ELSEVIER

Journal of Chromatography A, 823 (1998) 299–306

JOURNAL OF CHROMATOGRAPHY A

Selective identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of different types of gluten in foods made with cereal mixtures

Emilio Camafeita, Juan Solís, Patricia Alfonso, Juan Antonio López, Luis Sorell, Enrique Méndez*

Unidad de Análisis Estructural de Proteínas, Centro Nacional de Biotecnología, CSIC, Cantoblanco, E-28049 Madrid, Spain

Abstract

The gluten toxic fractions responsible for the mucosal damage in coeliac disease (CD), so-called gliadins, hordeins, secalins and avenins from a large number (30–40) of wheat, barley, rye and oats cultivars respectively, have been mass analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS). Gliadin, secalin and avenin characteristic mass profiles are nearly identical amongst distinct cultivars from the corresponding cereal, while hordeins profiles show more variability depending on the particular barley cultivar. On the basis of these four distinguishable characteristic mass patterns spreading within the 20 000–40 000 Da range, MALDI-TOF-MS has permitted the direct and simultaneous visualization of gliadins, hordeins, secalins and avenins in foods elaborated with cereal mixtures of wheat, barley, rye and oats. This capacity has been demonstrated by mass analyzing foods made with these four cereals in varying ratios. Thus MALDI-TOF-MS can be preliminarily established as a unique system with the ability to discriminate the specific type of gluten toxic fractions present in food samples.

Keywords: Cereals; Gliadins; Hordeins; Secalins; Avenins; Proteins; Gluten

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become a main tool for the analysis of complex cereal protein mixtures [1–7]. We have focused on the employ of this technique in the characterization and analysis of the complex toxic protein fractions present in wheat, barley, rye and oats, so-called gluten, which provoke coeliac disease (CD) (gluten-sensitive enteropathy) [8,9]. We first reported the utility of MALDI-TOF-MS as a tool to identify gluten gliadins from wheat in food based on the direct observation of the characteristic gliadin mass pattern as revealed in mass spectra [1,5]. More recently we reported the development of the first non-immunological method to quantify gluten gliadins in food based on MALDI-TOF-MS [6,7]. This analytical method can be considered as a complement to the conventional immunological, epitope-dependent procedures presently employed which are not definitively reliable and sensitive enough to analyze gluten in food [10–20].

Although these studies have been mainly aimed to gluten gliadins, the mass spectrometric analysis of the remaining gluten toxic cereal components from barley (hordeins), rye (secalins) and oats (avenins)

*Corresponding author.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00621-9

and their identification in food samples has not yet been addressed.

We now study the capability of MALDI-TOF-MS to simultaneously and selectively identify gluten from wheat, barley, rye and oats in samples containing complex mixtures of these four cereals.

2. Experimental

2.1. Cereal cultivars

Wheat (*Triticum durum*, L. cv. *Senatore capelli*, L. cv. *Roqueño*, L. cv. *Jabato* and L. cv. *Artena*), barley (*Hordeum vulgare*: L. cv. *Distica cameo*, L. cv. *Distica cresta*, L. cv. *Borwina* and L. cv. *Hexastica tabaiba*), rye (*Secale cereale*, L. cv. *Merkator*, L. cv. *Petkus*, L. cv. *Raña* and L. cv. *Soron*) and oats (*Avena sativa* L. cv. *PA-105*, L. cv. *MH21*, L. cv. *M12* and L. cv. *M13*, kindly provided by Dr. Th. Mothes) cultivars were used.

2.2. Other materials

Bovine serum albumin (BSA) and horse heart cytochrome *c* (CC) were purchased from Sigma (St. Louis, MO, USA). Food samples employed in this study were kindly supplied by Jaime Pedró, Barcelona, Spain.

2.3. Reagents

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

2.4. Ethanol extraction from cereal cultivar flours and food samples

A 1-g sample was homogenized in 5.0 ml 60% (v/v) aqueous ethanol for 2 min and then centrifuged for 15 min at 1500 *g*. The supernatant was taken off and the pellet re-extracted as above. This procedure was performed at room temperature. The supernatants were combined, brought to a final volume of 10 ml with 60% ethanol, kept at room temperature and analyzed by MALDI-TOF-MS and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Sample preparation for MALDI-TOF-MS

Twenty μl of ethanol extract containing 4 μl of 50 m*M* octyl-β-D-glucopyranoside detergent were mixed with a matrix solution comprised of 100 μl of saturated sinapinic acid in 30% aqueous acetonitrile and 0.1% trifluoroacetic acid. A-0.5 μl volume of this mixture was then 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 (Bremen, Germany) Reflex II MALDI-TOF-MS system equipped with an ion source with visualization optics and a N_2 laser (337 nm). Mass spectra were recorded in linear positive mode at 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 2–3 selected target spots were considered. The equipment was externally calibrated employing singly, doubly and triply charged signals from a mixture of BSA (66 430) and CC (12 360).

2.6. One-dimensional SDS-PAGE

SDS-PAGE was performed under the same conditions as previously described [21]. After electrophoresis, the gels were stained overnight in 0.1% (w/v) Coomassie brilliant blue R in methanol–acetic acid–water (10:10:80) and destained with the same solution in the absence of Coomassie brilliant blue.

3. Results and discussion

Conventionally, cereal-containing foods are elaborated with mixtures of distinct cereals. Some of these cereals, such as wheat, barley, rye and probably oats, are extremely toxic to coeliac patients. Consequently,

susceptible individuals must consume either gluten-free foods or foods containing quantities of gluten strictly below the toxicity threshold [22].

Epitope-dependent methods, such as enzyme-linked immunosorbent assay (ELISA) [11–20], can only quantify total gluten in food and fail to discriminate the distinct type of cereals present. This is due to the fact that they rely on monoclonal and polyclonal antibodies which cross-react against all gluten protein components (gliadins, secalins, avenins and hordeins) which are highly immunologically related. A high resolution system capable of differentiating the type of cereals present in a food sample is therefore of great interest.

We previously showed that ethanol extracts of gliadins, hordeins, secalins and avenins yield a characteristic protonated MALDI-TOF mass pattern within distinct mass ranges [1,5]. Given that these studies were carried out only for a single cultivar, it is then critical to demonstrate the occurrence of the typical mass signal patterns in a large number of cultivars. If so, the system could in principle be used to differentiate complex mixtures containing gliadins, hordeins, secalins and avenins.

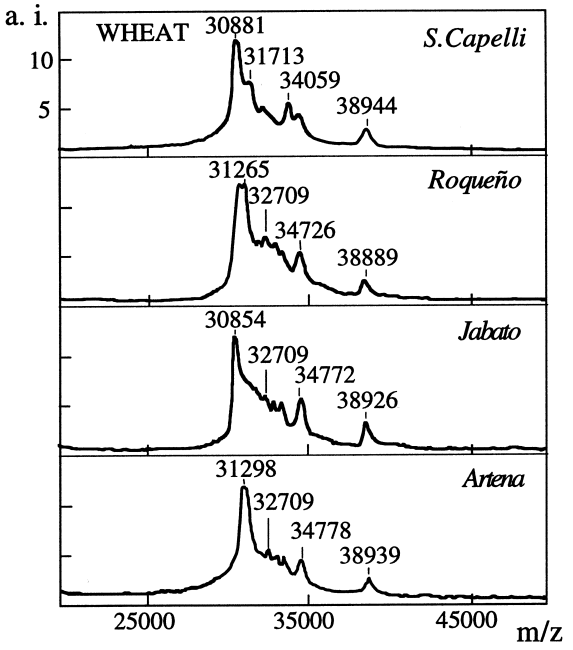

Fig. 1. MALDI-TOF mass spectra of the gliadin extracts (100 mg/ml) from four different wheat cultivars (top to bottom: *Senatore capelli*, *Roqueño*, *Jabato* and *Artena*). Information on molecular mass is provided only for some selected peaks within the selected gliadin mass range.

3.1. MALDI-TOF-MS analysis of different wheat, barley, rye and oats cultivars

Fig. 1 shows MALDI-TOF spectra of the gliadin extracts from four different wheat cultivars. These samples yield very similar characteristic mass patterns in the 30 000–40 000 Da range, in which lie the typical molecular masses of α- and γ-gliadins [1,5]. In all four cases, the most intense mass signal corresponds to an α-gliadin at around 31 000 Da.

Correspondingly, Fig. 2 displays the characteristic avenin profiles of the ethanol extracts from four distinct oats cultivars as revealed by MALDI-TOF-MS. Around 10 mass peaks occur in the 20 000–30 000 Da range with minor differences amongst the four cultivars.

On the other hand, MALDI-TOF mass spectra of the ethanol extracts from four different rye cultivars (Fig. 3) display three typical mass signals: one base peak at around 32 000 Da and two minor peaks at around 39 000 and 71 000 Da. It is interesting to note that the relative intensity amongst the three main stained bands (Figs. 5 and 7) differs from that of the corresponding MALDI-TOF mass peaks. The reason the mass signals at 39 000 and 71 000 Da appear very weak is unknown; nevertheless, in the case of ω-1 secalin (39 000 Da) this could be partially ascribed to the metachromasia phenomenon described for this protein [23].

Fig. 4 displays four of the most representative types of hordein mass patterns found in the MALDI-TOF analyses of a large number of barley cultivars. The four hordein patterns comprise different mass ranges and signals (Fig. 4, top to bottom): (i) two well-resolved peaks within the 34 000–38 000 Da range; (ii) two main peaks at around 31 and 32 kDa and some less defined peaks up to 38 000 Da, including a peak at about 34 000 Da similar to that of (i); (iii) two groups of signals: one nearly identical to (ii) and another comprising a main mass signal at around 45 000 Da; and (iv) a wide distribution of mass signals spreading over the 26 000–

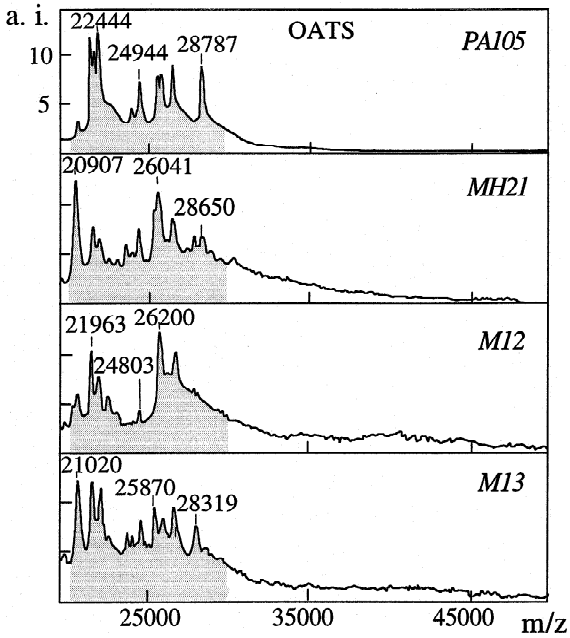

Fig. 2. MALDI-TOF mass spectra of the avenin extracts (100 mg/ml) from four different oats cultivars (top to bottom: *PA-105*, *MH21*, *M12* and *M13*). Information on molecular mass is provided only for some selected peaks within the selected avenin mass range.

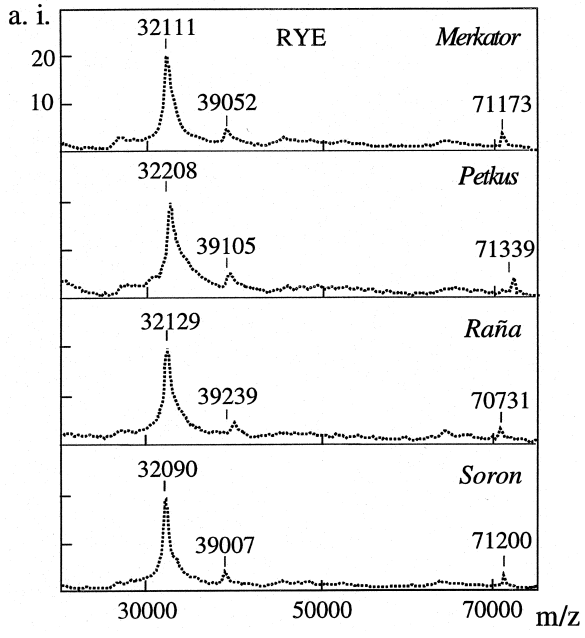

Fig. 3. MALDI-TOF mass spectra of the secalin extracts (100 mg/ml) from four different rye cultivars (top to bottom: *Merkator*, *Petkus*, *Raña* and *Soron*). Information on molecular mass is provided only for some selected peaks within the selected secalin mass range.

45 000 Da which comprises most of the peaks also present in (i), (ii) and (iii).

As a result, while gliadin, avenin and secalin mass profiles appear to be very similar in different cultivars from the corresponding cereal (Figs. 1–3), hordein mass patterns differ markedly depending on the particular barley cultivar (Fig. 4). Additional studies performed in a large number (30–40) of cultivars further confirm this observation (data not shown).

Fig. 5 shows the comparison of gliadins, avenins, secalins and hordeins mass spectra displaying their typical mass ranges and most relevant mass peaks. It can be observed that while avenin mass signals are well separated from the remaining components, gliadins, secalins and hordeins overlap within the 30 000–40 000 kDa region. This combination of mass patterns could in principle be expected when analyzing samples containing mixtures of all four types.

3.2. Direct identification by MALDI-TOF-MS of mixtures containing gliadins, avenins, secalins and hordeins

Fig. 6 displays MALDI-TOF-MS analyses of samples made with mixtures of wheat, barley, rye and oats in varying ratios. Only the most outstanding mass peaks from secalins and hordeins, which overlap with the gliadin mass profile, have been highlighted and their mass indicated. As expected, avenins are easily discernible from the remaining components (Fig. 6A, and D–H). Likewise, gliadins themselves, which comprise a wide mass range with numerous well defined mass peaks, yield a clearly distinguishable profile [1,5].

In foods elaborated with wheat and either rye or barley, secalins and hordeins, which overlap with gliadin mass signals (Fig. 5), can be easily identified by the corresponding increased intensity in the gliadin mass pattern: (i) Fig. 6C, and E–G display

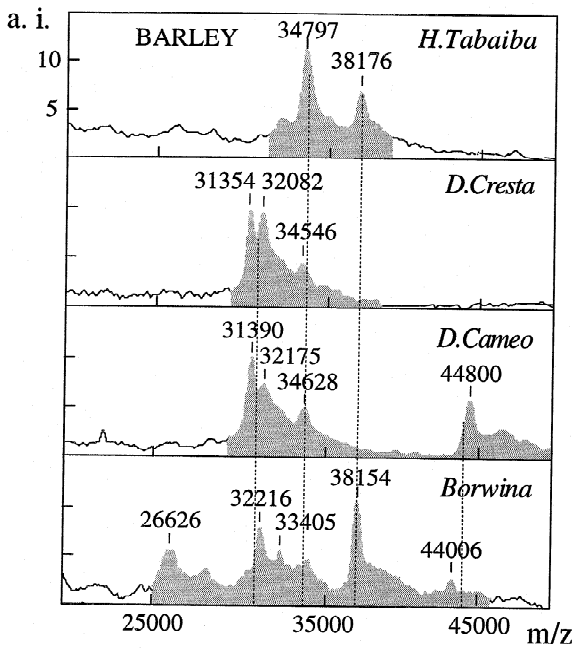

Fig. 4. MALDI-TOF mass spectra of the hordein extracts (100 mg/ml) from four different barley cultivars (top to bottom: *H. tabaiba*, *D. cresta*, *D. cameo* and *Borwina*). Information on molecular mass is provided only for some selected peaks within the selected hordein mass range. Dashed vertical lines are located around common groups of mass signals amongst distinct cultivars.

the location in the gliadin profile of the main and minor secalin mass signals at around 32 000 and 39 000 kDa (Fig. 3) and (ii) the hordein mass signals at around 34 000 and 38 000 kDa occur in the gliadin mass pattern (Fig. 6B, D and G), while the hordein mass peak at around 45 000 kDa shows in Fig. 6H, indicating that these hordein-containing samples have been elaborated with different barley cultivars similar to those in Fig. 4, top and bottom.

Despite the high complexity of individual gliadin, hordein, secalin and avenin extracts, their protein components can be partially resolved and sufficiently identified by SDS-PAGE (Figs. 5 and 7). Nevertheless, when analyzing more complex extracts from foods elaborated with mixtures of wheat, barley, rye and oats, SDS-PAGE fails to adequately identify the corresponding gliadin, hordein, secalin and avenin components (Fig. 7). Another limitation of this technique is its low sensitivity as compared with MALDI-TOF-MS. Hence, mass spectra in Figs. 1–6 were attained using an amount of sample 250-times lower than that employed for SDS-PAGE (Figs. 5 and 7). Such low amounts would have been undetectable by SDS-PAGE (data not shown). As an alternative, MALDI-TOF-MS is becoming a high-performance tool for the analyses of these cereal protein components which offers several important advantages: (i) higher sensitivity and resolving power [19,24] and (ii) more precise information about the molecular masses of individual protein components [24], as ilustrated in Figs. 1–6.

Unlike SDS-PAGE (Fig. 7), results presented in this work clearly show MALDI-TOF-MS to be capable of accurately identifying gliadin, hordein, secalin and avenin components when simultaneously present in foods (Fig. 6). This has been demonstrated for eight food samples (Fig. 6) in which the visualization of both the avenin and gliadin mass pattern is straightforward. Secalins and hordeins, whose mass signals overlap with those of gliadins when simultaneously present, can be also easily identified by observing not only their mass peaks outstanding from the gliadin mass profile, but also their molecular masses, which nearly match those of the corresponding rye and barley cultivars (Figs. 3 and 4).

The data point out that MALDI-TOF-MS is a useful system for the simultaneous identification of all gluten toxic components in foods by observing their corresponding characteristic protonated mass patterns. Therefore, this spectrometric technique suggests the possibility to develop a quantification procedure of the total gluten components (i.e., gliadins, hordeins, secalins and avenins) in foods. The system should be based on the measurement of the areas under the corresponding mass profile in the 20 000–45 000 kDa range. A procedure to quantify total gluten is under study and will improve our previous system, which was selectively aimed to quantify gluten gliadins in food by measuring the height of the most prominent α-gliadin mass signal at around 31 000 kDa [6,7]. Also, in view that the avenin mass range (20 000–30 000 kDa) is free of contributions from gliadin, hordein and secalin mass signals (Figs. 4 and 5), gluten avenins could be quantified separately and without interference. The estimation of either the total gluten content or gluten avenins exclusively will depend on the standard

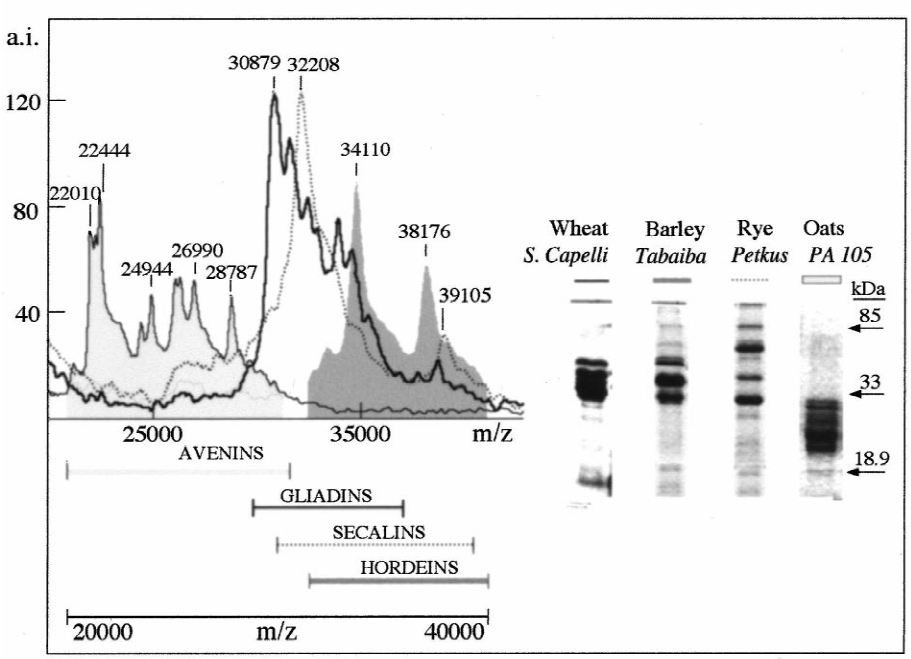

Fig. 5. Overlayed characteristic protonated mass patterns from gliadin, hordein, secalin and avenin extracts. MALDI-TOF mass spectra together with SDS-PAGE of the extracts from *S. Capelli* (Fig. 1), *H. tabaiba* (Fig. 4), *Petkus* (Fig. 3) and *PA-105* (Fig. 2) are presented. The whole mass range along with the corresponding to gliadin, hordein, secalin and avenin extracts are indicated. Mass patterns of the corresponding extracts have been highlighted according to those in Figs. 1–4.

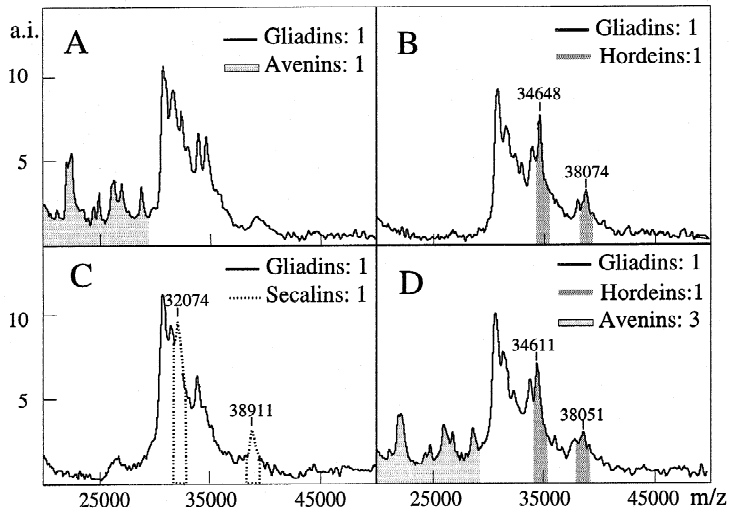

Fig. 6. MALDI-TOF mass spectra of food samples containing mixtures of gliadins, hordeins, secalins and avenins in varying ratios as indicated. Mass patterns have been highlighted according to those in Figs. 1–5. For comparison, several molecular masses of secalins and hordeins have been included.

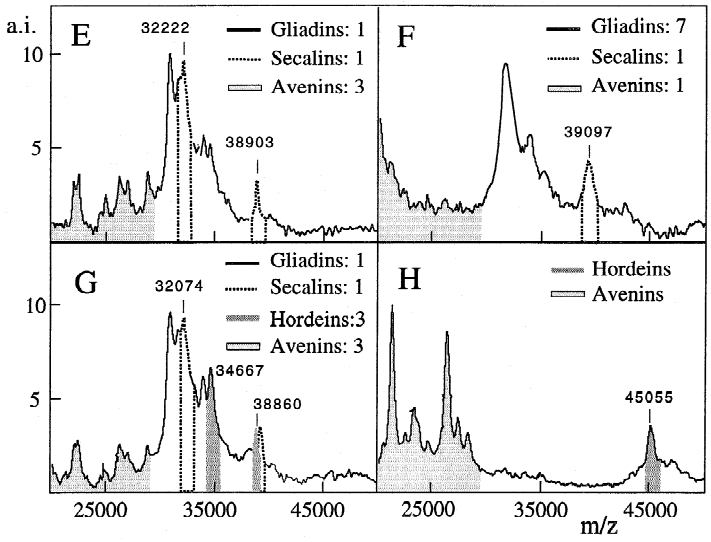

Fig. 6. Continued.

chosen, i.e., a mixture of total gluten for the former and gluten avenins for the latter.

A system to selectively quantify avenins would have special relevance since, at present, all the ELISA systems based on monoclonal and polyclonal antibodies employed to quantify gluten in food detect gliadins, secalins and hordeins well and avenins poorly [8,9]. As a result of this bias, ELISA data

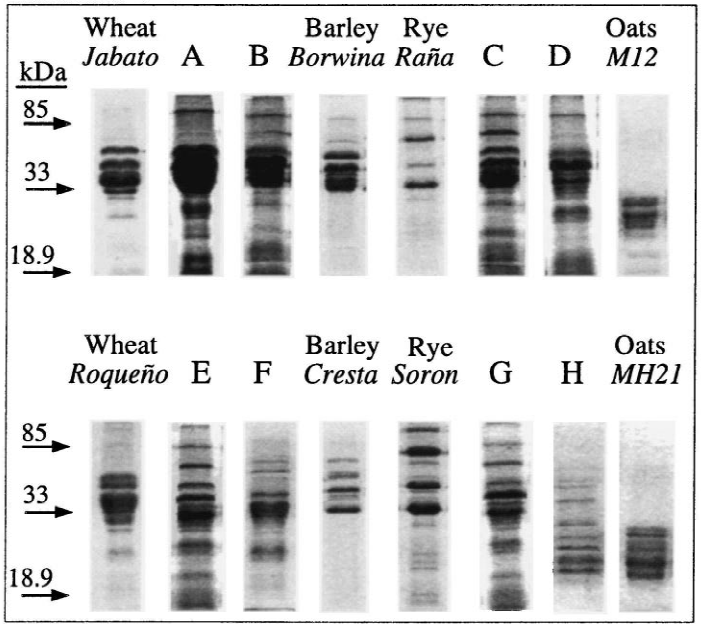

Fig. 7. SDS-PAGE analyses of two different gliadin, hordein, secalin and avenin extracts together with the eight food sample extracts in Fig. 6.

could result in an underestimation of total gluten in food. Taking into account that to date there is no agreement about whether gluten avenins from oats are toxic or not [25–27], the development of a procedure capable of quantifying avenins is of great interest.

Acknowledgements

This work was supported by grants from CICYT, BIO97-0076 and BIO95-2070-E. The authors wish to express their gratitude to Mr. J. Pedró Casanova and Ms. P. de la Villa Vela (Jaime Pedró, Barcelona, Spain) for kindly providing food samples.

References

[1] E. Méndez, E. Camafeita, J.S. Sebastián, Y. Valle, J. Solís, F.J. Mayer-Posner, D. Suckau, C. Marfisi, F. Soriano, J. Mass Spectrom., Rapid Commun. Mass Spectrom. S123 (1995) S123–S128.
[2] J.B. Turner, D.B. Gordon, G.V. Garner, G.A. Lord, C.A. Smith, Biochem. Soc. Trans. 22 (1994) 403S.
[3] T.A. Egorov, A.K. Musolyamov, A.A. Kochergin, J.S. Andersen, P. Roepstorff, J. Cereal Sci. 21 (1994) 107.
[4] D.R. Hickmann, P. Roepstorff, A.R. Clarke, A.S. Tatham, P.R. Shewry, J. Cereal Chem. 22 (1995) 99.
[5] E. Camafeita, P. Alfonso, B. Acevedo, E. Méndez, J. Mass Spectrom. 32 (1997) 444.
[6] E. Camafeita, P. Alfonso, T. Mothes, E. Méndez, J. Mass Spectrom. 32 (1997) 940.
[7] E. Camafeita, P. Alfonso, J.S. Sebastián, E. Méndez, J. Pediat. Nutr. 24 (1997) 464.
[8] M.N. Marsh, Gastroenterology 102 (1992) 330.
[9] P.R. Shewry, A.S. Tatham, D.D. Kasarda, in: M. Marsh (Ed.), Coeliac Disease, Blackwell, Oxford, 1992, Ch. 11, p. 305.
[10] F.W. Jansen, G. Voortman, J.A. de Baaij, J. Agric. Food Chem. 35 (1987) 563.
[11] S.U. Friis, Clin. Chim. Acta 178 (1988) 261.
[12] A.R. Freedman, G. Galfre, E. Gal, H. Ellis, P. Ciclitira, J. Immunol. Methods 98 (1987) 123.
[13] J.H. Skerritt, A.S. Hill, J. Agric. Food Chem. 38 (1990) 1771.
[14] H. Ellis, A.P. Doyle, H. Weiser, R.P. Sturgess, P. Day, P. Ciclitira, J. Biochem. Biophys. Methods 28 (1994) 77.
[15] F.G. Chirdo, M.C. Añón, C.A. Fossati, Food Agric. Immunol. 7 (1995) 333.
[16] E.N.C. Mills, G.M. Brett, S. Holden, J.A. Kauffman, M.J. Tatton, M.A.R. Morgan, Food Agric. Immunol. 7 (1995) 189.
[17] P.D. Howedle, M.S. Losowsky, Gut 31 (1990) 712.
[18] Th. Mothes, presented at the 11th Meeting of the Working Group on Prolamin Analysis and Toxicity, Tecklenburg, 1996.
[19] H. Wieser, presented at the 11th Meeting of the Working Group on Prolamin Analysis and Toxicity, Tecklenburg, 1996.
[20] J.H. Skerritt, A.S. Hill, J. Assoc. Off. Anal. Chem. 74 (1990) 257.
[21] A. Rocher, F. Soriano, E. Molina, G. González-Limas, E. Méndez, Biochim. Biophys. Acta 1247 (1995) 143.
[22] Codex Alimentarius Commission, Proposed Draft Revised Standard for Gluten-Free Foods, May 1995.
[23] A. Rocher, M. Calero, F. Soriano, E. Méndez, Biochim. Biophys. Acta 1295 (1996) 13.
[24] S.A. Carr, R.S. Annan, in F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidmann, J.A. Smith, K. Struhl (Eds.), Current Protocols in Molecular Biology, Wiley, New York, 1997, Ch. 10, p. 21.1.
[25] D. Branski, M. Shine, New Engl. J. Med. 334 (1996) 865.
[26] U. Srinivasan, N. Leonard, E. Jones, D.D. Kasarda, D.G. Weir, C. O'Farrelly, C. Feighery, Br. Med. J. 313 (1996) 1300.
[27] J. Schmitz, Br. Med. J. 314 (1997) 159.