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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