliminary experiments allowed us to determine the most favourable conditions for the chromatographic purification of transferrin contained in D1 fractions. Under the conditions previously mentioned (pH 6.4 and 1.8 mS/cm in conductivity), most of the IgG were found in a breakthrough peak. Transferrin was contained in the peak eluted by the 0.01 *M* $NaH_2PO_4$, 0.055 *M* NaCl buffer (pH 5.6). The peak eluted by 1 *M* NaCl was composed of $\alpha$- and $\beta$-globulins and albumin. The protein content of the two last washes was not determined. A typical chromatogram obtained during the transferrin purification is shown in Fig. 2.

Owing to the variability of the protein composition of D1 fractions, a study of the binding ca-

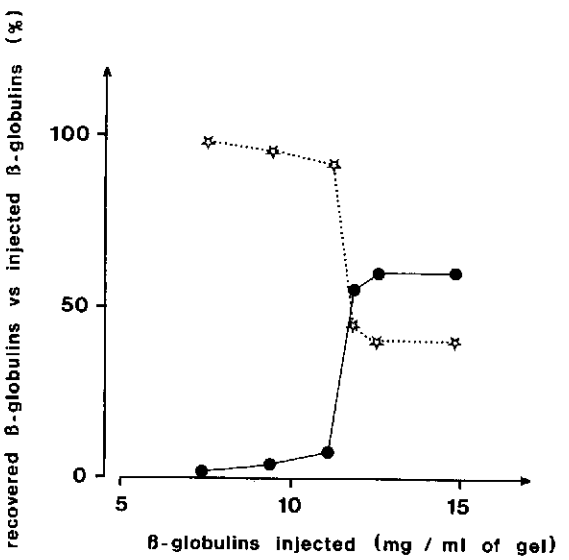


Fig. 3. Study of the binding capacity of the DEAE-Spherodex exchanger (5 ml). (●) $\beta$-globulins in the breakthrough peak; (☆) $\beta$-globulins in the transferrin-containing peak.

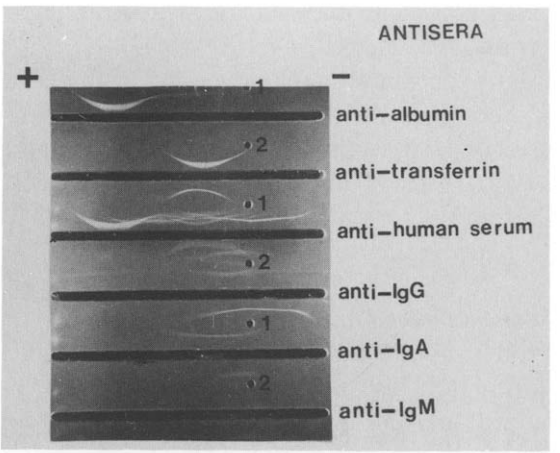


Fig. 4. Immunoelectrophoretic analysis of the transferrin fraction isolated by ion-exchange chromatography on DEAE-Spherodex: wells 1, standard human serum; wells 2, isolated transferrin (60 g/l).

pacity of the ion exchanger used had been made, using a small 5-ml column. Fig. 3 shows that 11 mg of $\beta$-globulins could be fixed on 1 ml of DEAE-Spherodex without contamination of the breakthrough peak with $\beta$-globulins. The working capacity will be 6.6 mg of $\beta$-globulins per ml of gel, *i.e.* 60% of the optimal capacity, a normal value for use on a large scale.

In order to check the reproducibility of this process, five purifications were carried out on the laboratory scale, and the transferrin solutions were tested for their purity and physicochemical properties. The homogeneity of transferrin solutions (concentrated to 60 g/l) was evaluated by cellulose acetate electrophoresis. One single symmetrical peak with a $\beta$ mobility was obtained (Fig. 1B). FPLC analysis showed one symmetrical peak eluted at 0.23 *M* NaCl in 20 m*M* triethanolamine buffer (pH 7.7) like the standard transferrin. Immunoelectrophoresis analysis revealed that the main constituent of the solution was transferrin (Fig. 4). Even at a concentration as high as 60 g/l, only two contaminants were identified as IgG and IgA. Data obtained after specific assays of IgG and IgA showed that transferrin was 98% pure, which is in agreement with the homogeneity found by cellulose acetate electrophoresis.

SDS-PAGE gave a relative molecular mass of 80 300, a value in agreement with those in the

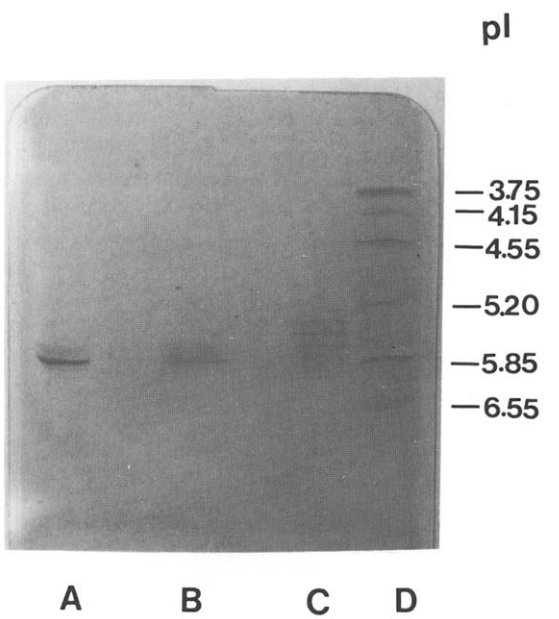


Fig. 5. Analysis by isoelectrofocusing of the transferrin fraction isolated by ion-exchange chromatography on DEAE-Spherodex in a pH 3–9 gel gradient. Lane A, standard iron-free transferrin from Sigma; lane B, transferrin isolated by DEAE-Spherodex chromatography; lane C, iron-saturated transferrin from Sigma; lane D, p*I* calibration proteins.

literature. Moreover, isoelectrofocusing showed one major band with a p*I* of 5.85, corresponding to the value obtained for the standard apotransferrin (Fig. 5).

The iron saturation coefficient was measured at different stages of the purification. If it was 37% in the starting plasma and Cohn supernatant II + III, it decreased to 10% in dialysed supernatant II + III and in $D_1$ fractions. The iron-saturation coefficient of final pure transferrin obtained by this process was 10%. In order to determine if transferrin kept its capacity for iron fixation, a titration curve was made. The calculation, taking into account a 10% initial iron saturation, showed that transferrin could bind 1.79 iron atoms per molecule.

*Virus inactivation*

$D_1$ starting fractions were subjected to solvent–detergent (SD) treatment as indicated in Experimental in order to inactivate viruses potentially present in the solutions. After the inactivation step, $D_1$ fractions were injected into the DEAE-Spherodex column. The presence of SD did not affect the chromatographic steps and did not alter the quality and yield of the transferrin obtained. The bulk of SD was found in the breakthrough peak containing IgG. Small amounts of SD were present in the transferrin fraction, but after dialysis and freeze-drying this contamination had largely disappeared (Table II).

TABLE II

ASSAYS OF SOLVENT AND DETERGENTS IN THE TRANSFERRIN SOLUTION, AFTER VIRUS INACTIVATION AND CHROMATOGRAPHIC PURIFICATION ON DEAE-SPHERODEX COLUMN

Values are μg of solvent or detergent per mg of transferrin.

| Preparation number | TNBP | | Tween 80 | | Sodium cholate | |
|---|---|---|---|---|---|---|
| | A[a] | B[b] | A[a] | B[b] | A[a] | B[b] |
| 1 | 3 | −[c] | 113 | 6 | | |
| 2 | 8 | −[c] | 58 | 9 | | |
| 3 | −[c] | 0.5 | | | 62 | 11 |
| 4 | −[c] | 1.1 | | | 165 | 8 |

[a] Before dialysis and freeze-drying.
[b] After dialysis and freeze-drying.
[c] Undetectable.

TABLE III

PURIFICATION OF TRANSFERRIN ON A PILOT SCALE

Industrial D1 fractions were obtained from the first step of chromatographic purification of albumin. Detailed chromatographic parameters are given in Experimental.

| | Preparation No. | |
|---|---|---|
| | 1 | 2 |
| *D1 fractions* | | |
| Volume (l) | 66.0 | 54.5 |
| Protein concentration (g/l) | 1.08 | 0.95 |
| β-Globulins (%)[a] | 75 | 65 |
| *Transferrin-containing peak* | | |
| Volume (l) | 37.0 | 34.2 |
| Protein concentration (g/l) | 0.98 | 0.79 |
| Quantity of transferrin (g) | 36.3 | 27.0 |
| Purity (%)[b] | 97 | 97 |
| Yield (%) | 68 | 80 |

[a] Evaluated by cellulose acetate electrophoresis.
[b] Determined after specific assays of IgG and IgA contaminants.

*Scaling up*

A scaling up of the method (150 times) was carried out using a DEAE-Spherodex column (95 cm × 14 cm I.D.) at a pilot plant. Data obtained for two purification cycles (Table III) show that the $D_1$ fractions were very dilute solutions (*ca.* 1 g protein per l) and contained a high proportion of β-globulins, mainly transferrin. Transferrin was generally recovered in a volume smaller than the volume of the starting solution with an average yield of 75% and with 97% purity. There is no detectable change of the biological and physicochemical properties of the transferrin obtained on this larger scale, *e.g.* the transferrin quality is the same as that of the product obtained on the laboratory scale.

DISCUSSION

Transferrin is a standard additive to cell culture media. For several reasons, human transfer-

rin is preferable to transferrin of animal origin. First, human transferrin is more effective than bovine transferrin in hybridoma cultures [7]. Secondly, proteins present in the cell culture medium must be removed from the cell culture end-product. For use in humans it is preferable that potential contaminant proteins of the final product be of human origin. Moreover, transferrin prepared from animal plasma could potentially contain unknown toxic contaminants: *e.g.* the problem of bovine spongiform encephalopathy cannot be ignored.

Human plasma is fractionated on the industrial scale according to a method developed by Cohn. The main products prepared by this method are albumin and immunoglobulins. Many by-products remain unexploited. Transferrin has been isolated from the Cohn fraction IV [16,17], which is a very complex mixture containing alcohol and lipid particles. Moreover, some problems are raised by the solubilization of this precipitated fraction. Recently, chromatographic methods have been used for plasma fractionation which also give rise to a large amount of by-products.

The subfraction D1 of the Spherodex-Spherosil process for human albumin chromatographic purification is a very interesting source material for purified human transferrin. At this stage, 50% of the plasma transferrin is recovered and this protein represents the majority of the proteins found in this fraction. The main advantage of this D1 fraction is that it is recovered in the form of a solution that can be directly passed through the column after its equilibration in terms of conductivity and pH. As we have seen above, D1 fractions are variable in their composition. This variability takes place in the Cohn supernatant II + III (data not shown) and is not due to the chromatographic preparation of D1. In a single chromatographic step, a pure and functional transferrin with physicochemical properties in a good agreement with the values given in the literature is obtained. Large amounts (*ca.* 30 g per cycle) of transferrin could be obtained on a pilot scale of a purity similar to that obtained at the laboratory scale.

Pure transferrin obtained according to this method is *ca.* 10% saturated with iron. Whereas in source material the iron saturation coefficient was 37%. This decrease could be explained because the starting material was dialysed against a phosphate buffer at pH 5.2. It is known that the presence of phosphate ions at acid pH could decrease the iron saturation coefficient [25]. We have shown that purified transferrin keeps its capacity to fix about two iron atoms per molecule, *i.e.* the protein is not denaturated during the chromatographic treatment.

Biological substances may become contaminated with viruses, and the Commission of the European Communities has established rules for virus inactivation of biological products [27]. The efficiency of solvent–detergent mixtures has been demonstrated by several studies carried out at the New York Blood Center [21,26]. An exta step of virus inactivation was included in the preparative process in order to guarantee the safety of the final product. When the TNBP–detergent mixture was used for this purpose, the solvent and detergent were found in very low amounts in the purified transferrin in a freeze-dried form. The transferrin concentration used in the cell culture experiments was 50 μg/ml, a concentration generally used in culture media. Under these conditions the culture medium contained *ca.* 0.03 μg/ml TNBP, and either 0.3 μg/ml Tween 80 or 0.5 μg/ml sodium cholate. These concentrations of solvent and detergent are far below the cytotoxic levels (data to be published).

## REFERENCES

1 R. T. A. McGillivray, E. Mendez, S. K. Sinha, M. R. Sutton, J. Lineback-Zins and K. Brew, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 2504.
2 D. Legrand, J. Mazurier, J. Montreuil and G. Spik, *Biochimie*, 70 (1988) 1185.
3 J. H. Brock, T. Mainou-Fowler and L. M. Webster, *Immunology*, 57 (1986) 105.
4 J. Kovar and F. Franek, *Folia Biol. (Praha)*, 31 (1985) 168.
5 M. S. Tsao, G. H. S. Sanders and J. W. Grisham, *Exp. Cell Res.*, 171 (1987) 52.
6 F. Martel, J. Dante, H. Jouishomme, C. Blourde and D. Rigal, in J. F. Stoltz and C. Rivat (Editors), *Biotechnology of Plasma Proteins*, Vol. 175, Editions INSERM, Paris, 1989, p. 123.

7 C. R. Ill, T. Brehm, B. K. Lydersen, R. Hernandez and K. G. Burnett, *In vitro Cell Develop. Biol.*, 24 (1988) 413.
8 A. Bezkorovainy, M. E. Rafelson and J. R. Likhite, *Arch. Biochem. Biophys.*, 103 (1963) 371.
9 R. C. Roberts, D. G. Makey and U. S. Seal, *J. Biol. Chem.*, 241 (1966) 4907.
10 W. E. Roop and F. W. Putnam, *J. Biol. Chem.*, 242 (1967) 2507.
11 N. Frenoy, Y. Goussault and R. Bourrillon, *Clin. Chim. Acta*, 32 (1971) 243.
12 P. A. M. Werner, R. M. Galbraith and P. Arnaud, *Arch. Biochem. Biophys.*, 226 (1983) 393.
13 N. D. Cook, R. J. Simpson, K. Osterloh and T. J. Peters, *Anal. Biochem.*, 149 (1985) 349.
14 J. S. Baumstark, *J. Biochem. Biophys. Methods*, 14 (1987) 59.
15 J. Shalan, S. A. Al Mashikhi and S. Nakai, *J. Dairy Sci.*, 71 (1988) 1756.
16 R. Schmitthaeusler, H. Ratefiarivelo and J. M. Meyer, in A. Faure, C. Doinel and J. F. Stoltz (Editors), *Technologies de Purification des Protéines*. DIPC-INPI, Nancy, 1985, p. 561.
17 B. Buatois, M. Allary, B. Mille, E. Boschetti and J. Saint-Blancard, in A. Faure, C. Doinel, M. Hours, Y. Fabert and J. F. Stoltz (Editors), *Technologies de Purification des Protéines*, CRCIL-GRBP-INNOBIO, Nancy, 1987, p. 239.
18 F. Hasko, M. Salamon, K. Bartha and P. Dodo, in S. Karger (Editor), *Development in Biological Standardization, Symposium on Standardization in Blood Fractionation Including Coagulation Factors*, Vol. 67, Academic Press, Basel, 1987, p. 39.
19 J. F. Stoltz, C. Rivat, C. Geschier, P. Colosetti, Ph. Sertillanges, J. Tondon and V. Regnault, *Biosciences*, 6 (1987) 103.
20 J. F. Stoltz, C. Rivat, C. Geschier, P. Colosetti and Ph. Sertillanges, in J. F. Stoltz and C. Rivat (Editors), *Biotechnology of Plasma Proteins*, Vol. 175, Editions INSERM, Paris, 1989, p. 191.
21 C. A. Edwards, M. P. J. Piet, S. Chin and B. Horowitz, *Vox Sang.*, 52 (1987) 53.
22 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
23 G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry*, 28 (1965) 237.
24 G. Scheidegger, *Int. Arch. Allergy Appl. Immunol.*, 7 (1955) 103.
25 J. Mazurier and G. Spik, *Biochim. Biophys. Acta*, 629 (1980) 399.
26 B. Horowitz, M. E. Wiebe, A. Lippik and M. H. Stryker, *Transfusion*, 25 (1985) 516.
27 Commission of the European Communities, *Validation of Virus Removal and Inactivation Procedures, Note for Guidance, Biologicals*, 19 (1991) 247.