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High-performance gel permeation chromatographic analysis of immunoglobulin M produced by hybridoma cell culture

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ABSTRACT

A high-performance gel permeation chromatographic (HPGPC) method, using TSK-Gel SW_{XL} 3000 and TSK-Gel SW_{XL} 4000 columns installed in series, was developed for the analysis of immunoglobulin M (IgM) produced by hybridoma cell culture. The detection of this protein was achieved using ultraviolet absorption at 225 nm, yielding a detection limit of *ca.* 0.3 $\mu g\ ml^{-1}$. IgM-containing samples obtained from different culture systems (T-flask, roller bottle, spinner flask, bioreactor operated in batch, fed-batch or perfusion modes) and media (Dulbecco's Modified Eagle Medium or Protein-Free Hybridoma Medium) were evaluated by this chromatographic technique and also by conventional enzyme-linked immunosorbent assay (ELISA). Concentrations of IgM in culture samples determined by both techniques were always in the same range (± 10%). The main advantages of HPGPC over ELISA included improved reproducibility (relative average deviation of 1–3% compared with 10–20% for ELISA), high linearity range between the signal and the concentration [at least three decades (0.3–500 $\mu g\ ml^{-1}$) compared with one decade for ELISA (25–200 $ng\ ml^{-1}$)] and ease of operation.

INTRODUCTION

Monoclonal antibodies are used routinely for blood typing and have considerably improved our understanding of the various structures expressed at the surface of human erythrocytes [1]. Hybridoma cell culture represents the most interesting alternative for the production of monoclonal antibodies (MAbs). The technology being developed in our laboratory for the large-scale production of an immunoglobulin M (IgM) directed against a human blood type antigen required a simple, efficient and sensitive method for MAb quantification. Hemagglutination is often used as a rapid method for the determination of the titer of a given MAb in a solution. Erythrocyte aggregates are formed and detected by subjective visual inspection. Other commonly used procedures for detecting and quantify MAb include highly specific immunoassays and especially enzyme-linked immunosorbent assay (ELISA) [2,3]. However, these techniques, based on antigen–antibody interactions, are known for their poor reproducibility [4,5]. The development of large-scale MAb production processes requires accurate and reproducible methods to assess their performance. Semi-quantitative tests such as hemagglutination or ELISA do not allow a proper evaluation of the effect of culture conditions on MAb production. Chromatography, which represents a more reliable technique, was evaluated for this purpose. Among the chromatographic techniques available, high-performance gel permeation chromatography

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(HPGPC) for protein separation appeared to be a valuable approach because of its easy of operation and the information that it provided on the relative molecular weight (MW) of the proteins analyzed. Since the introduction of silica-based GPC columns that allowed efficient and rapid separation of large molecules, several studies have been published on the separation of small immunoglobulins, including IgG and IgA [6,7]. The main problem with large IgM immunoglobulins is that they are excluded from the packing of most of the silica-based columns available. Flapper *et al.* [8] recently presented a separation methodology for serum proteins using HPGPC. In comparing the elution behaviours of IgM, macroglobulin, IgG and albumin on three different column systems (TSK-Gel SW, Zorbax and Sepharose), they concluded that the best separation was achieved using TSK-Gel SW columns. However, their work, which emphasized the theoretical aspects of the elution behavior of the different proteins, did not report quantitative statistical analyses of actual culture samples.

This work addresses the development and characterization, in terms of detection limit, linearity range, reproducibility and selectivity, of an HPGPC method for the quantification of a specific IgM protein produced by hybridoma cell culture. The analysis was achieved using TSK-Gel SW_{XL} columns installed in series and ultraviolet detection. These columns were preferred to TSK-Gel SW columns, as they were found to yield better separations of proteins [9]. Culture samples obtained from different hybridoma culture systems were analyzed by HPGPC and compared with the highly specific ELISA analysis.

EXPERIMENTAL

Hybridoma cell culture

Mouse × mouse hybridoma cell lines secreting an IgM against the human blood type antigen Lewis b were kindly provided by Chembiomed (Edmonton, Canada). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St.-Louis, MO, USA) supplemented with 1% (v/v) fetal bovine serum (FBS) (Flow Labs., Mississauga, Canada) or in Protein-Free Hybridoma Medium (PFHM II) (Gibco, Burlington, Canada) at 37°C and under appropriate carbon dioxide partial pressure. Cells were grown in various systems [T-flasks, roller bottles, spinner flasks or bioreactors (2.4 l)] under different culture modes (batch, fed-batch and perfusion).

Sample preparation

Samples obtained from the different cultures were used as the experimental source of IgM. Samples of various culture ages were collected and centrifuged at 8 800 *g* for 2 min and the supernatants were frozen at −80°C for further analyses by HPGPC and ELISA.

HPGPC system

The HPGPC system used (Waters Assoc., Bedford, MA, USA) included a pump (Model 590), a manual injector (Model U6K) or an automatic sampler (WISP, Model 710) and a UV detector (Model 481) connected to a chart recorded (Model 2210, LKB, Gaithersburg, MD, USA). The protein separation was achieved using a TSK-Gel 4000 SW_{XL} column connected in series to a TSK-Gel 3000 SW_{XL} column (Toyo Soda, Tokyo, Japan). A filter (0.45 μm) was placed on-line before the two columns. The mobile phase was a phosphate-buffered saline (PBS) aqueous solution (0.2 *M* sodium chloride–0.2 *M* sodium phosphate) (Anachemia, Montreal, Canada) of pH 6.8. The flow-rate and inlet pressure were 0.5 ml min^{-1} and *ca.* 35 bar, respectively. A volume of 50 μl supernatant was injected directly into the HPGPC system.

A protein standard mixture made of five globular proteins of known molecular weight [thyroglobulin (670 000 dalton), IgG (158 000 dalton), ovalbumin (44 000 dalton), myoglobin (17 000 dalton) and cyanocobalamin (1300 dalton) was purchased from Bio-Rad Labs. (Richmond, CA, USA) (catalog No. 151–1901) and was used to evaluate protein separation. The presence of IgM in samples was determined by comparison with the retention time of an IgM standard which was an affinity purified antibody solution [1 mg ml^{-1} in PBS + 1% BSA (lot No. 8PLB-0002)] kindly provided by Chembiomed. This same standard was also used for ELISA calibration. The IgM was quantified from its peak height on UV detection at 225 nm. The peak width was constant for the range of concentrations studied (1–500 μg ml^{-1}).

ELISA

A PBS solution of pH 7.2 was prepared using 1.11 g l^{-1} sodium phosphate dibasic (Anachemia), 0.30 g l^{-1} potassium phosphate monobasic (Anachemia), 9.0 g l^{-1} sodium chloride (Anachemia) and 0.1 m*M* thimerosal (Sigma) in deionized water. A washing buffer solution (WB) was prepared by adding 0.1% (v/v) of Tween 20 (Bio-Rad Labs.) to the PBS. An antibody diluting buffer solution (DB) was prepared by adding 1.0% (w/v) bovine serum albumin (BSA) and 1.0% (v/v) Tween 20 to the PBS. The enzyme-substrate solution was made of 4.67 g l^{-1} sodium citrate (Fisher, Montreal, Canada), 7.90 g l^{-1} sodium phosphate monobasic (Sigma) of pH 5.5 and, prior to use, 3 mg ml^{-1} *o*-phenylenediamine (OPD) (Sigma) and 0.67 μl ml^{-1} 30% hydrogen peroxide (Sigma). A Dulbecco's PBS solution (D-PBS) (Gibco) was used to prepare the antigen solution. A purified Lewis b antigen supplemented with BSA was used for the plate coating for ELISA at a concentration of 0.5 μg ml^{-1} in D-PBS. An enzyme immunoassay (EIA)-grade affinity purified goat anti-mouse IgG horseradish peroxidase conjugate (HRP) (Bio-Rad Labs.) was diluted 1:8000 in the DB.

Microtitration plates with 96 flat-bottomed wells (Flow Labs.) were coated with the antigen by dispensing 100 μl per well of the antigen solution. These plates were incubated at room temperature overnight, washed six times with the WB using an automatic microplate washer (Titertek, Flow Labs.) and stored at 4°C. The affinity purified antibody used as standard was diluted with the DB in the 25–200 ng ml^{-1} concentration range for calibration. Culture samples were diluted (1:200 to 1:1000) to reach the same range of concentrations. The WB solution was aspirated from wells and a 100-μl volume of the diluted standard or sample solution was distributed in each well. Four wells were used for each dilution and three different dilutions were prepared for each sample tested. The plates were covered and incubated at room temperature for 1 h before rinsing six times with the WB. A 100-μl volume of goat anti-mouse HRP conjugate was dispensed in each well. The plates were incubated and rinsed as above. A 100-μl volume of the enzyme substrate solution was distributed in each well. The plates were covered with aluminum foil for 10–20 min. Thereafter, the absorbances of the 96 wells were read rapidly at a wavelength of 450 nm using a microplate reader (Titertek Multiskan, MCC, Flow Labs.). The results were transferred to a microcomputer for calculations.

RESULTS AND DISCUSSION

Performance of the HPGPC system

The separation characteristics of the TSK columns were determined using the standard protein mixture described in the previous section. As shown in Fig. 1, each protein eluted as a single, symmetrical peak and was resolved under the conditions of

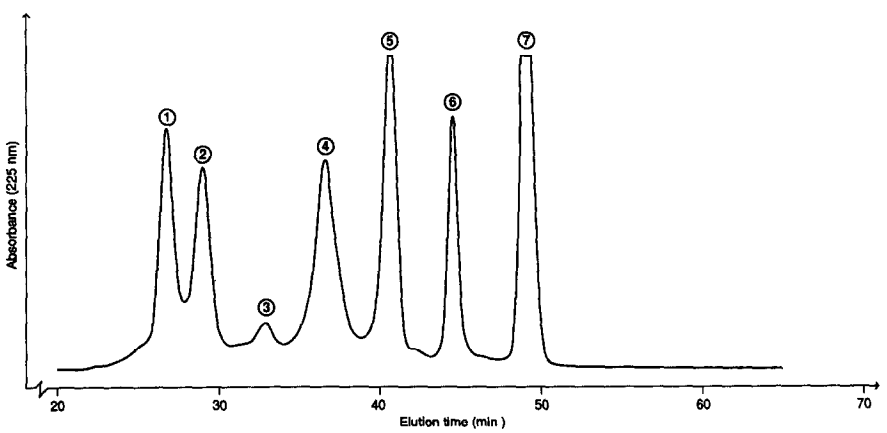

Fig. 1. HPGPC elution profile of a calibration mixture of proteins of different molecular weights with TSK-Gel 3000 SW_{XL} and TSK-Gel 4000 SW_{XL} columns connected in series. Conditions as described in the text (0.02 a.u.f.s.). Peaks: 1 = IgM (950 000 dalton); 2 = thyroglobulin (670 000 dalton); 3 = impurity; 4 = IgG (158 000 dalton); 5 = ovalbumin (44 000 dalton); 6 = myoglobin (17 000 dalton); 7 = cyanocobalamin (1300 dalton).

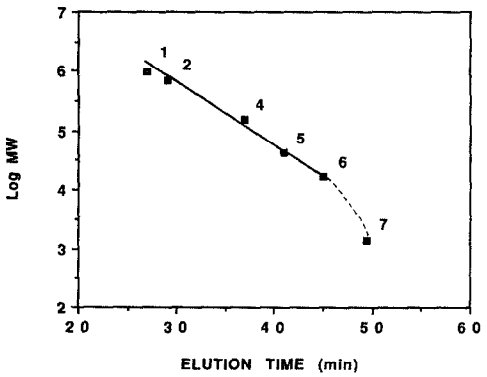

Fig. 2. Calibration graph: correlation between molecular weight (MW) and elution time obtained for TSK-Gel 3000 SW_{XL} and TSK-Gel 4000 SW_{XL} HPGPC columns connected in series. Peaks as in Fig. 1.

separation used. The elution time of these proteins as a function of their relative MW indicated that there is a linear relationship (Fig. 2) between log MW and elution time t_e (reported in min) for MW ranging from 17 000 to 950 000 dalton (correlation coefficient = 0.99):

$$\log MW = -0.0947\, t_e + 8.57 \quad (1)$$

Similar results were reported by Flapper *et al.* [8] using TSK Gel 3000 SW and TSK Gel 4000 SW columns. Cyanocobalamin (1300 dalton) eluted at the total volume of the columns (24.5 ml) and was not in the linear range of the calibration graph as expected from the lower MW limits given by the manufacturer for these columns (fractioning ranges of TSK gel 3000 SW_{XL} = 10 000–500 000 dalton and TSK Gel 4000 SW_{XL} = 20 000–10^7 dalton).

The detection limit of this technique evaluated at twice the noise level was 0.3 $\mu g\ ml^{-1}$. A linear relationship (correlation coefficient 0.99) between peak height h (reported in absorbance units) and IgM concentration c (expressed in $\mu g\ ml^{-1}$) was obtained for various IgM standards with concentrations varying from 1 to 500 $\mu g\ ml^{-1}$:

$$h = (1.52c + 1) \cdot 10^{-4} \quad (2)$$

The reproducibility of the analysis, defined as the relative standard deviation (R.S.D.) for five injections of the same IgM standard solution at 100 $\mu g\ ml^{-1}$, was 3% of the average value on manual injection and 1% on automatic injection.

GPC analysis of culture samples

Fresh DMEM medium contains known concentrations of salts, amino acids, vitamins and carbohydrates. Generally, this medium is supplemented with FBS. PFHM II medium is an unpublished formulation. In order to evaluate any interferences of the culture medium components with IgM determination, fresh samples of the medium were analyzed by HPGPC. The chromatograms obtained for serum-free DMEM, a 1% FBS solution in PBS and PFHM II are presented in Fig. 3a, b and c, respectively. These chromatograms showed several peaks at 50–60 min which exceeded the total volume of the columns. These small molecules may have penetrated the small pores of the gel phase and interacted with the residual silanol groups present, which caused their excessive retention in the columns [10]. The injection of a mixture of amino acids and a mixture of vitamins showed that some of these compounds eluted also after the total volume of the columns. The 1% FBS solution showed peaks at 29, 37 and 41 min (Fig. 3b). These peaks correspond to 660 000-, 168 000- and 68 000-dalton species and probably to thyroglobulin, IgG and albumin, three proteins usually found in FBS. As expected (Fig. 3c), the chromatogram of PFHM II showed no peak before 44 min (*i.e.*, MW > 17 000 dalton). Consequently, these analyses indicated that IgM determination should not be affected by compounds present in the medium used to grow hybridomas. This was confirmed by co-injection of IgM standard with different medium samples.

Fig. 4 illustrates typical chromatograms of actual culture supernatants. For DMEM 1% FBS supplemented samples, the IgM peak (26.5 min) was symmetrical and well resolved from the other peak at 29 min originating from 1% FBS solution (Fig. 3b). The peak pattern in the 45–65 min range of low-MW DMEM components was different from that obtained for a fresh sample of medium (Fig. 3a), indicating changes in the composition of the medium, probably as a result of nutrient consumption and cell metabolism. Similar results were obtained for PFHM II culture samples (Fig. 4b). In this instance, however, the IgM peak was sometimes slightly asymmetric, indicating co-elution of other

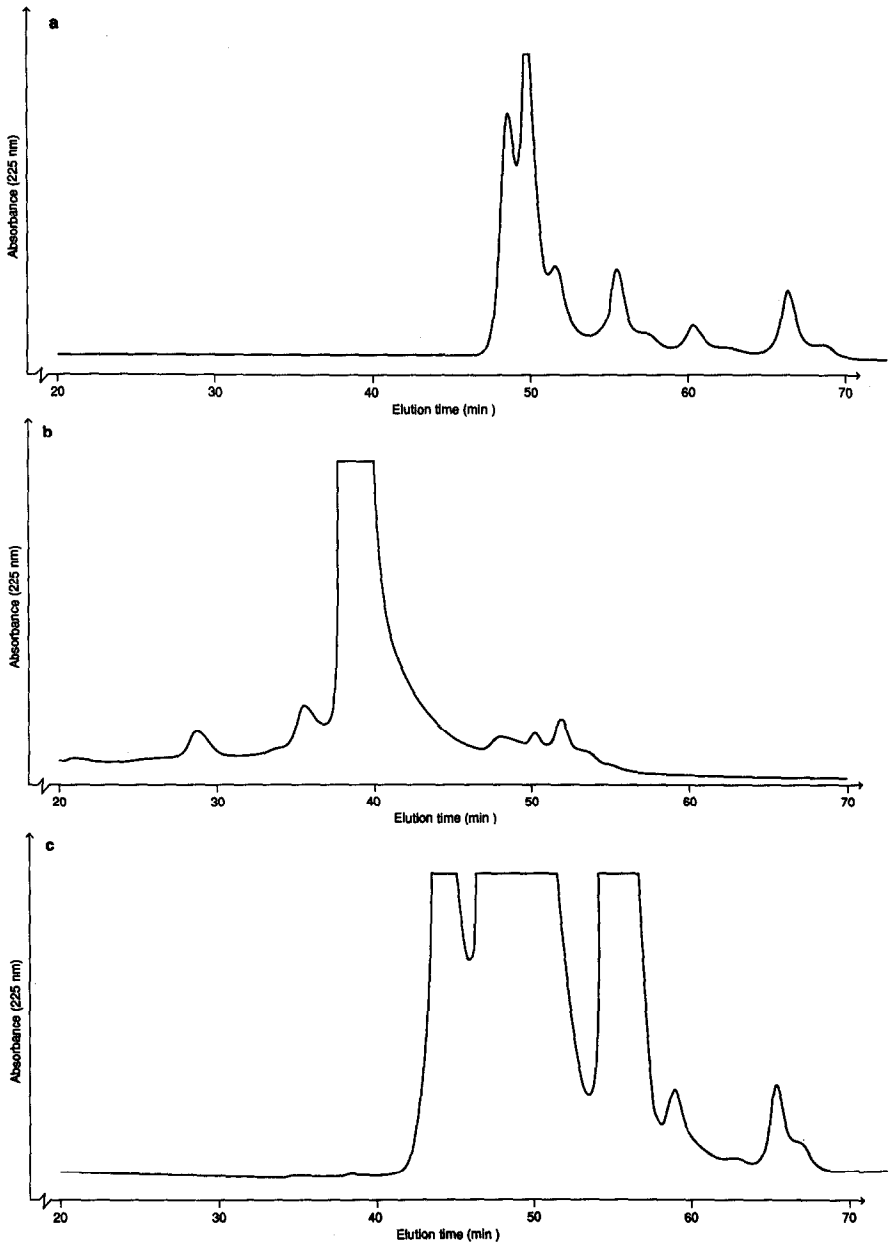

Fig. 3. HPGPC elution profiles of culture media and additives: (a) fresh DMEM (0.05 a.u.f.s.) (b) 1% FBS solution in PBS buffer (0.01 a.u.f.s.); (c) fresh PFHM II (0.01 a.u.f.s.). For experimental conditions, see text.

high-MW proteins or adsorption of the IgM molecule on the column. Previous injection of the IgM standard diluted in fresh PFHM II medium did not result in a similar asymmetric peak, indicating that the fresh medium does not contain compounds causing adsorption of the IgM molecule onto the columns. Future experiments will involve the characterization of the origin of this phenomenon.

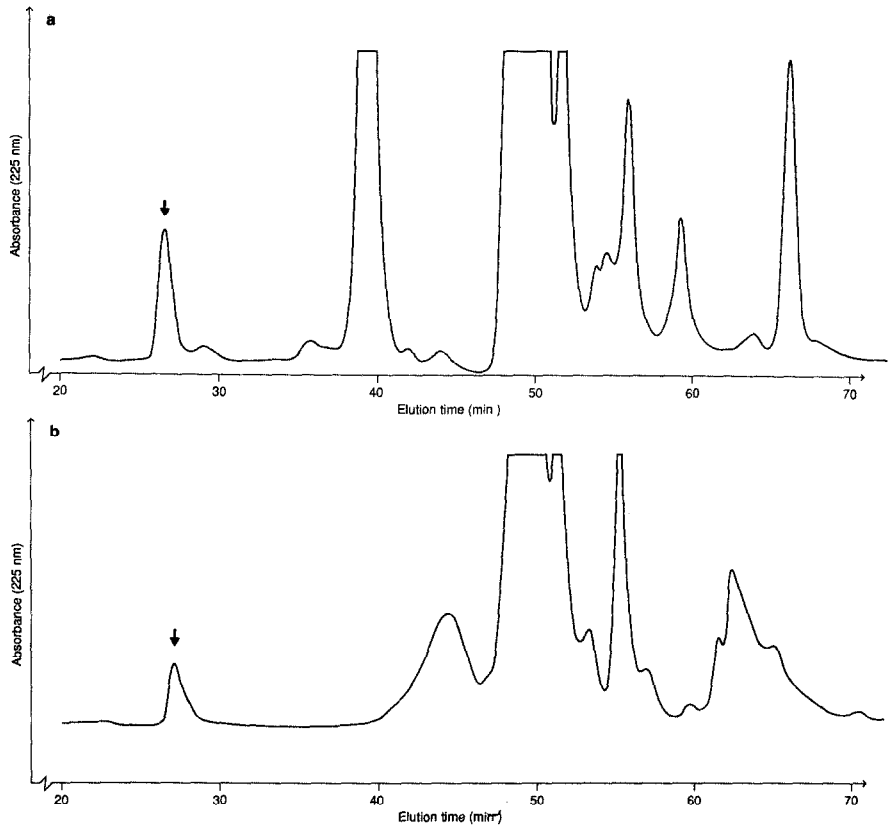

Fig. 4. Typical HPGPC elution profiles of IgM-containing samples from cell culture supernatants: (a) 40 μg ml^{-1} IgM produced in DMEM with 1% FBS (0.02 a.u.f.s.) and (b) 62 μg ml^{-1} IgM produced in PFHM-II (0.05 a.u.f.s.). For experimental conditions, see text.

Comparison of HPGPC and ELISA techniques

Results of the analysis of various IgM-containing samples produced using DMEM + 1% FBS and PFHM II media are presented in Table I. Both techniques measured similar concentrations of IgM in these samples. However, for 7 out of 22 samples (PFHM 4, 9, 11 and 12; DMEM + 1% FBS 4, 7 and 9) a significant difference (higher-to-lower average values *ca.* 20–50%) was observed between both methods, which decreased to 0–10% on taking into account the total spread of the data. As these differences were observed for the whole range of concentrations studied, and appeared randomly, they may be attributed to experimental errors. The HPGPC technique showed better reproducibility (relative average deviation *ca.* 1–2%) than the ELISA method (relative average deviation *ca.* 10–20%), which involves numerous steps and serial dilution, all contributing to the high variability of the results obtained.

The good agreement observed beween the two methods, based on molecular size exclusion and immuno-specific interaction, respectively, suggests that HPGPC is selective enough and represents a valuable quantitative technique for measuring IgM products obtained from protein-free and serum-containing cultures. It is now used to evaluate rapidly off-line, and eventually on-line, the performance of various culture processes. However, it remains a complementary technique to ELISA and hemagglutination, which allow the assesment of the quality and specificity of the MAbs produced.

CONCLUSION

The results clearly indicate that HPGPC is a reliable technique for the quantification of IgM contained in cell culture supernatants originating from either protein-free or serum-containing media. This

TABLE I

COMPARISON OF HPGPC AND ELISA MEASUREMENT FOR IgM CONTAINED IN DIFFERENT HYBRIDOMA CELL CULTURE SAMPLES

Average deviations (± values) calculated for 3–4 (ELISA) and 2–3 (HPGPC) independent measurements.

Culture medium	Sample No.	IgM concentration ($\mu g\ ml^{-1}$)	
		ELISA	HPGPC
PFHM II	1	3 ± 1	2.7 ± 0.3
	2	5 ± 1	6.0 ± 0.1
	3	9 ± 1	8.7 ± 0.2
	4	35 ± 6	27.8 ± 0.3
	5	24 ± 2	26.1 ± 0.2
	6	48 ± 2	54 ± 0
	7	115 ± 8	118 ± 2
	8	117 ± 23	115 ± 1
	9	128 ± 11	106 ± 1
	10	194 ± 23	211 ± 7
	11	287 ± 58	212 ± 7
	12	290 ± 58	220 ± 2
DMEM + 1% FBS	1	24 ± 5	27.2 ± 0.3
	2	27 ± 7	23.0 ± 0.9
	3	31 ± 2	33.4 ± 0.7
	4	22 ± 1	27.4 ± 0.3
	5	32 ± 4	34.0 ± 0.3
	6	35 ± 5	38.8 ± 0.7
	7	20 ± 10	33.0 ± 0.7
	8	32 ± 3	36.6 ± 0.4
	9	49 ± 2	40 ± 1
	10	54 ± 10	57 ± 1

method can be used to obtain reliable results in order to optimize culture processes for the production of IgM. It allows a rapid off-line analysis, which was not possible with ELISA because of its long and laborious protocol.

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