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To cite this article: S. Notermans , K. Wernars & J. Dufrenne (1991) Recognition of false positive results obtained with immunoassays by analyzing the reaction kinetics, Food and Agricultural Immunology, 3:2, 85-92, DOI: [10.1080/09540109109354734](https://doi.org/10.1080/09540109109354734)

To link to this article: <http://dx.doi.org/10.1080/09540109109354734>



Published online: 16 Sep 2008.



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Recognition of False Positive Results Obtained with Immunoassays by Analyzing the Reaction Kinetics

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(Received for publication 29 April 1991)

Sensitive immunoassays, such as the enzyme-linked immunosorbent assays (ELISA) and the latex agglutination assay are sensitive to non-specific reactions. Analysis of the reaction kinetics of the ELISA is highly useful in recognizing false positive results, as was demonstrated for assaying of staphylococcal enterotoxin A (SEA). The regression coefficient obtained after plotting the ELISA extinction values against the $\log_{10}$ of the reciprocal serial dilutions of the test sample was identical for SEA produced by several strains of Staphylococcus aureus. Regression coefficients obtained by the cross-reacting substances protein A and lysozyme differed significantly from that obtained for SEA. Also, the maximal extinction values obtained for, among others, the cross-reacting substances present in walnut extracts, differed significantly of that obtained for SEA. Food matrices were found to influence the regression coefficient only if the sample dilution factor was below 10.

Keywords: Immunoassays, reaction kinetics, false positive results

INTRODUCTION

For detection of microbial antigens in food, sensitive immunoassays such as the haemagglutination, radioimmunoassay, enzyme immunoassay and latex agglutination assays have been developed. All these techniques are based on a quantitative reaction of the antigen with its antibody. Both the enzyme immunoassays and the latex agglutination assay are simple techniques and can be used without extensive laboratory equipment. Each immunoassay, however, is sensitive to non-specific reactions. These reactions occur if substances other than the antigen bind to the antibodies used in the assay. Bacterial proteins capable of reacting with the Fc region of immunoglobulins (antibodies) have been detected in a number of staphylococcal and streptococcal strains (Mota *et al.*, 1987; Jürgen *et al.*, 1987; Chatwal & Globel, 1987). False positive

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results may also be caused by lysozyme, for instance in the assay of staphylococcal enterotoxins in mussels (Essink *et al.*, 1985). Recently we have observed that extracts of walnuts contain substances giving false positive latex agglutination results (Notermans & Kamphuis, 1990). Consequently, the immunological detection of microbial metabolites may not be reliable and it is necessary to check for false positive results.

Our own experience of routine application of the enzyme-linked immunosorbent assay (ELISA) has indicated that the reaction kinetics of the assay may provide information about reliability. Deviations of the regression coefficient, obtained after plotting the ELISA extinction against the $\log_{10}$ of the reciprocal dilution of the test sample, were found to be of a predictive value. The same was observed for the maximal extinction value obtained with the ELISA. In this paper results are described of a detailed study of the reaction kinetics to recognize false positive results. For this the ELISA for detection of staphylococcal enterotoxin A was applied.

MATERIALS AND METHODS

S. aureus strains

The *S. aureus* strains that were used included reference strains S6, ATCC 13565, FRI 913, FRI 722, 196E and some selected wild-type strains, set 24, V46-2 and 86 V13-15-1. All wild-type strains were isolated from food involved in staphylococcal enterotoxigenicoses. Strains were tested for enterotoxin production using the sac-culture method of Donnelly *et al.* (1967). After inoculation the sac-culture was incubated at 37°C on an orbital shaker (120 rev min⁻¹) for 40 h, and then centrifuged at 10 000g for 10 min. The supernatant was tested for the presence of enterotoxin using the optimal sensitive plate method as described by Robbins *et al.* (1974). Furthermore, the presence of the gene encoding SEA was tested by DNA-hybridization as described earlier by Notermans *et al.* (1988). All strains applied contained the gene encoding SEA. Strains V46-2, ATCC 13565, FRI 722, 196E and 86 V13-15-1 produced only SEA. Strain set 24 produced SEA, SEB and SED. Strain FRI 913 produced SEA, SEC and SEE, and strain S6 produced SEA and SEB.

SEA

Pure SEA applied in the experiments was a gift of Dr Anna Johnson Winegar, US Army Medical Research Institute, Fort Detrick, MD, USA.

Antibodies

Polyclonal antibodies (PABs) were produced by immunizing sheep (Notermans & Dufrenne, 1982). The immunoglobulin G (IgG) fraction was isolated from the serum with the aid of caprylic acid (Steinbuch & Audran, 1969) and stored freeze-dried. Hybridoma cell lines producing monoclonal antibodies (MABs) were produced according to the method described by Meyer *et al.* (1984). MABs were produced in about nine-week old BALB/c mice by intraperitoneal injection with the hybridoma cells. Ascites fluid was collected and the immunoglobulin was isolated by Na₂SO₄ precipitation.

ELISA Procedures

For detection of SEA the sandwich ELISA technique was used as described previously (Notermans *et al.*, 1983). Both an ELISA applying PAB and an ELISA applying MABs were used. For the PAB-ELISA, wells of polystyrene trays (Cooke, Dynatech cat. no. 1-220-25) were coated with 2 µg ml⁻¹ of anti SEA-IgG sheep. After sample incubation the amount of adsorbed toxin (or cross-reactive substance) was detected by using sheep anti SEA-IgG conjugated to peroxidase. For the MAB-ELISA as coating

antibodies MAB 1.55.5 was used at a concentration of $2 \mu\text{g ml}^{-1}$ and as conjugate MAb 1.33.3. The IgG peroxidase conjugate concentration was determined by checker-board titration. The enzyme reaction was determined spectrophotometrically at 450 nm after addition of 0.1 ml substrate (1 ml of 42 mM 3,5,3',5' tetramethylbenzidine in dimethyl sulphoxide was added under vigorous shaking to 100 ml 0.1 M sodium acetate buffer pH 5.6; just before use $7 \mu\text{l}$ of 30% H_2O_2 were added to the solution).

Foods

Food samples were purchased from local stores. Extracts were prepared by blending equal amounts of food and phosphate-buffered saline solution, pH 7.2 (PBS), and subsequent centrifuging ($10\,000g$ for 10 min). The supernatant was adjusted to pH 7.2 using either 1 M NaOH or 1 M HCl. The fluid and dilutions of the fluid were spiked with SEA. For dilution fluid, PBS containing 0.05% Tween 20 (PBS-Tween) was applied.

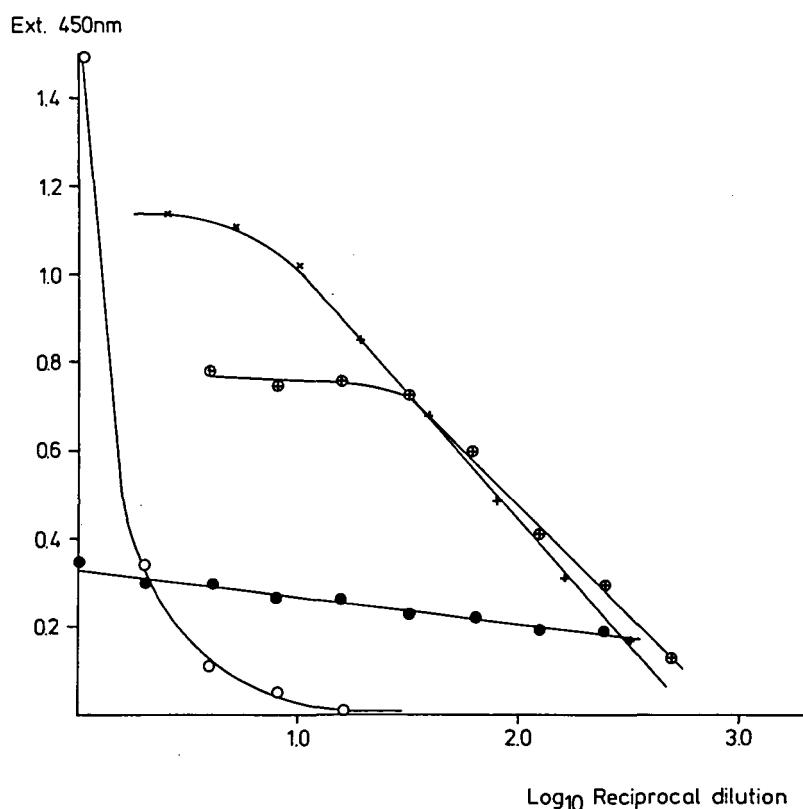


FIG. 1. ELISA using polyclonal antibodies performed with serial dilutions of SEA, lysozyme, walnuts extract and protein A. The starting concentrations were 200 ng ml^{-1} , 10 mg ml^{-1} , $1/2$ diluted extract and 1 mg ml^{-1} respectively. (+) SEA; (O) lysozyme; (⊕) walnuts; (●) protein A.

Lysozyme and Protein A

The lysozyme used was Sigma grade 1 lysozyme. Lysozyme was tested in ELISA experiments only after heating the enzyme at 100°C for 5 min (Essink *et al.*, 1985). Staphylococcal protein A was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

TABLE 1. Regression coefficients obtained with the ELISA (using either polyclonal antibodies, PAb or monoclonal antibodies, MAb) using serial dilutions of culture fluid of several *S. aureus* strains and some other products.

SEA, strains and toxins produced and other test products				PAb-ELISA			MAb-ELISA		
				<i>n</i>	Regression coefficient	SD	<i>n</i>	Regression coefficient	SD
SEA				6	-0.53	0.06	6	-0.85	0.06
Strain V46-2	A			3	-0.53		2	-0.87	
ATCC 13565	A			3	-0.55		2	-0.88	
FRI 722	A			3	-0.53		2	-0.83	
196E	A			3	-0.57		2	-0.89	
86V-13-15-1	A			3	-0.52		2	-0.90	
24	A, B, D			3	-0.31		2	-0.88	
FRI 913	A, C, E			3	-0.29		2	-0.83	
S6	A, B			3	-0.34		2	-0.90	
Lysozyme				2	-5.00				
Walnut extract				2	-0.48				
Protein A				2	-0.06				

Regression Coefficient

Regression coefficients (RC) were determined by plotting extinction values against the log₁₀ reciprocal dilution of the sample tested. Extinction values less than 0.15 and greater than the maximum extinction values 0.8 were excluded from the calculations.

TABLE 2. Regression coefficients obtained with the ELISA for SEA using polyclonal antibodies. SEA was added to extracts of foods diluted 1:2, 1:10 and 1:100 respectively. A serial dilution was made in the respective diluted extracts.

Food products used for dilutions	Ratio food product:PBS-Tween		
	1:2	1:10	1:100
Vanilla custard	-0.63	-0.58	-0.58
Raspberry custard	-0.53	-0.53	-0.58
Mon chou cheese	-0.43	-0.52	-0.52
Processed cheese	-0.43	-0.50	-0.51
String beans	-0.28	-0.48	-0.48
Macaroni	-0.49	-0.55	-0.49
Lasagne	-0.42	-0.58	-0.51
Lettuce	-0.50	-0.54	-0.55
Tomato soup	-0.58	-0.58	-0.55
Vegetable soup	-0.47	-0.55	-0.54

SEA in PBS-Tween: *n* = 5; regression coefficient, 0.53; SD = 0.06.

RESULTS

Reaction Kinetics of SEA and Some Cross-Reacting Substances

The reaction kinetics of SEA and some cross-reacting substances were determined by using the PAb-ELISA. In Figure 1, the results are presented of the extinction values obtained. Lysozyme gives a positive ELISA reaction at a concentration of 1 mg ml⁻¹ or more. A high extinction value was observed at a concentration of 10 mg ml⁻¹.

Protein A showed a reaction over the whole range of concentrations tested ($2 \mu\text{g ml}^{-1}$ to 2 ng ml^{-1}). The RC of both lysozyme and protein A differed significantly from the RC obtained for SEA (Table 1). Extract of walnuts also gives a positive result in the PAb-ELISA. The RC however, did not differ from that of SEA (Table 1). The maximal extinction value obtained within the walnut extract amounted 0.76 and for SEA 1.13 (Figure 1).

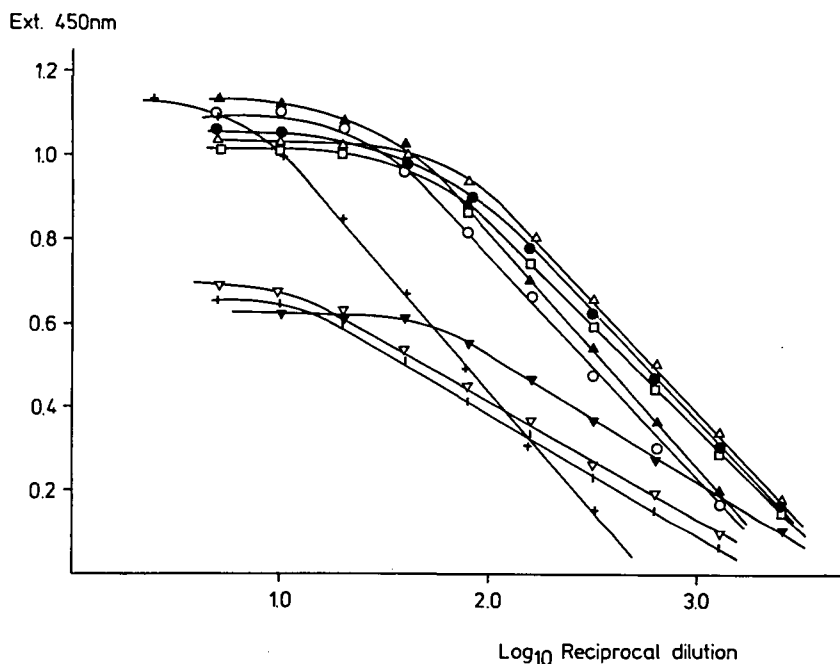


FIG. 2. ELISA using polyclonal antibodies performed with serial dilutions of SEA (starting concentration 200 ng ml^{-1}) and of culture fluid of several SEA-producing strains of *Staphylococcus aureus*. (+) SEA; (●) V46-2; (○) ATCC 13565; (△) FRI 722; (▲) 196E; (□) 86 V 13-15-1; (▽) S6; (—) set 24; (▼) FRI 913.

Effect of Diluent (Food Extract) on Reaction Kinetics

The effect of food products on the RC was tested by using food extracts as diluent. For this SEA was added to extracts of food diluted 1:2, 1:10 and 1:100, respectively, at a concentration of $200 \text{ ng SEA ml}^{-1}$. A serial dilution was made in the respective diluted extracts. If a serial dilution was made in 1:2 diluted extracts the RC differed in most cases from the RC obtained for SEA diluted in PBS-Tween (Table 2). The lowest RC was obtained with string beans extract diluted 1:2. The highest RC was obtained for vanilla custard. Both RCs differed significantly from that of SEA diluted in PBS-Tween.

If however, 1:10 and 1:100 diluted extracts were used the RC did not differ significantly from the RC obtained with SEA diluted in PBS-Tween.

Reaction Kinetics of Culture Fluids of Several *S. aureus* Strains

Culture fluids of several SEA-producing strains were tested using both the PAb-ELISA and the MAb-ELISA. For this serial dilutions of sac-culture fluid of the respective

strains were tested. As diluent, PBS-Tween was used. Results obtained with the PAb-ELISA are presented in Figure 2 and Table 1. *S. aureus* strains producing SEA as single enterotoxin showed a RC identical to pure SEA. The RC obtained for strains SET 24, FRI 913 and S6, producing several toxins, showed a different RC. Additionally the maximal extinction value obtained differed from that of pure SEA. If, however, culture fluids of the several strains were assayed by using the MAbs-ELISA no differences in RC and maximal extinction values were observed (see Figure 3).

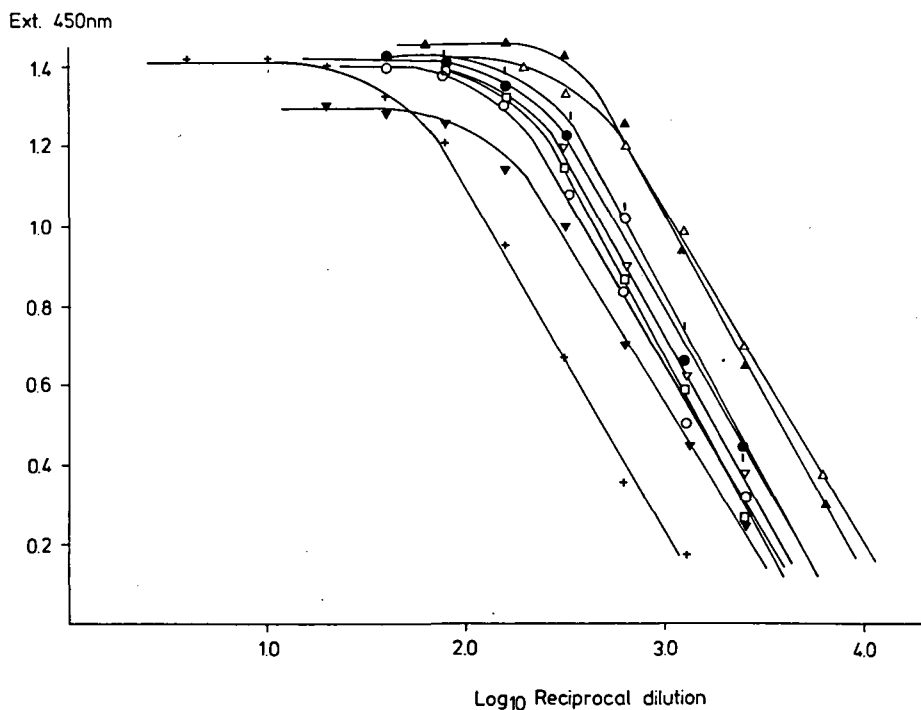


FIG. 3. ELISA using monoclonal antibodies performed on serial dilutions of SEA (starting concentration 200 ng ml^{-1}) and of culture fluid of several SEA producing strains of *Staphylococcus aureus*. (+) SEA; (●) V46-2; (○) ATCC 13565; (Δ) FRI 722; (▲) 196E; (□) 86 V 13-15-1; (∇) S6; (—) set 24; (▼) FRI 913.

Other Factors Affecting the Regression Coefficient

It was observed that the RC obtained by testing serial dilutions containing SEA was influenced by several parameters. Both the concentrations of the IgG used for coating and the dilution of IgG-peroxidase complex influenced the RC (results not presented). Also sample incubation time as well as enzyme reaction time was of influence on the RC. Even if experiments were carried out on different days, a slightly different RC was obtained.

DISCUSSION

Sensitive immunoassays, such as the ELISA and the latex agglutination assay, are increasingly used in the quality control of many food products. Most methods, however, suffer from the drawback that there is a lack of testing of the reliability of results obtained. In a study of Van Netten *et al.* (1990), for example, a latex agglutination assay was applied to the detection of enterotoxin produced by *Bacillus*

cereus strains. Based on the results obtained the authors claimed that *B. cereus* produces enterotoxin at 4°C, although they noted that the specificity of the assay was open to debate. However, in the absence of an adequate test to recognize false positive results such a debate is irrelevant.

For testing false positive results more possibilities exist. One is the specific blocking of immunoglobulins used in the assay. It was demonstrated that for this purpose either synthetic epitopes (Kamphuis *et al.*, 1989) or anti-idiotypic antibodies (Notermans *et al.*, 1989) can be used. After such specific blocking of the immunoglobulins no antigen can be bound and a positive assay result has to render a negative result. If not, the test result has to be considered as false positive.

In the study described in this paper the reaction kinetics were tested as a tool to recognize false positive results. The sandwich ELISA for detection of SEA was used as a model system. From the results obtained it can be concluded that the reaction kinetics can indeed be applied to recognize false positive results. Both the regression coefficient, obtained after plotting the ELISA extinction values against the $\log_{10}$ of the reciprocal sample dilution, and the maximal extinction values obtained are parameters to discriminate false positive results. However, these parameters do not have constant values for a given antigen. Firstly, the batch of immunoglobulin influenced the reaction kinetics. In our results clear differences were observed with the assays using either PAb or MAb. Secondly, food matrices affect the regression coefficient. To circumvent this problem the food extract tested has to be diluted at least five- to 10-fold. Finally, the regression coefficient is determined by the performance of the assay. Therefore the assay performance has to be standardized as well. A practical solution for this problem is the use of a standard serial dilution of the antigen in each ELISA tray applied.

Another problem arose in testing culture fluid of several *S. aureus* strains. By using PAb, the regression coefficient of three of the nine strains tested deviated from that of pure SEA. These three strains produce SEA in combination with other enterotoxins, whereas the other six strains produce only SEA. Such a difference was not found by applying MAb. In the absence of evidence for the existence of immunological subtypes of SEA, the different reaction kinetics may be explained by masking of a part of the SEA molecule. Since this was only observed with strains producing several enterotoxins it can be speculated that these other toxins are responsible for the masking effect.

From the results obtained it can be concluded that the reaction kinetics observed by testing serial dilutions of a sample can provide information about the reliability of the results obtained. A deviation of either the regression coefficient or maximal extinction value is indicative of a possible false positive reaction.

REFERENCES

- CHATWAL, G. S. & GLOBEL, H. (1987) Isolation and properties of a novel IgG-binding protein from streptococci of serological group U, *Medical Microbiology and Immunology*, **176**, 1-12.
- DONELLY, C. B., LESLIE, J. E., BLACK, L. A. & LEWIS, K. U. (1967) Serological identification of enterotoxigenic staphylococci from cheese, *Applied Microbiology*, **15**, 1382-1387.
- ESSINK, A. W. G., ARKESTEIJN, G. J. M. & NOTERMANS, S. (1985) Interference of lysozyme in the sandwich enzyme-linked immunosorbent assay (ELISA), *Journal of Immunological Methods*, **80**, 91-96.
- JÜRGEN, D., STERZIK, B. & FERHRENBACH, F. J. (1987) Unspecific binding of group B streptococcal cocytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity, *Journal of Experimental Medicine*, **165**, 720-732.
- KAMPHUIS, H. H., NOTERMANS, S., VEENEMAN, G. H., BOOM, J. H. VAN & ROMBOOTS, F. M. (1989) A rapid and reliable method for the detection of moulds in foods: using the latex agglutination assay, *Journal of Food Protection*, **52**, 244-247.

- MEYER, R. F., MILLER, L., BENNET, R. W. & MACMILLAN, J. D. (1984) Development of a monoclonal antibody capable of interacting with five serotypes of a *Staphylococcus aureus* enterotoxin, *Applied Environmental Microbiology*, **47**, 283–287.
- MOTA, G., GHETTIE, V. & SJÖQUIST, J. (1987) Characterization of the soluble complex formed by reacting IgG with protein A of *S. aureus*, *Immunochemistry*, **15**, 639–642.
- NETTEN, VAN P., MOOSDIJK, VAN DE A., HOENSEL VAN DE P., MOSSEL, D. A. A. & PERALES, I. (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin, *Journal Applied Bacteriology*, **69**, 73–79.
- NOTERMANS, S. & DUFRENNE, J. (1982) A simple purification method for enterotoxin F produced by *Staphylococcus aureus* and some properties of the toxin, *Antonie van Leeuwenhoek*, **48**, 447–455.
- NOTERMANS, S. & KAMPHUIS, H. H. (1990) Detection of moulds in food by latex agglutination: a collaborative study, *Food and Agricultural Immunology*, **2**, 37–46.
- NOTERMANS, S., HEUVELMAN, K. J. & WERNARS, K. (1988) Synthetic enterotoxin B DNA probes for detection of enterotoxigenic *Staphylococcus aureus* strains, *Applied and Environmental Microbiology*, **54**, 531–533.
- NOTERMANS, S., BOOT, R., TIPS, P. D. & DE NOOY, M. P. (1983) Extraction of staphylococcal enterotoxins (SE) from minced meat and subsequent detection of SE with enzyme linked immunosorbent assay (ELISA), *Journal of Food Protection*, **46**, 238–241.
- NOTERMANS, S., ALBER, G., SAILER-KRAMER, B. & HAMMER, D. K. (1989) The use of an anti-idiotypic monoclonal antibody for reliable detection of Staphylococcal enterotoxins, *Food and Agricultural Immunology*, **1**, 11–17.
- ROBBINS, R., GOULD, S. & BERGDOLL, M. S. (1974) Detecting the enterotoxigenicity of *Staphylococcus aureus* strains, *Applied Microbiology*, **28**, 946–950.
- STEINBUCH, M. & AUDRAN, R. (1969) The isolation of IgG from mammalian sera with the aid of caprylic acid, *Archives of Biochemistry and Biophysics*, **134**, 275–283.