1 **Comparative metabolomic study of transgenic versus conventional**

2 **soybean using capillary electrophoresis-time-of-flight mass**

3 **spectrometry**

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17

18   **Abstract**
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20   In this work, capillary electrophoresis - time-of-flight mass spectrometry (CE-
21   TOF-MS) is proposed to identify and quantify the main metabolites found in transgenic
22   soybean and its corresponding non-transgenic parental line both grown under identical
23   conditions. The procedure includes optimization of metabolites extraction, separation by
24   CE, on-line electrospray-TOF-MS analysis and data evaluation. A large number of
25   extraction procedures and background electrolytes are tested in order to obtain a highly
26   reproducible and sensitive analytical methodology. Using this approach, a large number
27   of metabolites was tentatively identified based on the high mass accuracy provided by
28   TOF-MS analyzer, together with the isotopic pattern and expected electrophoretic
29   mobility of these compounds. In general, the same metabolites and in similar amounts
30   were found in the conventional and transgenic variety. However, significant differences
31   were also observed in some especific cases when the conventional variety was
32   compared with its corresponding transgenic line. The selection of these metabolites as
33   possible biomarkers of transgenic soybean is discussed, although a larger number of
34   samples needs to be analyzed in order to validate this point. It is concluded that
35   metabolomic procedures based on CE-MS can open new perspectives in the study of
36   transgenic foods in order to corroborate (or not) the equivalence with their conventional
37   counterparts.
38
39   Keywords: Soybean; Metabolites; Genetically modified organisms (GMOs); Capillary
40   electrophoresis; Time of flight.
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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 km^2 of GM crops were cultivated worldwide. GM plants are obtained by inserting manipuled 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 familiy 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 μA.

The extraction protocol was as follows: soybean seeds were finely grounded at 5ºC using a mill and maintained in the fridge at 4ºC. 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 5ºC. The supernatants were separated in three aliquots of 4 mL and evaporated in a concentrator. The residue was dissolved in 500 μL of the solvent and analysed by CE-UV. It is important to keep the temperature below 20ºC 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 ºC, 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 μm i.d. and 375 μm 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 N_2 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

192 50-1000 m/z. Transfer parameters were optimised for high sensitivity while keeping the
193 resolution to better 10000. The accurate mass data of the molecular ions were processed
194 by DataAnalysis 3.3. software (Bruker Daltonik). It provides a list of possible elemental
195 formulae by means of the Generate Molecular Formula editor (GMF), which uses a
196 CHNO algorithm. This provides information about elemental composition, sigma and
197 m/z values. The calibration of the MS was performed using sodium formiate.

198 All new capillaries were conditioned before their first use by rising for 30 min
199 with 0.1 M sodium hydroxide followed by water for 10 min and then running buffer for
200 20 min. Capillary conditioning between runs was carried out by flushing for 10 min
201 with 0.1 M sodium hydroxide, then for 5 min with water and finally for 10 min with
202 running buffer. At the end of the day the capillary was rinsed with water for 15 min and
203 dried with air for 5 min.

204
205 **3. Results and discussion**

206
207 *3.1. Optimization of metabolites extraction from soybean samples.*

208
209 The possibility to obtain a good extraction procedure of metabolites from
210 soybean was deeply explored. To do this, the different extraction procedures above
211 described were investigated in order to determine the extraction conditions that led to a
212 higher number of peaks in a reproducible way, assuming this would mean a higher
213 number of metabolites extracted. CE-UV was initially employed to monitorize at 200
214 nm the results of the extraction. The soybean bought in a local market was selected for
215 the extraction optimization. As the aim of this part was to extract the highest number of
216 possible metabolites, extraction solutions able to cover a wide range of polarities were
217 frequently used, including ethanol:water and methanol:water mixtures at different
218 concentrations. The results obtained using the different extraction solvents are given in
219 Fig 1. The best extraction procedures were obtained with methanol and ethanol with
220 different percentages of water. The intensity of some peaks decreased at both higher
221 (90% v/v) and smaller (60% v/v) percentages of organic solvent. Finally, the selected
222 extract solvent consist of methanol/water (80/20, v/v) since it provided the highest
223 number of metabolites extracted with high intensity as can be seen in Fig. 1(iii).

224
225 *3.2. CE-UV analysis of soybean metabolites.*

226
227 The objective in this part of the work was to obtain a BGE compatible with the
228 subsequent ESI-MS analysis and able to provide fast separation with high resolution
229 and sensitivity. The effect of different separation parameters on resolution, sensitivity,
230 analysis time, and peak shape was studied using a methanol/water (80/20, v/v) extract
231 from commercial soybean. Preliminary experiments were carried out using CE with UV
232 detection in order to find a suitable background electrolyte (BGE) compatible with CE-
233 ESI-MS [45,46]. For this reason, in this work only volatile BGEs were tested at
234 different pH values and ionic strengths. Initially, the conditions tested were type,
235 concentration and pH of buffer. First, ammonium hydrogencarbonate and ammonium
236 acetate at 100 mM and pH 10 were tested. In general the profiles obtained using
237 carbonate-containing BGEs were better than the obtained with acetate. Finally
238 ammonium hydrogencarbonate was selected because of the short analysis time provided
239 by this BGE (data not shown).

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

242 efficiency, resolution and analysis speed. The effect of the BGE ionic strength was next
243 studied testing concentrations of 25, 50 and 100 mM. The best results were obtained
244 with 50 and 100 mM of ammonium hydrogencarbonate at pH 9 observing slightly better
245 resolution with 100 mM while, as expected, the analysis time was shorter with 50 mM.
246 To improve the resolution between peaks, different percentages of organic solvent
247 (acetonitrile, ACN) were added to the two buffers (50 and 100 mM ammonium
248 hydrogencarbonate). The best results in terms of peak shape and resolution were
249 obtained with the following 3 BGEs: 50 mM ammonium hydrogencarbonate at pH 9, 50
250 mM ammonium hydrogencarbonate at pH 9 + 20% v/v ACN, and 100 mM ammonium
251 hydrogencarbonate at pH 9. As no significant differences were found in CE-UV
252 between these three buffers, they were selected for a posterior study by CE-ESI-TOF-
253 MS choosing then the most appropiate BGE.
254   Based on these conditions different voltages: 20, 25, 28 and 30 kV were applied
255 and we found that in general a voltage of 28 kV shortened the analysis time and also
256 gave good resolution and acceptable electrical current values (around 35 μA with the 50
257 mM ammonium hydrogencarbonate buffer, 28 μA with 50 mM ammonium
258 hydrogencarbonate + 20% v/v ACN and 65 μA with 100 mM ammonium
259 hydrogencarbonate). The injection were made at the anodic end using N_2 pressure of
260 3570 Pa for 3, 5, 10 and 15 s, selecting 10 s as optimum injection in terms of sensitivity,
261 resolution and stability of the separation.
262   The repeatability of the optimised CE-UV method, using 50 mM ammonium
263 hydrogencarbonate at pH 9 as BGE, was then evaluated by carrying out three replicate
264 determinations with the same sample and with three different extracts and was
265 expressed by the relative standard deviation (RSD) for the migration time and for the
266 peak area. The RSDs of analysis time and peak area were determined for three of the
267 peaks present in the extracts. The results can be observed in Table 1 showing that the
268 procedure is reproducible.
269
270 *3.3. Optimization of ESI-MS parameters*
271
272   In order to select the BGE most compatible with CE-ESI-TOF-MS the three
273 mentioned buffers were tested using as sheath liquid isopropanol/water (50/50, v/v) +
274 0.1% v/v ammonium hydroxide (Fig. 2). The best results in terms of sensitivity were
275 obtained with 50 mM of ammonium hydrogencarbonate (Figure 2(i)). Therefore, these
276 conditions were chosen for the subsequent ESI optimization.
277   Sheath liquid composition, sheath liquid flow rate, nebulizer pressure, dry gas
278 flow rate and ESI chamber temperature were then optimized, selecting the MS intensity
279 of several peaks as optimization criterion. The choice of these variables represented a
280 compromise between maintaining efficient and well-resolved electrophoretic separation
281 and improving ionisation performance. Sheath-liquid isopropanol/water (50/50) was
282 tested together with different quantities (0.1 and 0.5% v/v) of TEA and ammonium
283 hydroxide trying to improve the ionisation yield. The highest MS signal was achieved
284 using a sheath-liquid containing 0.1% v/v ammonium hydroxide. Therefore, the sheat
285 liquid selected was composed of water-2-propanol (50/50, v/v) and 0.1% v/v
286 ammonium hydroxide. As optimum sheath liquid flow a value of 0.24 ml/h was selected
287 because lower flows reduced the ionization yield due to the instability of the spray,
288 while at higher flows dilution of the electrophoretic bands emerging from the capillary
289 was too high and the intensity of the MS signal for these compounds was reduced. The
290 other ESI parameters were chosen according to the sheath liquid flow of 0.24 ml/h and

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

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

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

304
305   *3.4. Identification of compounds*
306

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

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

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

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

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

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

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

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

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

352   Although these isoflavones are the predominant flavonoids in soybean seeds,
353 other flavonoids have been tentatively identified in this work since flavonoids are one of
354 the largest and most widespread groups of plant secondary metabolites [52]. The first
355 two phenolic compounds tentatively identified as dihydro tetramethoxyflavone
356 triglucoside and medicarpin 3-O-glucoside showed peaks migrating near the EOF, in
357 good agreement with its low ionizable character. Daidzin and genistin migrated later
358 than the EOF due to a partial ionization of the free –OH groups at the basic running
359 buffer. Other flavonoids identified were: taxifolin 3- ramnoside, naringenin
360 7- glucoside, 6-methoxytaxifolin and formononetin.

361   Besides it is very common to find in plant extracts flavonoid glycosided acylated
362 with acids such as malonic, acetic, coumaric etc [53,54] as e.g., liquiritigenin
363 6- coumarylglucoside, tentatively identified in soybean extract.

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

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

377
378 *3.5. Comparison between conventional and transgenic soybean*
379
380   A comparison between the metabolomic profile obtained with the transgenic
381 soybean and its isogenic wild variety was next carried. As can be seen in Table 3,
382 comparing the two samples (conventional and GM soybean), certain compounds were
383 detected in both soybeans, although there were interesting differences in the intensity of
384 their signals. Thus, the GM soybean produced higher amounts of some metabolites (as
385 e.g., liquiritigenin 6-coumaroylglucoside, naringenin 7-O-glucoside and 6-
386 methoxytaxifolin), while for other metabolites (as e.g., proline, histidine, asparagine,
387 gluconic acid, and trihydroxypentanoic acid) the conventional soybean produced higher
388 amounts. However, the main qualitative difference between GM and wild soybeans was
389 found in the compound 6 tentatively identified as 4-hydroxy-L-threonine (m/z 134). Fig.
390 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 narigenin 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.

441 **References**

[1] M. Qaim, D. Zilberman, Science 299 (2003) 900.
[2] D. Rosellini, F. Veronesi, J. Phys. Condens. Matter 19 (2007) 1.
[3] A.G. Gao, S.M. Hakimi, C.A. Mittanck, Y. Wu, B.M. Woerner, D.M. Stark, D.M. Sha, J. Liang, C.M.T. Rommens, Nat. Biotechnol. 18 (2000) 1307.
[4] M. Johanns, S.D. Wiyatt (Editors), National Agriculture Statistics Service in Acreage, US Department of Agriculture, Washington, DC, 2005, p.6.
[5] D.L. Nelson, M.M. Cox, Lehninger Principles of Biochemistry, Worth Publishers, New York, 3rd ed., 2000.
[6] G.R. Heck, C.L. Armstrong, J.D. Astwood, C.F. Behr, J.T. Bookout, S.M. Brown, T.A., Cavato, D.L. DeBoer, M.Y., Deng, C. George, J.R. Hillyard, C.M. Hironaka, A.R. Howe, E.H. Jakse, B.E. Ledesma, T.C. Lee, R.P. Lirette, M.L. Mangano, J.N. Mutz, Y. Qi, R.E. Rodriguez, S.R. Sidhu, A. Silvanovich, M.A. Stoecker, R.A. Yingling, J. You, Crop. Sci. 45 (2005) 329.
[7] T. Funke, H. Han, M.L. Healy-Fried, M. Fischer, E. Schonbrunn, Proc. Natl. Acad. Sci. USA. 103 (2006) 13010.
[8] European Commission (EC) Regulation No. 641/2004, Off. J. Eur. Union L102 (6 April 2004) 14-25.
[9] E. Anklam, F. Gadani, P. Heinze, H. Pijnenburg, G. Van Den Eede, Eur. Food Res. Technol. 214 (2002) 3.
[10] J.W. Stave, Food Control 10 (1999) 367.
[11] M. Lipp, E. Anklam, J.W. Stave, J. AOAC Int. 83 (2000) 919.
[12] A. Cifuentes, Electrophoresis 27 (2006) 283.
[13] C. García- Ruiz, M.C. García, A. Cifuentes, M.L: Marina, Electrophoresis 28 (2007) 2314.
[14] G.L. Erny, M.L. Marina, A. Cifuentes, Electrophoresis 28 (2007) 4192.
[15] M.C. Ruebelt, M. Lipp, T.L. Reynolds, J.J. Schmuke, J.D. Astwood, D. DellaPenna, K.H. Engel, K.D. Jany, J. Agric. Food Chem. 54 (2006) 2169.
[16] M.C. Ruebelt, M. Lipp, T.L. Reynolds, J.D. Astwood, K.H. Engel, K.D. Jany, J. Agric. Food Chem. 54 (2006) 2162.
[17] E. Gachet, G.G. Martin, F. Vigneau, G. Meyer, Trends Food Sci. Technol. 9 (1999) 380.
[18] V. García-Cañas, A. Cifuentes, R. González, Crit. Rev. Food Sci. 44 (2004) 425.
[19] V. García-Cañas, R. González, A. Cifuentes, Trends Anal. Chem. 23 (2004) 637.
[20] C. Giovannoli, L. Anfossi, C. Tozzi, G. Giraudi, A. Vanni, J. Sep. Sci. 27 (2004) 1551.
[21] V. García-Cañas, R. Gónzalez, A. Cifuentes, J. Agri. Food Chem. 50 (2002) 4497.
[22] L. Sánchez, R. González, A.L. Crego, A. Cifuentes, J. Sep. Sci. 30 (2007) 579.
[23] V. García-Cañas, R. Gónzalez, A. Cifuentes, J. Agri. Food Chem. 50 (2002) 1016.
[24] V. García-Cañas, R. Gónzalez, A. Cifuentes, J. Sep. Science 25 (2002) 577.
[25] L. Petit, F. Baraige, A.M. Balois, Y. Bertheau, P. Fach, Eur. Food Res. Technol. 217 (2003) 83.
[26] H.J. Brunnert, F. Spener, T. Börchers, Eur. Food Res. Technol. 213 (2001) 366.
[27] E. Ibañez, A. Cifuentes, Crit. Rev. Food Sci. 41 (2001) 413.
[28] V.T. Forte, A. Di Pinto, C. Martino, G.M. Tantillo, G. Grasso, F.P. Schena, Food Control 16 (2005) 535.
[29] D. James, A.M. Schmidt, E. Wall, M. Green, S. Masri, J. Agri. Food Chem. 51 (2003) 5829.
[30] P.J. Obeid, T.K. Christopoulos, P.C. Ioannou, Electrophoresis 25 (2004) 922.
[31] Y. Zhou, Y. Li, X. Pei, Chromatographia 66 (2007) 691.
[32] V. García-Cañas, R. González, A. Cifuentes, Electrophoresis 25 (2004) 2219.
[33] J. Xu, S. Zhu, H. Miao, W. Huang, M. Qiu, Y. Huang, X. Fu, Y. Li, J. Agric. Food Chem. 55 (2007) 5575.
[34] C. Peano, M.C. Samson, L. Palmieri, M. Gulli, N. Marmiroli, J. Agric. Food Chem 52 (2004) 6962.
[35] M. Vaïtilingom, H. Pijnenburg, F. Gendre, P. Brignon, J. Agric. Food Chem. 47 (1999) 5261.
[36] V. García-Cañas, A. Cifuentes, R. González, Anal. Chem. 76 (2004) 2306.
[37] G. Dinelli, A. Bonetti, I. Marotti, M. Minelli, M. Navarrete-Casas, A. Segura-Carretero, A. Fernández-Gutiérrez, Electrophoresis 27 (2006) 4029.
[38] Q.K. Wei, W.W. Jone, T.J. Fang, J. Food and Drug Analysis 12 (2004) 324.
[39] Y. Goda, H. Akiyama, E. Suyama, S. Takahashi, J. Kinjo, T. Nohara, M. Toyoda, J. Food Hyg. Soc. Japan 43 (2002) 339.
[40] T.L. Mounts, S.L. Abidi, K.A. Rennick, J. Am. Oil Chem. Soc. 73 (1996) 581.
[41] O. Mellenthin, R. Galensa, J. Agric. Food Chem. 47 (1999) 594.
[42] T. Aussenac, S. Lacombe, J. Daydé, Am. J.Clin. Nutr. 68 (1998) 1480S.
[43] Q. Wu, M. Wang, W.J. Sciarappa, J.E. Simon, J. Agric. Food Chem. 52 (2004) 2763.

[44] C. Cavaliere, F. Cucci, P. Foglia, C. Guarino, R. Samperi, A. Laganá, Rapid Commun. Mass Spectrom. 21 (2007) 2177.

[45] C. Simó, H. Cottet, W. Vayaboury, O. Giani, M. Pelzing, A. Cifuentes, Anal. Chem. 76 (2004) 335.

[46] C. Simó, P. López Soto-Yarritu, A. Cifuentes, Electrophoresis 23 (2002) 2288.

[47] M. Ibáñez, J.V. Sancho, O.J. Pozo, W. Niessen, F. Hernández, Rapid Commun. Mass Spectrom. 19 (2005) 169.

[48] T. Kind, O. Fiehn, BMC Bioinformatics 7 (2006) 234.

[49] T.K. McGhie, J Chromatogr. 634 (1993) 107.

[50] S.R. Padgette, N.B. Taylor, D.L. Nida, M.R. Bailey, J. McDonald, L.R. Holden, R.L. Fuchs, J. Nutr. 126 (1996) 702.

[51] T.K. McGhie, K.R. Markham, Phytochem. Anal. 5 (1994) 121.

[52] X.G. He, J. Chromatogr. A 880 (2000) 203.

[53] T. Iwashina, J. Plant Res. 113 (2000) 287.

[54] F. Cuyckens, M. Claeys, J. Mass Spectrom. 39 (2004) 1.

[55] S. Tahara, S. Orihara, J.L. Ingham, J. Mizutani, Phytochemistry 28 (1989) 901.

[56] N.K. Lee, K.H. Son, H.W. Chang, S.S. Kang, H. Park, M.Y. Heo, H.P. Kim, Arch. Pharm. Res. 27 (2004) 1132.

[57] C.J. Morris, J.F. Thompson, Biochemistry 1 (1962) 706.

[58] G. Dinelli, A. Bonetti, L.F. D'Antuono, S. Elementi, P. Catizone, in F. Yildiz (Editor.), Phytoestrogens in Functional Food, CRC Press, Boca Raton, FL, 2005, p. 19.

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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 180ºC, 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 180ºC. 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.