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Reversed-phase high-performance liquid chromatography–electrospray mass spectrometry profiling of transgenic and non-transgenic maize for cultivar characterization

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ABSTRACT

A reversed-phase high-performance liquid chromatography–electrospray mass spectrometry (RP-HPLC–ESI-MS (ion trap)) method is developed, for the first time, for profiling transgenic and non-transgenic maize with the aim of cultivar characterization. To optimize chromatographic conditions the following parameters were studied: column, gradient, and ion-pairing reagent. Moreover, the influence in the MS signal of the variation of the capillary voltage and the accumulated ions in the trap was also studied. The developed method was applied to the profiling of different protein fractions (albumin, globulin, prolamin, and glutelin) isolated from Bt transgenic and non-transgenic maize cultivars. Moreover, different maize samples, namely, maize cultivars from different geographical origins (USA, Canada, France, and Spain), transgenic maize samples with certified GMO content, and three transgenic Bt maize cultivars with their isogenic non-transgenic counterparts (Aristis Bt vs. Aristis, PR33P67 vs. PR33P66, and DKC6575 vs. Tietar) were profiled by the developed method. Mass spectra obtained for certain peaks in the maize cultivars studied resulted, in some occasions, useful for cultivar characterization and differentiation. The comparison of UV and MS profiles and mass spectra corresponding to the protein fractions with those of the whole seeds enabled the assignment of some peaks.

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops worlwide, constituting a cheap source of food and feed. The huge genetic diversity of this crop, the differences regarding quality and characteristics of every maize variety, the development of maize improvement programs, and the protection of maize genetic diversity are only some of the pursued goals by plant breeders and scientific programs. On the other hand, advances in genetics have enabled the introduction of heterologous DNA sequences into the maize genome to improve its resistance to certain plagues such as the European corn borer (*Ostrinia nubilalis*) and the tolerance to certain herbicides [1–3]. Nevertheless, the development of these new cultivars is surrounded by a great controversy concerning its safety, environmental and ethical impact, potential negative effect on human health, etc. [3,4].

The growing interest in evaluating and genetically improving the quality of maize and the implementation of regulations controlling development, use, and growth of transgenic maize have made the development of methodologies for maize characterization necessary [5]. Scientific communities have developed methodologies based on plant morphological and agronomical characters that have resulted in limited usefulness since they are strongly affected by environmental conditions in which the plant has grown. The characterization of maize varieties has also been carried out using molecular markers based on DNA analysis. First actions were focused on DNA analysis using different PCR (polymerase chain reaction) methods and separation techniques. Significant advances to overcome the problems derived from the use of classical PCR methodologies, especially its qualitative character, have been developed such as real-time PCR or competitive PCR [6–12]. Main weaknesses of this PCR-based methodology are the fact that the DNA extraction procedure could affect the quantification of genetically modified maize that the efficiency of PCR depends on the quality and purity of DNA in turn determined by its length, integrity grade, and the presence of substances that inhibit the amplification reactions [13,14].

Another strategy has been the analysis of proteins. Maize contains around 10% proteins classified according to their solubility in albumins, globulins, glutelins, and the most abundant called zeins or prolamins. Zeins analysis by MALDI–TOF-MS (matrix assisted laser desorption ionization–time of flight-mass spectrometry)

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has been conducted in nine different (non-transgenic) varieties concluding this could be a suitable analytical tool for genotype identification [15,16]. Moreover, specific monoclonal and polyclonal antibodies have been developed for the application of immunochemical assays to the analysis of transgenic maize [11,17,18]. Main limitations of the application of immunochemical methodologies are the dependency of accuracy and precision on sample matrix and that detection could be compromised by the limited amount of transgenic protein and their degradation due to thermal treatment and processing.

We have developed different methodologies for the characterization of maize products and maize cultivars [19–21], based on the use of chromatographic profiles, that have also been applied with success to the characterization and quantitative estimation of transgenic maize [22,23]. In all cases, rapid chromatographic methodologies using perfusion and monolithic stationary phases were employed together with UV detection. In this work, we propose, for the first time, the development of a methodology using HPLC with MS detection for profiling maize cultivars and the study of the applicability of the obtained profiles for the characterization of maize cultivars including transgenic and non-transgenic ones.

2. Experimental

2.1. Chemicals and samples

HPLC grade acetonitrile (ACN) (Merck, Darmstadt, Germany), HPLC grade water (Milli-Q system, Millipore, Bedford, MA, USA), trifluoroacetic acid (TFA) (Sigma, St. Louis, MO, USA), and acetic acid (HAc) (Merck) were employed for preparing mobile phases. 2-Mercaptoethanol, tris(hydroxymethyl)-aminomethane (Tris) (both from Merck), ammonium acetate, hydrochloric acid, potassium chloride, etilendiaminotetraacetic acid (EDTA), and 1-propanol (all from Panreac, Barcelona, Spain) were used for preparing maize extracts.

Corn gluten meal (purity, 60%) from Sigma and zein F4000 (purity, 92%) from Freeman Industries LLC (Tuckahoe, NY, USA) were employed. Different reference materials containing Bt-11 maize (<0.12 g/kg, 4.90 g/kg, 19.60 g/kg, and 48.90 g/kg), Bt-176 maize (<0.14 g/kg, 5 g/kg, and 20 g/kg), MON810 maize (<0.2 g/kg and 50 g/kg), GA 21 maize (<0.8 g/kg and 42.9 g/kg), NK 603 maize (<0.4 g/kg and 49.1 g/kg), MON 863 maize (<1.0 g/kg and 98.5 g/kg) or 1507 maize (<0.5 g/kg and 98.6 g/kg) certified for GMO content by the Institute for Research Materials and Measurements (IRMM) and marketed by Sigma were also used. Ten different inbred maize cultivars (CM109LP from Canada, EZ7LP and EZ8LP from Spain, A639LP, A239LP, Va26LP, B84LP, W64LP, and Mo17LP from USA, and F212LP from France) were kindly donated by Estación Experimental Aula Dei (CSIC, Zaragoza, Spain).

Conventional and MON810 transgenic varieties were obtained from a field assay carried out in Estación Experimental Agrícola Mas Badía in Tallada d'Empordá (Girona, Spain) using commercial varieties. Namely, in order to skip any influence from the growing conditions, Aristis maize (wild type and its Bt transgenic variety); Tietar maize (wild type) and its Bt transgenic variety, DKC6575; and PR33P66 maize (wild type) and its Bt transgenic variety, PR33P67, were grown under the same field conditions and investigated in this work. The transgenic and no transgenic nature of all these maize samples were confirmed by the methodology based on PCR and capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF) described elsewhere [24–28], using *Mon F* and *Mon R* primers for event-specific MON810 maize detection [29].

Maize protein fractions from Aristis, Tietar, and PR33P66 maize (both wild type and their Bt transgenic varieties) were sequentially prepared following the method of Osborne with some modifications [22,30]. Three liquid fractions (albumin, globulin, and prolamin) and a pellet (glutelin fraction) were obtained for each cultivar. Liquid fractions were directly injected in the chromatographic system while the pellets were dissolved in the extracting solution (0.5% (v/v) 2-mercaptoethanol, 0.5% (m/v) ammonium acetate, and 45% (v/v) acetonitrile) and the resulting solutions were sonicated for 3 min and centrifuged for 10 min (3362 × *g* at 25 °C).

Maize proteins extracts were prepared following a procedure previously optimized [19]. Maize cultivars were ground using a domestic miller. Known amounts of ground samples were dissolved in the extracting solution which consisted of 0.5% (v/v) 2-mercaptoethanol, 0.5% (m/v) ammonium acetate, and 45% (v/v) acetonitrile. Solutions were sonicated for 3 min and centrifuged for 10 min (3362 × *g* at 25 °C).

2.2. High-performance liquid chromatography

Two Agilent Technologies 1100 Series liquid chromatographs (Agilent Technologies, Pittsburgh, PA, USA) equipped with a degasser system, a thermostated column compartment, an automatic injector, and a variable wavelength detector were employed. One of the chromatographs was equipped with a quaternary pump while the other chromatograph was equipped with a binary pump and coupled with the mass spectrometer detector. Maize extracts were separated using two different columns: a POROS R2/H perfusion column (100 mm × 2.1 mm ID and 10 μm particle size) from Perseptive Biosystems (Framingham, MA, USA) and a C18 Zorbax Poroshell column (75 mm × 1 mm ID) from Agilent Technologies. The optimum flow-rate for every column was 0.5 and 0.1 mL/min, respectively. Mobile phases in both cases consisted of an ion-pairing reagent in water (mobile phase A) and in acetonitrile (mobile phase B). The injection volume was established in both cases in 5 μL and UV detection was performed at 280 nm. Separations were performed at 25 °C. All HPLC experiments were performed, at least, by duplicate.

2.3. Mass spectrometry

An ion trap mass spectrometer (model 1100) with an orthogonal electrospray interface (ESI, model G1607A) from Agilent Technologies (Palo Alto, CA, USA) was used. All mass spectrometry experiments were performed in the positive ionization mode. The conditions employed with the C18 1 mm ID column were: 350 °C as dry temperature, 40 psi of nitrogen for nebulization, and 8.0 psi for dry gas. For the POROS 2.1 mm ID column, the dry temperature was 350 °C, the nebulizer pressure was 50 psi, and the dry gas pressure was 10 psi. MS spectra were obtained in the mass range 600–2200 *m/z*. Control and acquisition of data was performed with the LC/MSD Trap Software 5.2. Other parameters were: compound stability, 100%; trap drive level, 100%; maximum accumulation time, 300 ms. Ion charge control (ICC) was activated for automatically adjustment of the accumulation time during the elution. All MS experiments were performed, at least, by duplicate.

3. Results and discussion

3.1. RP-HPLC–MS method development

A new RP-HPLC methodology was developed for profiling maize cultivars using both UV and mass spectrometry detection. With this aim a 30 mg/mL solution of corn gluten meal (CGM) was employed. Two different commercial columns were tested: a perfusion column (100 mm × 2.1 mm ID) working at an optimum flow-rate of 0.5 mL/min and a C18 column (75 mm × 1 mm ID) working at an

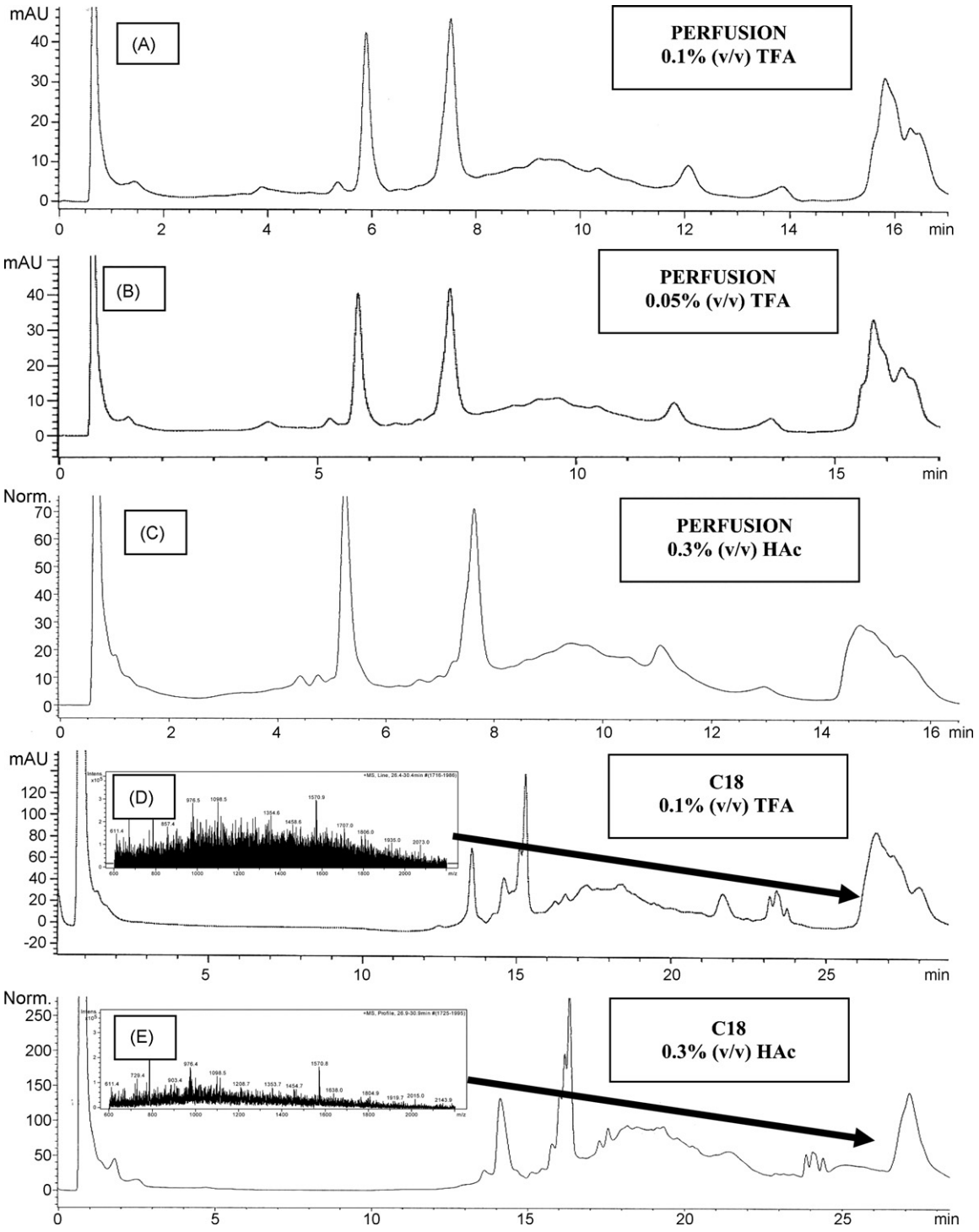

Fig. 1. Separations obtained from a 30 mg/mL corn gluten meal solution with a perfusion 100 mm × 2.1 mm ID column (A–C) and a C18 75 mm × 1 mm ID column (D and E) using different ion-pairing reagents: 0.1% (v/v) TFA, 0.05% (v/v) TFA, and 0.3% (v/v) acetic acid. Chromatographic conditions used with the perfusion column: Gradient, 5–50.2% B in 14.4 min; 50.2–65.4% B 0.98 min, and 65.4–5.0% B in 3 min; flow-rate, 0.5 mL/min. Chromatographic conditions used with the C18 column: Gradient, 5–90% B in 30 min; flow-rate, 0.1 mL/min. Mobile phases were water + ion-pairing reagent (mobile phase A) and ACN + ion-pairing reagent (mobile phase B). UV detection at 280 nm and separation temperature at 25 °C. Mass spectra corresponding to the peak indicated with the arrow are added in parts D and E for noise comparison in MS detection.

optimum flow-rate of 0.1 mL/min. Different gradient conditions for the perfusion 2.1 mm ID column using UV detection were first investigated. The best separations in terms of analysis time and resolution were achieved under the following conditions: 5–50.2% B in 14.4 min, 50.2–65.4% B in 0.98 min, and 65.4–5.0% B in 3 min, being the mobile phase A, 0.1% (v/v) TFA in water, and the mobile phase B, 0.1% TFA in ACN. The gradient employed with the C18 1 mm ID column was also optimized. The selected gradient conditions were from 5% to 90% B in 30 min using the same composition for mobile phase A and B as for the perfusion column. Separations obtained using UV detection at 280 nm are shown in Fig. 1A and D observing a better separation with the C18 column.

Next investigations were focused on the coupling of the optimized chromatographic separation with the ESI-MS detection. Despite TFA usually is the best ion-pairing reagent in reversed-phase chromatographic separations, it is also known as a strong

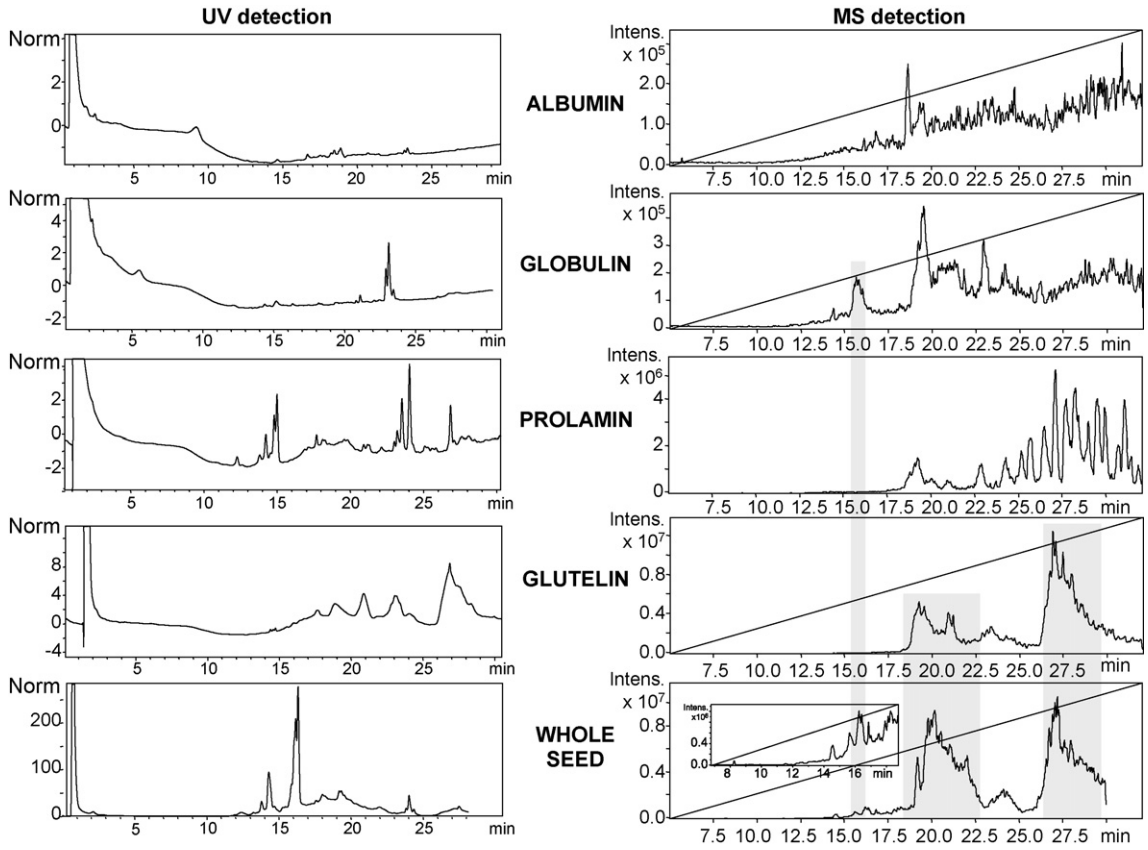

Fig. 2. UV and mass spectrometric profiles of albumin, globulin, prolamin, and glutelin fractions and of the whole protein extract corresponding to the Aristis cultivar.

signal suppressor when using ESI-MS detection. Therefore, the use of percentages of TFA lower than 0.1% or the use of an alternative ion-pairing reagent was evaluated. Based on the authors own experience, acetic acid was tried as an alternative to TFA [31–33]. Separations obtained with the perfusion 2.1 mm ID column with the optimized gradient conditions and using 0.05% (v/v) TFA or 0.3% (v/v) acetic acid are shown in Fig. 1B and C, respectively. Separations under these conditions were very similar regardless of using TFA or acetic acid. Similar results were also observed using the C18 1 mm ID column (see Fig. 1E). However, the use of 0.1% and 0.05% (v/v) TFA provided a much lower sensitivity and a noisier mass spectrum than that obtained when using acetic acid (see mass spectra added to Fig. 1D and E). Consequently, 0.3% (v/v) acetic acid was chosen. Moreover, the comparison of separations obtained with both columns enabled the selection of the 1 mm ID column for further studies due to the higher number of peaks separated with this C18 column.

Different MS parameters were then studied: capillary voltage (3.5 and 5 kV) and maximum number of ions accumulated in the trap (ICC target) (from 30,000 to 120,000). A capillary voltage of 5 kV enabled a more sensitive detection not observing any fragmentation. Despite the ICC being activated for the automatic control of the accumulation time in the trap, an ICC target limiting the maximum number of accumulated ions in the trap was also required. The highest sensitivity was observed for the lower target (30,000) that could be due to a lower suppression effect of the matrix. The target mass (1400 m/z) was set in the middle of the selected scan range (600–2200 m/z) and the lens system was adjusted automatically for obtaining the maximum signal. The other ESI parameters were chosen according to the flow-rate used with the selected column (0.1 mL/min): dry temperature, 350 °C; nebulizer pressure, 40 psi; dry gas pressure, 8 psi.

3.2. Application of the optimized methodology to the profiling of maize protein fractions

In order to assign some peaks in the whole seed profile of maize cultivars, maize protein fractions were isolated following the Osborne method (see Section 2). The application of this method to the three Bt maizes and their corresponding isogenic non-transgenic cultivars enabled to obtain four different protein fractions (prolamin (zein), glutelin, globulin, and albumin) from every cultivar. All these fractions were analyzed by the developed method using UV and MS detection. As an example, Fig. 2 shows the UV and mass spectrometry profiles corresponding to these four fractions and to the whole seed in the case of the Aristis cultivar. Albumins are the less abundant maize proteins and their assignment was not possible in the whole seed profile using neither UV nor MS detection. All globulin fractions presented a small peak at 15–16 min that was also observed in the whole seed and in all maize cultivar studied (see peak 2 in Fig. 3). The confirmation of the identity of this peak in the maize cultivars was possible by comparison of its mass spectrum in the globulin fractions with those in the whole seeds. In fact, this peak in the globulin fractions showed spectra corresponding to molecules of 9044 Da (in the PR33P66/PR33P67 and Tietar/DKC6575) or 9016 Da (in the Aristis and Aristis Bt cultivars) that were identical to the observed in the whole seeds. Prolamins eluted from 12 to 30 min in the UV profile, however, early eluting peaks (retention times between 11 and 16 min) were only observed in UV profiles, while most of the last eluting peaks (from 18 min onwards) could be observed only in the mass profiles (prolamin fraction in Fig. 2). The comparison of chromatographic analysis with UV detection of prolamin fraction with those obtained from the whole seeds enabled to confirm that peaks with reten-

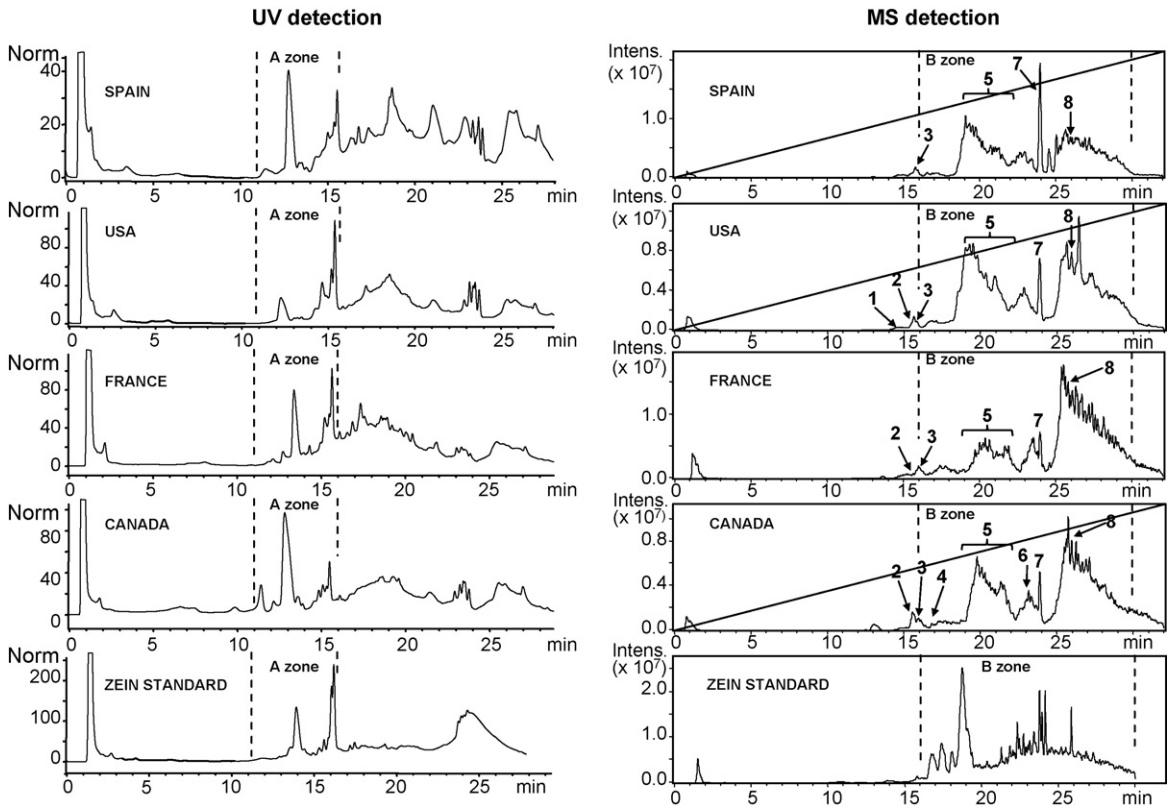

Fig. 3. UV and mass spectrometric profiles obtained for four maize cultivars (whole protein extracts) from Spain (EZ8LP), USA (A639LP), France (F212LP), and Canada (CM109LP) and for the zein standard using the optimized conditions. Peak descriptions are provided in the text.

tion times ranging from 11 to 16 min were prolamins. Moreover, glutelin fraction showed signals eluting from 18.0 to 30 min in both, the UV and mass profiles (glutelin fraction in Fig. 2). The signals appearing between 19.0 and 22.5 min in the whole seed (Fig. 2), corresponding to molecules of 17,520 and 24,815 Da were related with the glutelin fraction, since these mass spectra were also observed in glutelin fraction. Finally, signals in the chromatographic band between 26.5 and 30.0 min (whole seed in Fig. 2) were mainly assigned to glutelins since the analysis of glutelin fractions showed intense signals with the same retention times (glutelin fraction, Fig. 2) and identical mass spectra, associated with a molecule of 1570 Da. These signals and mass spectra were also observed in the analysis of all the cultivars under study (peak 8 in Fig. 3). Retention time and molecular weight of some characteristic peaks obtained in protein fractions are grouped in Table 1.

3.3. Application of the optimized methodology to the profiling of maize cultivars from different geographical origin

The optimized conditions were applied to the analysis of 10 different maize cultivars from USA, Canada, Spain, and France. As an example, Fig. 3 shows the chromatographic analysis with UV and MS detection of the zein standard and the protein fractions obtained from cultivars of different origins. All UV and MS chromatographic profiles showed similar number of peaks, but with different areas. Peaks ranging from 11 to 16 min (A zone, delimited by dotted lines in chromatograms of Fig. 3) were predominant when using UV detection. The comparison of these UV profiles with that corresponding to the zein standard enabled the probable identification of these peaks as zeins. Surprisingly, the peaks in this area of the chromatograms (A zone) were negligible when using MS detection, while the majority of detectable signals from 16 to 30 min (B

Table 1
Retention times and molecular weight of some characteristic peaks in protein fractions and maize cultivars analyzed.

Sample	Retention time (min)	Molecular weight (Da)	Cultivar
Globulin fraction	15.0–16.0	9016	Aristis, Aristis Bt
		9044	Tietar, DKC6575, PR33P66, PR33P67
Glutelin fraction	19.0–22.5	17520, 24815	All cultivars
	26.5–30.0	1570	All cultivars
Whole protein extract	12–12.7	10533	PR33P67
	14.9–15.3	1343	1507
	15.0–16.0	7182, 7152	USA cultivar
	15.5–16.0	4249	Aristis, Tietar
	16.0	8803	French F212LP
	16.0–16.3	5346	Aristis, Tietar
	17.0	8604	Canadian CM109LP
	23.0	28926	Canadian CM109LP

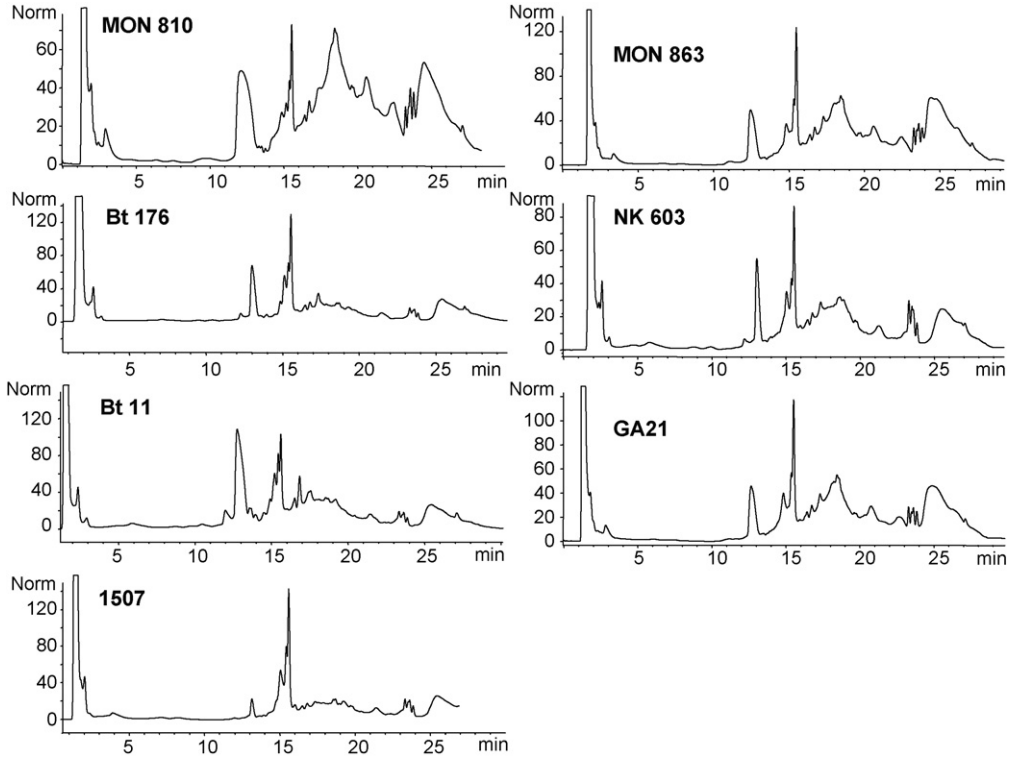

Fig. 4. Protein profiles obtained using UV detection at 280 nm for different transgenic maize cultivars (whole protein extracts): MON810 (50 g/kg), Bt-176 (20 g/kg), Bt-11 (48.9 g/kg), MON 863 (98.5 g/kg), NK 603 (49.1 g/kg), GA 21 (42.9 g/kg), 1507 (98.6 g/kg).

zone, delimited by dotted lines in chromatograms of Fig. 3), presented less intense signals in UV detection. This fact had already been observed for soybean and was attributed to the modification of the mobile phase composition during gradient elution [33]. In fact, electrospray production is highly affected by mobile phase composition being more favored with higher proportions of organic modifier than with water.

The study of mass spectra extracted from every peak enabled to observe certain common characters among cultivars. For instance, some similarities detected among the investigated cultivars included the detection of a signal at ~16.0 min (peak 3, Fig. 3) with mass spectra corresponding to a molecule of 9579 Da that was tentatively assigned to a minor zein [34,35]. Similarly, the detection of signals with retention times close to 24 and 26 min (peaks 7 and 8, Fig. 3), showed a unique mass spectra signal of 1040 m/z and a mass profile which corresponded to a molecule of 1570 Da, respectively. Moreover, a number of spectral signals, detected between 19.0 and 22.5 min (band 5, Fig. 3) were obtained in the analysis of all cultivars, presenting mass spectra corresponding to molecules of 17,520 and 24,815 Da that were identified as glutelin components [16,36]. Besides, the analyses of all cultivars, except the ones of the Spanish EZ7LP, French F212lP, American Va26LP and B84LP cultivars, showed signals between 15.0 and 16.0 min (peak 2, Fig. 3) with mass spectra related to a molecule of 9044 Da. On the other hand, the analysis of the latter American cultivars revealed the presence of a molecule of 9016 Da in peak 2. Contrary to the similarities found among the investigated maize cultivars, there were other distinctive signals that appeared only in those cultivars with the same origin. Thus, peak 2 (Fig. 3) with a spectrum associated with masses of 7182 or 7152 Da was only observed in all USA cultivars, except in A639LP and Mo17LP cultivars. Another difference was found in the analysis of the French F212LP cultivar, which showed mass spectra data corresponding to a molecule of 8803 Da with a retention time close to 16.00 min (peak 2, Fig. 3) while neither molecules of 9044 Da nor 9016 Da were detected in this cultivar. Canadian CM109LP cultivar yielded mass spectra corresponding to molecules of 8604 and 28926 Da at around 17.0 and 23.0 min, respectively (peaks 4 and 6, Fig. 3), which were not observed in any other cultivar. Table 1 groups the retention time and molecular weight of some characteristic peaks observed in maize cultivars from different origins.

3.4. Application of the optimized methodology to the profiling of transgenic maize cultivars

The developed methodology was also applied to the analysis of transgenic maize cultivars. Two different kinds of transgenic samples were studied: 17 standards with certified GMO content ranging from 0% to 10% and corresponding to seven different transgenic cultivars (Bt-11, Bt-176, MON 810, GA21, MON 863, NK 603, 1507) and three Bt maizes (100% of transgenicity) and their isogenic non-transgenic counterparts (PR33P67 vs. PR33P66, Aristis Bt vs. Aristis, DKC6575 vs. Tietar).

UV profiles corresponding to the seven standards with the highest GMO content are shown in Fig. 4. These profiles were, in general, similar to those shown in Fig. 3 being the most different one the corresponding to the transgenic cultivar 1507. The examination of mass spectra obtained for all these standards and their comparison with the signals corresponding to the blanks (samples with no transgenic content) showed that they were also very similar. Only in the 1507 maize cultivar, the transgenic variety presented an additional peak close to 15.0 min with a unique signal at 1343 m/z that did not appear in the non-transgenic one (see Table 1).

Regarding the three Bt maizes with 100% of transgenic content and their non-transgenic counterparts, UV profiles and base peak chromatograms observed (data not shown) were, from a qualitative point of view, very similar to the ones observed previously in Figs. 3 and 4. Nevertheless, the examination of the mass spectra obtained for every peak in every transgenic and its isogenic non-

transgenic cultivar did show certain differences. Thus, in the case Aristis vs. Aristis Bt and Tietar vs. DKC6575, the non-transgenic cultivars (Aristis and Tietar) presented signals corresponding to molecules of 4249 (15.5–16.0 min) and 5346 Da (16.0–16.3 min) that did not appear in the transgenic varieties (Aristis Bt and DKC6575) (see Table 1). In the pair PR33P66 vs. PR33P67, the transgenic variety (PR33P67) presented an additional peak whose signals corresponded to a molecule of 10,553 Da (12.0–12.7 min) that did not appear in the non-transgenic cultivar (PR33P66) (see Table 1). Neither of these molecules was observed in the reference materials.

4. Conclusions

This is the first time RP-HPLC–ESI-MS has been applied to the profiling of maize cultivars. The optimization of the experimental conditions involved the selection of a suitable separation column as well as the evaluation of the elution gradient, the ion-pairing reagent, and the mass spectrometry parameters. The optimized chromatographic parameters for a 1 mm ID C18 column were: flow-rate, 0.1 mL/min; elution gradient: 5–90%B in 30 min; mobile phase, water–acetonitrile–0.3% (v/v) acetic acid. The optimized mass spectrometry parameters were: capillary voltage, 5 kV (positive ionization mode); maximum number of accumulated ions in trap, 30,000. The methodology was applied to the characterization of maize cultivars from USA, Canada, France, and Spain observing that there were spectral signals that seemed to be characteristic of cultivars with a same geographical origin. The optimized methodology was of interest for the investigation of possible differences between different Bt maize cultivars (100% transgenic) and their isogenic non-transgenic counterparts (PR33P67 vs. PR33P66, Aristis Bt vs. Aristis, DKC6575 vs. Tietar). Mass spectra obtained from albumin, globulin, prolamin, and glutelin fractions isolated from different transgenic and non-transgenic maize cultivars enabled their assignment in the whole seed profile. In conclusion, in this work, the potential application of RP-HPLC–ESI-MS analysis for the profiling of maize cultivars and transgenic varieties is demonstrated. Using this methodology, some similarities and differences among cultivars have been observed. However, in order to determine if the differences have statistical significance, a larger number of samples should be analyzed.

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