**Comparative metabolomic study of transgenic versus conventional
soybean using capillary electrophoresis-time-of-flight mass
spectrometry**

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Abstract

In this work, capillary electrophoresis - time-of-flight mass spectrometry (CE-TOF-MS) is proposed to identify and quantify the main metabolites found in transgenic soybean and its corresponding non-transgenic parental line both grown under identical conditions. The procedure includes optimization of metabolites extraction, separation by CE, on-line electrospray-TOF-MS analysis and data evaluation. A large number of extraction procedures and background electrolytes are tested in order to obtain a highly reproducible and sensitive analytical methodology. Using this approach, a large number of metabolites was tentatively identified based on the high mass accuracy provided by TOF-MS analyzer, together with the isotopic pattern and expected electrophoretic mobility of these compounds. In general, the same metabolites and in similar amounts were found in the conventional and transgenic variety. However, significant differences were also observed in some specific cases when the conventional variety was compared with its corresponding transgenic line. The selection of these metabolites as possible biomarkers of transgenic soybean is discussed, although a larger number of samples needs to be analyzed in order to validate this point. It is concluded that metabolomic procedures based on CE-MS can open new perspectives in the study of transgenic foods in order to corroborate (or not) the equivalence with their conventional counterparts.

Keywords: Soybean; Metabolites; Genetically modified organisms (GMOs); Capillary electrophoresis; Time of flight.

1. Introduction

Genetic engineering has been applied to plants since the mid-1980s and since then the use of genetically modified organisms (GMOs) has seen a great increase in agriculture and food science [1]. As an example, in 2005 more than 900.000 km² of GM crops were cultivated worldwide. GM plants are obtained by inserting manipulated fragments of DNA from a different organism in order to improve some characteristic of the original crop, such as its resistance to plagues, pesticides or extreme environmental conditions, to provide better nutritional properties, etc [2, 3].

The transgenic soybean variety tolerant to glyphosate is one of the most extended GM crops in the world. In 2005, 87% of U.S. soybean fields were planted with glyphosate resistant varieties [4]. Glyphosate binds to and blocks the activity of 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS), an enzyme of the aromatic amino acid biosynthetic pathway (shikimate pathway) [5]. The glyphosate inhibition of EPSPS prevents the plant from producing the aromatic amino acids (phenylalanine, tyrosine, tryptophan) essential for protein synthesis. Some microorganisms have a version of 5-enolpyruvoylshikimate-3-phosphate synthetase that is resistant to glyphosate inhibition. The version used in genetically modified crops was isolated from *Agrobacterium tumefaciens* strain CP4 (CP4 EPSPS) that was resistant to glyphosate [6, 7]. This CP4 EPSPS gene was cloned and transfected into soybeans, and in 1996, such genetically modified soybean was made commercially available. This greatly improved the ability to control weeds in soybean fields since glyphosate could be sprayed on fields without affecting the crop.

In the last decades, several aspects of GMOs have been criticized and scientific and public debate is open about their influence on the environment and their safety as food and feed. As a result, many countries have implemented regulations regarding the development, growing, and commercialization of these genetically modified products. The European Union (EU) has dedicated special attention to customer information, and food products containing more than 0.9% of genetically modified soybean and/or maize must be labelled as transgenic [8].

Therefore, the development of fast, sensitive and informative analytical methods is of paramount importance not only to fulfil the labelling requirements but also to evaluate other possible alterations in GM grains and foods [9]. In this regard, there are two general approaches for the detection of GMOs, based on the detection of two types of macromolecules specific for the genetic modification: proteins and DNA. The new or modified proteins contained in novel foods and ingredients are mainly detected using immunoassay methods (enzyme-linked immunosorbent assays (ELISA) methods). However, protein detection by immunoassay requires the use of antibodies raised against the protein encoded by the transgene [10,11]. Capillary electrophoresis (CE) [12, 13] and 2D electrophoresis, where mixtures of proteins are separated based on their isoelectric point and molecular weight, have also been used to study the different protein profiles between some transgenic and non transgenic crops [14-16].

Regarding the detection of GMOs based on DNA analysis, the most frequent procedure is to apply polymerase chain reaction (PCR) methods, where DNA fragments are amplified, followed by agarose gel electrophoresis plus ethidium bromide staining of the amplification products [17]. To avoid the restrictions in sensitivity and resolution of the slab gel electrophoresis, capillary gel electrophoresis (CGE) has been recently used as an attractive alternative providing very high efficiency, resolution and sensitivity [18,19]. DNA separations are performed in capillaries using polymer solutions where the electroosmotic flow (EOF) is completely suppressed [20-24]. Other

method like PCR and immunoassay kits (PCR-ELISA) has also been described in the field of GMO analysis [25,26]. One of the last challenges in GMO detection is the development of multiplex PCR approaches able to detect multiple GMOs in a single analysis [27,28]. The separation and detection of multiplex-PCR products for the detection of GMOs were achieved by agarose gel electrophoresis (AGE) [29] and other innovative procedure like, capillary electrophoresis, microchip capillary electrophoresis (MCE) [30], capillary gel electrophoresis with laser-induced fluorescent detection (CGE-LIF) [31,32], and DNA arrays or biosensors [33]. One of the main deficiencies of the procedures based on DNA are related to the semiquantitative character of the PCR amplification, recently overcome by the development of real-time PCR (RT-PCR) [34,35] and competitive quantitative PCR (QC-PCR) techniques using capillary gel electrophoresis [36,37].

An alternative procedure is to investigate the substantial equivalence of a GMO and its isogenic counterpart using profiling (or shotgun) procedures. In this regard, the comparison of the content of secondary metabolites has been proposed as an interesting approach to carry out this type of comparative studies. In the case of soybean, the studies done so far have been focused on the analysis of a given family of compounds following a more targeted approach. Thus, isoflavones, the main secondary metabolites in soybean, have been determined by HPLC [38] and LC/MS [39] and the results did not show significant differences between GM and non-GM soybeans. In other publication, the content of tocopherols, sterols, and phospholipids in oils obtained from GM soybeans and non-GM soybeans were determined by normal- and reversed-phase HPLC and GC observing some difference in the phospholipids fraction [40]. Several methods for identifying secondary metabolites of soybean, mainly isoflavones, have been published based on HPLC and capillary electrophoresis techniques with UV [41,42] and mass spectrometry detection [43,44], however, these works were not dealing with GM vs. non-GM comparisons.

The aim of this study was to develop a new analytical strategy based on comparative metabolomics able to determine differences between GM soybean and its isogenic wild variety both grown under the same conditions. The proposed method is based on the following steps: i) soybean metabolites extraction, ii) CE separation, iii) on-line ESI-TOF-MS analysis and iv) data evaluation.

2. Experimental

2.1. Chemicals and samples.

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogencarbonate from Fluka (Buchs, Switzerland) ammonium acetate from Panreac (Barcelona, Spain) and ammonium hydroxide from Merck (Darmstadt, Germany) were used for the CE running buffers at different concentrations and pH values. Buffers were prepared by weighting the quantity indicated in doubly distilled water and adding ammonium hydroxide to adjust the pH. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). Triethylamine (TEA) (Sigma, St. Louis, MO, USA) and 2-propanol of HPLC grade (Scharlau, Barcelona, Spain) were used in the sheath liquid. For the extraction of the soybean compounds, methanol, ethanol, hexane, and acetonitrile from Lab-Scan (Dublin, Ireland) and ethyl acetate from Fluka (Buchs, Switzerland) were used.

The soybean used for the optimisation was obtained from an herbalist's shop (Madrid, Spain). The isogenic and transgenic soybean seeds used for the comparative

metabolomic study were supplied by Professor Giovanni Dinelli (University of Bologna). Isogenic and transgenic plants were grown under the same conditions in a growth chamber: at the end of growing cycle the seeds were collected and finely ground as flour. The transgenic and nontransgenic nature of all these soybean samples was confirmed based on their DNA using an analytical procedure developed in our laboratory and described elsewhere [21, 23, 24, 32, 36].

2.2. Extraction procedures.

To extract the major number of compounds from the soybean seeds different organic solvents together with different water percentages were tested: methanol (60, 80, 90 and 100% v/v), ethanol (60, 80, 90 and 100% v/v), ethyl acetate (100% v/v) and acetonitrile (70 and 100% v/v). All the extracts profiles were compared using the same initial CE-UV conditions: running buffer 100 mM ammonium hydrogencarbonate at pH 9 + 5% v/v acetonitrile, voltage of 28 kV, 5 s injection time, and detection wavelength at 200 nm. With these initial conditions the electric current was around 80 μ A.

The extraction protocol was as follows: soybean seeds were finely grounded at 5°C using a mill and maintained in the fridge at 4°C. After, 1 g of the milled samples was mixed with 15 mL of the different solvents and extracted during 30 min in the ultrasonic bath. The extracts were then centrifuged at 5750 g for 15 min at 5°C. The supernatants were separated in three aliquots of 4 mL and evaporated in a concentrator. The residue was dissolved in 500 μ L of the solvent and analysed by CE-UV. It is important to keep the temperature below 20°C because it is known that this temperature is the most suitable for extracting the compounds (specially isoflavones) from seeds with little or no modifications of its composition [42].

An alternative extraction procedure was tested removing the fat (mainly phospholipids) with hexane previously to the extraction. Thus, once the soybean was milled, 1 g was extracted with 10 ml of hexane during 15 min in the ultrasonic bath. The extracts were centrifuged at 5750 g for 10 min at 5 °C, the supernatant was eliminated and the solid was extracted with the solvent as described above. The use of hexane did not improve the results of the extraction.

2.3. CE-UV and CE-MS.

Analyses were performed in a PACE/2100 apparatus (Beckman, Fullerton, CA, USA) equipped with a UV-vis detector working at 200 nm and coupled to a Bruker Daltonik micrOTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray ionization (ESI) interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The CE instrument was controlled by a personal computer running the System Gold Software from Beckman. Bare fused-silica capillaries with 50 μ m i.d. and 375 μ m o.d. were purchased from Composite Metal Services (Worcester, England). The detection length to the UV detector was 84 cm and the total length was 90 cm (corresponding to the MS detection length). Injections were made at the anodic end using N₂ at pressure of 3570 Pa and the times indicated in each case.

Electrical contact at the electrospray needle tip was established via a sheath liquid pumped by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, IL, USA). The ESI-voltage of the micrOTOF is applied at the end cap of the transfer capillary to the MS (-4.2 kV) with the spray needle being grounded. The mass spectrometer was run in the negative mode. The micrOTOF was operated to acquire spectra in the range of

50-1000 m/z. Transfer parameters were optimised for high sensitivity while keeping the resolution to better 10000. The accurate mass data of the molecular ions were processed by DataAnalysis 3.3. software (Bruker Daltonik). It provides a list of possible elemental formulae by means of the Generate Molecular Formula editor (GMF), which uses a CHNO algorithm. This provides information about elemental composition, sigma and m/z values. The calibration of the MS was performed using sodium formate.

All new capillaries were conditioned before their first use by rising for 30 min with 0.1 M sodium hydroxide followed by water for 10 min and then running buffer for 20 min. Capillary conditioning between runs was carried out by flushing for 10 min with 0.1 M sodium hydroxide, then for 5 min with water and finally for 10 min with running buffer. At the end of the day the capillary was rinsed with water for 15 min and dried with air for 5 min.

3. Results and discussion

3.1. Optimization of metabolites extraction from soybean samples.

The possibility to obtain a good extraction procedure of metabolites from soybean was deeply explored. To do this, the different extraction procedures above described were investigated in order to determine the extraction conditions that led to a higher number of peaks in a reproducible way, assuming this would mean a higher number of metabolites extracted. CE-UV was initially employed to monitorize at 200 nm the results of the extraction. The soybean bought in a local market was selected for the extraction optimization. As the aim of this part was to extract the highest number of possible metabolites, extraction solutions able to cover a wide range of polarities were frequently used, including ethanol:water and methanol:water mixtures at different concentrations. The results obtained using the different extraction solvents are given in Fig 1. The best extraction procedures were obtained with methanol and ethanol with different percentages of water. The intensity of some peaks decreased at both higher (90% v/v) and smaller (60% v/v) percentages of organic solvent. Finally, the selected extract solvent consist of methanol/water (80/20, v/v) since it provided the highest number of metabolites extracted with high intensity as can be seen in Fig. 1(iii).

3.2. CE-UV analysis of soybean metabolites.

The objective in this part of the work was to obtain a BGE compatible with the subsequent ESI-MS analysis and able to provide fast separation with high resolution and sensitivity. The effect of different separation parameters on resolution, sensitivity, analysis time, and peak shape was studied using a methanol/water (80/20, v/v) extract from commercial soybean. Preliminary experiments were carried out using CE with UV detection in order to find a suitable background electrolyte (BGE) compatible with CE-ESI-MS [45,46]. For this reason, in this work only volatile BGEs were tested at different pH values and ionic strengths. Initially, the conditions tested were type, concentration and pH of buffer. First, ammonium hydrogencarbonate and ammonium acetate at 100 mM and pH 10 were tested. In general the profiles obtained using carbonate-containing BGEs were better than the obtained with acetate. Finally ammonium hydrogencarbonate was selected because of the short analysis time provided by this BGE (data not shown).

Next, the effect of BGEs at different pH values (8, 9 and 10) adjusted by adding ammonium hydroxide was studied. A pH value of 9 was selected as optimum in term of

efficiency, resolution and analysis speed. The effect of the BGE ionic strength was next studied testing concentrations of 25, 50 and 100 mM. The best results were obtained with 50 and 100 mM of ammonium hydrogencarbonate at pH 9 observing slightly better resolution with 100 mM while, as expected, the analysis time was shorter with 50 mM. To improve the resolution between peaks, different percentages of organic solvent (acetonitrile, ACN) were added to the two buffers (50 and 100 mM ammonium hydrogencarbonate). The best results in terms of peak shape and resolution were obtained with the following 3 BGEs: 50 mM ammonium hydrogencarbonate at pH 9, 50 mM ammonium hydrogencarbonate at pH 9 + 20% v/v ACN, and 100 mM ammonium hydrogencarbonate at pH 9. As no significant differences were found in CE-UV between these three buffers, they were selected for a posterior study by CE-ESI-TOF-MS choosing then the most appropriate BGE.

Based on these conditions different voltages: 20, 25, 28 and 30 kV were applied and we found that in general a voltage of 28 kV shortened the analysis time and also gave good resolution and acceptable electrical current values (around 35 μ A with the 50 mM ammonium hydrogencarbonate buffer, 28 μ A with 50 mM ammonium hydrogencarbonate + 20% v/v ACN and 65 μ A with 100 mM ammonium hydrogencarbonate). The injection were made at the anodic end using N₂ pressure of 3570 Pa for 3, 5, 10 and 15 s, selecting 10 s as optimum injection in terms of sensitivity, resolution and stability of the separation.

The repeatability of the optimised CE-UV method, using 50 mM ammonium hydrogencarbonate at pH 9 as BGE, was then evaluated by carrying out three replicate determinations with the same sample and with three different extracts and was expressed by the relative standard deviation (RSD) for the migration time and for the peak area. The RSDs of analysis time and peak area were determined for three of the peaks present in the extracts. The results can be observed in Table 1 showing that the procedure is reproducible.

3.3. Optimization of ESI-MS parameters

In order to select the BGE most compatible with CE-ESI-TOF-MS the three mentioned buffers were tested using as sheath liquid isopropanol/water (50/50, v/v) + 0.1% v/v ammonium hydroxide (Fig. 2). The best results in terms of sensitivity were obtained with 50 mM of ammonium hydrogencarbonate (Figure 2(i)). Therefore, these conditions were chosen for the subsequent ESI optimization.

Sheath liquid composition, sheath liquid flow rate, nebulizer pressure, dry gas flow rate and ESI chamber temperature were then optimized, selecting the MS intensity of several peaks as optimization criterion. The choice of these variables represented a compromise between maintaining efficient and well-resolved electrophoretic separation and improving ionisation performance. Sheath-liquid isopropanol/water (50/50) was tested together with different quantities (0.1 and 0.5% v/v) of TEA and ammonium hydroxide trying to improve the ionisation yield. The highest MS signal was achieved using a sheath-liquid containing 0.1% v/v ammonium hydroxide. Therefore, the sheath liquid selected was composed of water-2-propanol (50/50, v/v) and 0.1% v/v ammonium hydroxide. As optimum sheath liquid flow a value of 0.24 ml/h was selected because lower flows reduced the ionization yield due to the instability of the spray, while at higher flows dilution of the electrophoretic bands emerging from the capillary was too high and the intensity of the MS signal for these compounds was reduced. The other ESI parameters were chosen according to the sheath liquid flow of 0.24 ml/h and

the most suitable ones were: nebulizer pressure of 0.4 bar, dry gas flow equal to 4 l/min and dry gas temperature 180°C.

In summary, the best results were obtained by using the following CE-ESI-MS conditions: the running buffer was 50 mM ammonium hydrogencarbonate at pH 9, voltage 28 kV and 10 s of hydrodynamic injection at 3570 Pa. The sheath liquid consisted of water-2-propanol (50/50, v/v) with 0.1% v/v ammonium hydroxide pumped at 0.24 mL/h, used together with a nebulizer gas pressure at 0.4 bar and a dry gas flow rate of 4 l/min at 180°C. With these optimum conditions the electric current was around 35 μ A.

Fig. 3 shows the CE-MS base peak electropherogram of an extract of commercial soybean as well as the extracted ion electropherograms (in migration order) obtained under optimum CE-ESI-TOF-MS conditions. As can be seen, under these conditions a large number of metabolites could be detected.

3.4. Identification of compounds

The identification of the majority of metabolites can be performed by a careful interpretation of the mass spectra combined with the aid of their electrophoretic mobility. The assignment was later confirmed by soybean composition and metabolic pathways found in literature. The compounds tentatively identified are summarized in Table 2, including experimental m/z values, fragments detected, the error and sigma value (comparison of the theoretical with the measured isotope pattern) a list of possible compounds and references about these including soybean pathways.

A reduced number of possible elemental compositions are obtained from the accurate mass of the suspected peak combined with the correct determination of the isotopic pattern applying ESI-TOF-MS analyzer. These elemental compositions can then be matched against available databases using the deduced molecular formula as a search criterion [47,48].

Additional proves that corroborate the adequacy of the compounds given in Table 2 can be found from the electrophoretic mobility of these compounds. The total migration time for highly charged small molecules is longer than that for molecules of smaller charge and greater size [49].

The first group of peaks migrated very close to the EOF and most of them were identified as aminoacids (essential aminoacids present in soybean and others produced via secondary metabolism [50]) as e.g., proline, 4-hydroxy-L-threonine, leucine, tyrosine, asparagine. They have a carboxylic group that at pH 9 will be fully ionized and also have a primary amine susceptible to bear positive charge at that pH, giving rise to a net charge near to zero that explains this behaviour.

The last migrating peaks (as e.g., glutamic acid, aspartic acid) correspond to compounds with carboxylic groups that are totally ionized at the separation pH 9, providing to the molecules the highest negative electrical charge/size ratio, the lowest apparent electrophoretic mobility and therefore high migration time.

Major isoflavones previously observed in several studies [41, 42, 44] were also detected in the present work. Soybean was revealed to be more abundant in malonyl derivatives of genistin and daidzin, followed by genistin, daidzin and their aglicones (genistein and daidzein). In spite of their abundance in soybean seeds, malonyl forms are thermally unstable and are easily converted into their corresponding glycosides forms.

Interestingly, the migration order of the isoflavones, previously studied in literature [41, 42], is also in good agreement with our results. Thus, the glycosides

(daidzin and genistin) were detected earlier because of their higher molecular weights; then the malonyl derivatives migrated due to increased electrophoretic mobility with the introduction of a negative charge (malonate) to the sugar that overcompensate the increase in molecular mass. The smaller aglycone molecules with higher mobility toward the anode and against the EOF were detected latest. In all cases daidzein was detected before genistein because of the extra hydroxyl group of genistein [51].

The glycosides and their malonyl derivatives also contain the [M-H] ions corresponding to their aglicones forms produced in the electrospray (ESI-TOF) so, we can observe in the extracted ion electropherograms (EIEs) of daidzein (253,049) and genistein (269,044) three peaks: first two correspond to the fragments of the glycosides and malonyl derivatives and the last one is the aglycon.

Although these isoflavones are the predominant flavonoids in soybean seeds, other flavonoids have been tentatively identified in this work since flavonoids are one of the largest and most widespread groups of plant secondary metabolites [52]. The first two phenolic compounds tentatively identified as dihydro tetramethoxyflavone triglucoside and medicarpin 3-O-glucoside showed peaks migrating near the EOF, in good agreement with its low ionizable character. Daidzin and genistin migrated later than the EOF due to a partial ionization of the free -OH groups at the basic running buffer. Other flavonoids identified were: taxifolin 3- ramoside, naringenin 7- glucoside, 6-methoxytaxifolin and formononetin.

Besides it is very common to find in plant extracts flavonoid glycosides acylated with acids such as malonic, acetic, coumaric etc [53,54] as e.g., liquiritigenin 6- coumaroylglucoside, tentatively identified in soybean extract.

Small traces of other flavonoids in soybean extract were tentatively identified as: Kushenol M, Sophora-iso-flavanone D, Exiguaflavanone and Kushenol B that contain prenyl, geranyl, lavandulyl and similar groups. These results are in good agreement with the data found in the literature about plants belonging to the family of Fabacea [55,56] in which some isoflavones with prenyl groups or further O-heterocyclic rings were detected. Nevertheless, the high sigma and error values found for these compounds have to be also considered (see Table 2).

It was also possible to study other compounds present in this fraction of the soybean. Among them we found compounds from the linoleic acid metabolism as e.g., trihydroxyoctadec-11-enoic and epoxyoctadecenoic acid, other belonging to arachidonic acid metabolism as e.g. 2, 3- Dinor-8-iso prostaglandin F1-alpha and dipeptides as γ -L-glutamyl-L-tyrosine and γ -L-glutamyl-L-phenylalanine [57]. These and other compounds are listed in Table 2.

3.5. Comparison between conventional and transgenic soybean

A comparison between the metabolomic profile obtained with the transgenic soybean and its isogenic wild variety was next carried. As can be seen in Table 3, comparing the two samples (conventional and GM soybean), certain compounds were detected in both soybeans, although there were interesting differences in the intensity of their signals. Thus, the GM soybean produced higher amounts of some metabolites (as e.g., liquiritigenin 6-coumaroylglucoside, naringenin 7-O-glucoside and 6-methoxytaxifolin), while for other metabolites (as e.g., proline, histidine, asparagine, gluconic acid, and trihydroxypentanoic acid) the conventional soybean produced higher amounts. However, the main qualitative difference between GM and wild soybeans was found in the compound 6 tentatively identified as 4-hydroxy-L-threonine (m/z 134). Fig. 4 shows the comparison of the extracted ion electropherograms (EIEs) of some analytes

identified by CE-ESI-TOF-MS in the transgenic and conventional soybeans. Comparing the results for the two samples, we could not find 4-hydroxy-L-threonine in the electropherogram obtained with transgenic soybean. We have represented this compound with an asterisk and have compared it with other peaks that as can be seen are unchanged what eliminates any possible analytical artifact. Before this compound can be assigned as a possible indicator of this genetic modification, a large number of samples should be analyzed.

On the basis of obtained data, some biochemical considerations can be drawn. By comparing conventional and GM metabolite profiles a different expression of three free amino acids (i.e. proline, histidine and asparagine) and of one amino acid derivative (i.e. 4-hydroxy-L-threonine) was observed. Although proline, asparagine and threonine are biosynthesized in different anabolic pathways (glutamine-proline, alanine-asparagine and threonine-methionine pathways, respectively), they are interconnected sharing a common precursor. The common precursor of proline and asparagine is the glutamic acid, while the precursor of threonine is the aspartic acid [5]. It is to underline that glyphosate resistant soybean expresses both endogenous and transgenic EPSPS [6,7]. As a consequence considering that EPSPS is a key enzyme of shikimate pathway, it is plausible that the synthesis of aromatic amino acids (phenylalanine, tyrosine, tryptophan) may be differently regulated in GM soybean and corresponding isogenic line. The different allosteric properties of eukaryote (endogenous soybean enzyme) and prokaryote (*A. tumefaciens* enzyme) EPSPS may be the basis of different biosynthetic regulatory systems. The presumable different regulation of shikimate pathways can explain the higher relative content of liquiritigenin, naringenin and taxifolin derivatives observed in GM soybean. In fact, these three compounds share common precursors: the aromatic amino acids phenylalanine and tyrosine for the flavanones naringenin and liquiritigenin, and naringenin for the majority of flavonoids (including the flavonol taxifolin) [58]. Further studies are in progress in order to confirm at transcriptional level the observed metabolomic differences between GM and conventional soybean.

4. Conclusions

In the present work, a complete analytical method (including an extraction protocol, CE-ESI-TOF-MS analysis and data evaluation) has been developed to comparatively study the metabolic profile of conventional and GM soybean. This method allows the tentative identification of more than forty compounds, including, isoflavones, aminoacids, carboxylic acids, peptides and other analytes. The results show that some of the detected metabolites do not change, while other show significant quantitative differences in their intensities in the conventional and GM soybean. A compound tentatively identified as 4-hydroxy-L-threonine seems to disappear in the transgenic soybean compared to its parental non-transgenic line.

Acknowledgements

This work was supported by Projects AGL2005-05320-C02-01 and CONSOLIDER INGENIO 2010 CSD2007-00063 FUN-C-FOOD (Ministerio de Educación y Ciencia), S-505/AGR-0153 (ALIBIRD, Comunidad de Madrid) and HA2006-0057 (Ministerio de Educación y Ciencia).

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Figure legends

Fig. 1. CE-UV electropherograms of soybean extracts obtained using the following solvents: (i) methanol; (ii) methanol/water, 90/10; (iii) methanol/water, 80/20; (iv) methanol/water, 60/40; (v) ethanol, (vi) ethanol/water, 90/10; (vii) ethanol/water, 80/20; (viii) ethanol/water, 60/40; (ix) acetonitrile; (x) acetonitrile/water, 70/30; (xi) ethyl acetate. All the solvents were compared under the same initial conditions: fused-silica capillary with 50 μm i.d., 375 μm o.d., 90 cm total length, running buffer 100 mM ammonium hydrogencarbonate at pH 9 + 5% v/v acetonitrile, voltage: 28 kV, 5 s injection time, electrical current 80 μA . Detection wavelength: 200 nm.

Fig. 2. CE-TOF-MS electropherograms using as BGE: (i) 50mM ammonium hydrogencarbonate, (ii) 50mM ammonium hydrogencarbonate +20% v/v ACN, (iii) 100mM ammonium hydrogencarbonate. The rest of CE-ESI-MS conditions were: voltage: 28 kV; injection time: 10 s; sheath liquid: 2-propanol/water 50:50 (v/v) containing 0.1% v/v ammonium hydroxide, flow rate 0.24 mL/h, drying gas: 4 l/min at 180°C, nebulising gas pressure: 4 bar. MS analyses were carried out using negative polarity. MS scan 50-1000 m/z.

Fig. 3. CE-TOF-MS Base peak electropherogram (BPE) of commercial soybean, using the optimal conditions and CE-TOF-MS extracted ion electropherograms (EIEs) of the detected compounds. CE-MS conditions: Buffer: 50 mM ammonium hydrogencarbonate at pH 9. Voltage: 28 kV. Injection time: 10 s. Sheath liquid: 2-propanol/water 50:50 (v/v) containing 0.1% v/v ammonium hydroxide, flow rate 0.24 mL/h. Drying gas: 4 l/min at 180°C. Nebulising gas pressure: 4 bar. Electrical current 35 μA . MS analyses were carried out using negative polarity. MS scan 50-1000 m/z.

Fig. 4. Comparison of the CE-TOF-MS extracted ions electropherograms (EIEs) of some metabolites found in conventional and transgenic soybean. * compound that changes; 1, 2, 11, 12, 17 unmodified compounds. Peak numbers correspond to the compounds identified in Table 2. All the conditions as in Fig. 3.