Improved Protocols for ELISA Determination of Gliadin in Glucose Syrups

Stefania Iametti,[1,2] Chiara Cappelletti,[1] Antonio Oldani,[1] Laura Scafuri,[3] and Francesco Bonomi[1,4]

ABSTRACT

Cereal Chem. 81(1):15–18

Simple modifications of existing protocols for high-sensitivity detection of gluten proteins by immunochemical methods allowed rapid and sensitive determination of residual gluten in highly viscous samples of glucose and maltose syrups obtained from processing wheat starch. Dilution of the original syrup to no less than 15–20% in solids allowed retention of gluten proteins in a soluble form so that ELISA determination of gliadin was possible without an extraction step in aqueous ethanol. An ultrafiltration step may be added to concentrate residual gluten proteins in the diluted syrup samples and allow a further increase in sensitivity. The results are relevant for quality assessment of wheat starch derived syrups as raw materials for use in gluten-free foods for celiac individuals.

Celiac disease is an autoimmune disease associated with the consumption of gluten. Gluten represents the main water-insoluble protein fraction in several cereals such as wheat, rye, barley, and triticale. The consumption of gluten, and in particular the gliadin fraction, can have a toxic effect on the gastrointestinal tract (Ferguson 1996). The different forms of celiac disease affect more than one individual out of three hundred in Western Europe (Corazza and Gasbarrini 1995; Catassi et al 1997).

Celiac individuals must follow a gluten-free diet. However, the successful implementation of a gluten-free diet is complicated by the frequent use of gluten-containing grains, especially wheat and its derivatives, in numerous food products. Wheat and wheat-derivatives are used either alone or associated with other food ingredients. In the latter case, little if any attention is paid to the presence of residual wheat proteins that may represent a remarkable health threat to sensitive individuals.

The issue of the allowed daily intake of gluten in celiac patients is much debated. In Northern Europe, a food is considered to be gluten-free when its gluten content is <1,000 mg/kg. The Celiac Associations in Southern Europe have proposed a much lower limit for gluten-free food (10 mg/kg). The official limits described in the Codex Draft Revised Standard (2000) are 20 mg/kg for foodstuffs naturally gluten-free, 200 mg/kg for foodstuffs rendered gluten-free; solid foodstuffs on a dry matter basis, and liquid foodstuffs on the basis of the original product. Quantitative data on the gluten content of foods is of capital importance to ensure the well-being of celiac individuals. One typical case of such foods is wheat starch derivatives including glucose and maltose syrups.

In the absence of accurate data on the gluten content of sugars derived from wheat starch, most clinicians have banned all products containing glucose syrups from the diet of celiac patients. Concerns about the possible presence of gluten in wheat-derived sugars are also felt by the starch industry. Producers of starch derivatives are asked by the food industry to provide accurate estimates of residual gluten in the syrups that are used as ingredients in a countless number of popular consumer products.

As a routine, gluten is most conveniently analyzed by ELISA tests (see review in Denery-Papini et al 1999). The AOAC-approved test based on the monoclonal antibodies against ω-gliadins developed by Skerrit (Skerrit and Hill 1990, 1991) offers the advantage of detecting heat-stable proteins but has some limitations. The major criticism is that ω-gliadins are only a minor component in gluten and show a considerable variability among wheat cultivars (6–20% of total gliadins; the lower figure is characteristic of Western Europe wheat germplasm). To the best of our knowledge, all current ELISA protocols call for an initial step in which water-insoluble gliadins are extracted into aqueous 40–60% ethanol (by volume). (Ridascreen Gluten 2000; Enzyme immunoassay for the quantitative analysis of heat-stable omega-gliadins and corresponding prolamins. R-Biopharm AG, Dolivostr. 10, 64293 Darmstadt, Germany). In some cases, dilution in ethanol may follow a preliminary extraction into a cocktail solution of undefined composition (Ridascreen Gliadin 2003; Enzyme immunoassay for the quantitative analysis of gliadins and corresponding prolamins. R-Biopharm AG, Dolivostr. 10, 64293 Darmstadt, Germany) The recommended procedure typically uses 10 volumes of the extractant per unit mass of the food to be analyzed. Such a procedure is not very practical for highly viscous liquid samples such as starch-derived syrups and further lowers their protein content. A syrup containing 400 g of solids/kg typically contains <1 g of protein/kg.

We report here on simple pretreatment protocols that allowed a substantial increase in the sensitivity of the immunoenzymatic determination of gliadin in syrups and on the application of these protocols to a quantitative analysis of residual gliadin in some commercial syrups obtained in European plants by controlled hydrolysis of wheat starch.

MATERIALS AND METHODS

Gluten content was determined in nine different commercial samples of wheat-derived glucose syrups provided by major European companies. The range of solid content of the syrups was 370–480 g/kg and the range of protein content (Kjeldhal N × 5.75) was 200–700 mg/kg. The protein content was also evaluated by a dye-binding colorimetric method that is not affected by the possible presence of nonprotein nitrogen (Bradford 1976). Colorimetric protein measurements were made after 10-fold dilution of the original sample in water, and a calibration curve was constructed with bovine serum albumin (Sigma Chemical Co., St. Louis, MO), taking into account the different response of the colorimetric method when applied to wheat proteins (Eynard et al 1994). One of the syrups was gluten-free in preliminary tests and was used as a reference material for establishing matrix effects and for gliadin recovery studies.

Preparation of Samples for Gliadin Determination

Syrup samples were diluted at room temperature with water or with the dilution buffer supplied with a commercial ELISA kit (Transia Plate Gluten, item GL0301, Diffchamb Italia, San Giuliano Milanese, Italy), and stirred on a magnetic stirrer for 10 min. The diluted samples were then concentrated by ultrafiltration at room temperature in Amicon (Millipore, Billireca, MA) cells fitted with a 10-kDa nominal cut-off membrane. Both 200-mL and 50-mL

[1] Dipartimento di Scienze Molecolari Agroalimentari.
[2] Corresponding author. Phone: +39-02-5031-6819. Fax: +39-02-5031-6801. E-mail: stefania.iametti@unimi.it.
[3] Associazione Italiana Celiachia, Via Lombardia 11, 23888 Rovagnate, Italy.
[4] Centro Studi Celiachia, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy.

Publication no. C-2003-1110-04R.
© 2004 American Association of Cereal Chemists, Inc.

cells were used, with membrane diameters of 63 and 43 mm, respectively, and were operated under a nitrogen pressure of 0.2 MPa. The enrichment in protein content of the syrup samples was calculated by assuming complete protein recovery in the retentate and referring to the starting volume of the syrup sample before dilution. Residual sample volume in the ultrafiltration device was determined by weighing the device after filling it with a known volume of the solution to be concentrated, and reweighing it (with the remaining solution inside) at the end of the concentration step.

The recovery of gliadin during the ultrafiltration procedure was assessed by adding a known amount of a standard gliadin solution to a gluten-free syrup sample before the dilution step. The standard gliadin solution used as the internal standard in the procedure mentioned above was the same provided for calibration purposes in the kit and contained 0.5 mg/mL gliadin in 40% (v/v) aqueous ethanol. The effects of the syrup matrix on the ELISA determination of gliadin was evaluated by adding a known amount of the same standard gliadin solution mentioned above to the retentate obtained by ultrafiltration of a gluten-free syrup sample immediately before the ELISA procedure.

Determination of Gluten Content by ELISA

The gliadin content in the syrup samples was measured by using a commercial ELISA kit (Transia Plate Gluten, item GL0301, Diffchamb Italia, San Giuliano Milanese, Italy). The kit is based on a direct sandwich ELISA and the distributor gives a detection limit of 5 mg/kg of gliadin in solid samples. Given the general consensus on the fact that only 50% of gluten is available as gliadins, this represents a limit of 10 mg/kg of gluten. The solid support of the reaction is a microtiter plate coated with a monoclonal antigliadin antibody. This test is based on monoclonal antibodies raised against ω-gliadin (Skerrit and Hill 1990) and has gained official recognition by the Association of Official Analytical Chemists (Skerrit and Hill 1991), in spite of some intrinsic limitations discussed above.

The ELISA assay was performed according to the standard procedure suggested by the supplier, except for the preparation of the syrup samples. A fixed volume (typically 0.2 mL) of the concentrated protein solution obtained by dilution and ultrafiltration of the syrup samples was diluted with an equal volume of the dilution buffer provided in the kit, and serial twofold dilutions were prepared starting from this original material. Then, 0.1 mL of each serial dilution were placed in the antibody-coated microplate wells, and the remainder of the test procedures were carried out as recommended by the kit supplier.

At the end of the assay procedure, the optical density in each well was measured at 405 nm in a 3550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Absorbance figures were corrected for average blank readings, and Bio-Rad software was used to calculate absorbance values and to correlate them with the gliadin content in the samples.

The gliadin content in all the glucose syrup samples were detected in two duplicate independent measurements, using two different lots of the same ELISA kit. Therefore, each figure for gluten content in the commercial glucose syrups represents the average of quadruplicate determinations.

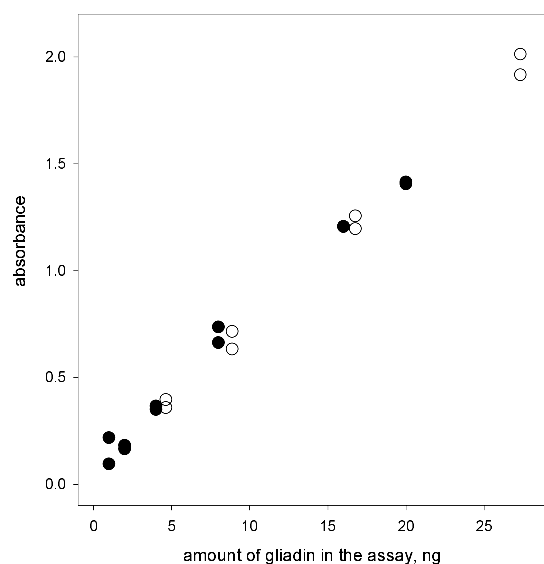

Fig 1. ELISA response curves for a standard solution of gliadin (0.5 mg/mL in 40% aqueous ethanol) dissolved in dilution buffer (solid symbols) or in a diluted and ultrafiltered gluten-free syrup (15% total sugars, open symbols). Final gliadin concentration in both samples before sequential dilution for the ELISA assays was 13.5 mg/L. Each value is the average of duplicate determinations from two different ELISA kits.

RESULTS AND DISCUSSION

Relevance of Matrix Effects

The high viscosity of the glucose syrups at room temperature requires that they be diluted before any subsequent handling. A threefold dilution of the original syrup with water was sufficient for conducting the subsequent ultrafiltration steps in a convenient time. Typically, 10 mL of syrup were diluted to 30 mL with water and concentrated overnight to a final volume of 3 mL in a standard 50-mL Amicon device fitted with a 43-mm membrane under a nitrogen pressure of 0.2 MPa. The residual retentate, in which the original protein concentration had been increased by a factor of 3.3, still contained ≈150–200 g/L of solids, depending on the solids content in the original syrup.

Therefore, it seemed necessary to verify the absence of any interference with the ELISA procedure of this sugar-rich matrix and of the accompanying proteins. By assuming that all proteins were retained by the ultrafiltration membrane, the original proteins (as total nitrogen × 5.75) had been concentrated to 0.6–2.5 g/L. To this purpose, ELISA tests were conducted with a gliadin standard diluted either with the kit-supplied dilution buffer or with the retentate obtained by ultrafiltration of a gluten-free glucose syrup sample. Appropriate aliquots of the kit-supplied standard gliadin were added to the gluten-free ultrafiltered syrup to give a final

TABLE I
Recovery of Gliadin Added in Different Concentrations to a Gluten-Free Syrup as a Function of the Diluting Solvent[a]

Gliadin Added Before Ultrafiltration (mg/L)	Gliadin Detected by ELISA in the Ultrafiltration Retentate (mg/mL)		Gliadin Recovery (%)
	Dilution in Water	Dilution in the Kit-Dilution Buffer	
10	7.18 ± 1.18a	· · ·	72
1	0.13 ± 0.06a	· · ·	13
0.1	0	· · ·	0
1	· · ·	1.11 ± 0.34a	100
0.1	· · ·	0.94 ± 0.02a	94

[a] Gliadin was added to gluten-free glucose syrups from a stock 0.5 mg/mL solution in 40% (v/v) aqueous ethanol. Spiked syrups (10 mL) were diluted with two volumes of solvent and concentrated to a final volume of 3 mL by ultrafiltration. Gliadin content of retentate is corrected for concentration to reflect the gliadin content in spiked syrups; standard deviation, $n = 4$.

concentration of 13.5 mg/L. As shown in Fig. 1, no matrix effect is evident even at the highest solids concentration, corresponding to a 40-fold dilution of the gliadin-spiked ultrafiltered syrup. The highest solids concentration in the samples loaded in the microplate wells in these experiments was ≈3.3 g/L of sugar and 0.022 g/L of protein. These figures were calculated by assuming that the ultrafiltration membrane was totally permeable to sugars and totally impermeable to proteins.

Defining a Proper Protocol for Protein Enrichment

A threefold dilution of the original syrup was used in our preliminary tests, such as those reported above. Recovery of gliadin added to a gluten-free syrup before the ultrafiltration step decreased dramatically when increasing the amount of water used in dilution of the original syrup. Whereas a threefold dilution of a syrup sample spiked with 10 mg/L of gliadin before the ultrafiltration step allowed a 72% (w/w) recovery of the added gliadin in the retentate, the gliadin recovery dropped to 32% (w/w) when the same gliadin-spiked syrup was diluted 10-fold before ultrafiltration. This suggests that the sugars in the syrup either improved gliadin solubility or prevented irreversible interaction of the water-insoluble, hydrophobic gliadin with the components of the ultrafiltration cell, including the membrane itself.

The effects of the medium composition on gliadin recovery became impressive when the gliadin concentration in the spiked syrup was lowered to <10 mg/L of gliadin, that is the sensitivity threshold for ELISA tests conducted with the standard procedure recommended for this kit.

Table I shows that gliadin recovery was very low or nil after a threefold dilution with water of a spiked syrup containing <1 mg/L of gliadin. However, a significant improvement in gliadin recovery was achieved simply by substituting the kit-supplied dilution buffer for water when diluting the syrup before ultrafiltration. Although information on the composition of dilution buffers is kept proprietary for the ELISA kits available at the time of this study (RIDASCREEN Gluten and Transia Plate Gluten), it seems reasonable that these buffers were designed to keep gliadin soluble in a predominantly aqueous environment for a reasonable time, without interfering with the immunochemical reactions.

Figure 2 shows the efficacy of the protein-enrichment procedure using the kit-supplied buffer from the early steps of sample preparation The results obtained in ELISA tests conducted on ultrafiltration retentates of a gluten-free syrup spiked with either 0.1 or 1 mg/L of gliadin are overlapping. Also, at either gliadin concentration, there is only a modest deviation from the ideal response represented by the dotted line in Fig. 2. Thus, no loss of gliadins occurs in these conditions.

It is noteworthy that the point corresponding to an expected gliadin content of 16.5 ng of gliadin, and taken from a syrup sample spiked with 0.1 mg/L of gliadin, is aligned with the other points in Fig. 2. This sample originated from a 1:1 dilution of the ultrafiltration retentate. Thus, the high solids content in this sample (≈80 g/L of sugars) did not interfere with recognition of gliadin by antibodies on the surface of the wells in the microtiter plate.

We suggest the following steps for protein enrichment in syrup samples to be analyzed by ELISA: 1) dilute 10 mL of syrup with 20 mL of the kit-supplied dilution buffer; 2) concentrate proteins in the thinned syrup by ultrafiltrafiltration to a retentate volume of 3 mL or less (a protein concentration factor of 3.3 with respect to the original sample); 3) conduct the ELISA assay, starting with a 1:1 dilution of the retentate with the kit-supplied dilution buffer as the initial point for serial dilution.

Comparison Between Protocols

From a practical standpoint, the lowest gliadin level that can be confidently detected by the commercial ELISA kit is 5 ng of gliadin in 0.1 mL of the solution loaded in each well of the microtiter plate (Figs. 1 and 2). Therefore, the minimum gliadin

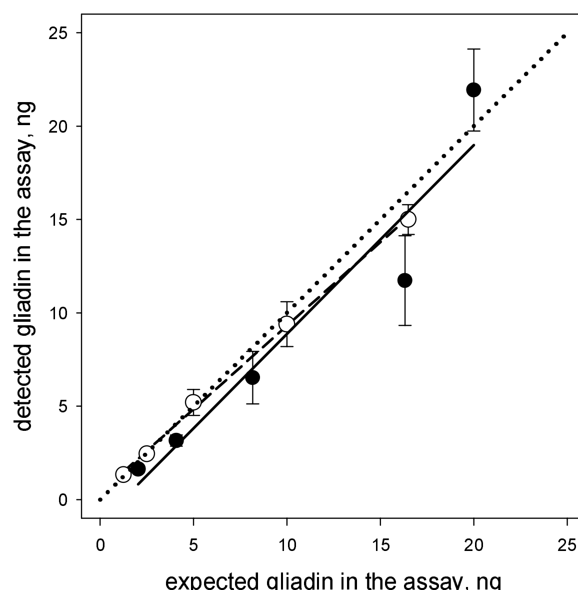

Fig. 2. Recovery of gliadin added to syrup before ultrafiltration. A known amount of a gliadin standard solution (0.5 mg/mL in 40%, v/v, aqueous ethanol) was added to a gluten-free glucose syrup to a final gliadin concentration of 1 mg/L (solid symbols, solid line) or 0.1 mg/L (open symbols, dashed line). Each spiked syrup (10 mL) was then diluted with 20 mL of the kit-supplied dilution buffer and concentrated to a final volume of 3 mL by ultrafiltration. Aliquots from each ultrafiltration retentate were then diluted to the indicated expected gliadin content in 0.1 mL—the volume loaded in each well of the ELISA plate. Each value is the average of duplicate determinations from two different ELISA kits. Standard error is given for each point. Dotted line with a unity slope is for reference only.

TABLE II
Gluten Content in Commercial Glucose Syrups Obtained from Wheat Starch[a]

Sample	Protein Content (mg/kg)	Gluten Content (mg/kg)	
		Dilution Protocol	Protein-Enrichment Protocol
A	344	325.7 ± 10.2a	203.1 ± 18.5a
B	282	20.8 ± 5.4a	12.0 ± 0.5a
C	35	nd	nd
D	<10	nd	nd
E	<10	0.38 ± 0.08a	0.41 ± 0.06a
F	34	nd	nd
G	20	nd	nd
H	28	nd	nd
I	<10	nd	nd

[a] Samples were analyzed by using either the dilution protocol or the protein-enrichment protocol. Each value is the average of two duplicate determinations from different ELISA kits. Protein content was measured by a colorimetric method after a 10-fold dilution in water of the original samples; standard deviation, $n = 4$; nd, not detected.

concentration that can be detected with confidence by using the protein enrichment protocol corresponds to: 5×10^{-9} g $(10^{-4}\ L)^{-1} \times$ 2 (the lowest dilution of the ultrafiltrate for the assay)/3.33 (the concentration factor achieved by ultrafiltration) = 30 µg/kg. Given the consensus on gliadin being ≈50% of total gluten protein, these figures become 60 µg/kg (0.06 mg/kg) gluten; about 160-fold lower than the strictest recommended limit for gluten-free products (10 mg/kg) suggested by the Southern Europe Celiac Associations.

Conversely, the conventional procedure based on hydro-alcoholic extraction (1 g of sample in 10 mL of extractant, followed by a minimum dilution of 1:50 before the actual test) gives a minimum detectable gliadin concentration of: 5×10^{-9} g $(10^{-4}\ L)^{-1} \times 10$ (the sample dilution in the extraction step) × 50 (the lowest dilution of the extract for the assay) = 25 mg/kg.

If only the syrup solids (350–500 g/L) were used for analysis, the figure given above could be improved only by a factor of 1.2 – 1.5. Also, bringing a 400 g/L solution of sugars to dryness may take a lot longer than it takes to remove soluble sugars by ultrafiltration. High temperatures cannot be applied to these samples, in view of the likely occurrence of Maillard-like reactions between proteins and carbohydrates.

Considering the lack of matrix effects after a minimum sixfold dilution of the original syrup (Fig. 2), an alternative protocol could rely simply on a threefold dilution with water of the original syrup, followed by a minimum twofold dilution with the kit-provided dilution buffer. In this case, the minimum concentration of gliadin that could be detected with confidence should be 5×10^{-9} g $(10^{-4}\ L)^{-1} \times$ 3 (sample dilution with water) × 2 (lowest dilution for the assay) = 300 µg/kg. At a lowest hypothetical detection limit of 300 µg/kg of gliadin, this simplified procedure is 10× less sensitive than that involving an ultrafiltration step but has the advantage of minimal sample handling and of low analysis time.

Application of the Protocols

Both the dilution and the protein-enrichment protocols were applied to the determination of the gluten content in various samples of commercial glucose syrup. These syrup samples are representative of a number of processes applied to the treatment of wheat starch by different European starch-processing companies. The results of these determinations are summarized in Table II and show that either method can be applied with confidence.

In protein-rich samples, the dilution protocol gave higher figures than the one involving a protein-enrichment step. There are two possible explanations for the ≈40% difference between the two protocols. First, immunoreactive peptides may originate from gluten proteins during the starch hydrolysis process and they may be small enough to permeate the ultrafiltration membrane. Second, high-temperature steps in starch processing may modify the protein structure so that some immunoreactive proteins may adhere to the membranes or form precipitates during ultrafiltration of the diluted syrups. This did not occur with the standard gliadin used in our recovery studies (Table I). However, no difference is evident when the two protocols were applied to samples with very low protein content (Table II, sample E). This suggests that the proteins in individual samples may have different properties.

Significant quantities of gluten were detected only in two samples. Another sample had a gluten content close to the limit of reliable detection. No gluten was detected in the other syrup samples, indicating that these glucose syrups can be used safely as ingredients for the preparation of gluten-free products.

The gluten content in positive samples was not related to the total protein content. One sample had an extremely low gluten content, much below even the strictest proposed limit for a gluten-free product. Another sample had a gluten content still below the suggested threshold for a gluten free-product, despite having a high content in residual proteins. A third sample was by any standards not suitable for the production of gluten-free foods. Gluten proteins represented ≈95% of the protein in this sample.

CONCLUSIONS

We demonstrated the feasibility of applying ELISA tests to gliadin detection in syrups obtained from the hydrolysis of wheat starch. Two protocols were developed for the analysis of these materials. The simplest protocol relies on mere dilution of the original syrup in appropriate solvents and could detect with confidence ≈0.3 mg of gliadin/L (0.6 mg of gluten/L). This figure could be improved at least 10-fold by adding a ultrafiltration step for protein concentration before the ELISA assay. Either procedure represents a marked improvement in sensitivity with respect to conventional protocols that rely on hydro-alcoholic extraction of the original sample.

In principle, the protein-enrichment procedure reported here may be used for improving gluten detection in a number of foods and beverages with very low protein concentration, unless extensive protein hydrolysis occurs.

As for the practical relevance of our findings, one can consider the following. A celiac individual drinking every day 0.5 L of a beverage formulated with 20% by volume of a syrup containing 20 mg of gluten/L (which is below the detection threshold by a conventional protocol) could be taking up as much as 2 mg of gluten/day. This may be significant, in view of the additive effects of gluten assumption by celiac individuals. On the other hand, if the same drink were produced by using a syrup that tests as devoid of gluten by applying the protein-enrichment protocol (that is, containing <0.05–0.06 mg gluten/L), the daily gluten uptake by the same celiac individual will be lower than 5–6 µg. This is about 400-fold less the amount taken up by consuming the same drink prepared with a syrup tested to be gluten-free by the current, low-sensitivity procedure.

In other words, should syrups be tested by the improved protocols proposed here, it will take a celiac patient one whole year to consume the amount of gluten someone could consume inadvertently in one day by consuming products containing syrups that tested gluten-free with current protocols.

ACKNOWLEDGMENTS

We wish to thank Roberto Comitti (Diffchamb Italia; now with R-Biopharm, San Giuliano, Italy) for partial support of these studies and for useful discussion.

LITERATURE CITED

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Catassi, G. R., Fabiani, E., Ratsch, I. M., Coppa, G. V., and Giorgi, P. L. 1997. Celiac disease in the general population: Should we treat asymptomatic cases? J. Pediatr. Gastr. Nutr. 24:810-813.

Corazza, G. R., and Gasbarrini, G. 1995. Celiac disease in adults. Baillieres Clin. Gastroenterol. 9:329-348.

Denery-Papini, S., Nicolas, Y., and Popineau, Y. 1999. Efficiency and limitations of immunochemical assays for the testing of gluten-free foods. J. Cereal Sci. 30:124-131.

Eynard, L., Guerrieri, N., and Cerletti, P. 1994. Determination of wheat proteins in solution by dye binding in flour, dough, and bread crumb. Cereal Chem. 17:434-438.

Ferguson, A. 1996. Gluten-sensitive enteropathy (celiac disease). Pages 287-301 in: Food Allergy: Adverse Reactions to Food and Food Additive, 2nd Ed. Blackwell Publishing: Malden, MA.

Skerrit, J. H., and Hill, A. S. 1990. Monoclonal antibody sandwich enzyme immunoassay for determination of gluten in foods. J. Agric. Food Chem. 38:1771-1778.

Skerrit, J. H., and Hill, A. S. 1991. Enzyme immunoassays for determination of gluten in foods: A collaborative study. J. Assoc. Off. Anal. Chem. 71:257-264.

[Received January 29, 2003. Accepted June 26, 2003.]