Talanta 85 (2011) 2391–2396

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Usefulness of gold nanoparticles as labels for the determination of gliadins by immunoaffinity chromatography with light scattering detection

M.A. Molina-Delgado, M.P. Aguilar-Caballos, A. Gómez-Hens*

Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry (IAQFN) Campus of Rabanales, Marie-Curie Building (Annex), University of Córdoba, 14071 Córdoba, Spain

ARTICLE INFO

Article history:
Received 15 April 2011
Received in revised form 19 July 2011
Accepted 23 July 2011
Available online 29 July 2011

Keywords:
Immunoaffinity chromatography
Light scattering detection
Gold nanoparticle
Gliadins

ABSTRACT

A simple and fast immunoaffinity method is proposed for the determination of gliadins for the first time using gold nanoparticles (AuNPs) as labels. The tracer used consists in a gliadin–AuNP conjugate prepared by the adsorption of gliadins onto the nanoparticle surface. Two AuNP sizes with diameters of 10 nm and 20 nm were assayed to compare the behaviour of the corresponding tracer in the assay. The method relies on the injection in a commercial Protein G column of a preincubated mixture containing gliadins, polyclonal anti-gliadin antibodies, and the gliadin–AuNP tracer. This approach allows the separation of free and bound tracer fractions without any additional elution step, and the direct measurement of the resonance light scattering intensity of the free tracer through the peak height of the immunochromatogram, which is proportional to the analyte concentration. The immunocolumn can be used up to 25 times without eluting and it can be regenerated for at least 20 times. The dynamic ranges of the calibration graphs and the detection limits are 0.5–15.0 and 1.5–15.0 $\mu g\,mL^{-1}$ gliadins, and 0.2 $\mu g\,mL^{-1}$ and 0.8 $\mu g\,mL^{-1}$ gliadins, using 20-nm and 10-nm Au-NPs as labels, respectively. The precision, expressed as relative standard deviation, ranges between 2.7% and 2.9% using 20-nm AuNPs and 4% and 6.1% for 10-nm AuNPs. The method has been applied to the determination of the prolamin fraction in beer samples, obtaining recovery values in the range 71.2% and 101.7%.

1. Introduction

The optical and electrochemical properties of gold nanoparticles (AuNPs) have opened a wide research activity for the development of new immunoassays in which these NPs are used as alternative labels to conventional enzymes or fluorescent compounds [1]. A high number of competitive and sandwich formats have been described in which the characteristic plasmon absorption band of AuNPs or their capability to produce Rayleigh or Raman scattering signals are measured. Also, the sensitivity of direct electrochemical immunosensors has been improved by using AuNPs as efficient three-dimensional nanoscaffolds to increase the binding capacity of the sensor surface [2,3]. Most immunoassays involving AuNPs have been applied to the determination of analytes of clinical interest such as tumor markers [4], but their use in other analytical areas such as food analysis has been limited.

Light scattering measurements have been often used in immunoassays involving AuNPs as labels [5–9]. These NPs have been recently described to develop nanoparticle aggregation immunoassays for the monitoring and determination of protein complexes aggregates as potential cancer biomarkers [5,6]. These assays rely on the use of dynamic light scattering of the complexes owing to the increase of the size of antibody–AuNP conjugate upon binding a protein antigen, using the average particle size distribution as the analytical parameter. Also, the enhanced aggregation observed through the formation of an immunocomplex using AuNPs has been previously used to develop homogeneous immunoassays with light scattering detection for the determination of IgG [7] and soy protein [8] in serum and food samples, respectively. In these assays, AuNPs were used as aggregation enhancers for the determination of macromolecular antigens in their binding to their corresponding antibody. These NPs acted in a similar way as latex microparticles do in agglutination assays, but they present the advantage of providing lower background signals, which can improve the detection limits obtained.

The method presented here is the first attempt to show the usefulness of AuNPs as labels in immunoaffinity chromatography (IAC), using a competitive format, gliadins as model analyte, and a tracer synthesized by adsorbing gliadins onto AuNPs. A preincubated mixture of the analyte, tracer and anti-gliadin antibodies is injected onto a Protein G column, which allows the separation of free and bound tracer fraction. The free tracer is measured through the scattering peak height of the immunochromatogram, which is

* Corresponding author.
E-mail address: qa1gohea@uco.es (A. Gómez-Hens).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.07.083

directly proportional to gliadin concentration. Fluorescence detection has been previously described in a similar approach developed for the determination of the linear alkylbenzenesulfonates (LASs) in environmental samples using a fluorescein derivative [9] and a long-wavelength fluorophor [10] as labels.

The proposed method has aimed to expand the use of immunoassay techniques for gliadin determination, which are the recommended techniques by the Codex Alimentarius [11]. Gliadins, hordeins and secalins are the prolamin fraction of wheat, barley and rye, respectively, which are responsible of the autoimmune celiac disease. The only treatment of this disease consists of a gluten-free diet to avoid this allergic response that can degenerate in ultimate instances into intestinal cancer after a repeated gluten intake. Gluten-free foods fall into two categories: gluten-free foods or rendered gluten-free foods, the Codex Alimentarius Standard having set up limits of 20 mg kg^{-1} and 100 mg kg^{-1} gluten, respectively [11]. Several ELISA methods have been developed for the determination of gliadins [12–18], being the official method for the determination of these proteins in foods a sandwich ELISA that uses R5 antibodies [16], which are monoclonal anti-gliadin antibodies. These antibodies also react with hordeins and secalins owing to their similar amino acid composition. Two immunoassay methods with fluorometric detection, using lanthanide [19] and ruthenium [20] chelates as alternative labels to enzymes have demonstrated their usefulness for the analysis of gluten-containing and gluten-free foods.

The competitive immunoaffinity method described here has been applied to the determination of the prolamin fraction in barley malt beer samples bearing in mind that the anti-gliadin antibodies used have a level of reactivity with hordeins similar to that with gliadins. Competitive ELISA gives better results than sandwich ELISA for the analysis of foods that contain hydrolyzed gluten, such as beers [18], since the recognition of just a single epitope is required.

2. Materials and methods

2.1. Instrumentation

The instrumental design used for the development of the method is schematised in Fig. 1. A Gilson Minipuls 3 (France) peristaltic pump was used to provide the system with a buffer solution at a flow rate of 1.0 mL min^{-1}. Teflon tubing used was 1/8 in. with a 0.8-mm internal diameter. It was connected to a Rheodyne 5011 six-position rotary valve used as injection valve with a 500-μL loop, and to the 1-mL Protein G column, supplied by GE Healthcare (Uppsala, Sweden). The observation cell was a flow-through 100-μl Hellma fluorescence cell, which was inserted into the cell compartment of a SLM-Aminco (Urbana, IL, USA) model 8100 photon-counting spectrofluorometer, equipped with a 450 W xenon arc source and a R928 photomultiplier tube. The instrument was controlled by the associated electronics to obtain the immunoaffinity chromatogram.

2.2. Reagents

All reagents used were of analytical grade. Anti-gliadin polyclonal antibodies raised in rabbits (6.2 mg mL^{-1}) (Sigma–Aldrich, Steinheim, Germany, ref. G-9144) were used and diluted with a phosphate buffer solution (0.05 M, pH 7.2). A gliadin stock solution of 1 mg mL^{-1} was prepared dissolving the appropriate amount of gliadins (Sigma, ref. G-3375, MW 50,000) in 70% aqueous ethanol solution, stirring the suspension for 12 h, and centrifuging at 3500 rpm for 10 min. Working solutions of 200 and 20 μg mL^{-1} were prepared by diluting the supernatant with phosphate buffer solution (PBS) (0.05 M, pH 7.2). This buffer solution was prepared by dissolving the appropriate amount of potassium dihydrogen phosphate (Merck, Darmstadt, Germany) in water and adjusting the pH using hydrochloric acid. AuNPs dispersions (0.01% in $HAuCl_4$) (Sigma–Aldrich) with nominal diameters of 10 and 20 nm were used as purchased. A carbonate buffer solution (0.05 M, pH 9.55) was prepared by dissolving the appropriate amount of sodium carbonate (Sigma–Aldrich) in distilled water and adjusting the pH using hydrochloric acid. Absolute ethanol (UV–IR–HPLC) (Panreac, Castellar del Vallès, Barcelona, Spain) was used to perform the sample treatment.

2.3. Procedures

2.3.1. Synthesis of gliadin–AuNP tracers

Two different tracers were synthesized, using AuNPs with diameters of 10- and 20-nm, respectively, by the following procedure: a volume (100 μL) of a 200 μg mL^{-1} gliadin solution prepared from the stock solution previously treated with 0.5 M sodium hydroxide for 10 min and then, diluted in carbonate buffer (0.05 M, pH 9.55) was mixed with 1 mL of AuNPs and the suspension was stirred for 15–30 s, and then, let to stand for 15 min at room temperature. After this time, the mixture was centrifuged for 45 min (10-nm AuNPs) or 20 min (20-nm AuNPs), at 10,000 rpm and 20 °C. The supernatant, with a pale pink color, was discarded and the precipitate was reconstituted in 1 mL of PBS (0.05 M, pH 7.2). The suspension was sonicated for about 15 s to achieve the complete re-dispersion of the precipitate.

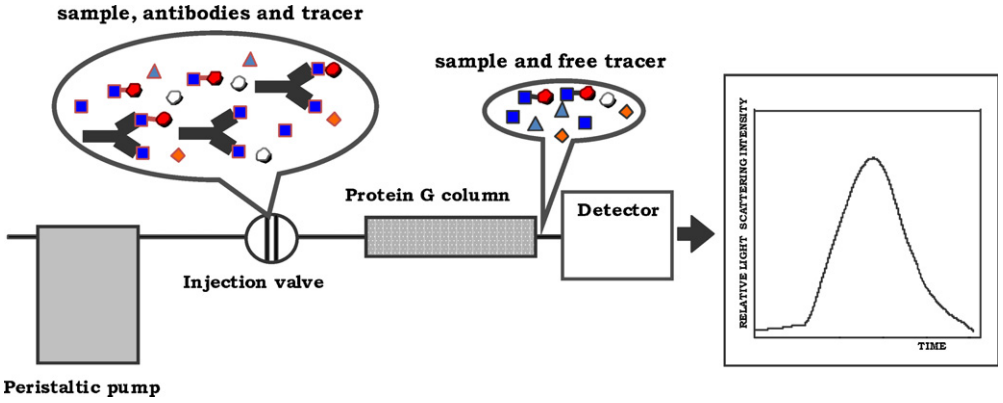

Fig. 1. Scheme of the immunoaffinity system used for gliadin determination.

2.3.2. Determination of gliadins

A volume (80 μl) of 4×10^{-7} M gliadin tracer, the suitable volume of gliadin standards, to achieve final concentrations in the range of 0.5–15 μg mL^{-1}, and 400 μL of 4×10^{-8} M polyclonal anti-gliadin antibodies were diluted to 2 mL with PBS. The mixture was left to stand 10 min at room temperature and, afterwards, it was injected in the Protein G column by means of an injection valve. The variation of the light scattering intensity with time was monitored for 4 min by placing both excitation and emission monochromators at λ = 535 nm.

2.3.3. Analysis of beer samples

Each beer sample was degassed by magnetic stirring for 30 min and an amount (0.5 g) was mixed with 1 mL of absolute ethanol. The mixture was homogenized and kept in the freezer until the precipitation of alcohol insoluble compounds. Then, the mixture was centrifuged at 12,000 rpm for 20 min and the supernatant was taken. An appropriate volume (500 μL) of this solution was subjected to the above mentioned procedure for the determination of gliadins.

3. Results and discussion

3.1. Immunoaffinity system

The system presented here uses commercial pre-packed Protein G columns that work in a reproducible way since the potential irregularities between batches of home-made columns is minimized or eliminated. The procedure allows repeated injections of pre-incubated mixtures of tracer, analyte and antibodies, which considerably enhances the speed of the system because it is not necessary to elute between subsequent injections until the saturation of the column is reached. This approach increases the life-time of the column as the elution–regeneration cycles affect negatively the long-term stability of the column. Also, higher flow rates than those required for the on-line retention of analytes onto the column can be used since the formation of the immunocomplexes is not the limiting step. Both features are possible owing to the strong affinity of polyclonal antibodies towards Protein G and the relatively high capacity of Hi Trap Protein G columns to immobilize either polyclonal or monoclonal IgG. Another advantage of this methodology is that the column reusage is expanded because less elution–regeneration cycles, which affect the long-term stability of the column, are required.

3.2. Scattering properties of gliadin–AuNP tracers

Light scattering measurements were performed using a conventional spectrofluorometer and placing both excitation and emission monochromators at the same wavelength. This wavelength was selected by obtaining the synchronous spectra of the tracer, which showed a high light scattering band at 520–570 nm, with a maximum signal at about 535 nm. This band can be ascribed to the AuNPs scattering as it was not shown in the synchronous spectra obtained for gliadins, at the same concentration level than that of the tracer. The scattering signal obtained for the tracer involving 10-nm AuNPs as label was about two times lower than that obtained using 20-nm AuNPs, at the same concentration level, which was expected since the scattering signal depends on the size of the dispersed particles.

Fig. 2 shows several immunoaffinity chromatograms obtained at different gliadin concentrations for the 20-nm AuNP–gliadin tracer. As it can be seen, the peak height of the immunoaffinity chromatogram is directly proportional to gliadin concentration, since the free tracer concentration increases as the gliadin concentration increases. The potential influence of gliadins on the light scattering intensity of the system was studied by injecting onto the column

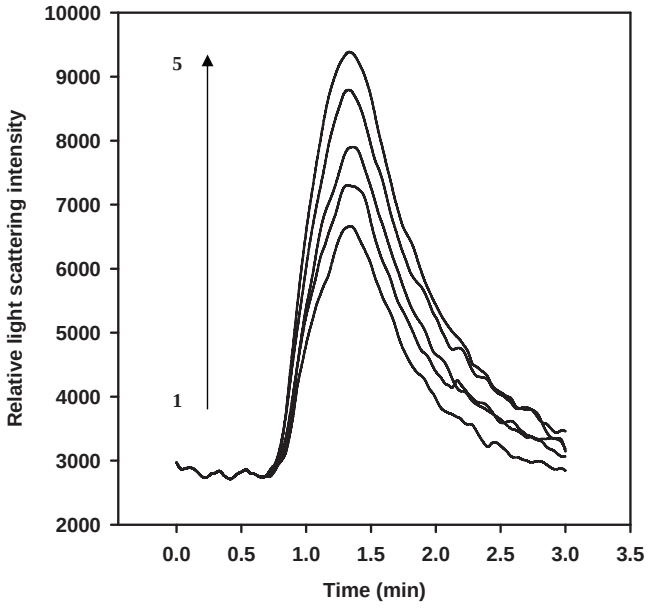

Fig. 2. Immunoaffinity chromatograms obtained at (1) 0, (2) 1, (3) 5, (4) 10 and (5) 15 μg mL^{-1} gliadins. Experimental conditions: [anti-gliadin antibodies] = 8×10^{-9} M, [tracer] = 1.6×10^{-8} M, [phosphate buffer] = 0.05 M, pH 7.2, flow-rate = 1 mL min^{-1}, diameter of AuNPs = 20 nm.

solutions of tracer, gliadins, and the mixture of tracer and gliadins, at the same concentrations, in the absence of anti-gliadin antibodies. Fig. 3 shows the immunochromatograms obtained, from which can be seen that the tracer alone gives the same signal that in the presence of gliadins, whereas gliadins alone do not give any signal. This assay shows that the signals obtained in the presence of antibody (Fig. 2) correspond to free tracer concentrations, which increase as a result of the immunochemical reaction of the antibody with gliadins.

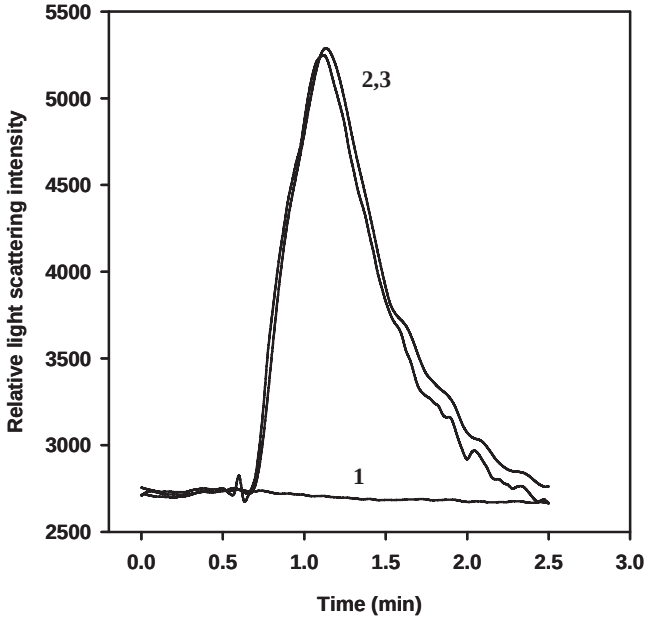

Fig. 3. Blank immunoaffinity chromatograms obtained for (1) 2 μg mL^{-1} gliadins, (2) 8×10^{-9} M tracer and (3) 2 μg mL^{-1} gliadins together with 8×10^{-9} M tracer. pH 7.2, [phosphate buffer] = 0.05 M, flow-rate = 1 mL min^{-1}, diameter of AuNPs = 20 nm.

3.3. Optimisation of the system

The variables affecting the system were optimised by the univariate method, using the difference in the relative scattering intensity obtained in the presence (B) and absence (B_0) of gliadins as the analytical parameter. Each result was the average of three measurements.

Two tracers were synthesized using a 200 μg mL^{-1} gliadin solution and 20-nm and 10-nm AuNPs as supplied. Different volumes of the gliadin solution, from 0.05 mL to 0.6 mL, were mixed with variable volumes of AuNPs, until a total volume of 1.1 mL. The best results were obtained using 0.1 mL of the gliadin solution with 1.0 mL of AuNP solution, decreasing the light scattering intensity when a lower amount of AuNPs was used. The formation of the tracer was visually observed because the AuNPs color turned more reddish and intense than that exhibited by AuNPs alone. The synthesis was carried out using a 0.05 M carbonate buffer solution of pH 9.55, which has been previously described to obtain conjugates by means of the protein adsorption onto AuNPs [7,8]. The incubation time of the synthesis reaction was checked from 5 min to 30 min, finding that 10–15 min were enough to obtain the tracer. The increase of the incubation time did not provide any increase in the light scattering intensity from the tracer. The study of the centrifugation conditions showed that 15 min were enough to separate the gliadin–AuNP conjugate from the AuNPs free in solution, when 20-nm NPs were used. However, the separation required 45 min for 10-nm AuNPs. All centrifugations were carried out at a temperature of 20 °C to avoid the excessive heating of the mixture, which could cause AuNPs aggregation and destabilization. The conjugate was re-dispersed using ultrasonication in a relatively short time, not much longer than 15 s, to avoid the formation of a dark violet precipitate that cannot be re-dispersed, which could be ascribed to the tracer decomposition and AuNPs aggregation.

The system was optimised by assaying the two tracers prepared from both 20-nm and 10-nm AuNPs, obtaining similar optimum values for the experimental variables in both instances. The study of the performance of the Protein G column and its capability to retain the immunocomplexes of the analyte and the tracer showed that the system remained practically unaltered after the sequential injection of 25 pre-incubated mixtures and that elution–regeneration cycles could be done at least for 20 times. The influence of the flow-rate was evaluated in the range of 0.4–1 mL min^{-1}, finding that the signal increased as the flow-rate increased. Values higher than 1 mL min^{-1} were not assayed since this is the optimum flow-rate recommended by the manufacturer for the proper functioning of Protein G column.

Several assays were carried out to obtain the optimum tracer and antibody concentrations in the presence of 2 μg mL^{-1} gliadin concentration. Fig. 4 shows the influence of the antibody concentration, which was studied in the range 2.6×10^{-9}–2×10^{-8} M, in the presence of 1.6×10^{-8} M tracer concentration. An 8×10^{-9} M antibody concentration was chosen as the optimum value because, although higher antibody concentrations gave lower blank signals, the differences obtained in the presence of the analyte were lower. The study of the influence of the tracer concentration in the range 4×10^{-9}–4×10^{-8} M and in the presence of the optimum antibody concentration showed that a 1.6×10^{-8} M tracer concentration was suitable for the development of the method.

The influence of the pH on the assay was evaluated in the presence of 0.05 M phosphate buffer solution in the range 6.0–8.0, which is the typical working range of most immunoassays. This study showed that a pH range of 7.0–7.5 was adequate to obtain the best differences between the signals obtained in the presence and absence of the analyte.

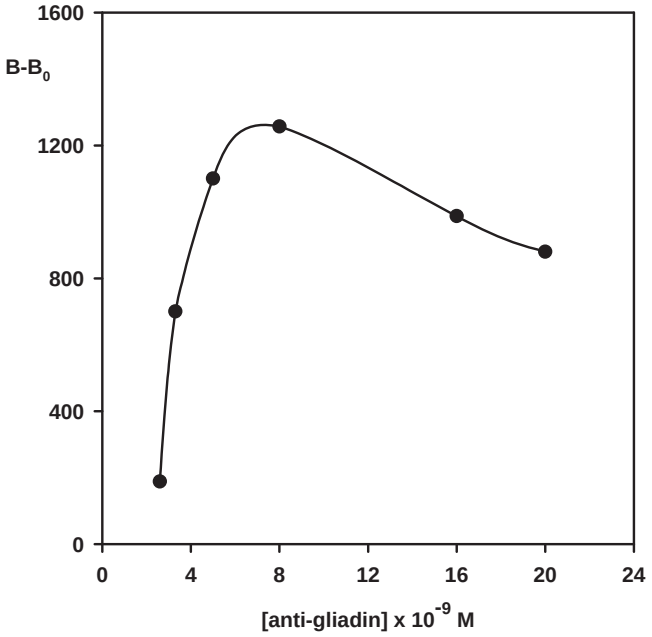

Fig. 4. Influence of antibody concentration on the system. Conditions: [tracer] = 1.6×10^{-8} M, [gliadin] = 2 μg ml^{-1}, [phosphate buffer] = 0.05 M, pH 7.2, flow-rate = 1 mL min^{-1}, diameter of AuNPs = 20 nm.

3.4. Analytical features of the system

Fig. 5 shows the calibration curves obtained in the presence of each tracer using light scattering measurements obtained by placing both excitation and emission monochromators at λ = 535 nm and the difference in the scattering peak height of the immunochromatograms obtained for the eluted free tracer in the presence and in the absence of gliadins ($B - B_0$) as the analytical parameter. The calibration curve obtained with the tracer prepared from 20-nm AuNPs (Fig. 5A) has a wider linear range (0.5–15.0 μg mL^{-1}) than that using the tracer from 10-nm AuNPs (Fig. 5B) (1.5–15.0 μg mL^{-1}). Both calibration curves were adjusted to a four-parameter logistic regression curve by plotting the analytical parameter vs the gliadin concentration expressed in μg mL^{-1}. The values for the four parameters of the regression equation ($y = y_0 + a/(1 + (x/x_0)^b)$) were $y_0 = 430 \pm 80$, $x_0 = 7 \pm 2$, $a = 2700 \pm 400$ and $b = -1.2 \pm 0.2$ for the calibration curve obtained with 20-nm AuNP–gliadin tracer, and $y_0 = 160 \pm 60$, $x_0 = 8 \pm 1$, $a = 1660 \pm 20$ and $b = -2.3 \pm 0.7$ for the calibration curve obtained using the 10-nm AuNP–gliadin tracer. The correlation coefficients were 0.9992 and 0.997 for 20-nm and 10-nm AuNP–gliadin tracers, respectively, which suggest a good fit of the experimental data to the regression curve. The detection limits, calculated according to IUPAC recommendations [21], were 0.2 and 0.8 μg mL^{-1} for tracers with 20-nm and 10-nm AuNPs, respectively. The precision was assayed at two gliadin concentration levels and expressed as the percentage of relative standard deviation. The values obtained using the 20-nm AuNP–gliadin tracer at 1 and 8 μg mL^{-1} gliadin concentrations were 2.7% and 2.9%, respectively, whereas those for 10-nm AuNP–gliadin tracer were 4.0% and 6.1%, when assayed at 1.5 and 8 μg mL^{-1} gliadin concentrations. The polyclonal antibodies used in this study, which were developed in rabbit, can react with prolamins fractions of rye, barley and oat, but they do not react with maize, potato or rice prolamins. Thus, these antibodies can detect prolamins from different cereals, which are toxic for patients suffering from coeliac disease.

The comparison of the analytical features of the methods developed by using both tracers shows that the method using 20-nm AuNPs features a higher dynamic range of the calibration graph,

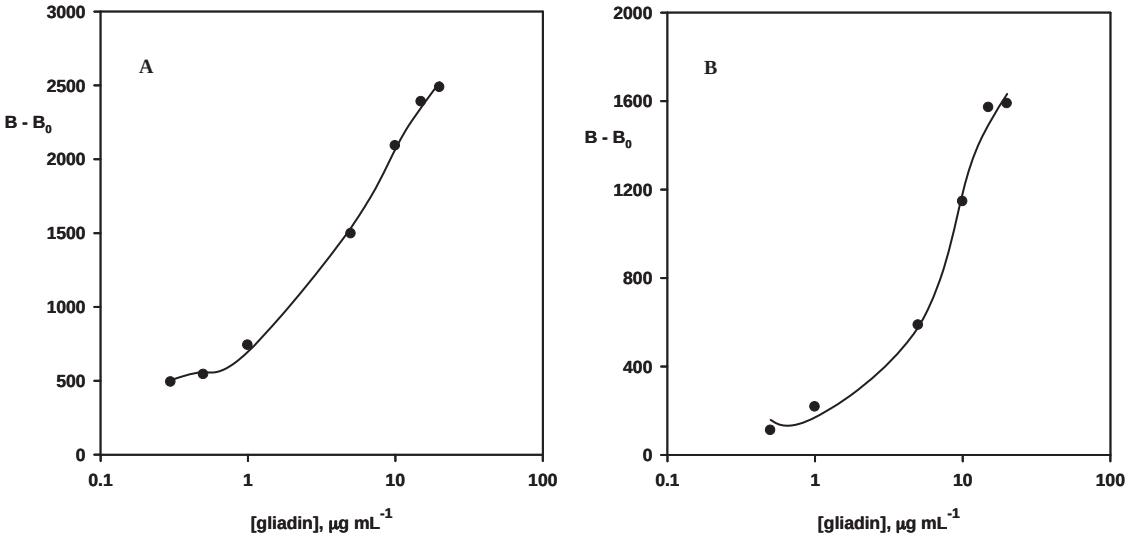

Fig. 5. Calibration curves obtained using the tracers prepared from 20-nm AuNPs (A) and 10-nm AuNPs (B). Experimental conditions: [anti-gliadin antibodies] = 8×10^{-9} M, [tracer] = 1.6×10^{-8} M, [phosphate buffer] = 0.05 M, pH 7.2, flow-rate = 1 mL min^{-1}.

Table 1
Determination of prolamins in beer samples.

Sample	Prolamin content[a] (mg kg^{-1})	Recovery study		
		Added (mg kg^{-1})	Found[a] (mg kg^{-1})	Recovery (%)
Gluten-free beer	–	24	19 ± 1	79.2
		60	61 ± 4	101.7
		84	71 ± 6	84.5
Gluten-containing beer	10.3 ± 0.9	24	17.1 ± 0.8	71.2
		60	50 ± 5	83.3
		84	80 ± 3	95.2

[a] Mean ± SD.

lower detection limit and better precision than the method involving the 10-nm AuNP–gliadin tracer.

3.5. Applications

The method was applied to the analysis of two barley malt beer samples. One of them was a gluten-free beer (<6 ppm gluten) and the other was a gluten-containing beer. The sample treatment used was quite simple and it was intended to remove potential interferences from the sample matrix. Table 1 shows the results obtained and the recovery study, which was carried out by adding three different amounts of gliadins to the samples and subtracting the results obtained from similarly treated non-spiked samples. The values obtained were in the range 71.2% and 101.7%, which are similar to those provided by some competitive ELISA methods [17,18] described for this purpose.

4. Conclusions

The present method constitutes, to the best of our knowledge, the first application of AuNPs as labels for the indirect detection in immunoaffinity chromatography. This study contributes to expand the versatility of AuNPs in bioassays, and more specifically, to the applications of protein–AuNPs interactions. A very simple immunoaffinity procedure with a relatively high sample throughput has been achieved by using commercial pre-packed Protein G columns and pre-incubated mixtures of the immunoreagents and samples. The results obtained by assaying two different tracers involving AuNPs with diameters of 10 and 20 nm as labels showed that the NPs of higher size provide the immunoaffinity method with better analytical features. An interesting feature of this method is that it avoids the multiple incubation and washing steps that are needed for the performance of competitive and sandwich ELISA methods often used for the determination of gliadins.

Acknowledgments

Authors gratefully acknowledge financial support from the Spanish Ministerio de Ciencia e Innovación, MICINN (Grant No. CTQ2009-08621) and from the Junta de Andalucía and the FEDER-FSE Program (Grant No. P09-FQM4933). M.A.M.D. thanks the Spanish Ministerio de Ciencia e Innovación, MICINN (Grant No. CTQ2009-08621) for the financial support of her research contract.

References

[1] A. Gómez-Hens, J.M. Fernández-Romero, M.P. Aguilar-Caballos, Trends Anal. Chem. 27 (2008) 394–406.
[2] M. Pumera, S. Sanchez, I. Ichinose, J. Tang, Sens. Actuators B 123 (2007) 1195–1205.
[3] J. Wang, Electroanalysis 19 (2007) 769–776.
[4] A. Gómez-Hens, J.M. Fernández-Romero, M.P. Aguilar-Caballos, Mini Rev. Med. Chem. 9 (2009) 1064–1074.
[5] J. Bogdanovic, J. Colon, C. Baker, Q. Huo, Anal. Biochem. 405 (2010) 96–102.
[6] Q. Huo, Colloids Surf. B: Biointerfaces 78 (2010) 259–265.
[7] B. Du, Z. Li, Y. Cheng, Talanta 75 (2008) 959–964.
[8] M.L. Sánchez-Martínez, M.P. Aguilar-Caballos, A. Gómez-Hens, Anal. Chim. Acta 636 (2009) 58–62.
[9] M.L. Sánchez-Martínez, M.P. Aguilar-Caballos, S.A. Eremin, Anal. Chim. Acta 553 (2005) 93–98.
[10] M.L. Sánchez-Martínez, M.P. Aguilar-Caballos, S.A. Eremin, Anal. Bioanal. Chem. 386 (2006) 1489–1495.
[11] Codex Standard for foods for special dietary use for persons intolerant to gluten. CODEX STAN 118-1979 (amended in 2008). http://www.codexalimentarius.net/.
[12] D. Gabrovska, J. Rysova, V. Filova, J. Plicka, P. Cuhra, M. Kubik, S. Barsova, H. Bulawová, F.G. Chirdo, A. Dermühl, S. Iametti, S. Kirinčič, J. Pavelka, H. Tlaskalová, J. AOAC Int. 89 (2006) 154–160.
[13] Y. Nicolas, S. Denery-Papini, J.P. Martinant, Y. Popineau, Food Agric. Immunol. 12 (2000) 53–65.
[14] L. Sorell, J.A. López, I. Valdés, P. Alfonso, E. Camafeita, B. Acevedo, F. Chirdo, J. Gabilondo, E. Méndez, FEBS Lett. 439 (1998) 46–50.
[15] W. Seilmeier, H. Wieser, Eur. Food Res. Technol. 217 (2003) 360–364.

[16] F. Kahlenberg, D. Sánchez, I. Lachmann, L. Tuckova, H. Tlaskalova, E. Mendes, T. Mothes, Eur. Food Res. Technol. 222 (2006) 78–82.
[17] B. Gessendorfer, P. Koehler, H. Wieser, Anal. Bioanal. Chem. 395 (2009) 1721–1728.
[18] P. Dostálek, I. Hochel, E. Méndez, A. Hernando, D. Gabrovská, Food Addit. Contam. 23 (2006) 1074–1078.
[19] M.L. Sánchez-Martínez, M.P. Aguilar-Caballos, A. Gómez-Hens, Anal. Chim. Acta 523 (2004) 35–41.
[20] M.L. Sánchez-Martínez, M.P. Aguilar-Caballos, A. Gómez-Hens, Anal. Chem. 79 (2007) 7424–7430.
[21] G. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712A–724A.