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# Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometric Micro-analysis: the First Non-immunological Alternative Attempt to Quantify Gluten Gliadins in Food Samples

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**The first epitope-independent procedure for rapidly quantifying gluten gliadins in food by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) based on the direct observation of the characteristic gliadin mass pattern is presented. This pattern was identified in both processed and unprocessed gluten-containing food samples. The procedure allows the micro-quantification of gluten in food samples below levels toxic for coeliac patients, with a linear response in the 0.4–10 mg per 100 g range and a high detection sensitivity similar to that of enzyme-linked immunosorbent assay (ELISA) systems. Food samples simultaneously analyzed by MALDI/TOF-MS and a highly sensitive laboratory-made sandwich ELISA revealed a good correlation between the two techniques. In addition, MALDI/TOF-MS provides a rapid screening system to determine the presence of gliadins in food samples by directly monitoring the occurrence of the protonated gliadin mass pattern. The procedure also permits the study of the alteration of gliadins in food during the baking process, providing data on the heat effect by changes in protein mass signals.** © **1997 by John Wiley & Sons Ltd.**

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## INTRODUCTION

Coeliac disease (CD) (gluten-sensitive enteropathy) is a permanent intolerance to gluten prolamins from wheat (gliadins), barley (hordeins), rye (secalins) and oats (avenins). The small intestine of coeliac patients undergoes severe mucosal lesions upon ingestion of these toxic cereal prolamins.[1,2] The only treatment for coeliac patients is a strict diet of foods with gluten contents below the toxicity threshold (5 mg per 100 g of food).[3] Therefore, very sensitive methods for the measurement of gluten in foods are required.

Several conventional immunological procedures including immunoblotting[4] and laboratory-made and commercial enzyme-linked immunosorbent assay (ELISA) formats using different monoclonal or polyclonal antibodies against a variety of gliadin components[5–14] are commonly used in an attempt to quantify gluten in food. However, comparison of ELISA data, especially in the low range of gluten contents close to toxic levels, are inconsistent[13] since these are epitope-dependent methods. Therefore, these systems are not reliable, sensitive methods for the measurement of gluten content in food. Consequently, a complementary, non-immunological method would be of great interest.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) has become a powerful technique in the life sciences for the rapid screening of even very crude biological extracts[15–18] and cereal protein extracts.[19–22] Recently, we demonstrated that direct MALDI/TOF-MS analysis of unfractionated alcohol-soluble prolamin fractions of gluten from wheat, barley, rye and oats yields the characteristic protonated mass pattern of gliadins, hordeins, secalins and avenins, respectively,[19] thus allowing the identification of these highly complex cereal prolamin fractions.[23–25] On the basis of these results, a procedure was developed to detect gluten gliadins directly in food samples by observing the characteristic gliadin mass pattern (ranging from 25 to 40 kDa) in food which allowed the measurement of gliadin concentrations at toxic levels and opened up the possibility of using the technique as a quantitative tool.[26]

We now present a first attempt to quantify gluten in food by means of MALDI/TOF-MS micro-analysis. The method, based on the rapid and easy detection of gliadins via the direct visualization of the characteristic gliadin mass pattern and its subsequent measurement, permits the determination of gliadin concentrations with a sensitivity similar to those of the most efficient conventional ELISA formats currently employed to

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quantify gluten in food.[8,9,14]

## EXPERIMENTAL

### Materials

Bovine serum albumin (BSA) and horse heart cytochrome *c* (CC) were purchased from Sigma (St Louis, MO, USA). Wheat (*Triticum durum* L. cv. *Araldun*) and maize were employed. An albumin- and globulin-free gliadin standard from *Triticum durum* L. cv. *Rektor*, prepared as described,[27] was kindly supplied by Dr H. Wieser (Munich, Germany). Plain bread, wheat starch samples (kindly supplied by the Association des Amidonneries de Céréales de la CE) and commercially available gluten-free foods were employed.

### Reagents

Acetonitrile and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany), ethanol from Scharlau (Barcelona, Spain) and sinapinic (*trans*-3,5-dimethoxy-4-hydroxycinnamic) acid and octyl-$\beta$-D-glucopyranoside from Fluka (Buchs, Switzerland). Ultra-pure water from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used in the preparation of all solutions.

### Sample extraction for micro-analysis of gluten-free food samples by MALDI/TOF-MS and ELISA

A sample of 1 g was homogenized in 5.0 ml of 60% (v/v) aqueous ethanol for 2 min and then centrifuged for 15 min at 1500 *g*. The supernatant was removed and the pellet re-extracted as above. The procedure was carried out at room temperature. The supernatants were combined, brought to a final volume of 10 ml with 60% ethanol and immediately analyzed by MALDI/TOF-MS and ELISA.

Aliquots of the supernatant of low-gluten content food, such as gluten-free foods, are used directly for MALDI/TOF-MS since the mass signals generally lie in the linear response range (0.4–10 $\mu g\ ml^{-1}$). Sample dilutions ranging from 1:5 to 1:50 are required for ELISA. Supernatants of samples containing high levels of gliadins require dilutions (1:500 to 1:2000) for MALDI/TOF-MS and ELISA to obtain data within the linear range of response. Gluten content values are expressed as twice the gliadin content levels.[14]

### Laboratory-made sandwich ELISA

An IgG-AGA laboratory-made sandwich ELISA was performed as described.[12] This assay consists of a sandwich ELISA which uses an IgG affinity-purified gliadin polyclonal antibody for the coating and the same antibody biotinylated.

### Sample preparation for MALDI/TOF-MS

A 160 µl volume of ethanol extract containing 16 µl of 50 mM octyl-$\beta$-D-glucopyranoside detergent was mixed with a matrix solution consisting of 100 µl of saturated sinapinic acid in 30% aqueous acetonitrile and 0.1% trifluoroacetic acid. Since high crystal density in dried sample spots facilitates laser irradiation, this matrix–sample mixture was concentrated as follows. Duplicate 25 µl aliquot samples of the mixture were concentrated in a Speed-Vac centrifuge down to 3–4 µl (10–15 min) and brought to a final volume of 5 µl with the above acetonitrile–trifluoroacetic acid solution. A 1 µl volume of each duplicate aliquot was then deposited on a stainless-steel probe tip and allowed to dry at room temperature for 5 min.

Samples were measured on a Bruker (Bremen, Germany) Reflex II MALDI/TOF mass spectrometer equipped with an ion source with visualization optics and a nitrogen laser (337 nm). Mass spectra were recorded in the linear positive mode at a 30 kV acceleration voltage and 2 kV in the linear detector by accumulating 70 spectra of single laser shots under threshold irradiance. Only highly intense, well resolved mass signals arising from 5–7 selected target spots were considered. The equipment was externally calibrated by employing singly, double and triply charged signals from a mixture of BSA (66 430 Da) and CC (12 360 Da).

## RESULTS

### Sample concentration for the optimized analysis of gliadins by MALDI/TOF-MS

Recently, we described a modified sample preparation procedure for gliadin analysis in food by MALDI/TOF-MS in which we employed an 80 µl volume of sample with a 100 µl volume of saturated solution of sinapinic acid used as a matrix.[26] This improvement permitted the detection of gliadin levels down to 10 mg per 100 g of food, which is slightly above the toxic limit in gluten-free foods (5 mg of gliadins per 100 g of food) for coeliac patients.

Seeking greater detection sensitivity, larger sample volumes, 80–200 µl with a constant 100 µl volume of matrix solution, were used for the MALDI/TOF mass analyses of an ethanol gliadin complex protein extract from the wheat cultivar L. cv. *Araldun* (2 $mg\ ml^{-1}$). Although the intensity of gliadin mass signals increased with increase in sample volume, the dilution effect made it progressively more difficult to obtain mass spectra owing to the decrease in matrix/sample crystals density in the dried target spots (data not shown). Consequently, the task of finding appropriate crystals became very

time consuming or impossible.

To address this dilution effect, a concentration step was included in the sample preparation procedure prior to MALDI/TOF-MS. Reduction of the sample–matrix mixture to one fifth or one sixth of the initial volume allows rapid high-quality mass spectra to be obtained. Aliquots of 25 μl of sample–matrix mixture were concentrated in a Speed-Vac centrifuge down to a final volume of 5 μl, of which 1 μl was directly applied on to the sample probe. Figure 1 shows the intensity of the gliadin mass pattern obtained with this concentration procedure in the 2–200 μl range for the ethanol extract from *Triticum durum* L. cv. *Araldun*. Data include standard deviations corresponding to three independent sample preparations showing an acceptable reproducibility. The increase in mass signal intensity for volumes up to 160–200 μl permits gliadin detection with a markedly greater sensitivity than the previously reported method which uses 80 μl.[26] This opens up the possibility of visualizing the gliadin mass pattern at concentrations below the toxicity limit of 5 mg per 100 g of food.

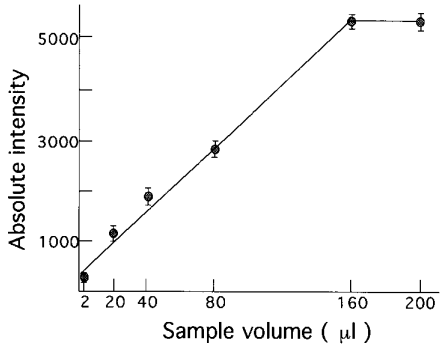


**Figure 1.** MALDI/TOF mass pattern intensity vs sample volume of a 60% ethanol wheat gliadin extract from *Triticum durum L.* cv. *Araldun* (2 mg $ml^{-1}$) by the concentration procedure. As a measure of the mass pattern intensity, the absolute intensity of the most intense mass signal around 31 kDa [indicated by asterisk in the gliadin mass spectra in Fig. 2 (B)] was selected. Error bars are standard deviations of three measurements from 5–7 selected target spots.

## Linear range and detection limit of gliadins

To establish the detection sensitivity for gliadins by MALDI/TOF-MS including the above concentration procedure, dilutions of a gliadin standard ranging from 0.05 to 100 μg $ml^{-1}$ were mass analyzed. The characteristic protonated gliadin mass pattern could be visualized down to a concentration of 0.4 μg $ml^{-1}$ [Fig. 2(B)]. As we reported previously,[26] the intensity of the most intense α-gliadin mass signal around 30 kDa [Fig. 2(B), asterisk] was chosen to measure the gliadin mass pattern intensity. A linear response within the 0.4–10 μg $ml^{-1}$ range [Fig. 2(A)] was obtained and the characteristic gliadin mass pattern at four gliadin concentrations within this linear range is shown in Fig. 2(B). A slight variation is observed for three independent sample preparations, as revealed by standard deviation data included in Fig. 2(A), which demonstrates acceptable reproducibility. This corresponds to 0.4–10 mg of gliadins per 100 g of food according to food sample dilutions and therefore permits the analysis of gluten-free foods below the toxic threshold.

It is noteworthy that this procedure yields a linear response for gliadin detection in the same range as the most sensitive ELISA systems used to quantify gluten in food.[8,9,14] To our knowledge, this is the first non-

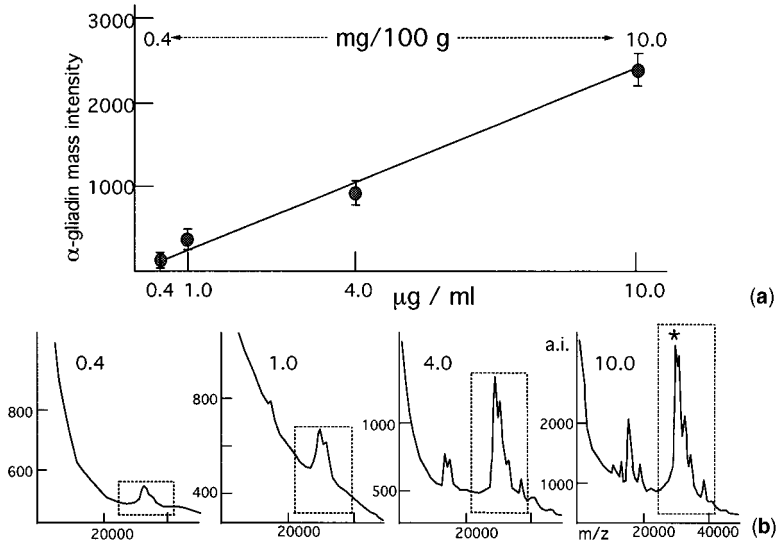


**Figure 2.** (A) Calibration graph from 0.4 to 10.0 μg $ml^{-1}$ using the gliadin standard obtained from *Triticum durum* L. cv. *Rektor*. The equivalent range expressed as mg per 100 g of food is indicated by a dashed line. (B) MALDI/TOF mass spectra corresponding to the above gliadin standard concentrations. The asterisk indicates the mass signal around 31 kDa selected as a measure of the gliadin mass pattern intensity. The major characteristic gliadin mass pattern ranging from 25 to 40 kDa is indicated by a box. Error bars are standard deviations of three measurements from 5–7 selected target spots.

 

immunological system that can detect gliadins at such low concentrations.

### Rapid screening of gliadins in foods by MALDI/TOF-MS

This procedure could also be used as a routine qualitative method to test rapidly and directly for the presence of gliadins in food samples by visualizing the corresponding gliadin mass pattern, provided that its occurrence is demonstrated in all types of gluten-containing food samples.

Unexpectedly, gliadins were found as contaminants in maize and chickpea flours used in the diet of coeliac patients. A gliadin-contaminated maize sample displays a characteristic gliadin mass pattern, not present in pure maize [Fig. 3(A)].

It is noteworthy that the typical gliadin mass pattern could also be identified in a variety of processed samples including 9 wheat starch samples, 11 commercial gluten-free products and 10 bread samples. This is illustrated by two selected starches [Fig. 3(B)], four gluten-free samples [Fig. 3(C) and (D)] and four bread samples (Fig. 4). The fact that the characteristic gliadin mass profile is revealed with all unprocessed and processed gluten-containing samples suggests that this pattern could in principle be employed as a measure of gliadin content.

### Quantitative MALDI/TOF-MS analysis of food samples

On the basis of the high sensitivity achieved for MALDI/TOF-MS in the detection of gliadins at toxic level, the method was used as an alternative technique to quantify gluten gliadins in food samples by comparing the gliadin mass pattern intensity in each sample with that of the gliadin calibration graph [Fig. 2(A)]. Quantification data for 30 samples were compared with those from a laboratory-made sandwich ELISA performed simultaneously. Tables 1, 2 and 3 give the calculated average gluten contents with standard deviations (SD).

Table 1 lists the gluten content of nine wheat starch samples as determined by laboratory-made ELISA and MALDI/TOF-MS. Seven starches had a gliadin mass pattern which allowed quantification within the linear range and two were not detected. There was a good correlation factor of $1.1 \pm 0.6$ between the laboratory-made ELISA data obtained simultaneously using an antigliadin polyclonal antibody and MALDI/TOF-MS data. Table 2 lists the results for 11 processed commercial gluten-free food samples. Four samples showed

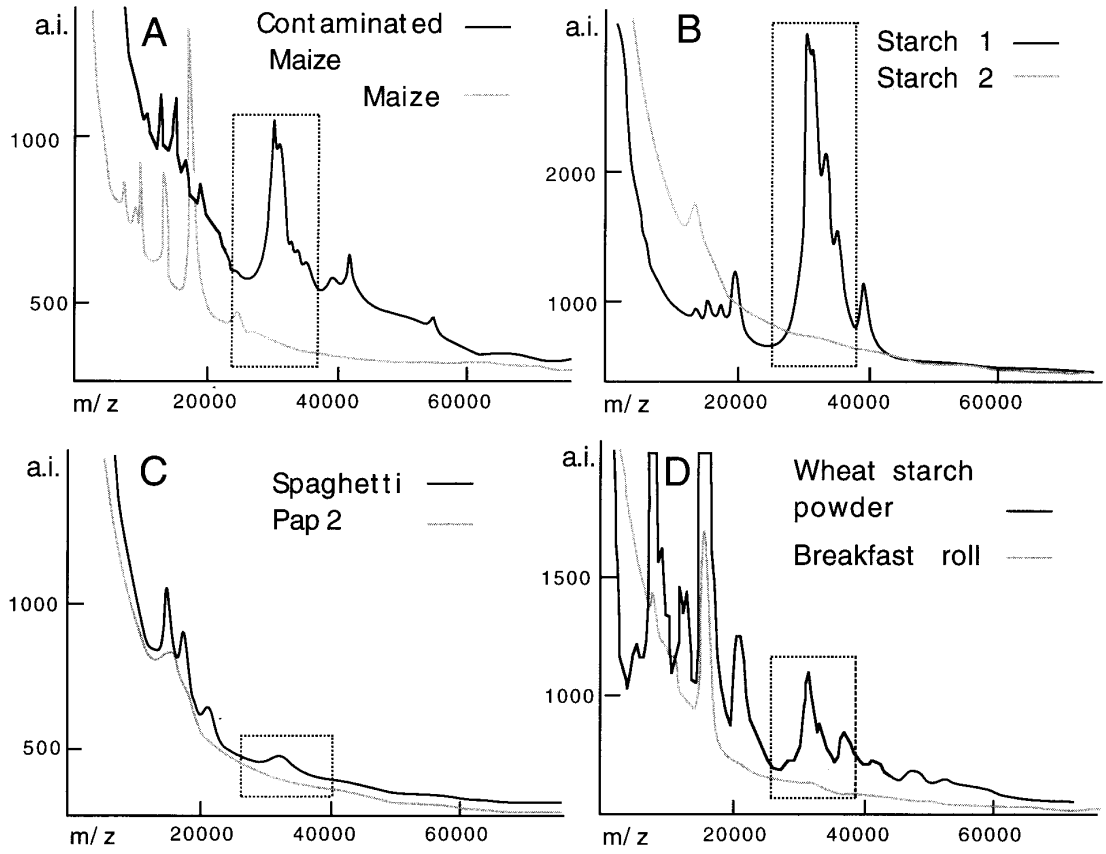


**Figure 3.** MALDI/TOF mass spectra of the ethanol extract from food samples. Unprocessed: maize and contaminated maize (A). Starches 1 (CE 20, Table 1) and 2 (CE 10, Table 1) (B). Processed: spaghetti and pap (C); wheat starch powder and breakfast roll (D). The major characteristic gliadin mass pattern is indicated by a box.

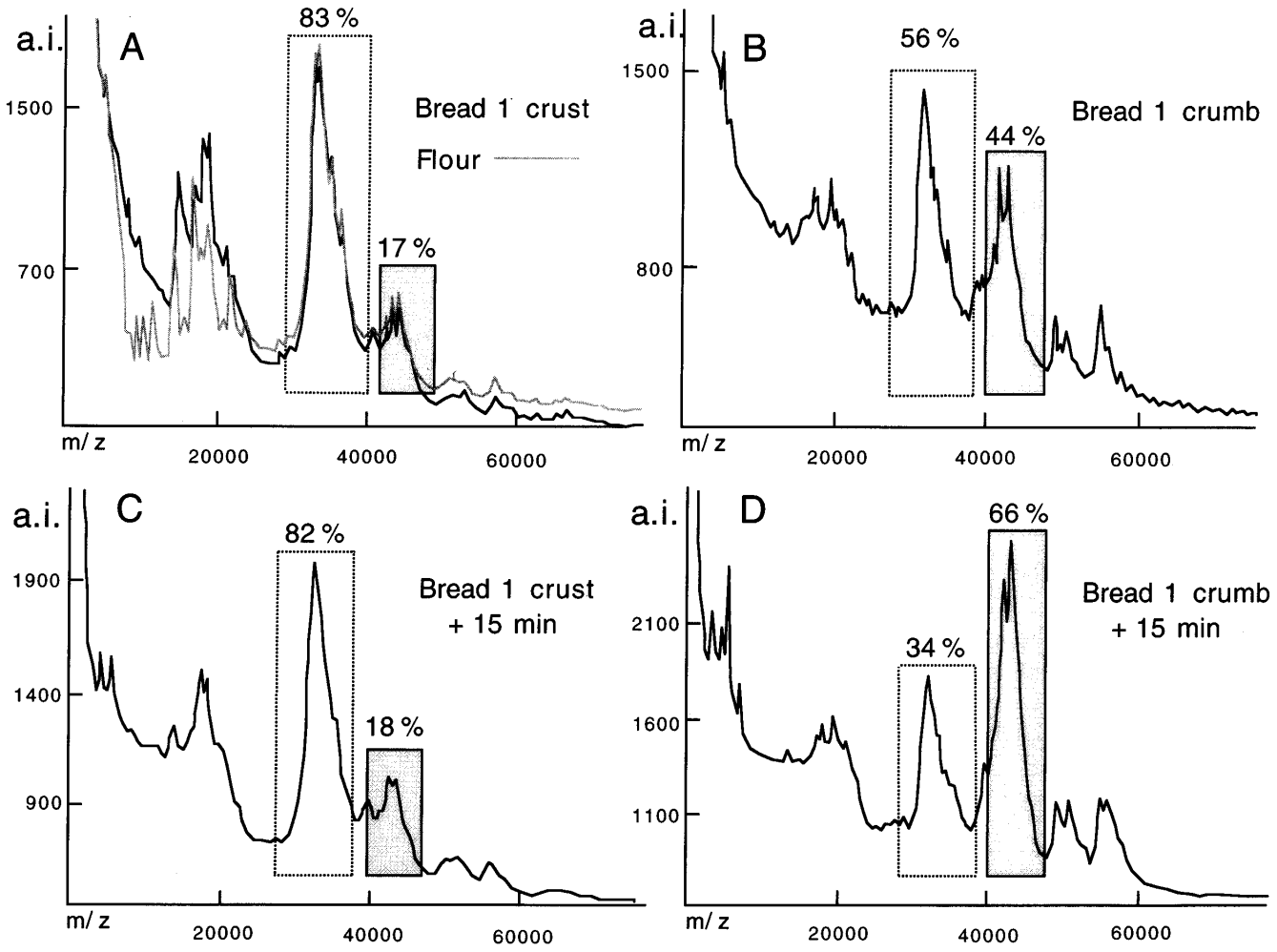


**Figure 4.** MALDI/TOF mass spectra of the ethanol extract from plain bread samples. Bread crust (A) and bread crumb (B) and the same samples (C and D, respectively) after an additional 15 min baking time. The major characteristic gliadin mass pattern is indicated by a box and a minor gliadin mass range by a dark box. The percentage intensity of the most intense mass signal in the major and minor gliadin patterns is indicated on top of the boxes. The mass signal profile of the original flour (not scaled) has been included for comparison (A).

**Table 1 Comparison of gluten content of starch samples as determined by laboratory-made ELISA and MALDI/TOF-MS**

| Sample | Laboratory-made ELISA[a] | MALDI/ TOF-MS[a] | SD | Laboratory-made ELISA MALDI/TOF-MS[b] |
|---|---|---|---|---|
| AM 10 | <0.1 | UD[c] | | |
| AM 20 | <0.1 | 0.9 | 0.1 | |
| AM 30 | <0.1 | 0.9 | 0.1 | |
| CE 10 | <0.1 | UD | | |
| CE 20 | 23.5 | 18.2 | 0.1 | (1.3) |
| LA 10 | <0.1 | 0.9 | 0.1 | |
| LA 20 | 7.3 | 10.1 | 0.5 | (0.7) |
| LA 30 | 18.1 | 9.2 | 0.8 | (2.0) |
| KR 10 | 2.7 | 8.6 | 0.6 | (0.3) |
| | | | | 1.1 ± 0.6[d] |

[a] Values are expressed as mg of gluten per 100 g.
[b] Values in parentheses are the ratios between laboratory made ELISA and MALDI/TOF-MS.
[c] UD: undetected samples.
[d] Represents the mean value of correlation factors ± standard deviation.

very faint gliadin mass signals and seven remain undetected. The MALDI/TOF and ELISA data indicate that these are gluten-free food samples. There was also a good correlation (1.1 ± 0.3) between MALDI/TOF-MS and laboratory-made ELISA values for the quantification of typical high-gluten content food samples (breads) (Table 3).

## Alteration of gliadins by baking effect in crust and crumb from bread samples

It is generally accepted that whereas $\omega$-gliadin fractions are relatively heat stable, $\alpha$-, $\beta$- and $\gamma$-gliadins are extremely labile.[13] Consequently, gluten levels of heat-processed samples decrease considerably when measured by epitope-dependent methods, such as ELISA, since antibodies fail to recognize these modified gliadins.

To study whether MALDI/TOF-MS can monitor this effect, 10 differently baked plain bread components

**Table 2 Gluten content of commercial gluten-free food samples analyzed by laboratory-made sandwich ELISA and MALDI/TOF-MS**

| Sample | Laboratory-made ELISA[a] | MALDI/ TOF-MS | SD |
|---|---|---|---|
| Spaghetti | 9.0[a] | 2.2 | 0.2 |
| Maize powder mixture for bakery | 1.2 | UD[c] | |
| Pap 1 | 1.6 | UD | |
| Pap 2 | 2.2 | UD | |
| Cookies | ND[b] | 7.2 | 0.8 |
| Coconut cookies | ND | 5.0 | 0.4 |
| Corn bread | 0.5 | UD | |
| Fiber bread | 0.8 | UD | |
| Wheat starch powder mixture for bakery | 2.5 | 4.3 | 0.1 |
| Breakfast roll | 0.4 | UD | |
| High-fiber crackers | <0.02 | UD | |

[a] Values are expressed as mg of gluten per 100 g.
[b] ND: non-determined samples.
[c] UD: undetected samples.

were mass analyzed and the results were compared with those of a laboratory made ELISA performed simultaneously. Results for 10 crusts and crumbs from three plain bread samples verified the aforementioned good correlation between the two techniques (Table 3). The data confirmed that both crust and crumb undergo a significant decrease in gluten content with respect to the original unprocessed flours owing to the heating effect (Fig. 5). It is noteworthy that crumbs have a lower gluten content than crusts, probably because internal crumbs retain heat longer than crust. (Table 3 and Fig. 5). An additional decrease in gluten content can be observed when the exposure to heat (baking) is increased by 15 min for both crust and crumb (Table 3 and Fig. 5).

The mass spectra (Fig. 4) revealed marked alterations in the percentage intensity between the most intense mass signal in the major and minor gliadin mass patterns of the crumb due to the heating effect [Fig. 4(B), see boxes]. This effect is increased with the time of exposure to heat [Fig. 4(D), see boxes]. Conversely, the

**Table 3 Gluten content of bread crusts and crumbs by laboratory-made sandwich ELISA and MALDI/TOF-MS**

| Sample | Laboratory-made ELISA[a] | MALDI/ TOF-MS[a] | SD | Laboratory-made ELISA MALDI/TOF-MS[b] |
|---|---|---|---|---|
| Bread 3 crust | 264.2 | 433.5 | 21.5 | (0.6) |
| Bread 3 crumb | 92.7 | 112.2 | 5.0 | (0.8) |
| Bread 1 crust | 326.5 | 454.2 | 16.5 | (0.7) |
| Bread 1 crumb | 171.6 | 126.9 | 4.1 | (1.4) |
| Bread 2 crust | 913.9 | 761.3 | 35.8 | (1.2) |
| Bread 2 crumb | 529.6 | 411.0 | 22.1 | (1.3) |
| Bread 1 crumb +15[c] | 43.7 | 30.1 | 5.3 | (1.4) |
| Bread 1 crust +15[c] | 44.0 | 30.2 | 1.2 | (1.5) |
| Bread 2 crust +15[c] | 36.7 | 52.9 | 3.6 | (0.7) |
| Bread 2 crumb +15[c] | 38.1 | 41.9 | 3.4 | (0.9) |
| | | | | 1.1 ± 0.3[d] |

[a] Values are expressed as mg of gluten per 100 g.
[b] Values in parentheses are ratio between laboratory-made ELISA and MALDI/TOF-MS.
[c] 15 min additional baking time.
[d] Represents the mean value of correlation factors ± standard deviation.

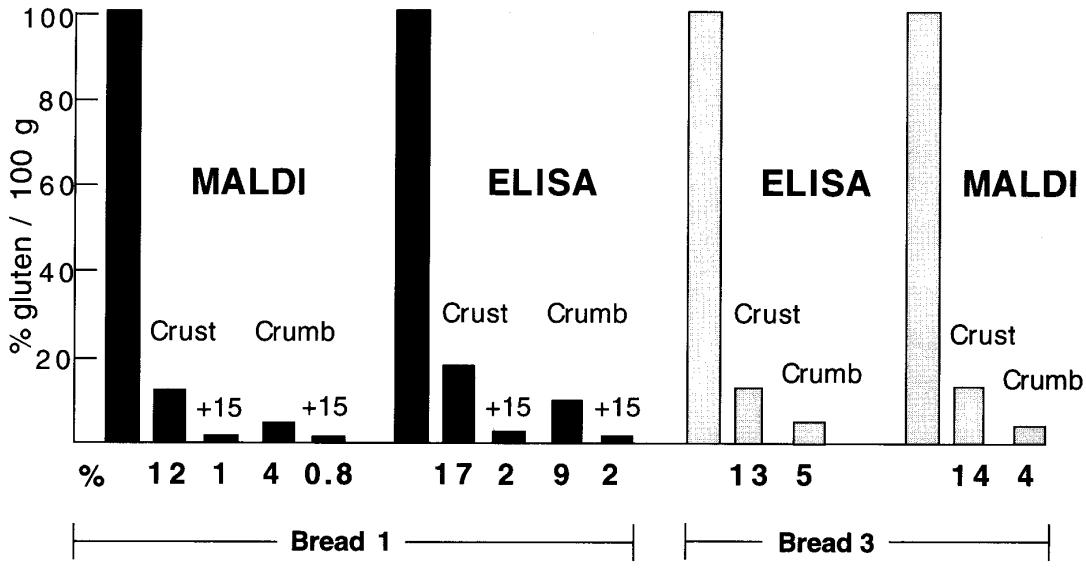


**Figure 5.** Comparison of gluten content in flour, crust and crumb from two plain breads as determined by MALDI/TOF-MS and ELISA. Gluten content data after an additional 15 min baking time are included. Values are given as percentage, taking flour as 100%.

major and minor gliadin mass patterns of crust [Fig. 4(A)] are unaffected [Fig. 4(C)] and are comparable to those of the original flour [Fig. 4(A), see light spectrum, not scaled]. The basis of this observation is unclear and further studies are required.

The changes in the gliadin mass signal ratio must be related to the different heat stabilities of α-, β- and γ- and ω-gliadins. However, the precise identification in the spectra of corresponding mass signals of different gliadin types has not yet been achieved.

The above results demonstrate that MALDI/TOF mass spectra provide valuable additional information on changes in gliadin composition ratios that are unattainable by ELISA.

## DISCUSSION AND CONCLUSIONS

Based on the direct observation of the characteristic gliadin mass pattern by MALDI/TOF-MS (within the 25–40 kDa range), we have developed a non-immunological method for the identification and quantification of wheat gliadins in food.

The procedure includes a concentration step and permits the detection of gliadins down to 0.4 μg $ml^{-1}$ (0.4 mg per 100 g of food), i.e., below the toxic limit for coeliac patients, with an acceptable linear response within the 0.4–10 μg $ml^{-1}$ range. This linear response is in a correspondingly low range to the most sensitive ELISA described to quantify gluten in food. The variation observed for three independent measurements indicated good reproducibility.

Based on the observation that the gliadin mass pattern occurs in all types of gliadin-containing foods, the procedure was used for the rapid screening of food samples for the presence of gliadins and to quantify gluten in both unprocessed and processed food samples. There was an acceptable correlation between MALDI/TOF-MS and ELISA determinations of gluten levels in food samples, despite the small number of samples analyzed. Results for a large number of additional samples performed in other studies (data not shown) further support this correlation.

MALDI/TOF-MS has been used successfully for the study of decreased gliadin levels due to heat processes. However, although quantitative mass spectrometric values were nearly identical with those obtained by ELISA, MALDI/TOF-MS reveals modifications in gliadin composition ratios, probably due to heat denaturation, which are not detected by ELISA. These denatured gliadin fractions are not detected either under these experimental conditions by MALDI/TOF-MS or by antibodies in ELISA systems. It is also possible that these denatured gliadin fractions are not soluble in alcohol; therefore, they are not extracted and consequently undetected by both systems.

The main advantages of using MALDI/TOF-MS to quantitate gluten gliadins in foods are (a) rapidity (a few minutes), (b) simple operation and interpretation of spectra (c) easy and reproducible sample handling, (d) high mass accuracy and (e) relatively low cost of analyses.

MALDI/TOF-MS in comparison with ELISA has the following advantages for the quantification of gluten in foods: (a) low-gliadin containing samples can be analyzed directly from alcohol extracts, whereas ELISA has a low alcohol tolerance;[9] (b) compatibility with many buffers, salts, non-ionic detergents and reducing agents;[26] and (c) MALDI/TOF-MS, unlike ELISA, is free from errors arising from buffer handling, choice of antibodies and standards, plate coating and storage, stability of conjugates, incubation and color intensity reading. Conversely, a MALDI/TOF mass equipment is more expensive and the sample processing rate is lower than that of ELISA. Although the gliadin fraction is selectively extracted by means of the ethanol extraction procedure generally used,[3] the possibility that accompanying non-gliadin components from complex formula gluten-containing foods might be co-extracted cannot be completely discarded. Taking into account the high resolving power exhibited by MALDI/TOF-MS, these co-extracted components could result in overestimated measurements only if they appear very close to the 31 kDa signal employed for measurement. Nevertheless, this seems not to be the case in view of the good correlation observed between MALDI/TOF-MS and ELISA values, at least for the samples analyzed so far. Additional analyses will confirm whether relevant contaminants can be expected depending on the sample type.

This preliminary study constitutes a first effort to establish the MALDI/TOF-MS technique for the detection of gluten in food. Nevertheless, the success of this task will depend on the development of additional experimental improvements, such as (i) increase in detection sensitivity to 0.1 mg of gliadins per 100 g of food; (ii) the confirmation of a consistently good correlation between MALDI/TOF-MS data and values from distinct ELISA formats; (iii) the establishment of an efficient, quantitative gliadin extraction procedure (possibly by using reducing agents, non-ionic detergents, etc.) along with a method to remove co-extracted components; (iv) the use of a cereal prolamin as an internal standard which will allow the determination of the percentage recovery and also an additional internal standard to monitor better the reproducibility of mass spectra; and (v) comparison of a large number of gliadin standards to determine the most appropriate one according to reproducibility and sensitivity.

Once all the above challenges have been addressed, MALDI/TOF-MS will probably be considered as a general procedure for quantifying gluten in food complementary to conventional immunological methods. Furthermore, MALDI/TOF-MS can in principle be used to quantify other gluten toxic cereal prolamins from barley (hordeins), rye (secalins) and oats (avenins) since these prolamin extracts also yield a characteristic protonated prolamin mass pattern.[19] All these questions are currently being addressed.

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