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Article *in* FEBS Letters · December 1998

DOI: 10.1016/S0014-5793(98)01336-2 · Source: PubMed

FEBS Letters 439 (1998) 46–50 FEBS 21092

An innovative sandwich ELISA system based on an antibody cocktail for gluten analysis

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Received 7 September 1998; received in revised form 7 October 1998

Abstract A cocktail sandwich ELISA based on the employ of two monoclonal antibodies (MAbs) as coating antibodies and a third MAb conjugated to horseradish peroxidase has been developed for the analysis of gluten in foods. Given that each MAb displays a wide specificity spectrum for wheat, barley, rye and oats prolamins, their combination for ELISA ensures a high crossreactivity with most of the potentially toxic gliadin, hordein, secalin and avenin protein family. One of the unprecedented features of the cocktail sandwich ELISA is that it permits for the first time analysis of barley hordeins in foods, which is unattainable using conventional or commercial ELISA kits. Besides, gliadins, hordeins and secalins are recognised to the same extent. The system provides a high detection sensitivity for gliadins, hordeins, secalins and avenins (1.5, 0.05, 0.15 and 12 ng/ml, respectively). The working linear range comprises 3–100 ng/ml with a gliadin detection limit of 1.5 ppm. This limit of detection is even better than that demanded in the latest Codex recommendation, 10 ppm. Cocktail ELISA data were contrasted with those of commercial ELISA kits and confirmed by mass spectrometry, a non-immunological technique which provides evidence for the occurrence of false positive results with the commercial kits.

Key words: Coeliac disease; Gluten analysis; Gluten-free food; MALDI-TOF mass spectrometry; ELISA

1. Introduction

To date one of the enigmas in coeliac disease is to clear up the precise wheat, barley, rye and oats gluten toxic components [1]. Therefore the development of a detection procedure aimed to such target toxic components in gluten-free foods is an unresolved task. Despite that compliance to a toxic gluten-free diet is the only treatment for coeliac patients, the availability of a reliable in vitro or in vivo system to analyse toxicity in food samples to control diet is still far from the scope. In the meantime, as an alternative to a toxicity assay, immunological approaches, mainly ELISA [2–10], as well as a non-immunological approach by mass spectrometry [11,12], have been utilised to analyse gluten in food samples. Despite that several ELISA systems have been described since, only one ELISA based on a MAb against a wheat ω-gliadin has been ringtested [10] and commercialised in three different kits with detection limits of 10 and 80 ppm. This ELISA, as well as others described, based on Abs generated against gliadin fractions [5,7] or peptides derived of [6,13], mainly recognises prolamins from wheat and rye. Systems capable to detect as well the presence of toxic gluten prolamins from barley and oats in gluten-free products and with a detection limit of a few ppm are still demanded. In this sense, we recently reported a highly sensitive mass spectrometric system to quantify oats avenins in foods [13]. We now describe a cocktail sandwich ELISA system based on three MAbs which recognises for the first time barley as well as wheat and rye glutens.

2. Materials and methods

2.1. Materials

Wheat, barley, rye and oats cultivars employed in this study are indicated in Fig. 2. Commercially available foods (some of them kindly provided by J. Pedró, La Campesina, Barcelona, Spain), gluten-free foods and wheat starches were employed.

2.2. Preparation of standard protein extracts and food samples

After pre-extraction with 0.15 M NaCl to remove the albumin-globulin fraction, gliadin extracts from wheat (Senatore Capelli) or Sigma (G-3375) were dissolved in 60% (v/v) aqueous ethanol at 220–500 μg/ml and used for the standard curve. Other gliadins, hordeins, secalins, avenins and zeins (Fig. 2) were extracted from wheat, barley, rye, oats and maize flours as above. A 1-g food sample was extracted with 10 ml of 60% (v/v) aqueous ethanol using an Ultra-Turrax (Janke and Kunkel, Ika Labortechnick) for 1 min. Samples were centrifuged at $2500 \times g$ at room temperature and the supernatant collected. Protein concentration was determined according to the Kjeldahl method ($N \times 5.7$).

2.3. Commercial ELISA kits

Two commercial kits, Transia and r-Biopharm Ridascreen, with a detection limit for gliadins of 80 and 10 ppm, respectively, were used following the supplier's instructions.

2.4. Production of MAbs and screening of hybridomas

BALB/c female mice (6–8 weeks old) were immunized subcutaneously three times with 100 μg of wheat, rye or oats ethanol extracts. The first dose was emulsified with Freund's complete adjuvant and the other two with Freund's incomplete adjuvant. A final boost without adjuvant was administered intraperitoneally three days before the isolation of splenocytes. These were fused with P3/X63-Ag.653 myeloma cells as previously described [14]. Hybridomas were grown in 96-microwell cultures and selected in HAT medium. The identification of antibody-containing supernatants was done by ELISA using either secalins, gliadins, hordeins, avenins or zeins (as a negative control) at 10 μg/ml adsorbed to wells. Selected hybridomas were grown as ascites in pristane-primed BALB/c mice. The described 13B4 MAb [15] was employed in this study.

2.5. Purification of MAbs

MAbs were purified from ascites by affinity chromatography in a protein A-Sepharose column (Pharmacia). One MAb (Rye 3), which recognized gliadins, secalins, hordeins and avenins in direct ELISA and immunoblotting (see below), was conjugated to HRP by using the periodate method [16].

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PII: S0014-5793(98)01336-2

2.6. Cocktail sandwich ELISA

Polystyrene EIA/RIA flat bottom plates (Costar) were coated overnight at 4°C with 0.6 μg each of 13B4 and Rye 5 MAbs in 100 μl of 0.1 M sodium bicarbonate. Plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with PBS-T plus 1% BSA (blocking solution) for 1 h at room temperature. Plates were incubated for 1 h with sample extracts and gliadin standard diluted in the blocking solution. Dilutions were 1:50 for gluten-free foods. After washing, HRP-Rye 3 conjugated was added (1:1500 in the blocking solution) and incubated 1 h at room temperature. Plates were washed again and the substrate solution (0.05% *o*-phenylenediamine and 0.015% hydrogen peroxide in 0.15 mM phosphate-citrate buffer, pH 5.0) was added. The reaction was stopped 10 min later with 2.5 M sulfuric acid. Absorbances at 492 nm were measured in a microplate reader.

2.7. Reactivity of MAbs by immunoblotting

After mono-dimensional SDS-PAGE, gliadin, hordein, secalin, avenin and zein extracts were transferred onto PVDF membranes. The blots were incubated with MAbs (1 μg/ml) and then with anti-mouse IgG horseradish peroxidase-conjugated antibodies and developed with diamine benzidine tetrahydrochloride.

2.8. Analysis of food samples by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS)

Food samples extracts in 60% (v/v) aqueous ethanol were mass analysed on a Bruker Reflex II MALDI-TOF mass spectrometer as described elsewhere [11,17].

3. Results

3.1. Characterisation of MAbs

The generated MAbs raised against secalins (Rye 1, Rye 3, Rye 4 and Rye 5) and avenins (Oat 1) and the described 13B4 [13] against gliadins were assayed. Direct ELISA and immunoblotting analysis indicated that most of the MAbs displayed a wide crossreactivity spectrum with gliadins, hordeins and secalins and some of them also with avenins, but failed to recognise zeins (data not shown). Rye 3 showed the highest crossreactivity with most of the potentially toxic gliadin, hordein, secalin and avenin protein family (Fig. 1, insert). Therefore this antibody was selected to be conjugated to horseradish peroxidase as labelled antibody in sandwich ELISA studies.

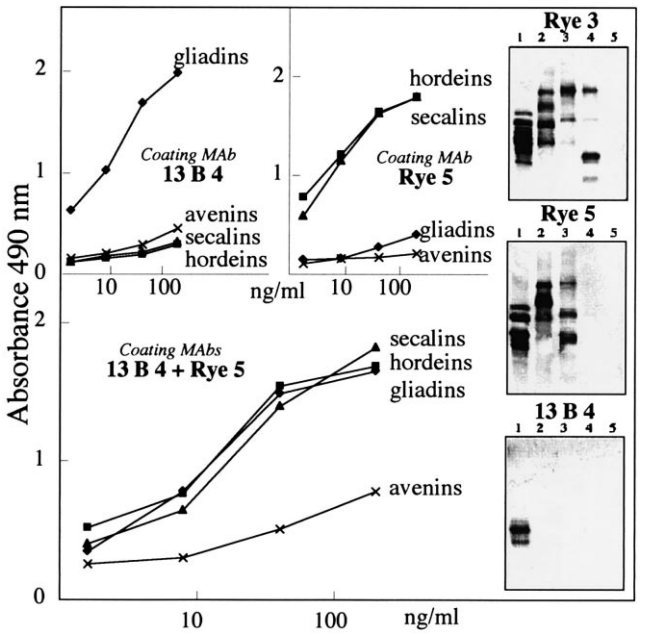

Fig. 1. Optimization of cocktail sandwich ELISA. Microtiter ELISA plates were coated with MAb 13B4 (upper left) or Rye 5 (upper right) or with a mixture of 13B4 plus Rye 5 antibodies (bottom), incubated with gliadin, hordein, secalin, avenin and zein extracts and developed with HRP-Rye 3 as conjugated antibody. Immunoblotting analysis of the three MAbs against gliadins (1), hordeins (2), secalins (3), avenins (4) and zeins (5) are inserted.

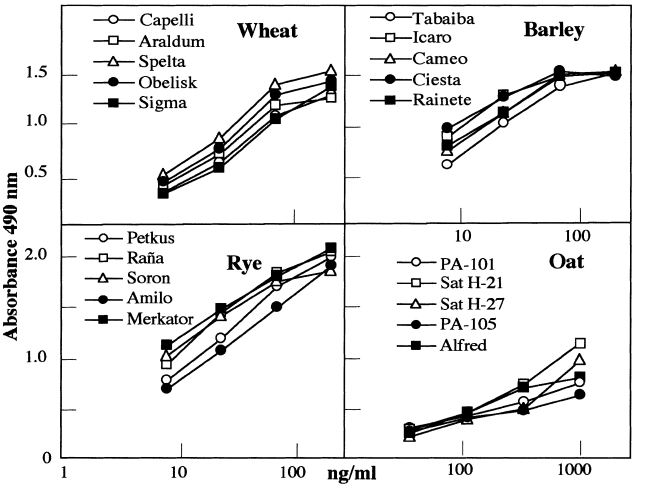

Fig. 2. Comparative reactivity of prolamins by cocktail sandwich ELISA. Five different ethanol extracts from wheat, barley, rye and oats cultivars were assayed.

3.2. Selection of MAbs for sandwich ELISA

The six MAbs were individually assayed as coating antibodies with HRP-Rye 3 MAb as labelled antibody in a sandwich ELISA. Most of the MAb combinations displayed marked specificity against either one or two types of prolamin extracts, as is the case when using 13B4, which permits to selectively recognize gliadins, while with Rye 5 secalins and hordeins are recognized (Fig. 1, top). None of these combinations enabled detection of gliadins, hordeins, secalins and avenins to the same extent.

3.3. Cocktail sandwich ELISA

In view of the above results, to enhance the recognition specificity against all types of prolamin extracts, mixtures of two distinct MAbs were assayed as coating antibodies using HRP-Rye 3 MAb as labelled antibody. Amongst all antibody mixtures, the best results were accomplished when using the combination of 13B4 and Rye 5. Results are easily explained by the behaviour of 13B4 and Rye 5 when used separately as single coating antibodies in sandwich ELISA (Fig. 1, top). This antibody cocktail permitted the recognition of gliadins, secalins and hordeins to the same extent in the 3–200-ng/ml range, while the sensitivity against avenins was much lower (Fig. 1, bottom). A noticeable feature of this ELISA system with respect to others previously reported, which mainly detect gliadins and secalins, is that this system allows for the first time a highly sensitive detection of barley hordeins as well. We have named this sandwich ELISA system as cocktail sandwich ELISA. It should be noted that no relevant differences were found in the recognition behaviour when using gliadin, secalin, hordein and avenin extracts from distinct cereal cultivars (Fig. 2). This suggests that the system performance will not be significantly affected by the choice of a stand-

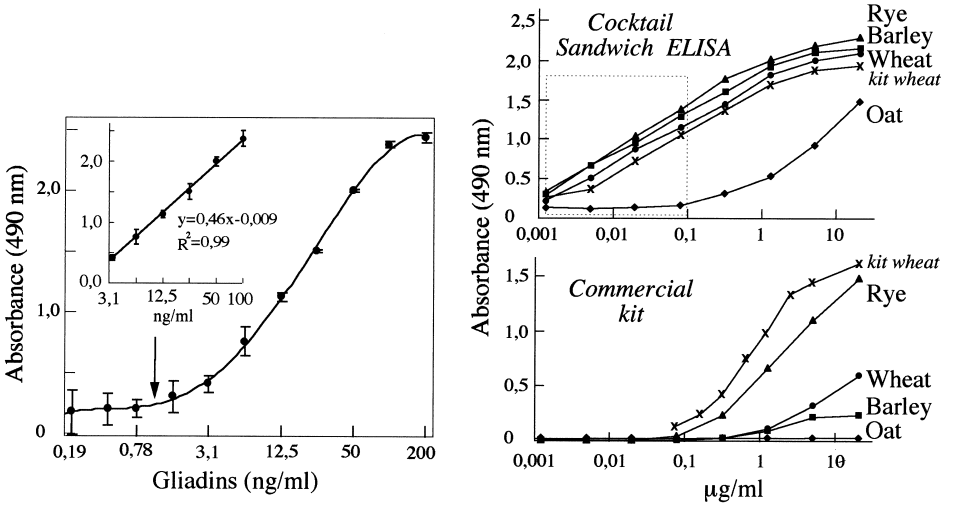

Fig. 3. Left: Standard curve for gliadin determination with the cocktail sandwich ELISA. Vertical bars represent standard errors for eight independent assays. The 3.1–100 ng of gliadins/ml linear range is inserted. The arrow points out the lower detection limit. Right: Comparative analyses of wheat gliadin, rye secalin, barley hordein and oats avenin extracts and the kit wheat standard at varying concentrations by cocktail sandwich ELISA and commercial kit. The working linear range is indicated by a box.

ard. In fact, no relevant differences were found when employing a gliadin standard from either Sigma or Senatore Capelli (data not shown).

3.4. Sensitivity of the cocktail sandwich ELISA

To establish the standard curve for gliadins (Senatore Capelli) in the cocktail sandwich ELISA, the lower detection limit was defined as the minimum gliadin concentration giving an absorbance value distinguishable from that of the blank according to a *t*-test at 99% confiability. This detection limit was 1.5 ng/ml as estimated from 30 independent experiments (Fig. 3, left). However, the working linear range of the standard curve was 3–100 ng/ml. This working lower detection limit of 3 ng/ml permits to analyse food samples down to 0.15 mg of gliadins per 100 g of dry food, i.e. 1.5 ppm of gliadins when working with a 1:50 minimum sample dilution. When determining the standard curve under the above conditions for gliadins, the lower detection limits for hordeins, secalins and avenins were 0.05, 0.15 and 12 ng/ml, respectively.

To determine the reproducibility of the cocktail sandwich ELISA, inter- and intra-assay coefficients of variation (c.v.) were calculated. For the inter-assay, 8 independent gliadin standard curves were determined and the absorbance c.v., calculated at each point of the curve, was in the 3–8% range. In addition, the inter-assay absorbance c.v. was also calculated using three distinct gluten-free samples (containing 0.4, 1.5 and 5 mg of gliadins per 100 g) in 12 independent measurements. The c.v. was 4, 6.6 and 4.5%, respectively. For the intra-assay, 12 independent measurements on the above three gluten-free samples yielded a c.v. around 4% in all three cases.

3.5. Cocktail sandwich ELISA vs. the commercial ELISA kit

In order to compare sensitivity and selectivity, varying concentrations of gliadin, secalin, hordein and avenin extracts, as well as the commercial kit wheat standard in the 0.01–20-µg/ml range were tested by both ELISA systems. While all extracts, including the commercial kit wheat standard, were equally recognised by the cocktail sandwich ELISA in the whole concentration range (Fig. 3, right, top), on the contrary by the commercial kit these are distinctly recognised (Fig. 3, right, bottom). In addition, it should be noted that the cocktail sandwich ELISA can clearly detect barley (Fig. 3, right, top), which remains nearly undetected by the commercial kit (Fig. 3, right, bottom), as expected [10]. This is exemplified in Fig. 4, insert, where the cocktail sandwich ELISA quantitatively detects hordeins in a gluten-free food sample to which increasing amounts of barley hordeins were added. Again, the commercial kit fails to detect these hordeins, even at high concentrations. Furthermore, the working range for the cocktail sandwich ELISA, from 0.001 to 0.1, is markedly superior to that of the commercial kit, 0.1–20 µg/ml (Fig. 3, right).

3.6. Analysis of food samples by cocktail sandwich ELISA

The validation of the cocktail sandwich ELISA was made by testing gluten-free foods and food samples whose results were compared with those of two commercial kits. Results for some selected samples are presented in Tables 1 and 2. De-

Table 1
Analysis of gliadins (ppm) in gluten-free foods and starch samples by commercial kits, cocktail ELISA and mass spectrometry

Sample	Commercial kits[a]	Cocktail	Mass spectrometry[b]
18	<40	7.0	ND
20	<40	10.5	ND
1002	<40	17.0	ND
1134	<40	18.0	ND
1145	<40	5.0	ND
Starch 1	<40	4.5	10.5
Starch 2	63	40.5	59.0
Starch 3	200	57.0	48.5
Starch 4	865	195.0	107.0
Starch 5	120 (200)	23.0	21.5
1188	200 (1035)	28.5	R
1114	290 (105)	7.0	R
1102	<40 (34) 1765[c]	4.5	R

[a]Transia; Ridascreen (in parentheses).
[b]ND, not detected.
[c]From a different laboratory.
R: Only rice prolamin mass pattern detected.

spite that the first six samples in Table 1 are shown by both systems to be gluten-free samples, the high sensitivity of the cocktail sandwich ELISA permits quantification of a few ppm gliadins, while the commercial kit cannot discriminate values below 40 ppm.

Most of the remaining samples yield varying values by the commercial kits which turn out to be several times higher than those provided by the cocktail sandwich ELISA, while a good correlation is obtained between sandwich ELISA data and mass spectrometric data (Table 1, starches 2–5). On the other hand, samples 1188, 1114 and 1102 yield high, distinct values by both commercial kits which the cocktail sandwich ELISA reveals much lower. These low values are in agreement with the absence of gliadin, secalin, hordein or avenin mass signals in the corresponding mass spectra (Fig. 4). Only rice prolamin mass peaks (15 kDa, not comprised in the figure) were obtained for these samples, thus supporting the possibility that the commercial kits results constitute false positives, as previously reported [18]. In this sense, sample 1102 yields completely opposite values (<40 and 1765 ppm) when analysed with two different batches of the same commercial kit.

3.7. Analysis of barley by cocktail sandwich ELISA

At present, the hordein content in barley-contaminated gluten-free foods cannot be determined by means of the available commercial kits since both display a very low sensitivity for barley (Figs. 1 and 4). Because of the high sensitivity of the cocktail sandwich ELISA for hordeins (Figs. 1 and 4), the system has enabled to determine barley content in food samples (Table 2). Barley content values were measured by the cocktail sandwich ELISA not only in three gluten-free foods, one of them elaborated with barley starch, but also in a group of commercial barley-containing foods. Data in Table 2 indicate noticeable differences between the two systems. While the cocktail ELISA yields very high barley content values for all samples, the commercial kit could detect barley only in two extremely high barley content samples. The presence of barley in all these samples was confirmed by mass spectrometry (Fig. 4, samples D and 90).

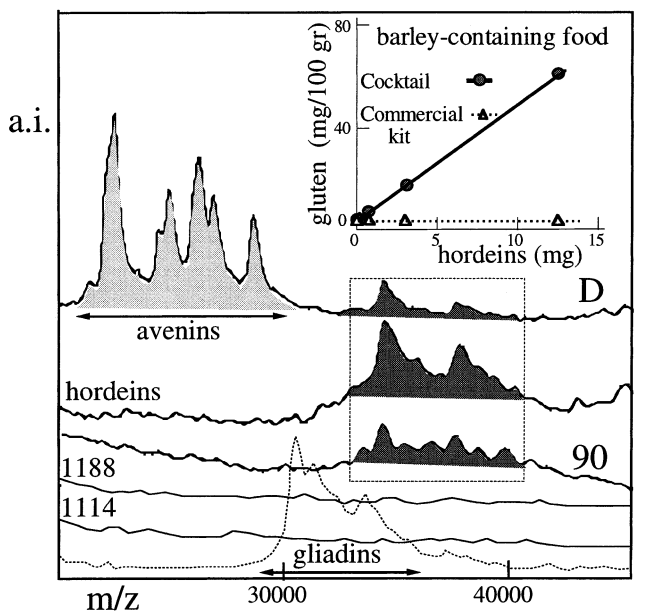

Fig. 4. MALDI-TOF mass spectra of the ethanol extracts from barley-containing food samples. Barley- and oat-containing foods and barley starch-containing food (Samples 1184, 1111, D and 90 in Tables 1 and 2). Mass spectra of hordein and gliadin standards have been included for comparison. The hordein mass range is in a box. Barley gluten quantification in a gluten-free food sample after the addition of increasing amounts of hordeins by cocktail sandwich ELISA and commercial kit is inserted.

Table 2
Analysis of barley-containing foods by a commercial kit and cocktail ELISA

Sample	Commercial kit	Cocktail
90[a,b]	<40	1020
1046[a]	<40	125
64[c]	281	>5000
A[c]	70	2700
B[a]	<40	235
C[d]	<40	935
D[c]	<40	210
E[c]	<40	1175

[a]Gluten-free food.
[b]Barley starch-containing gluten-free food.
[c]Barley and oats-containing food.
[d]Barley-containing food.
Hordeins were revealed by mass spectrometry in all samples. Values in ppm as in Table 1.

Samples presented in Tables 1 and 2 were selected from a group of around 400 foods, mainly gluten-free samples, analysed by the cocktail sandwich ELISA and the commercial kit. All of these samples were analysed using a gliadin standard from Senatore Capelli. However, to address the effect of changing the gliadin standard, a large group of them was assayed using a different gliadin standard (Sigma) as well. A mean ratio of 1.3 with a correlation factor 0.993 was found between values measured using these two gliadin standards. Results indicate that values measured do not undergo relevant shifts when changing the gliadin standard, as suggested by Fig. 2.

4. Discussion

Present epitope-dependent methods based on polyclonal and monoclonal antibodies mainly recognise wheat and rye glutens, to a lesser extent barley and fail to detect oats gluten. The lack of specificity together with the limited sensitivity of these methods makes it difficult to lower the limits presently accepted for gluten-free products by the Codex Alimentarius, 200 ppm gluten equivalent to 100 ppm gliadins [19]. Moreover, this entails one of the main problems when attempting to control diet, since coeliac patients often become silent consumers of toxic substances from barley or oats when these cereals are present as contaminants in gluten-free products.

We have developed a sandwich ELISA based on two coating MAbs and a third HRP-conjugated MAb whose high crossreactivity against the prolamin family has permitted detection of wheat, barley and rye glutens to the same extent and more weakly oats gluten. In addition, the combination of these MAbs provides a high crossreactivity that must be responsible for the very low recognition variability observed when analysing different wheat, barley, rye and oats varieties, in contrast to the two commercial kits r-Biopharm and Transia, which yield highly varying results depending on both the cereal type and variety [20,21]. Consequently, quantification results by the cocktail ELISA are expected not to depend significantly either on the particular cereal varieties employed

to elaborate the food sample or the choice of a standard; in fact, nearly identical values were found when analysing a large group of food samples employing a gliadin standard from either Sigma or Senatore Capelli.

The cocktail ELISA is suitable for the analysis of thermally processed gluten-free foods, since no alteration in the quantification measurements by this system was observed when heating gliadin, hordein and secalin solutions (1–100 ng/ml) and avenin solutions (50–1000 ng/ml) at least to 120°C up to 30 min (data not shown).

The comparative analyses performed in a large number of samples with the cocktail ELISA and the commercial kits, shown for some selected samples in Table 1, have permitted to reach the following conclusions: (i) for low gliadin content gluten-free foods, both the cocktail ELISA and the commercial kits have permitted to classify such samples as gluten-free, although the cocktail ELISA allows measurement of gliadins at the low ppm level; (ii) for higher gliadin content foods, values are generally higher by the commercial kits, while those by cocktail ELISA and mass spectrometry are quite similar; and (iii) a third group of samples yields very low values by the cocktail ELISA and very high values by the two commercial kits. Despite that both commercial kits are based on the same MAb raised against ω-gliadin, the discrepancy of data between both systems is noteworthy. The possibility that some of these inconsistent high values could be due to false positive results is supported by the mass spectrometric data, which reveal the absence of wheat, barley, rye and oats glutens in agreement with the low values yielded by cocktail ELISA. The occurrence of possible false positive results by the commercial kits when analysing maize flours as well as millet had already been claimed [18]. In this sense, we frequently found higher values by the commercial kits than by cocktail ELISA when analysing gluten-free foods elaborated with maize flour (data not shown) whose mass spectra showed only maize prolamin signals.

One of the outstanding features of the cocktail ELISA is that it allows the analysis of barley gluten to the same extent as wheat in contrast to most ELISA systems [5–7,13], including commercial kits, which are nearly insensitive to barley gluten. Thus the cocktail ELISA has permitted to analyse for the first time the content of barley in food samples.

To date, commercial ELISA kits and Western blot immunological methods have been routinely employed for the analysis of gluten in foods with the inconvenience of the lack of specificity for barley of the aforementioned ELISA systems and their limited sensitivity together with the high time consumption of Western blot. As an alternative, we have successfully employed the highly sensitive cocktail ELISA together with the rapid mass spectrometric technique, with a low detection limit around 5–10 ppm gliadins [11,13]. Since mass spectrometry allows selective identification of wheat, barley, rye and oats glutens, this non-immunological technique facilitates confirmation of the reliability of ELISA data and provides evidence for false positive results arising from antibody crossreactivity, thus helping clear up suspicious values.

In conclusion, this innovative cocktail ELISA permits the analysis of wheat, barley and rye glutens to the same extent down to 1.5 ppm, i.e. around the limit desirable for gluten-free foods as stated by the Codex Alimentarius [20] but not yet recommended since to date no commercial kit is capable of detecting gluten at this level.

Acknowledgements: This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología Bio95-2070-E and Bio97-0076.

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