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Screening of Gluten Avenins in Foods by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

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The first procedure capable of analysing gluten avenins in gluten-free food samples aimed at the diet control of coeliac patients is described. The method is based on the direct observation of the characteristic avenin mass pattern, around 20–30 kDa, as revealed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS). The mass range where avenin signals appear is free from mass peaks arising from wheat gliadin, barley hordein and rye secalin protein components, which are also toxic to coeliac patients. Therefore, avenins can easily be screened in complex formula food samples elaborated with mixtures of wheat, barley, rye and oats. In addition, a procedure to quantify avenins in food samples is described on the basis of avenin mass area measurement with a detection limit of 0.4 mg of avenins per 100 g of food. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; coeliac disease; gluten avenins; gluten-free

INTRODUCTION

It is generally accepted that wheat gliadins, barley hordeins, rye secalins and oat avenins constitute the toxic gluten components which provoke the damage of the small intestine in coeliac patients,[1,2] despite the fact that the precise protein components in these cereals responsible for this phenomenon are still unidentified. It is currently under discussion whether avenins are toxic or not; nevertheless, the data reported[3–5] are not conclusive and further studies are required to resolve this point. Consequently, until this question is finally answered, avenins must in principle be considered toxic and therefore be avoided in the diet of coeliac patients.

To control the gluten content in the diet of these patients, most of the immunological methods employed, e.g. enzyme-linked immunosorbent assay (ELISA), are currently based on antibodies which recognize mainly wheat gliadins and rye secalins and to a much lesser extent barley hordeins, while they fail to detect avenins.[6–13] Hence the development of non-immunological alternative procedures to determine the four types of toxic gluten in foods is of great interest. The MALDI/TOF technique has been demonstrated to be an efficacious, powerful technique for the analysis of wheat gliadin, barley hordein, rye secalin and oats avenin protein components.[14–21] We recently reported the use of MALDI/TOF-MS as the first non-immunological method to quantify gluten gliadins in food samples.[18,19] Based on the fact that oats avenins exhibit a typical mass profile in the 20–30 kDa range, which is free of gliadin, hordein and secalin mass signals,[20] we have now developed a mass spectrometric procedure for the analysis of avenins both for screening and quantification in food samples.

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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. *Senatore Capelli*), rye (*Secale cereale* L. cv. *Raña*), oats (*Avena sativa* L. cv. *MH21*, *MH27*, *PA-101* and *PA-105*) and maize (*Zea mays* L. cv. *Golda*) cultivars were used. Food samples elaborated with a mixture of different cereals supplied by Jaime Pedró (Barcelona, Spain) and commercially available oat-containing foods, some of them kindly provided by Dr Feighery (St James Hospital, Dublin, Ireland), were employed.

Reagents

Acetonitrile and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany), ethanol from Scharlau (Barcelona, Spain), sinapinic acid (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid) and octyl β-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.

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Ethanol extraction from oat-containing foods

A 1 g amount of sample or cereal flour was homogenized in 10.0 ml of 60% (v/v) aqueous ethanol using an Ultra-Turrax (Janke & Kunkel, Ika Labortechnick) for 2 min. Samples were centrifuged at 2500*g* and the supernatant was collected. The procedure was carried out at room temperature.

Preparation of the oats avenin standard

The oats cultivar *Avena sativa* L.cv. PA-101 was selected to prepare the avenin standard. After pre-extraction of the oats flour with 0.15 M NaCl to remove the albumin–globulin fraction, avenins were extracted with 60% (v/v) aqueous ethanol at 1 mg ml^{-1}. Samples were centrifuged at 2500*g* for 10 min at room temperature after each extraction step. Protein concentration was determined by acid hydrolysis in 5.7 M HCl containing 0.05% 2-mercaptoethanol in evacuated sealed tubes followed by analysis using a Beckman System 6000 high-performance amino acid analyzer.[22] Alternatively, the Kjeldahl (N × 5.7) method was used. Gliadin, hordein and secalin ethanol extracts employed in this study were also prepared as above.

Sample preparation for MALDI/TOF-MS

A 160 μl volume of the ethanol extract was mixed with 32 μl of 50 mM octyl β-D-glucopyranoside detergent and 100 μl of saturated sinapinic acid in 30% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid used as a matrix solution. A 75 μl volume of the matrix–sample mixture was dried in a Speed-Vac centrifuge (30–35 min) and the residue was dissolved in 6 μl of 60% aqueous ethanol and 0.1% TFA. A 0.5 μl volume of sample–matrix mixture was deposited on a Bruker (Bremen, Germany) Multiprobe 20011 lathe-tooled stainless-steel probe tip and allowed to dry at room temperature for 5 min.

Samples were measured on a Bruker 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 30 kV acceleration voltage and 2 kV in the linear detector by accumulating 100 spectra of single laser shots under threshold irradiance. Only highly intense, well resolved mass signals arising from 3–5 selected target spots were considered. The equipment was externally calibrated employing singly, doubly and triply charged signals from a mixture of bovine serum albumin (66 430 Da) and CC (12 360 Da).

RESULTS AND DISCUSSION

Despite the fact that there is much debate about the place of oats in the gluten-free diet, their status in this diet has not yet been resolved.[3–5] Although several immunological systems capable of quantifying wheat and rye glutens in foods are available, no methods for determining and quantifying oats gluten have been reported. Oats avenins represent a group of protein components in the 20–30 kDa range which can be easily analysed by distinct mass spectrometric techniques such as electrospray ionization[15] and MALDI/TOF.[20] Different oats cultivars yield very similar characteristic protonated avenin mass patterns with only minor mass differences from cultivar to cultivar. Taking into account that oats avenins can be easily differentiated from gliadins, secalins and hordeins by MALDI/TOF-MS,[20] we investigated whether this mass spectrometric technique permits the identification and quantification of oats avenins in food samples.

Sample preparation optimization for the analysis of avenins by MALDI/TOF-MS

Having developed previously a sample preparation optimization for gliadin analysis,[19] we utilized a similiar procedure for the analysis of avenins. The sample : matrix volume ratio was optimized searching for the highest mass signal area for avenins extracts. Aliquots of 2–240 μl of the oats avenin standard *PA-101* at 10 mg ml^{-1} containing 5 mM octyl-β-D-glucopyranoside detergent were mixed with a fixed 100 μl volume of the aforementioned saturated sinapinic acid solution used as a matrix and mass analysed as described in the Materials and Methods section. Figure 1, bottom, displays the avenin mass pattern area versus avenin standard volume. Measurements were carried out by calculating the area of avenin mass signals arising from 3–5 selected target spots in the 18–33 kDa mass range. The highest mass area : sample volume ratio is reached at 160 μl of ethanol extract volume added. Hence this was the ratio employed for the subsequent mass analyses of food samples. To illustrate the effect of increasing sample volume on the avenin mass pattern, MALDI/TOF mass spectra corresponding to two limiting sample volumes of 20 and 160 μl are shown (Fig. 1, top).

Rapid screening of avenins in foods by MALDI/TOF-MS

Taking advantage of the fact that the avenin mass peaks appear in a mass range far from those of the remaining toxic gliadin, secalin and hordein protein components, we investigated whether MALDI/TOF-MS could be used to identify the presence of avenins in food samples.

Figure 2 displays the MALDI/TOF mass spectra of three oat-containing food samples in which the typical avenin mass pattern ranging from 20 to 30 kDa, as ilustrated for an oat cultivar, can be rapidly distinguished. These avenin mass profiles are very similar and resemble that of an oat cultivar (Fig. 2, bottom, right) despite the fact that minor mass differences are observed among the characteristic avenin mass patterns from these samples owing to their being elaborated with different oat cultivars.[20] A gliadin mass pattern from a wheat cultivar was included in the mass spectrum of one of the

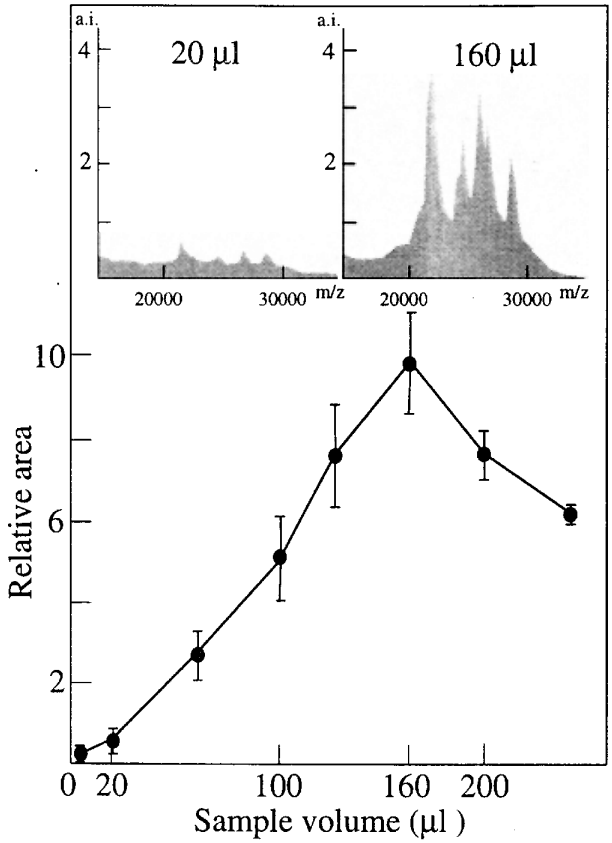

Figure 1. Bottom: MALDI/TOF mass pattern area versus sample volume of the ethanol oat avenin extract from *Avena sativa* L. cv. *PA-101* (10 mg ml^{-1}) by the concentration procedure. The avenin mass area within the 18–33 kDa range was measured. Error bars are standard deviations of three independent duplicate measurements from 3–5 selected target spots. Top: MALDI/TOF mass spectra of the avenin extract at two different sample volumes.

oat samples (Fig. 2, top, left) to indicate the absence of gliadin mass signals in these food samples. Secalins and hordeins, which would lie in the 30–40 kDa region, are also absent. Figure 2 (top, right) shows the mass spectrum of an unrelated grain used as a control which is free of gliadin, secalin, hordein and avenin mass peaks.

We investigated whether avenins could be also identified in complex foods elaborated with mixtures of cereals such as wheat, barley, rye, oats and maize. To facilitate the identification of gluten components in these samples, the overlayed mass spectra of gliadins, secalins and avenins and the theoretical mass profile expected for a food sample elaborated with a mixture of wheat, rye and oats are shown in Fig. 3 (top), despite the fact that proteins in a mixture can influence MALDI mass spectra significantly. The characteristic avenin mass pattern is unambiguously recognized in two complex oat-containing samples whose composition includes also wheat, rye or barley (Fig. 3, bottom). The avenin mass signals for these two samples show slight differences owing to their being elaborated with distinct oats cultivars and are similar to six other avenin mass profiles displayed in Figs 2 and 4. These data reveal the usefulness of MALDI/TOF-MS for identifying rapidly avenins in oat-containing food samples even when other cereals are present.

Detection of avenins in gluten-free foods

Gliadins, hordeins, secalins and avenins constitute the protein components which are toxic to coeliac patients. Unfortunately, the commercial ELISA kit most used for

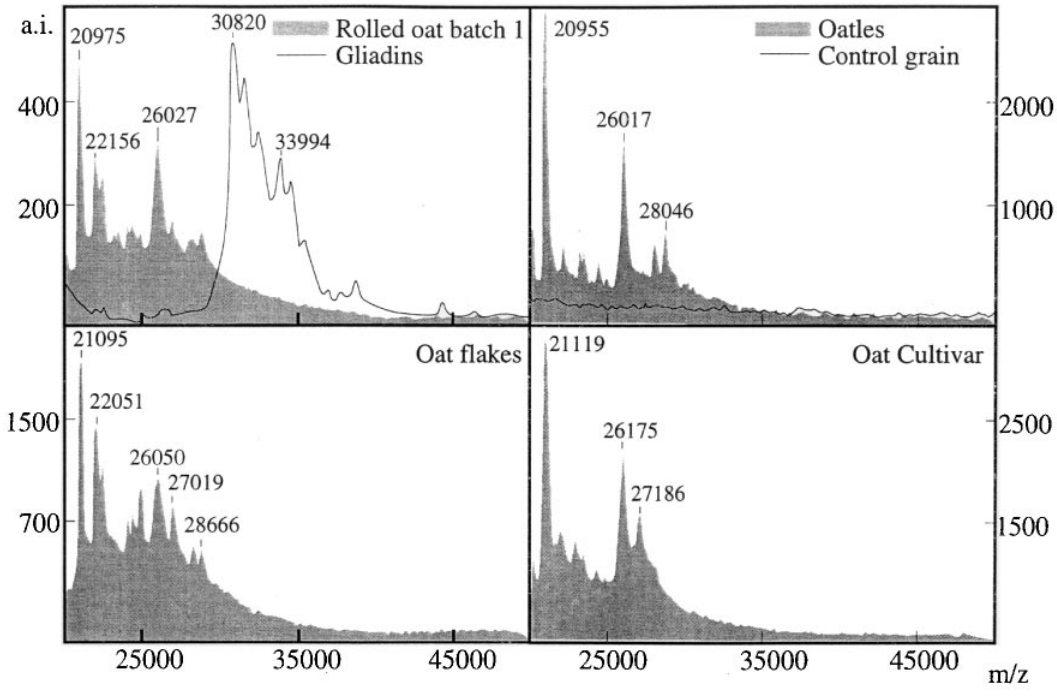

Figure 2. MALDI/TOF mass spectra of the ethanol extracts (100 mg ml^{-1}) from three different oat-containing food samples. Several main peaks within the highlighted typical mass range of avenins (20–30 kDa) have been labelled for comparison. The MALDI/TOF mass spectra of the ethanol extracts from the wheat cultivar *Senatore Capelli* (top, left) and the oat cultivar *MH21* (bottom, right) as well as that of a grain used as a control (top, right) are displayed.

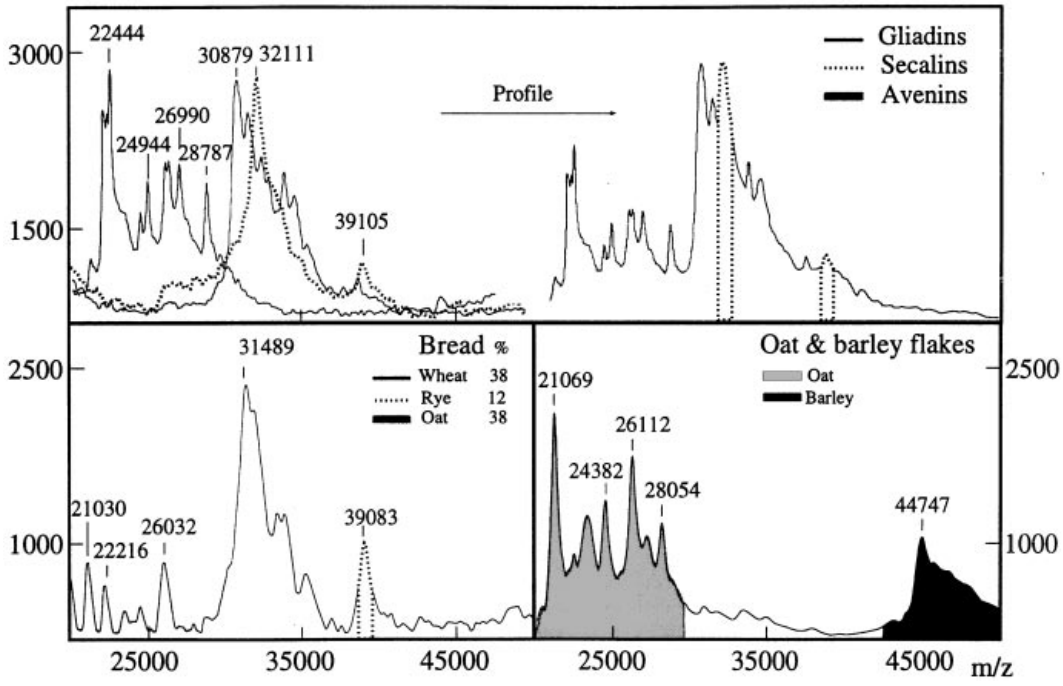

Figure 3. Top: overlayed MALDI/TOF mass spectra of gliadins secalins and avenins (left) and mass profile expected in the ethanol extract from a food sample elaborated with a mixture of wheat (*Senatore Capelli*), rye (*Rañ*a) and oats (*PA-105*) (right). Bottom: MALDI/TOF mass spectra of the prolamine extracts (100 mg ml^{-1}) from two different food samples containing mixtures of wheat, rye and oats. Several main peaks within the highlighted typical mass ranges of gliadins (30–45 kDa) and avenins (20–30 kDa) and also relevant mass peaks from secalins (32 and 39 kDa) have been labelled for comparison.

the analysis of gluten fails to recognize oats gluten components and, consequently, avenins would remain undetected in oats-contaminated gluten-free products when analyzed with the commercial kit. Hence a system which enables one to identify oats gluten in gluten-free foods is of great interest in monitoring the diet of coeliac patients.

The suitability of MALDI/TOF-MS for this purpose is exemplified in Fig. 4 by the mass analyses of two batches of a gluten-free maize food in which the commercial ELISA kit failed to detect any traces of gluten (data not shown). Since, in principle, the only cereal employed to elaborate this product is maize starch, only maize zein mass signals would be expected. Indeed,

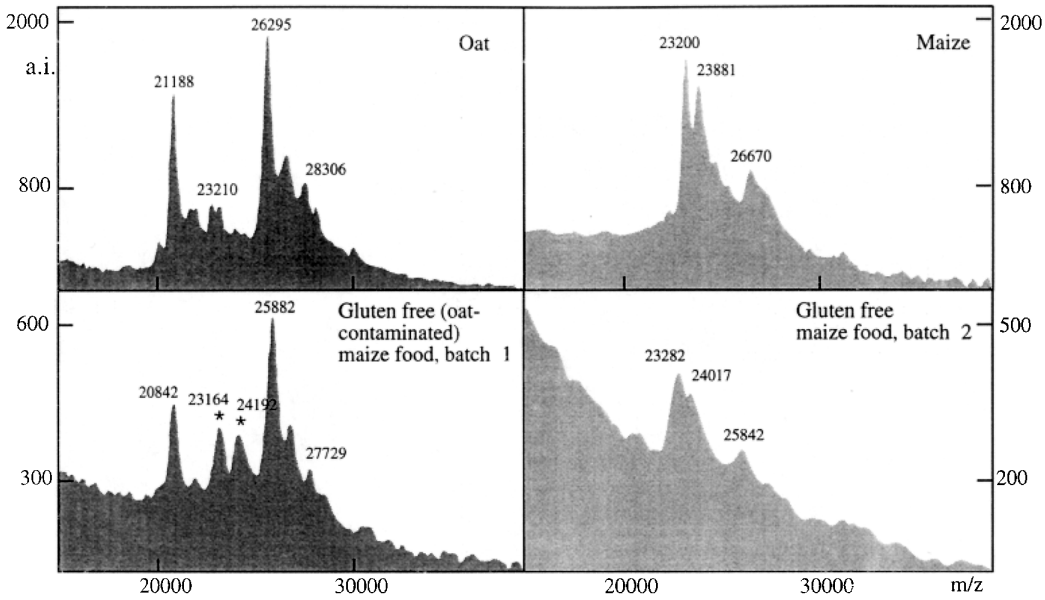

Figure 4. MALDI/TOF mass spectra of the ethanol extracts (100 mg/ml^{-1}) from two different batches of a gluten-free maize food: oats-contaminated (bottom, left) and free from contamination (bottom, right). The MALDI/TOF mass spectra of the ethanol extracts from the oat cultivar *MH27* (top, left) and the maize cultivar *Golda* used as a control (top, right) are displayed for comparison. Several main peaks within the typical mass range of avenins (20–30 kDa) and also those of zeins have been labelled. Identified zein mass peaks have been labelled with an asterisk.

batch 2 (Fig. 4, bottom, right) displays only the three characteristic maize zein mass signals at about 23, 24 and 26 kDa (Fig. 4, top, right). In contrast, batch 1 shows a mass pattern which can be unambiguously assigned to a typical avenin mass pattern together with zein mass signals (Fig. 4, bottom, left). The results indicate that MALDI/TOF-MS is at present the only system capable of detecting oats contamination in gluten-free foods.

Detection limit for the mass analyses of avenins

We studied whether MALDI/TOF-MS provides a detection sensitivity sufficient to detect oats gluten in food samples at levels close to the toxicity threshold.[23] Dilutions of the oats avenin standard *PA-101* ranging from 0.1 to 10.0 mg ml^{-1} were mass analysed employing the 160:100 sample:matrix volume ratio as described above. Figure 5 demonstrates that the characteristic protonated avenin mass pattern can be reliably detected down to a concentration of 0.4 mg ml^{-1}. This lower limit is nearly identical with that reported for gliadins.[19] A linear response between the avenin standard concentration and either the avenin mass area (20–30 kDa) or the main avenin peak height (around 22 kDa) was obtained within the 0.4–10 mg ml^{-1} range corresponding to 0.4–10 mg of avenins per 100 g of food. For routine analyses, we chose avenin mass area measurement instead of peak heights, since relying on the measurement of a single avenin mass peak could lead to quantification errors as the main avenin peak might vary from cultivar to cultivar. Figure 5 (inset) shows the calibration graph obtained when avenin mass area measurement is utilized with good reproducibility as revealed by the standard deviation data included.

As a result, this procedure could in principle be used to quantify avenins in food samples.

Mass spectrometric quantification of avenins in food samples

The calibration graph described above (Fig. 5) was used to quantify gluten avenins in food samples by measuring the avenin mass area in each food sample and matching it against that of the avenin standard. Table 1 gives the results of the analyses of three groups of samples with different oats content. Four products elaborated with oats (Fig. 2) as the only cereal and with a high oats content yielded high values. Two oats-containing foods elaborated with mixtures of distinct cereals (Fig. 3) with a lower oats content yielded correspondingly lower values; 1:100–1:10 dilutions were required for these two groups of samples to be measured in the linear range described in Fig. 5.

Finally, two batches of a gluten-free food elaborated with maize starch (Fig. 4) were also analysed. Only batch 1 showed the typical avenin mass pattern (Fig. 4, bottom), which permitted the quantification of the oats gluten contamination in this sample. Nevertheless, since maize zein mass peaks overlap the avenin mass area, this quantification value is overestimated. This illustrates that when both avenins and zeins are present in a

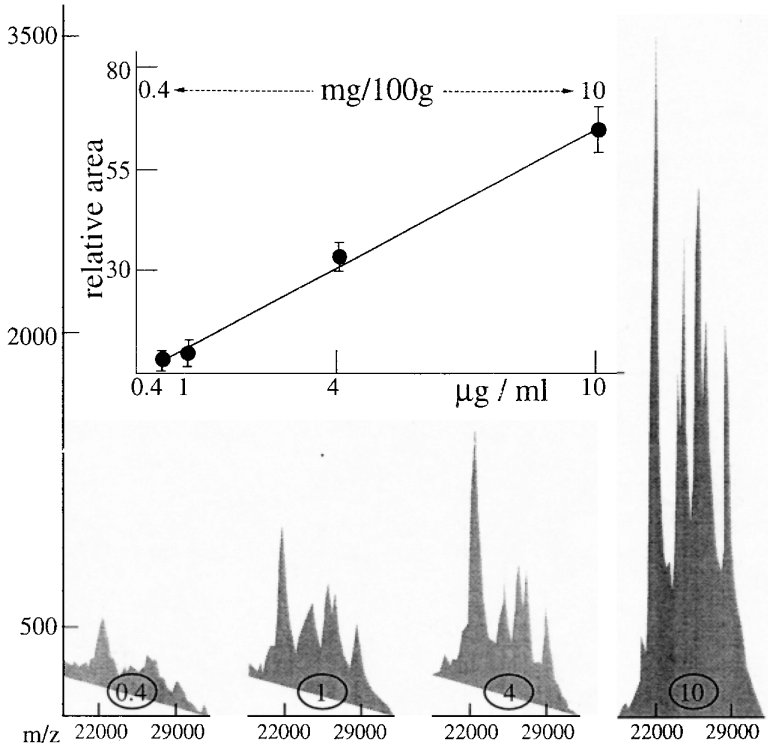

Figure 5. Calibration graph from 0.4 to 10 mg ml^{-1} using the ethanol oat avenin extract from *Avena sativa* L. cv. *PA-101*. The equivalent range expressed as mg per 100 g of food is indicated by a dashed line (inset). The 18–33 kDa range employed to measure peak areas of the MALDI/TOF mass spectra corresponding to the above avenin standard concentrations has been highlighted. Error bars are standard deviations of three independent duplicate measurements from 3–5 selected target spots.

Table 1. Quantitative analysis of gluten[a] in oats-containing samples by MALDI/TOF-MS

Sample	Amount gluten per 100 g (mg)	SD[b] (mg)	Fig.
Rolled oats (batch 1)	551	38	2
Rolled oats (batch 2)	684	50	
Oatles	284	22	2
Control grain	UD[c]		2
Oat flakes	732	37	2
Oat and barley flakes	52	5	3
Oats-containing bread	60	8	3
Gluten-free maize food (batch 1)	2.8[d]	—	4
Gluten-free maize food (batch 2)	UD[c]		4

[a] Gluten values are expressed as twice the avenin levels.
[b] SD, standard deviation.
[c] UD, undetected.
[d] Overestimated.

food sample, this mass spectrometric procedure is not fully reliable from the quantitative point of view, but only as a screening method.

Analyses of these samples with the commercial ELISA kit failed to reveal the presence of oats gluten (data not shown). To our knowledge, the present approach is the first system for quantifying oats gluten in food samples.

In conclusion, this study has demonstrated that MALDI/TOF-MS is a suitable tool for the screening and quantification of oats avenins in foods. The system permits, for the first time, analyses of oats gluten toxic components. This leads to an improved diet control of coeliac patients which is unattainable using immunological methods such as ELISA. The detection sensitivity of this mass spectrometric system is comparable to that reported for gliadins based on the same technique. The procedure is strongly recommended for screening for oats contamination in gluten-free foods. The efficiency of this procedure has already been proved (data not shown) by testing a large number of gluten-free food samples and is currently being extended. On the other hand, limitations have been noted from the quantitative point of view which demand further study.

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