

## Chemotaxonomic markers of organic, natural, and genetically modified soybeans detected by direct infusion electrospray ionization mass spectrometry

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The crude methanolic extracts of a single bean from samples of organic, natural or genetically modified (GM) soybeans [*Glycine max.* (Merrill) L.] were analyzed by direct infusion electrospray ionization mass spectrometry (ESI-MS). These extracts, containing the most polar natural products of soybeans (free aglycones, monoglucosides, diglucosides and esters including isoflavones and flavones) provide characteristic fingerprinting mass spectra owing to different proportions or sets of components. Spectra distinctiveness is confirmed by chemometric multivariate analysis of the ESI-MS data, which place the three-types of beans into well-defined groups. When ESI-MS is applied, these polar components constitute therefore unique chemotaxonomic markers able to provide fast soybean typification.

### Introduction

Soybean [*Glycine max* (Merrill) L.] has played for centuries an important role in human nutrition in Eastern Asia. Owing to its widely-recognized health benefits, this crop has gained global acceptance, being now largely grown also in Western countries such as Brazil, USA and Argentina, with a global consumption exceeding 200 million tons per year.<sup>1</sup> Soybeans are beneficial for human health as a main source of isoflavones,<sup>2</sup> a class of important natural phytochemicals. Genistein and daidzein are the major isoflavones in soybean, in which they occur predominantly in glucosidic forms. These heterocyclic phenols form a class of chemotaxonomic markers<sup>3</sup> with close similarity in structure to estrogens and a diphenolic character similar that of lignans. For humans, isoflavones have been proposed to influence sex hormone metabolism and to display many important biological activities.<sup>4</sup> Environmental factors are known to affect isoflavone concentrations in plants, and isoflavones in soybeans are highly concentrated in the beans.<sup>5</sup>

Modern biotechnology assays for genetic transformation have been used to develop genetically modified (GM) organisms such as corn and soybeans with a number of improved characteristics.<sup>6</sup> However, the genetically modified organisms (GMO) have not yet gained worldwide acceptance because of concerns related mainly to potential gene flow to other organisms, the destruction of agricultural diversity, allergenicity, antibiotic resistance and gastrointestinal problems.<sup>7</sup>

Recently, the electrospray ionization mass spectrometric technique (ESI-MS)<sup>8</sup> has revolutionized the way polar molecules are ionized and subjected to mass spectrometers for mass and structural analysis,<sup>9–11</sup> and mass spectrometers able to perform such

experiments are today readily available worldwide. ESI-MS with direct infusion has also shown as a powerful technique for the fast fingerprinting characterization of mixtures of polar natural products, such as those found in plant extracts,<sup>12</sup> whisky,<sup>13</sup> wine,<sup>14</sup> petroleum,<sup>15</sup> beer,<sup>16</sup> and propolis.<sup>17</sup> In ESI-MS of a chemical mixture, the most polar molecules (often the ones bearing acidic or basic sites) are transferred to the gas phase as a single ion, either in their protonated [M+H]<sup>+</sup> or deprotonated [M–H]<sup>–</sup> forms.

GMO are commonly identified by detecting the inserted genetic material at DNA level, the mRNA transcribed from the newly introduced gene, the resulting protein or alternatively its metabolite or phenotype. The analytical tests are generally performed either with the polymerase chain reaction (PCR method) to detect the inserted DNA, immunological assays to detect the resulting protein (e.g., the enzyme-linked immunoassay – ELISA, and the lateral flow sticks), or using bioassays to detect the resultant phenotype (e.g., herbicide bioassays).<sup>18,19</sup> Other analytical techniques also based on protein or DNA analysis are emerging for GMO detection using mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices and DNA chip microarray technology.<sup>20</sup> So far, the molecular PCR-based method is the one that found the broadest application in GMO detection and is the generally accepted method for regulatory purposes.<sup>20</sup>

Using organic, natural and GM soybeans as a proof-of-principle case, we describe herein a novel method for distinguishing GM plants from their naturally-grown conventional counterparts based on simple extraction and fast and effective detection by direct infusion ESI-MS of sets of polar natural products that function therefore as soybean chemotaxonomic markers.

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## Experimental

### Collection of soybean grains

Commercial organic soybean varieties from certified organic farms and conventional and transgenic soybean varieties were selected from the available soybean germplasm from CENA-USP and Monsanto, respectively. The soybean extracts were prepared by rough maceration of a single bean, followed by ethanol addition (1 ml), shaking and fast centrifugation. An aliquot (500  $\mu$ l) of the supernatant was then immediately analyzed by direct infusion ESI-MS.

### Electrospray ionization mass spectrometry (ESI-MS)

ESI mass and tandem mass spectra were acquired in the negative ion mode using a Micromass (Manchester – UK) QToF instrument of ESI-QqToF configuration. The following typical operating conditions were used: 3 kV capillary voltage, 40 V cone voltage and de-solvation gas temperature of 100 °C. Tandem ESI-MS/MS spectra were collected after 5–20 eV collision induced

dissociation (CID) of mass-selected ions with argon. Mass-selection was performed by Q1 using a unitary  $m/z$  window, and collisions were performed in the rf-only quadrupole collision cell, followed by mass analysis of product ions by the high-resolution orthogonal-reflectron TOF analyzer.

## Results and discussion

### Electrospray ionization mass spectrometry (ESI-MS)

Figure 1 shows typical and distinguishable ESI-MS in the negative ion mode of the methanolic extracts of each of the three types of soybeans. In the ESI-MS of three different varieties of organic soybeans, as that shown in Fig. 1a, by far the most characteristic and abundant ion is that of  $m/z=279$ . An expansion of the  $m/z$  region in which the main soybean isoflavones daidzein, genistein, and glicitin (Fig. 2) are expected to be detected in their deprotonated forms, that is, by the  $[M-H]^-$  ions of  $m/z=253$ , 269 and 283, only a very minor ion of  $m/z=283$  is observed for the organic soybean extracts.

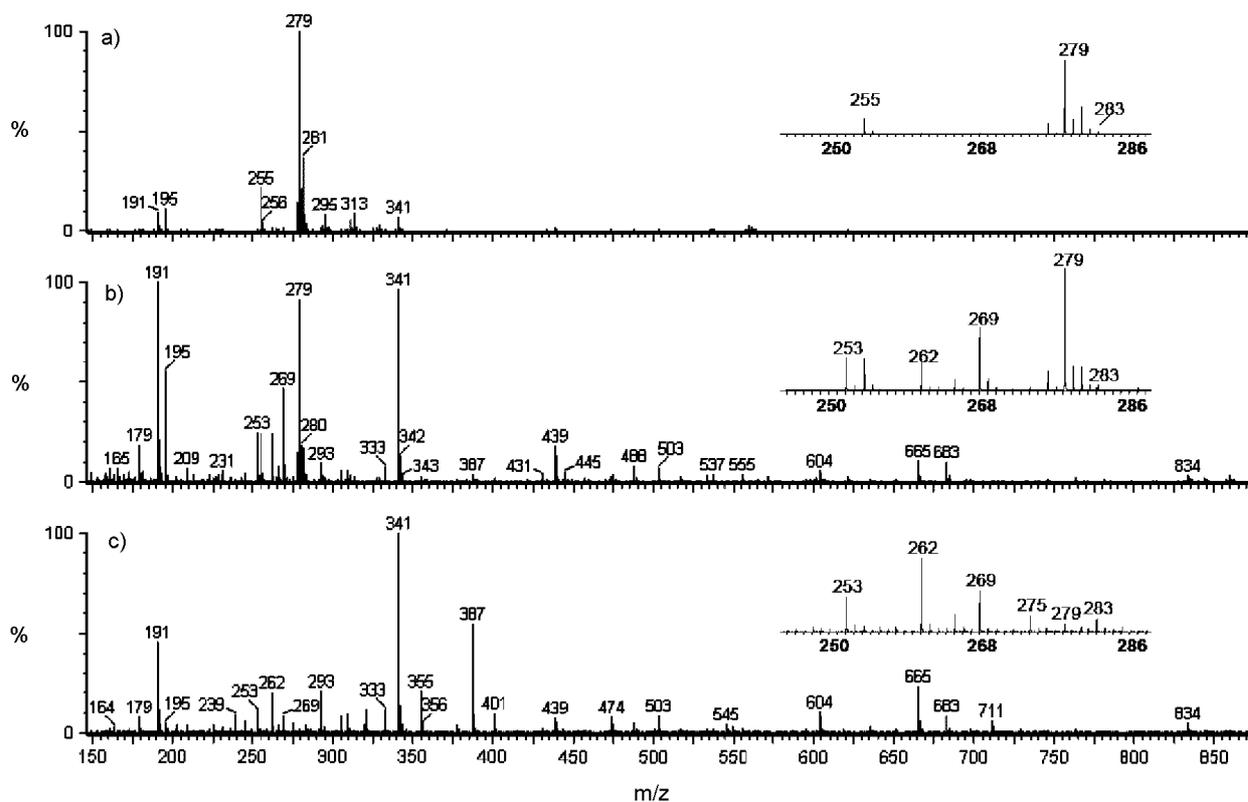


Fig. 1. ESI-MS in the negative ion mode for the methanolic extracts of organic (a), natural (b) and GM (c) soybeans

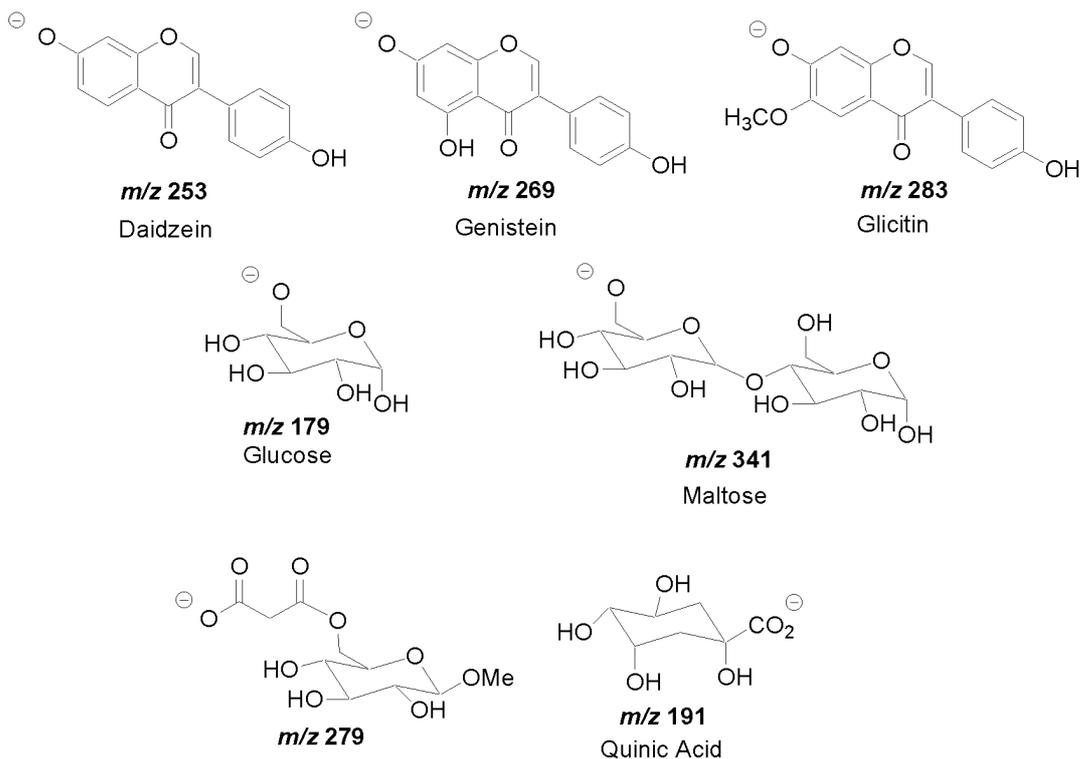


Fig. 2. Structures of the main soybean isoflavones daidzein, genistein, and glicitin

In the ESI-MS of the methanolic extracts of three varieties of natural soybeans, as exemplified by that of Fig. 1b, the ion of  $m/z=279$  so abundant for the organic soybean extracts is also detected as a major peak, but a number of other major ions spread concurrently through the whole  $m/z=150-850$  range with three prominent ions of  $m/z=191$ , 193 and 341. For the set of three soybean isoflavones, both daidzein and genistein are also clearly detected by corresponding  $[M-H]^-$  ions of  $m/z=253$  and 269, whereas deprotonated glicitin of  $m/z=283$  is still minor.

The ESI-MS of the extracts of the six varieties of GM soybeans are also typical (Fig. 1c). The major ions detected for the natural soybean extracts which spread through the whole  $m/z=150-850$  range are also abundant for the GM soybeans, but several variances in relative abundances are clear, as that of the ion of  $m/z=387$ . But by far the most characteristic feature of the ESI-MS of the GM soybean extracts is the very minor ion of  $m/z=279$ , which is otherwise so abundant for the natural and particularly organic soybean extracts.

#### Seasonality effects

To investigate seasonality effects, natural and organic modified soybeans cultivated and harvested at the Center of Nuclear Energy in Agriculture (CENA-University of São Paulo) in different seasons of the year (2003–2004) were analyzed. The ESI-MS of such grains were found not to be nearly the same, that is, not substantially affected by seasonality effects.

#### ESI tandem mass spectra

Although definitive structural attribution of all chemotaxonomic markers forming the deprotonated molecules detected in the ESI-MS of Fig. 1 (which is not a crucial issue for screening purposes) will require more refined analysis preceded by chromatographic separation and combined with spectroscopy data, ESI tandem mass spectra (ESI-MS/MS) provide clue information about their origins. ESI-MS/MS of the ions of  $m/z=253$ , 269 and 283 shows the same dissociation patterns as that of the deprotonated molecules of daidzein, genistein, and glicitin standards, as Fig. 3 exemplifies for genistein.

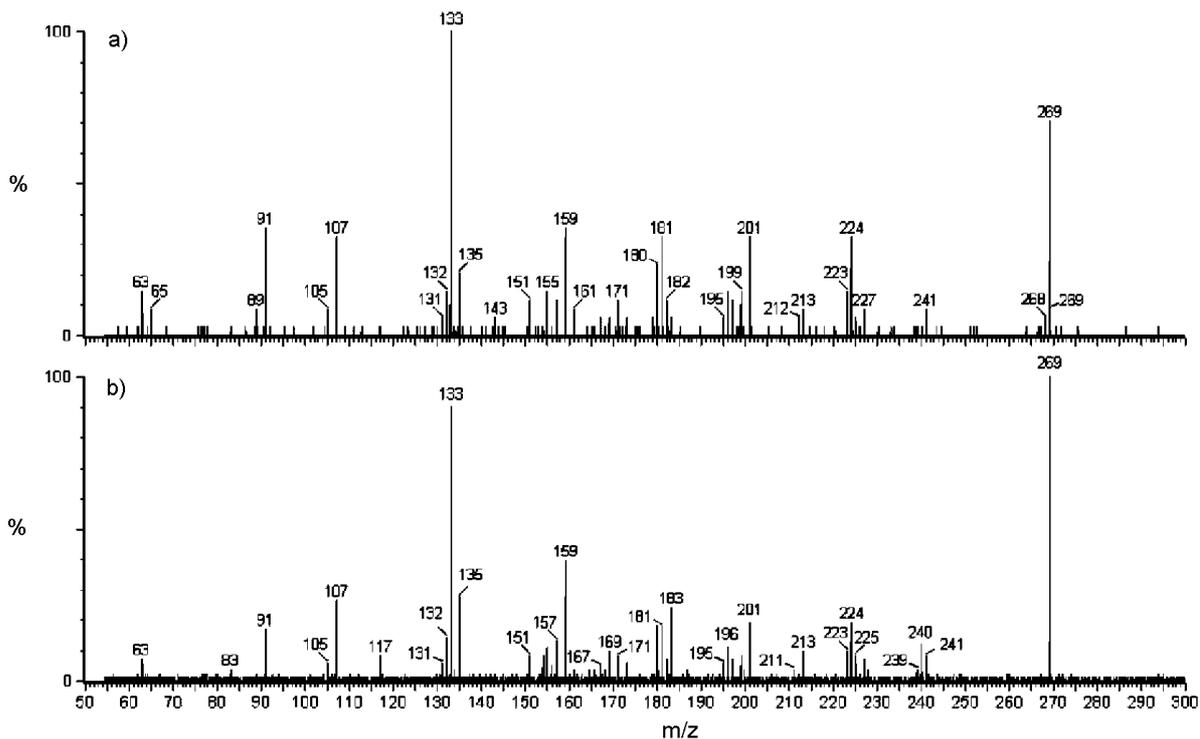


Fig. 3. ESI-MS/MS for the ion of  $m/z = 269$  from a deprotonated genistein standard (a) and that detected in the methanolic extract of a GM soybean (b)

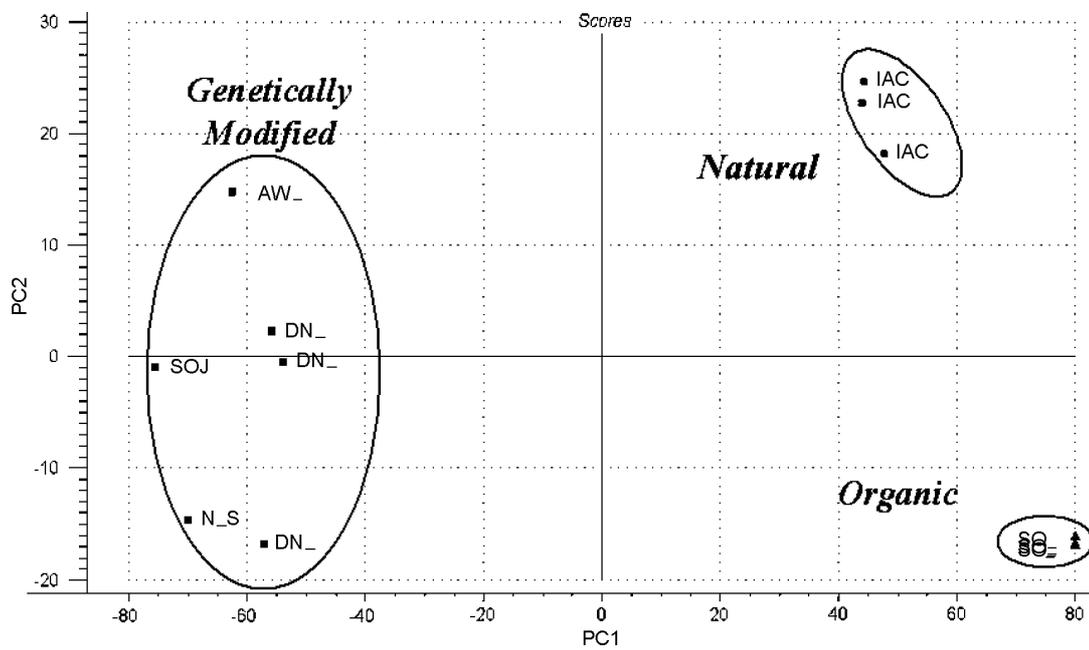


Fig. 4. PCA analysis of ESI-MS data showing scores for major anions detected in the methanolic extracts of different varieties of organic, natural and GM soybeans

The ion of  $m/z=279$ , so characteristic for both the organic and natural varieties of soybeans, is likely the  $[M-H]^-$  ion of methylated glycosyl malonate (Fig. 2), although its high resistance towards dissociation have precluded secure structural elucidation via tandem MS. Similarly, the ions of  $m/z=179$ , 341 (Fig. 2), 503, and 665 are likely the deprotonated molecules of glucose, maltose, maltotriose, and maltotetraose, respectively, as indicated by spectra comparison using standards, whereas the ion of  $m/z=191$  appears to be the deprotonated molecule of quinic acid (Fig. 2). The ion of  $m/z=387$ , which is so abundant in the ESI-MS of the GM soybean extracts, dissociates upon collisions to the ion of  $m/z=341$ , and then to nearly the same set of fragments as those observed in the ESI-MS/MS of the ion of  $m/z=341$ . This similarity indicates that the molecule corresponding to the  $[M-H]^-$  ion of  $m/z=387$  is likely a derivative of maltose.

#### *Chemometric data analysis*

To test chemometrically the distinctiveness of the ESI-MS, PCA has been employed.<sup>17</sup> The PCA plot (Fig. 4), which covers 92% of the total data variance, is found to place indeed the samples into three well-defined groups comprised of the different varieties of organic, natural and GM soybeans.

#### **Conclusions**

Direct infusion ESI-MS of simple methanolic extracts with the detection of the most polar components (ESI-MS chemotaxonomic markers) is demonstrated to be an effective fingerprinting method for high-throughput typification of organic, conventionally-grown and GM soybeans. We are currently validating further the methodology by growing the three types of soybeans under well-controlled and similar conditions to eliminate any of such effect on ESI-MS data.

The method offers an outlook for expansion of applicability to other GMO's, but case-by-case it has to be tested whether other organisms contain similar different sets of chemotaxonomic markers as here described for soybeans.

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#### **References**

1. H. D. BERLITZ, W. GROSCH, P. SCHIEBERLE, *Food Chemistry*, 3rd ed., Springer-Verlag, Germany, 2004.
2. W. MAZUR, H. ADLERCREUTZ, *Pure Appl. Chem.*, 70 (1998) 1759.
3. T. IWASHINA, *J. Plant Res.*, 113 (2000) 287.
4. R. VAN RHIJN, J. VANDERLEYDEN, *Microbiol. Rev.*, 59 (1995) 124.
5. I. LEROUGE, J. VANDERLEYDEN, *FEMS Microbiol. Rev.*, 26 (2002) 17.
6. R. N. BEACHY, *Science*, 285 (1999) 335.
7. A. G. HASLBERGER, *Science*, 287 (2000) 431.
8. J. B. FENN, M. MANN, C. K. MENG, S. F. WONG, C. M. WHITEHOUSE, *Science*, 246 (1989) 64.
9. S. D. FUERSTENAU, W. H. BENNER, J. J. THOMAS, C. BRUGIDOU, B. BOTHNER, G. SIUZDAK, *Angew. Chem. Intern. Ed.*, 40 (2001) 542.
10. A. A. SABINO, A. H. L. MACHADO, C. R. D. CORREIA, M. N. EBERLIN, *Angew. Chem. Intern. Ed.*, 43 (2004) 2514.
11. K. J. KOCH, F. C. GOZZO, S. C. NANITA, M. N. EBERLIN, A. R. G. COOKS, *Angew. Chem. Intern. Ed.*, 41 (2002) 1721.
12. P. MAURI, M. MINAGGIO, P. SIMONETTI, C. GARDANA, P. PIETTA, *Rapid Commun. Mass Spectr.*, 16 (2002) 743.
13. J. K. S. MOLLER, R. R. CATHARINO, M. N. EBERLIN, *Analyst*, 130 (2004) 890.
14. H. J. COOPER, A. G. J. MARSHALL, *Agric. Food Chem.*, 49 (2001) 5710.
15. C. A. HUGHEY, R. P. RODGERS, A. G. MARSHALL, *Anal. Chem.*, 74 (2002) 4145.
16. A. A. ARAUJO, L. L. R. ROCHA, M. N. EBERLIN, *Analyst*, 130 (2004) 884.
17. A. C. H. F. SAWAYA, D. M. TOMAZELA, I. B. S. CUNHA, V. S. BANKOVA, M. C. MARCUCCI, M. N. EBERLIN, *Analyst*, 129 (2004) 739.
18. W. M. FREEMAN, D. J. ROBERTSON, K. E. VRANA, *Biotechniques*, 29 (2000) 1042.
19. S. KAY, G. VAN DEN EEDE, *Nature Biotech.*, 19 (2000) 405.
20. I. T. JOLLIFFE, in: *Principal Component Analysis*, Springer Verlag, New York, 1996.