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Marek Trojanowicz[ab]; Anna Latoszek[a]; Ewa Poboży[a]
[a] Department of Chemistry, University of Warsaw, Warsaw, Poland [b] Institute of Nuclear Chemistry and Technology, Warsaw, Poland

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Separations

ANALYSIS OF GENETICALLY MODIFIED FOOD USING HIGH-PERFORMANCE SEPARATION METHODS

Marek Trojanowicz,[1,2] Anna Latoszek,[1] and Ewa Poboży[1]
[1]*Department of Chemistry, University of Warsaw, Warsaw, Poland*
[2]*Institute of Nuclear Chemistry and Technology, Warsaw, Poland*

Many recent aspects of genetic modification in food are discussed. Changes of genes during modification cause alteration in a protein's biosynthesis, which may result in expression of new proteins and changes in metabolite pathways. This review, based on over 100 literature references, reports applications of separation methods (liquid, gas chromatography, and capillary electrophoresis) for the identification of genetically modified food based on protein profiles and selected metabolites.

Keywords: GMO; Metabolites; Proteins; Separation methods

INTRODUCTION

Genetic modification (GM) of organisms is an outstanding scientific achievement of molecular biology and biotechnology in recent decades, but from another perspective, it is a widely discussed subject due to numerous social fears and controversies. The sources of these controversies can be related to consumer suspicions regarding the true intentions of biotechnological producers and mistrust in government bureaucracies. Some of the main environmental and public health issues of using recombinant DNA technology in development of transgenic plants used for food production are, for example, the potential gene flow to the other organism, the distortion of agricultural diversity, allergenicity, antibiotic resistance, and gastrointestinal problems (Ahmed 2002).

Genetic modification means generation of organisms by combining genes of different species using recombinant DNA technology. The obtained genetically modified organism (GMO) is called *transgenic* and, in comparison to non-transgenic one, a particular gene can be added or removed in its genome. For comparison, in traditional plant breeding based on crossing one plant variety with a related

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Address correspondence to Marek Trojanowicz, Department of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland. E-mail: trojan@chem.uw.edu.pl

plant to get the desired characteristics, the principal mechanism is a chromosomal recombination resulting in manipulations with hundred of genes, which makes it impossible to predict how much genetic information will be transferred. As potential for the application of metabolic engineering in plant biotechnology is enormous, GM food can resolve problems of nutrition in poor countries and reduce vitamin and nutrition deficiency on a global scale.

At its present state of development, genetic engineering of plant organisms used for food production achieves several very important aims that are widely discussed in biochemical and food chemistry literature, including several books (Engel, Takeoka, and Teranishi 1995; Schreiber and Bogl 1997; McHughen 2000; Nelson 2001). The first successful achievements in genetic modification of plants can be dated back to the 1980s. In parallel, a spectacular achievement was gained, which was the official approval of the first genetically engineered drug, human insulin, produced by bacteria. By the early 1980s, major grain biotechnological producers in North America introduced public relation programs to modify consumer concerns regarding newly developed food biotechnology (Gaskel et al. 1999). At the end of the 1990s, the first transgenic foods such as tomatoes, potatoes, canola, and squash appeared on the market (van Duijn et al. 1999); however, the main transgenic plants are soybean, maize, and cotton. This is also reflected in the research conducted in this field and illustrated by number of published papers in the last 15 years (Fig. 1). In 1999 in the USA, more than 40% of the corn, 50% of the cotton, and 45% of soybean planted have been genetically modified, and at least 60% of food products in the US supermarkets contained GMO (Beachy 1999). In 2007, biotech global crop areas achieved 114 million hectares (Global Status 2007).

Increased biotechnological interest in soybeans is the reason for the significant role of this plant in food production. As early as the 1950s, the crop became one of the most important sources of edible oil and protein for the animal industry.

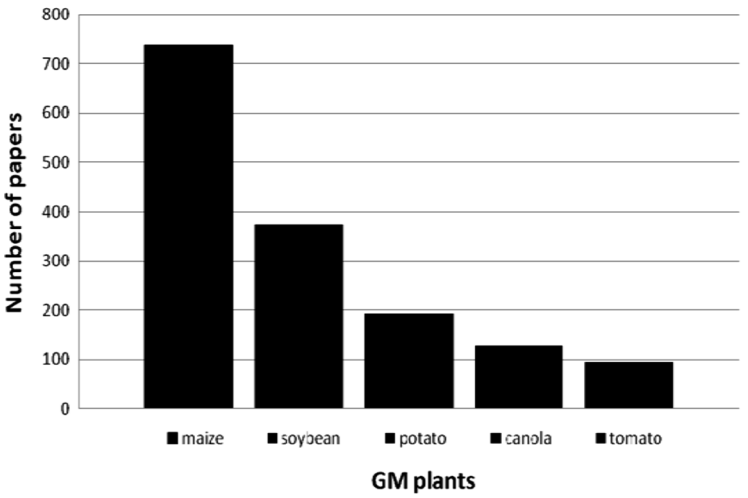

Figure 1. Number of papers dealing with various aspects of transgenic plants found in the period 1995–March 2009. Keyword used for search of citations: genetically modified maize, genetically modified soybean, genetically modified canola, genetically modified potato, and genetically modified tomato.

At present it is recognized as the most economical source of food proteins; hence, a lot of efforts have been focused on mutant genes controlling the production of soybean seed proteins and enzymes closely related to nutritional and food processing quality. This includes, for instance, genetic modification of seed storage proteins or genetic elimination of seed lipoxygenases that catalyze hyperoxidation of unsaturated fatty acids and polyunsaturated lipids (Kitamura 1995).

Genetically modified food can be classified into two groups (Celec et al. 2005). First generation brings various improvements in the production; whereas, the second one is oriented toward consumers. Genetic engineering is employed to improve resistance to plagues, herbicides, and also hydric or saline stress. It may result in improvement of technological properties during storage and processing, and it may lead to improvement of the sensorial and nutritional properties of food products such as starch quality, content of vitamins, or presence of essential amino acids. So, it seems obvious that the present role and increasing importance of genetic engineering in food production is an irreversible fact, and, therefore, it is not surprising that biological and biomedical aspects of GM food are the matter of continuous debates in the scientific community (i.e., Jain, Rivera, and Loke 1999; Lack 2002; Kuiper and Kleter 2003; Celec et al. 2005); the public relation campaigns and guidelines issued by national and international authorities and organizations (i.e., Kuiper et al. 2001; Hails and Kinderlerer 2003; Commission Regulation 2004); and the large interest on this subject in the public media.

In the European Union, GMO events are authorized as food ingredients for commercialization: maizes Bt11, DAS1507, GA21, MON810, MON863, NK603, and T25; rapeseeds GT73 and T45; and RoundReady soybean (Leimanis et al. 2006; European Commission Regulation 1829/2003). The EC requires labeling of foodstuffs containing GMOs and these regulations established a 0.9% threshold for contamination of unmodified foods with GM food products (Commission Regulation 2004); whereas, in the USA regulation does not stipulate obligatory labeling of GM foods. In addition to numerous other reasons that affect public acceptance, this is the main cause of the intensive development of analytical methods for identification and qualitative analysis of GM food.

Genetic modification of plants can be carried out by different methods of molecular biology widely discussed in literature. In each case, it results in the presence of recombinant DNA, which produces new proteins. Presence of new proteins, including enzymes, may then lead to change in metabolic pathways. The two commonly considered main groups of analytical methods employed for this purpose are determination of DNA and determination/profiling of new proteins expressed by recombinant DNA (i.e., Lüthy 1999; Kuiper 1999; Spiegelhalter 2001; Ahmed 2002). Increasing attention in recent years has also been focused on metabolic profiling, which is closely related to functional genomics (Trethewey 1999). Among metabolites that are determined are fatty acids, polyphenols, amino acids, and several other groups of compounds. From the point of view of food safety, this also includes examination of the safety of new proteins, the allergenicity of new proteins, and the role of new food in the diet (Kuiper et al. 2001). Metabolite profiling, from this point of view, should also include determination of key nutrients, toxicants, and allergens. In recent research, especially through the valuable review by Kuiper et al. (2001), one can find interesting lists of results of the compositional analysis of numerous GM crops which have been taken from studies carried out in 1990s.

The changes of metabolic profile by genetic modification are associated with the concept of substantial equivalence of GM food and its practical implications.

Additionally, analytical procedures for GMO identification may be oriented toward one particular analyte (targeted single compound analysis) or can be a non-targeted profiling analysis (Kuiper and Kleter 2003). The selection of a particular analyte for determination may concern determination of particular protein, e.g., by immunoassay or single nutritional or toxic compound to compare its content in GMO containing food product and its non-transgenic species. Non-targeted profiling may be employed as gene expression analysis with cDNA arrays, protein expression, or metabolite analysis using high-performance separation techniques, which is the main subject of this article. As DNA methods often involve a separation step prior to the detection, they will be also briefly reviewed.

METHODS OF DNA DETERMINATION IN GM PLANTS AND FOOD PRODUCTS

Determination of recombinant DNA is, obviously, the direct method of identification of genetic modification in plants or foodstuffs produced from GM plants. The DNA detection methods for GM foods are based mainly on the complementarity of two strands of DNA double helix that hybridize in a sequence-specific manner. The DNA that is engineered into a crop consists of several elements that govern its functioning. They typically include a promoter sequence, a structural gene, and a stop sequence for the gene. Fundamental methodology of such determination is the application of a polymerase chain reaction (PCR), which allows the selective amplification of specific DNA segments. The PCR method, developed in the middle of 1980s, is considered one of the most valuable tools in the life science field, including clinical medicine for diagnosis of infections, genetic disorders, and genetic traits. It is also widely used in food control for monitoring of microorganisms and food constituents. For the first time, to GM plants PCR was used for detection of of FlavrSavr tomatoes (Meyer 1995), and its applications were further reviewed (Meyer 1999; Ahmed 2002).

The PCR method is widely described in literature (Mullis et al. 1994; Dorak 2006; Van Pelt-Verkuil et al. 2009) and also for the detection of GMOs (Weighardt 2003). In its basic form after PCR amplification of determined DNA, the reaction products are separated by agarose gel electrophoresis according to the size of DNA fragments, which are then detected in UV light by measurements shown as a fluorescence band on the gels after staining with ethidium bromide. The main stages of determination are extraction from examined material, repeated cycles of denaturation-annealing of primers-extension of primers, and detection. A very essential step in performing the whole procedure is extraction of the DNA from analyzed material for GM quantification in food. There are two favorable methods for DNA isolation from raw plant material (Meyer 1999). A sample is incubated with hexadecyltrimethyl ammonium bromide (CTAB), extracted with chloroform, and the DNA is precipitated with isopropanol in the CTAB method. Another commonly used method uses a DNA-binding silica resin (Wizard^{TM}, Promega) to purify DNA directly from the solution after enzymatic reaction with proteinase K and SDS. Extraction is usually carried out in 100 mg samples, which results in obtaining about 100 mg DNA for PCR. There are also other methods developed for extraction from

plant material. Nine different methods were compared in extraction from soybean samples (Zimmermann, Lüthy, and Pauli 1998). In an interlaboratory project, eight national metrological institutes utilized four methods that were compared for genomic isolation from GM Bt176 corn powder (Corbisier et al. 2007). In addition to the two methods previously mentioned, commercial DNeasy Plant Mini Kit and Nippon Gene GM Quicker protocol were also examined. No significant difference was observed regarding the final result of BT176 content. The highest extraction yield and the best DNA quality were obtained in the CTAB method. It can also be noted that in addition to the widely used PCR method for DNA determination, the Southern blot method can be utilized, which is based on fixing isolated sample DNAs on appropriate membranes, probing with *ds*-labeled nucleic acid probes specific to GMO, and detecting hybridization radiometrically or using luminescence labels (Ross 1999).

In qualitative PCR determination of recombinant DNA, different methods can be used for verification of results, which vary in reliability, cost, and precision, and of which, as the simplest, is considered specific cleavage of the amplified product by restriction endonuclease digestion (Lipp et al. 1999).

The main drawback of conventional PCR in application for GMOs detection in food is the lack of an accurate quantitative method that is due to difficulties in evaluation of amplification efficiency. It changes both between different reactions and also between successive cycles of the same reaction. Qualitative determinations can be carried out with developed quantitative end-point PCR methods and real-time PCR methods. In recent years, the real-time PCR methods have gained in evident importance, and one can find several of their applications in analysis of GM plant material (e.g., Hernandez et al. 2004) and in references to other papers given herein. Those methods are based on parallel amplification of transgene specific sequences and reference genes, which serves as internal control for the quality of extracted DNA and evaluation of the total amount of target plant DNA present in the analyzed sample. They are based on fluorescence detection employing different fluorophores. Three of these methods were successfully used in quantification of maize line GA21, obtaining for the TaqMan® assay the detection limit 0.01% GA21 (Hernandez et al. 2004).

A general problem in application of these methods is limited availability of certified reference materials for calibration of real-time PCR methods (Trapman and Emons 2005). Those methods are sensitive to contamination from various sources, hence the necessity to verify amplified fragments (Meyer 1999) in comparison to protein-based methods discussed in the following as they are much more time-consuming and more expensive (Ahmed 2002). Another limitation in discussed applications is the fact that not all products derived from GM foods contain enough DNA. Although theoretically qualitative PCR can detect a single molecule of DNA, the detection limit of a quantitative PCR given as a percentage of analyzed GM material depends on the number of DNA molecules present in the sample and can be in the range of 0.01 to 1% (Spiegelhalter 2001), which is not always sufficient to detect 0.9% contents of GMOs.

It should also be noted that in addition to PCR usage in the past two decades, several other methods were developed for DNA amplification, which can potentially be used for determination of GMOs. They may amplify target DNA to a similar magnitude and exhibit LOD of less then 10 copies. For instance, in loop-mediated

isothermal amplification of DNA, the cyclic reaction continues with accumulation of 109 copies in less than one hour, with the advantage of much simpler instrumentation (Notomi et al. 2000).

As was mentioned previously, in addition to typical slab agarose gel electrophoresis for detection of amplified recombinant DNA, capillary electrophoresis (CE) can be employed. It is a very important area of combining measurement techniques of molecular biology with high-performance separation, which has already been reviewed for several different fields of application in food analysis (Garcia-Canas, Gonzales, and Cifuentes 2004). In the same review, earlier works on such applications of CE were presented. For CE separation of DNA, a capillary gel electrophoresis (CGE) mode is employed with use of background electrolyte (BGE) containing 2-hydroxyethyl cellulose as the sieving polymer. In such determinations for analysis of GM plants, both UV (Giovannolli 2004; Dinelli et al. 2006) and laser induced fluorescence (LIF) with different fluorophores (Garcia-Canas, Gonzales, and Cifuentes 2002; Sanchez et al. 2007) were applied. Optimized conditions of separation allowed determination of DNA of sizes amplified by PCR for detection of GMOs in food. In CGE-UV measurements, it is possible to accurately evaluate the length of DNA sequences (Giovannolli 2004). In a comparison of the detection of PCR amplified DNA by CGE with slab agarose gel electropho-resis, a lower limit of detection (LOD) was shown for CGE in analysis of RR-soybean (Dinelli et al. 2006). It was also demonstrated in the same work that, whereas all maize samples containing less than 1% of GMOs cannot be quantified for soybean quantitative determination of transgenic DNA, it was possible for samples containing minimum 0.5% GMO. Lower limits of detection, and hence possibilities of quantitative determinations were shown for CGE determinations with LIF detection. Fluorophore, in this case, can be covalently bound to DNA fragments or used as an intercalating agent in BGE. In determinations of DNA from transgenic maize in flours, the limit of detection for a 200 bp fragment is sufficiently low to detect 0.01% of transgenic maize (Garcia-Canas et al. 2002). The example electropherogram obtained in such determinations is presented in Fig. 2. It shows satisfactory

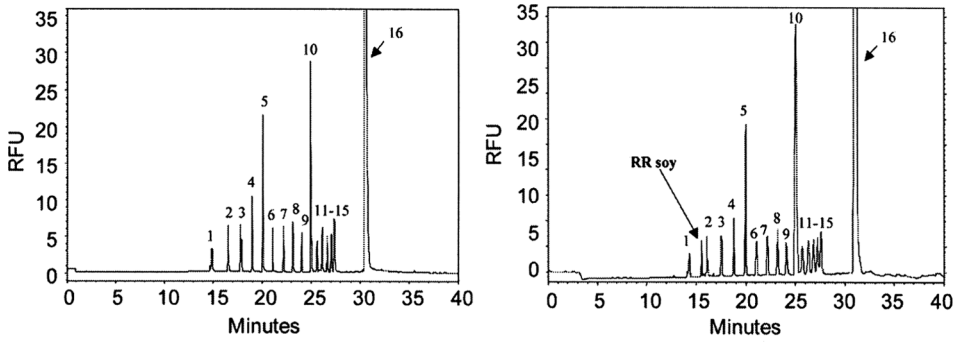

Figure 2. Example of CGE-LIF electropherograms used to determine the size of the amplicons obtained after PCR amplification. (a) Injection of 50 bp DNA ladder; and (b) coinjection of 50 bp DNA ladder and PCR sample (in this case containing the RRS amplicon from RR soy) (Sanchez et al. 2007).

separation of DNA fragments from 50 to 750 bp in about 30 min with well separated amplicon for RR-soybean.

Important developmental trends of capillary electrophoresis for various applications in recent decades are designed with miniaturized capillary systems described as microfluidic systems or CE chips. These measuring systems are equipped in almost the same peripheral devices as conventional CE instruments; however, separation is carried out in much shorter capillary channels fabricated of solid material. They are usually laboratory-fabricated but also increasingly commercially available. Numerous analytical methods were already developed for such systems (Henry 2006). Their applications have also been reported for analysis of PCR products for GMO analysis in food. For such purposes, satisfactory applications were reported for the commercial system LabChip^{TM} from Agilent Technologies for CE analysis with LIF detection (Birch et al. 2001; M. Burns 2003). In the initial works cited, for the determination of PCR products, the DNA 500 LabChip^{TM} was used, which is designed for determination of DNA 30 to 500 bp. Analysis required 1 μl of post-PCR solution, and on the same chip, simultaneous analysis of 12 samples could be carried out. Results of determination were comparable with those obtained on agarose gel. Determinations performed for GM soy standards and limits of detection for both examined methods were determined on a 0.1% level of GMO content in samples; however, the CE chip offered improvements in quantitation accuracy, objectivity, and ease of use.

For the same purpose, a laboratory-made CE chip with LIF detection was reported (Obeid, Christopoulos, and Ioannou 2004). Separation and measurement on the chip were carried out using CGE conditions, and determinations were reported for certified reference materials (CRM) of soy powder with GMO content from 0.1 to 5%. Reported determinations are significantly faster than commonly used techniques from GMO analysis and LOD was evaluated as 0.1% GMO, which was reported as a significant improvement compared to control measurements on the slab gel.

Another similar system reported for GMO determination in plant materials was the DSBCE-100 Microchip CE system from Digital Bio Technology in Korea (Kumar and Kang 2007). Determinations of PCR products were carried out, in this case, using a programmed field strength gradient (PFSG) with CGE procedure and application of various sieving gels. Analyses were performed for five transgenic maize varieties with LOD evaluated on 0.03–0.08%. Example recordings from reported determinations are shown in Fig. 3. It seems that it is currently one of the best methods developed with CGE for detection of PCR products reported for GM food analysis.

Although it is out of scope of this review, two other types of miniaturized analytical instruments will be shortly mentioned, as they may become truly competitive for routine analysis of GM food. They are microarray chips (Marshall and Hodgson 1998) and DNA biosensors. In the first case, on small surfaces, many different DNA sequences are immobilized and can be analyzed in parallel with hundreds of tests running simultaneously. Two such approaches developed for GM food analysis worth mentioning are based on commercialized technologies to produce microarray biochips. Using CodeLink slides with immobilized probes, they were employed for parallel detection of five transgenic events in foods (Bordoni et al. 2005) and arrays

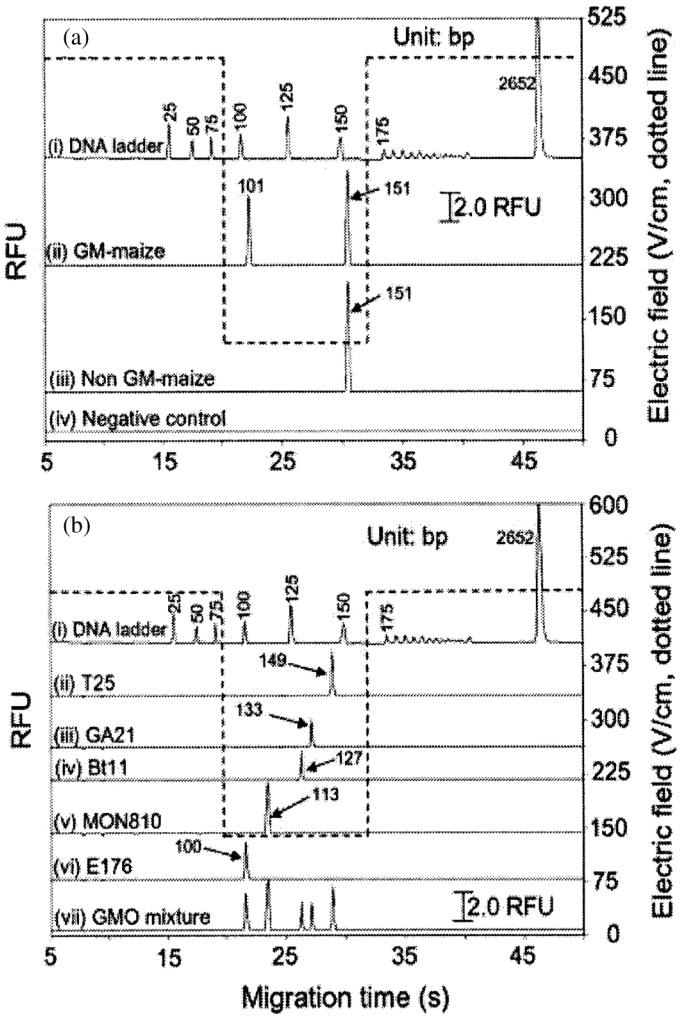

Figure 3. Representative ME-PFSG electropherograms of (a) GM and non-GM maize and (b) five transgenic maize along with the 25-bp DNA ladder under the PFSG. ME-PFSG applied separation voltage conditions: 470,6 V/cm for 20 s, 117,6 V/cm for 12 s, and 470,6 V/cm for 30 s (Kumar and Kang 2007).

based on technology developed by Eppendorf Array Technologies for simultaneous identification of nine GMO events (Leimanis et al. 2006). Due to satisfactory LOD, simplicity, and speed of post-PCR determinations such determinations may soon find very wide routine applications. The second kind of miniaturized devices are DNA biosensors, also named genosensors. They have been developed over almost half a century and are designed with various optical, electrochemical, or mass sensitive detections. An example of optical genosensor would be a surface plasmon resonance biosensor developed for real-time detection of GM RR-Soybean sequences (Feriotto et al. 2002). An especially large variety of designs and applications can be found for electrochemical genosensors (Drummond, Hill, and Barton 2003).

For a rapid detection of GMO, a recent development is the electrochemical genosensor, which employs the integration of isothermal PCR amplification with analysis on an electrochemical printed chip in one device (Ahmed et al. 2009). This unique device combining amplification and detection of genetic material may also provide perspectives for broad future routine applications.

PROTEIN PROFILING

A result of genetic modification of each organism is expression of novel proteins, and this property is utilized not only in biological and biochemical research, but also in the fundamental application in identification and determination of GMOs. Protein profiling in given material, which will be the main subject of this paragraph, is a relatively new approach for analytical purposes. As immunoassays have been predominately used for many years in the analysis of GMOs, they are considered main analytical methods for this purpose. They will be discussed as an introduction to the main subject.

The beginning of modern analytical immunochemical methods can be dated back to the 1950s when the first methods were developed for clinical diagnostics. These applications were and still are the area of use for immunoassays, but at present they are widely employed in environmental analysis, pharmaceutical, agricultural, and, for many years, they are well developed for food analysis (Paraf and Peltre 1991). Generally, it is a widely described, well documented, and commonly employed method of biochemical analysis. Very wide applications are based on several important advantages of immunoassays, which are also utilized in determination of proteins (Brett et al. 1999), namely very good selectivity and outstanding sensitivity with limit of detections on the level 10^{-12} M. Both those factors depend significantly, however, on a type of employed antibody (monoclonal, polyclonal). Progress in development of immunochemical analytical methods involves a search for new sources and methods of antibody protection and on the development of improved and simpler formats of determinations, as those factors affect their applications in routine analysis. An additional essential factor is the limitation of sample processing necessary for a particular format of determination.

Immunoassays for analysis of GM foods have been developed for about 10 years and designed with different formats (Stave 1999). One of the most common formats widely employed in different applications is enzyme-linked immunosorbent assay (ELISA) and is carried out in a microwell plate or coated tune formats with immobilized antibody. A commercial ELISA kit was employed for analysis of different food stuffs for the presence of the synthetic *CryIA(b)* gene as an ingredient of different events of transgenic maize (Margarit et al. 2006). The presence of transgenic Bt maize was found in 8 of 32 analyzed human and animal foodstuffs, as the largest content of protein CryIA(b) was determined at 0.1 ppm. In the same work, the Western blot format was employed with an application of immunopurified polyclonal antibodies, which allowed determination of not only target protein but also partially degraded protein due to food processing. The ELISA was also developed for the detection of a novel protein from RR-soybean, but for GMO content $>0.5\%$ only, an evidently higher signal was recorded compared to samples not containing GMO (Stave 1999). Then, the Western blot format was used for

immunoassays of the CP4 synthase in RR-soy with a similar range of LOD between 0.5 and 1% (van Duijn et al. 1999). Another kind of ELISA format, lateral flow strip, utilizes applications of strips instead of microtiter walls and was commercialized for detection of CP4 EPSPS proteins in soybean, canola, cotton, and sugar beet (Fagan et al. 2001). It was used to screen the presence of this protein in GM seed extracts and chromatographic fractions in development of mass spectrometry detection of this protein in GM soy and maize (Ocana et al. 2007). For determination of the same protein in GM maize flour, an electrochemical immunomagneticsensor was developed with a sandwich format using magnetic beads for immobilization of antibody and screen-printed electrode for detection (Volpe et al. 2006). The limit of detection for this biosensor was evaluated as 0.1 ng/ml and the relationship between the percentage of GM material and concentration of determined protein was reported.

Generally, immunochemical methods, which are very practical in application, have lower detection power than PCR based methods. Their main limitation is the limited availability of antibodies that have to be produced for each novel protein expressed by recombinant DNA in GM plants used as ingredients of food. They can be used successfully to screen raw material, although less efficiently in processed food where proteins can be degraded to a various degree.

Ever since early studies on mutations of maize and soybean, essential crops in food production on a global scale, the technological value of mutants that are important for plant breeders and plant scientists was the quality of proteins contained in plant material and their content. In early studies, protein distribution was investigated by successive extraction with water and other solutions (e.g., Mertz, Bates, and Nelson 1964; Robutti, Hoseney, and Deyoe 1974). Since the early 1990s, HPLC methods were successfully employed for protein distribution investigation (Paulis et al. 1991; Dombrink-Kurtzman 1994), and nowadays they are commonly used in the analysis of proteins in plant material used for human food production, which is evidenced in research on maize (Rodriguez-Nogales, Garcia, and Marina 2006a; Rodriguez-Nogales, Garcia, and Marina 2006b) and soybean (Saz 2007) proteins. For this purpose, size-exclusion HPLC, especially reversed-phase HPLC, methods are applied. These methods were widely employed for studies of nature and quantity of proteins in the early 1990s, and it was shown that HPLC can also be used for the study of zein proteins between parent maize and opaque maize mutants (Paulis et al. 1991, Dombrink-Kurtzman 1994). Reversed-phase HPLC utilizing perfusion columns was successfully employed for the characterization of commercial maize products for human consumption and differentiation between products with different technological processes (Rodriguez-Nogales, Garcia, and Marina 2006a). The HPLC methods can be successfully applied to identification of different plant genotypes (Smith and Smith 1987; Lookhart 2003). In HPLC analysis of European and North American maize inbred and hybrid line, data processing using linear discriminant analysis enabled classification of examined lines according to their geographical origin (Rodgriguez-Nagales 2006b). This large variety of earlier applications was the basis for the investigation of possible protein applications profiled with RP-HPLC methods for identification and characterization of transgenic and non-transgenic maize and soybeans. They were the subject of a series of papers from the research group of Marina (Rodriguez-Nogales et al. 2007; Rodriguez-Nogales et al. 2008; Garcia 2009). They described applications of HPLC systems with perfusion and monolithic

columns, UV or fluorimetric detection, and experimental data processing with various multivariate chemometric methods for identification and quantitation of GM foods. As far as detection is concerned, chromatograms with the largest number of resolved signals, which means potentially with the largest information content, was obtained for UV detection at 280 nm (Garcia 2009). The comparison of two kinds of columns employed indicated faster separation on the perfusion column, while for monolithic ones of different fractions of proteins, larger number of signals is recorded (Rodriguez-Nogales et al. 2007). Figure 4 shows comparison of chromatograms for extracts of non-transgenic and Bt Aristis maize seeds obtained with monolithic columns. Protein fractionation was obtained by extraction with different solvents and all fractions of transgenic sample signals were smaller and were not recorded at all at given detection conditions. Developed methods were also applied for

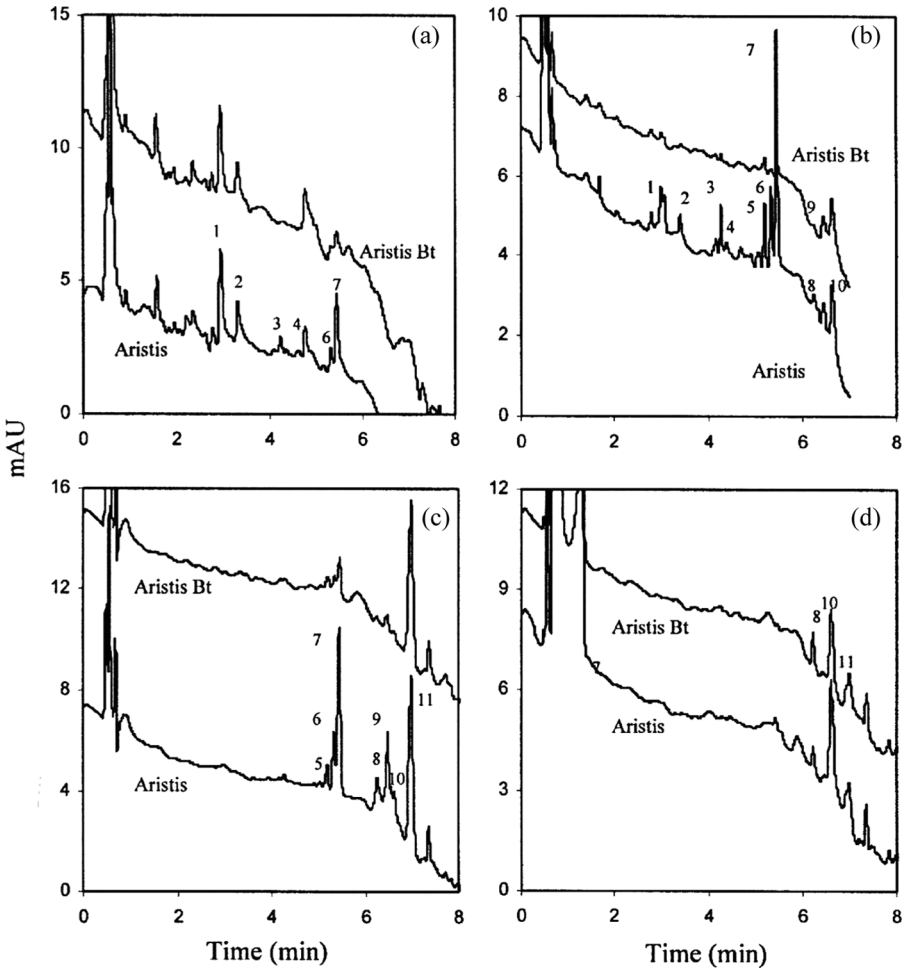

Figure 4. Monolithic RP-HPLC chromatograms of albumin (a), globulin (b), prolamin (c), and glutelin (d) fractions from Aristis and Aristis Bt (Rodriguez-Nogales et al. 2007).

quantitative determination of transgenic Bt maize in maize flour mixtures with the use of various chemometric methods for experimental data processing (Rodriguez-Nogales et al. 2008). The best results of all examined methods expressed by standard errors of prediction were reported for the linear regression method.

A high-performance separation method that usually allows better efficiency of separation in the liquid phase than HPLC is capillary electrophoresis (CE). It is widely employed with various detections in protein analysis, including maize proteins (Rodriguez-Nogales, Garcia, and Marina 2006a; Rodriguez-Nogales, Garcia, and Marina 2006b) and soybean proteins (Saz 2007). Its main obstacle in protein analysis is the problem of protein attachment onto walls of silica capillaries, but various preventive strategies are being developed to minimize this interference (Stutz 2009). In recent years, papers on the application of CE have been published on the identification of transgenic plants and foods containing GMOs. In the CE system with UV detection, several varieties of soybean were characterized and attempts were made to use this method for differentiation of transgenic and non-transgenic samples (Garcia-Ruiz et al. 2007). Example electropherograms for the extraction of transgenic samples and two non-modified products at 280 nm are shown in Fig. 5a. As one can see in all samples, practically the same signal is recorded; however, their magnitude is different and by the use of discriminate analysis based on signals 2, 4, and 5, 100% correct classification was obtained for differentiation of transgenic samples. Additionally, application in BGE laboratory-synthesized dendrimers with silicon cores allowed better differentiation recorded profiles of protein extracts, as well as a change of magnitude for several of them; also, some signals were characteristic of one kind of sample only (Fig. 5b) (Latoszek et al. 2009). In the aforementioned CE works, no attempts were made to identify separated proteins. In an Application Note of Agilent, the CE chip (Protein 200 Plus chip 14–200 kDa) and Agilent 2100 Bioanalyzer with LIF detection, characterization of transgenic seedless soybean by protein expression was described with measurements of primary seed storage proteins β-conglycynin (7S) and glycyninin (11S) (Dempsey and Jensen 2003). The identification of transgenic soybean was based on the concentration ratio of both target proteins.

The largest content of information on protein profiles for evaluation of genetic modification of food can potentially be expected in the application of the most important tools of modern proteomics, mass spectrometry. In recent years, intense progress in plant proteomics can be observed (i.e., Corpillo et al. 2004; Park 2004). Mass spectrometry was employed, for instance, in the comparative study of transgenic and non-GM potato (Careri et al. 2003). Protein fractionation using gel filtration, anion-exchange chromatography, and gel electrophoresis hyphenated to mass spectrometry was used for detection and characterization of the transgenic CP4 EPSPS present in RR-soy and maize (Ocana et al. 2007). Measurements were carried out in NanoLC/nanoESI-QTOF MS. Using MALDI-TOD MS, a tryptic peptide map of the examined protein was obtained. General analytical strategy based on enzymatic digestion of the GM protein and its identification showed comparable transgenic peptides map from GM soy and maize, and allowed identification of CP4 EPSPS from 0.9% soybeans. It was noted that soybean is of great agronomic and economic importance, but so far no complete genome sequence is known. Research towards a soybean proteome map should permit fast comparison of soybean cultivars, mutants, and transgenic lines (Komatsu 2009).

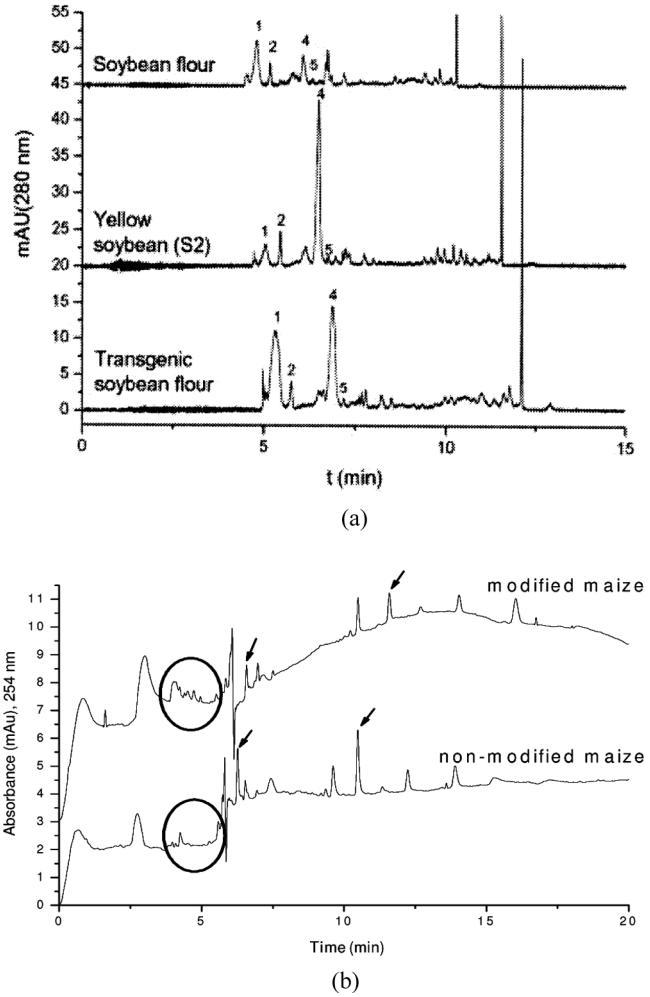

Figure 5. (a) Electropherograms corresponding to the injection of the protein extracts obtained from a commercial soybean flour (~40 mg/ml), a yellow soybean (~60 mg/ml), and a transgenic soybean flour (~60 mg/ml) using a solution of water/CAN(75:25 v/v) with 0.3% v/v acetic acid (Garcia-Ruiz et al. 2007); (b) Differences in electrophoretic profiles of protein extracts from corn (300 mg/g). Extraction solvent: ACN:H_2O (25:75) + 0,3% HAc. BGE: 80 mmol · L-1 phosphate buffer pH 2,5, 5% ACN, 0,01% dendrimer D16I; 25°C, −20 kV (Latoszek et al. 2010).

Mass spectrometry in recent years was also applied for detection in CE separations for determination of zein proteins in conventional and transgenic maize (Erny, Marina, and Cifuentes 2007; Erny et al. 2008). Both detections with ESI-MS as well as with ESI-TOF-MS did not exhibit significant differences in the presence of the zein proteins composition of three main lines and their corresponding transgenic lines. The CE-ESI-TOF-MS system allowed the identification of larger number of proteins.

METABOLITE PROFILING

Expression of new proteins as a result of genetic modification may create disturbances in existing metabolic pathways in parent organisms and may cause formation of new metabolites, which can be, in some cases, used as markers for GMO identification. Knowledge of this change of events has been evident since the beginning of genetic research. Then, it is also understandable that genetic modification can potentially be in some cases a source of toxicity, a resistance to antibiotics, a potential allergenicity from consumption of FM foods, or changes in nutritional quality of food, content of vitamins, antioxidants, etc. (Taylor 1997; WHO 2005). Investigation of metabolites is, therefore, an especially important part of the aforementioned functional genomics, determining a possibly wide range link between a gene sequence and the function of the metabolic network. So far, such studies of multitargeted profiling were mostly carried out for clinical detection of human diseases (e.g., Duez, Kumps, and Mardens 1996). The amount of similar investigations for the identification and studies of effects of GM food on human and animal organisms quickly increased in recent years, to some extent due to the results of public controversies about risk assessment of GMOs. Investigation of metabolites related to GM food involves numerous groups of compounds that are known to interact strongly with human organisms (Table 1).

Flavonoids

Flavonoids are groups of secondary metabolites that are present to some degree in most, if not all, plants. As they are known for their health-promoting properties such as antioxidant activity, which prevents cardiovascular or coronary heart disease or chronic inflammation, the level of interest in in GM plants and food seems to be very understandable. The flavonoid biosynthesis pathway in plants is regulated in response to developmental status, nutrient status, and numerous environmental stimuli. These factors significantly affect expression of flavonoid biosynthetic enzymes (Kubasek, Ausubel, and Shirley 1998). In genetic engineering each change of genetic material leading to the formation of GMOs may in various ways affect changes of content of various metabolites, and, obviously, genetic modifications are designed in order to obtain a particular change of properties of the parent organism. In the case of flavonoids in tomato, certain modifications have been carried out; for example, altering pigmentary flavonoids, which play an essential role in flower and leaf color (Mol, Grotewold, and Koes 1998). Overexpression of the gene encoding in tomato of Petunia chalcone isomerase, the enzyme involved in flavonoid biosynthesis, resulted in obtaining fruits with increased peel flavonoids up to 78 fold (Muir et al. 2001). Investigations into the change of flavonoid content in GM plant materials in this work and other discussed in the following study are carried out with HPLC with various detection methods. A large increase of flavonoid content resulted, mainly due to an accumulation of rutin, but increased levels were also observed for quercitin glycosides and kaempferol glycosides in fruit peel. The phenotype segregated with the transgene and demonstrated a stable inheritance pattern from subsequent generations tested. Whereas levels of rutin increased during ripening, kaempferol rutinoside was present in small amounts during all stages;

naringenin chalcone, intermediate in the biosynthesis of flavonols, was absent in green peels but increased during coloring of the fruit and declined in overripe stages. Processed high-flavonol tomatoes demonstrated that 65% of flavonols were retained in the processed paste. As a result, these new varieties offer opportunities for tomato-based food products with improved health benefit properties. Flavonoids in tomatoes are mainly located in the peel of the fruit, but it was also shown that the flavonoid accumulation in tomato flesh can be achieved by means of simultaneous overexpression of the maize transcription factors *LC* and *C1* (Le Gall, DuPont et al. 2003). Using HPLC methods with DAD spectrophotometric detections, it was shown that, for a particular modified line, the total flavonoid glucoside content of ripe transgenic tomatoes was about 10-fold higher than that of the controls, and kaempferol glycoside accounted for 60% of this. Figure 6 shows results of the HPLC analysis of transgenic and control tomato extracts at three stages of maturity. Although Le Gall, DuPont et al. (2003) has dealt with so called intended effects of the genetic modification, another paper deals with the high resolution ^{1}H NMR which has been used to detect any additional effect on metabolite composition following the overexpression of *LC* and *C1* genes (Le Gall, Colquhoun et al. 2003). In examination of tomato transgenic lines with altered carotenoid content, however,

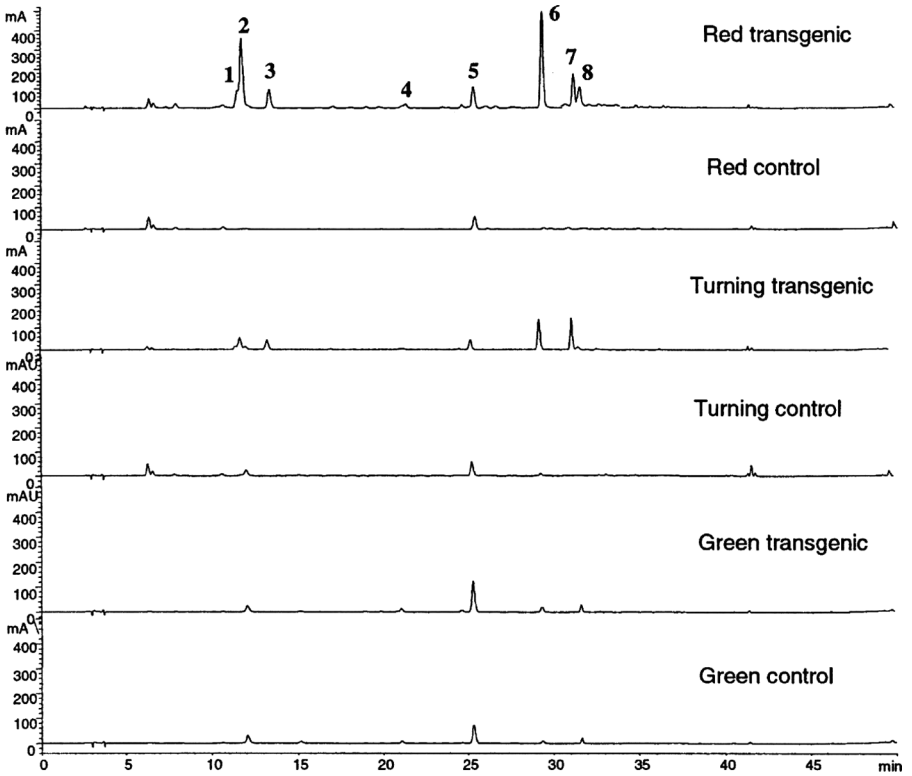

Figure 6. HPLC analysis of transgenic and control tomato extracts (mine 2059 +) at three stages of maturity (5 µl injection): Signal wavelengths at 270 nm (Le Gall, Dupont et al. 2003).

it was found that metabolic diversity in carotenoid content does not have a significant impact on flavonoid content (Long et al. 2006).

Changes of flavonoid content were also investigated with LC/MS for novel transgenic rice overexpressing anthocyanin synthase (ANS), which is one of the dioxygenases of the flavonoid biosynthetic pathway (Reddy et al. 2007). Examined transgenics showed approximately 10 and 4-fold increases in the ANS transcripts, which resulted in novel transgenic rice with a mixture of flavonoids, especially quercitin, and enhanced antioxidant potential. Using HPLC/UV measurements, the content of flavonoids in transgenic sugarcane leaves modified with two soybean proteinase inhibition genes was compared with non-modified plant samples (Colombo, Lancas, and H. Yariwake 2006). It was reported that, although no evident qualitative difference between flavonoids was found in the control and transgenic sugarcanes, the chemometric statistical comparison revealed that the total flavonoid content of control and transgenic sugarcane differed significantly.

One specific class of polyphenols, which are naturally occurring intermediates of flavonoid pathway are stilbenes. Their biosynthesis is controlled by the enzyme stilbene synthase, and the expression of the gene encoding this enzyme was obtained in several plants, including tomato, which caused the synthesis of trans-resveratrol and trans-piceid, leading to increase of total antioxidant capability (Giovinazzo et al. 2005). The transgenic tomato fruits were obtained by overexpression of a grapevine gene and were analyzed for the presence of stilbenes and flavonoids in either glycosylated or free forms by LC/MS with electrospray ionization in negative mode and reversed-phase HPLC with a narrow-bore column and DAD detection (Nicoletti et al. 2007). Stilbenes were not detected in the extracts of wild-type whole fruit, either at immature or at mature stages of ripening; they were found, however, in transgenic plants, both trans and cis forms of resveratrol and piceid. Some variations in the levels of rutin, naringenin, and chlorogenic acid was found in extracts from transgenic plants compared to control lines and seem also to be related to the genetic transformation. Earlier, in analysis of extracts of tomato fruits, the same transgenic line and trans-resveratrol-glucopyranoside was reported (Giovinazzo et al. 2005); also, not detected in either wild-type or vector-transformed plants. Using RP-HPLC also evidenced an increase in the levels of ascorbate and glutathione; the soluble antioxidants of primary metabolism were found as well as in the total antioxidant activity. Compared to previously cited work (Nicoletti et al. 2007), no significant effect on the content of naringenin was detected in transgenic plants.

Isoprenoids

Isoprenoids are also an important family of flavonoid compounds in the plant kingdom. This family includes over 20 thousands compounds such as carotenes, tocopherols, chlorophylls, xanthophylls, or plastoquinones that are derived from the same precursor isopentyl pyrophosphate. Their biosynthesis affects health-promoting properties of plants, hence a wide interest in determining and manipulating their levels in plants (Grusak and DellaPenna 1999). Therefore, there are numerous studies carried out on GM plants such as tomatoes with an increased content of these compounds in fruits.

The reversed-phase HPLC/DAD method employing C30 column was developed for simultaneous determinations of 40 analytes belonging to carotenes, xanthophylls, ubiquinones, toxcopherolos, and plastoquinones in plant extracts (Fraser et al. 2000). It was used in analysis of samples of transgenic and mutant tomato varieties with altered isoprenoid content. In determination of isoprenoids from transgenic ripe tomato fruit expressing an additional bacterial phytoene desaturase, a significant increase in β-carotene accumulation compared to the wild-type was found (Fig. 7), as well as some increase in lutein and cyclic carotenoids. Transgenic tomato lines expressing different genes show different content of biosynthetically related isoprenoids (Fraser et al. 2001), which means that enhancement of carotenoids is dependent on metabolic engineering of the pathway. For instance, ripe tomato fruits expressing the *Erwinia uredovora* phytoene desaturase (*crtI*) showed significant increases in β-carotene, but the carotenoid content decreased. The content of other related isoprenoids was not altered by manipulation to the carotenoid pathway. In another work of the same research group in metabolite profiling of carotenoid and phenol pathways in mutant and transgenic lines of tomato, it was

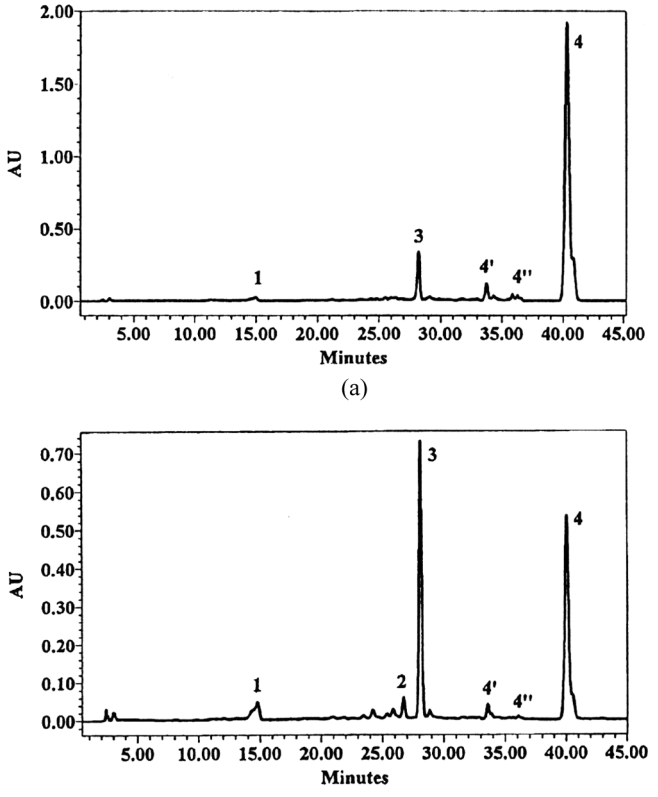

Figure 7. Chromatograms of (a) wild-type ripe tomato fruit, (b) transgenic crtl-containing ripe tomato fruit, recorded at 460 nm. Peaks are: (1) lutein; (2) α-carotene; (3) β-carotene; (4) all-trans lycopene; (4′) 13-cis-lycopene, (4″) 9-cis-lycopene (Fraser et al. 2000).

proven that altering the structural genes for carotenoid biosynthesis did not generally alter phenolic or flavonoid content (Long et al. 2006). Similarly to earlier works (Fraser et al. 2000; Fraser et al. 2001), determinations of isoprenoids in plant extracts were carried out using HPLC/DAD with reversed-phase C30 column, and also in normal phase system. Phenolic profiling was carried out with RP C18 columns. Those studies were carried out mostly in order to determine the possibility of occurrence of a cross-talk between pathways to terpenoids, phenolics, and alkaloids, which may affect a plant's secondary metabolism. Carotenoids, besides fatty acid content, total phenols, polyphenols, vitamin C, mineral composition, and total antioxidant activity were determined in assessment of the nutritional values of genetically modified wheat, corn, and tomato crops compared to the non-transgenic control (Venneria et al. 2008). Content of carotenoids, polyphenols, and vitamin C was determined by HPLC while for fatty acids GC with a FID detector was used. By this targeted nutritional analysis, it was shown that genetic modifications of examined lines did not produce significant changes in any of the nutritional and antioxidant components analyzed. This also includes, for example, transgenic maize, *Zea mays* (L.), containing the endotoxin Bt, which was developed to control pests of maize—corn borer. Reversed-phase profiles with C30 column of tocopherols and isoprenoid pigments (carotenoids, chlorophylls) were also measured for ten different commonly consumed fruits and vegetables that can be used to determine differences between genetically modified and non-transgenic plants (J. Burns, Fraser, and Bramley 2003). The same HPLC method was also applied to examine differences in the carotenoid content of ordinary citrus and carotenoid lycopene-accumulating mutants (Xu et al. 2006).

Other Metabolites as Markers of Genetic Modification

Among other groups of metabolites characterized in wild-type and transgenic plants used for human food, lipid and amino acids should be mentioned. Lipids (fatty acids, sterols, and tocopherols) were determined in extracts of conventional and GM maize Bt-176 (El-Sanhoty, Shahwan, and Ramadan 2006). Methyl esters of fatty acids were determined by GC with FID detector, sterols by gas liquid chromatography after saponification, while tocopherols by HPLC/UV in normal-phase systems. Total lipids were extracted in a Soxhlet apparatus, fractionated by column chromatography and analyzed for the presence of fatty acids, phytosterols, and tocopherols. The transgenic maize contained more polar lipids than the control

Table 1. Metabolites suggested as marker of genetic modification of selected plants

Plant	Marker	Reference
Tomato	β-carotene	Fraser et al. 2000
	Rutin	Muir et al. 2001
	Kaempferol glucosides	Le Gall et al. 2003a
	trans-resveratrol	Giovinazzo et al. 2005; Nicolletti 2007
Rice	Quercitin	Reddy et al. 2007
Maize	L-carnitine, stachydrine	Levandi et al. 2008

maize, but generally lipid distribution analysis showed a comparable composition of Bt-176 to that of the control maize.

Another group of metabolites determined in three varieties of conventional and transgenic maize, with micellar electrokinetic chromatography with LIF detection after derivatization with FITC, were chiral amino acids (Herrero et al. 2007). The optimized conditions of determination ensured a very efficient chiral separation. It is widely known in food chemistry that the content of D-amino acids is used to assess food quality, food adulteration, digestibility, and nutritional value of foods (Friedman 1999). Three lines of analyzed plants were grown under identical conditions. Enantiomers of Arg, Ser, Ala, Glu, and Asp, corresponding to the majority amino acids in maize extracts, were determined. Only in one line Tieter content of D-amino acid was it similar in conventional and transgenic plants. In the case of two other examined maize lines, significant differences ($P < 0.05$) were observed in a percent of D-amino acid content of Arg, Ser, and Asp between wild-type and transgenic lines, but only in one case (Ser in PR33P66 maize) D-amino acid content differed more than 100%. It seems that the observed differences may find potential use in identification of transgenic plants as GM food constituents.

Total Analysis of Metabolite Content

In an approach presented previously, schemes of analysis with high-performance separation methods were developed for selected groups of metabolites, belonging to a particular class of compounds of similar structure and properties. This allows tracing metabolic changes in transgenic plants used for food production and, simultaneously, providing a basis for selection of a marker for detection of GMOs in food for commercial purposes. Instruments of modern metabolomics involving efficient separation methods, identification of analytes with high resolution mass spectrometry methods, and data processing with advance chemometric methods allow profiling of much larger numbers of analytes in analyzed plant extracts, and hence provide more reliable differentiation between wild-type and transgenic plant material.

A separation method of outstanding resolution is capillary gas chromatography. In spite of some obvious limitations, GC/MS was applied, e.g., in metabolite profiling of four *Arabidopsis* genotypes (two homozygous ecotypes and a mutants of each type (Fiehn et al. 2000), and to analysis of metabolites in potato tuber soil-, in vitro-grown, and to transgenic lines modified in sucrose catabolism or starch synthesis (Roessner et al. 2000). In the first case, methanolic plant leaf extracts yielded 326 quantifiable compounds. The first step was fractionation into lipid and polar phases. The lipid phase, after derivatization, was used for analysis of total fatty acids, fatty alcohols, sterols, and aliphatics, while the polar one was for analysis of hydroxyl- and amino acids, sugars, sugar alcohols, organic monophosphates, amine, and aromatic acids. Of all analyzed metabolites, 101 polar and 63 lipophilic analytes were identified. It was found that the loss of activity of a single enzyme in mutation resulted in a dramatic change in the metabolite composition, as levels of 153 out of 326 metabolites were significantly different. In extracts of another mutant, 41 metabolites had significantly different levels. By the use of principal component analysis taking into account all metabolite data, it was possible to assign plant samples to groups defined predominantly by different genotypes.

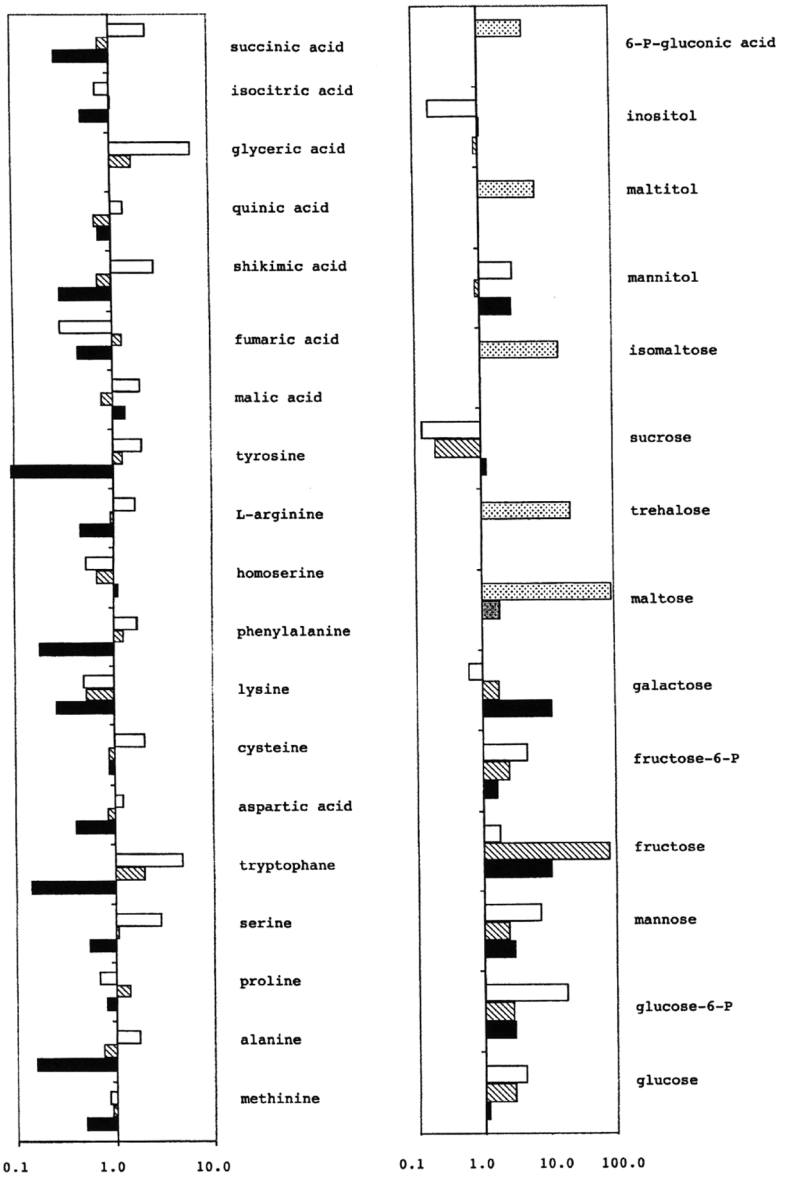

Figure 8. Comparison of metabolite in wild-type developing potato tubers with those in tubers of transgenic potato plants. Transgenic plants exhibiting antisense repression of ADP-glucose pyrophosphorylase (AGP93), black bars), or overexpressing a yeast invertase in the apoplast (U-IN1–33, grey bars) or in the cytosol (U-IN2–30, white bars). The quotients of the mean relative response ratio from transgenic tuber samples ($n = 6$) and from wild-type tuber samples ($n = 6$) ale plotted using a logarithmic scale. Only changes in metabolite levels that were evaluated to be significantly different from the wild type are shown ($P < 0.05$). Dotted bars indicate metabolites which were detected in the transgenic tubers but were below the detection limit in wild-type tubers. In these cases the numerical value of the detection limit of the respective compound in the soil-grown tuber samples was used in order to estimate a representative quotient. Metabolites that did not show significant changes between the four genotypes were leucine, isoleucine, beta-alanine, ornithine, valine, asparagines, glycine, glutamine, threonine, glutamic acid, and GABA (Roessner et al. 2000).

Pronounced differences in profiles of wild-type and mutants were found for some amino acids, indole derivatives, fatty acids, and amines, which was also reported in other works, and they can be employed as markers of genetic modifications. Authors of this work have mentioned, however, that the most dramatic changes occured in unknown metabolites, hence further analyses can be focused on a small number of compounds and structure elucidation.

In the second example mentioned previously, GC/MS was employed for metabolite profiling in potato tuber extracts from non-genetically modified and transgenic lines and a total of 70 compounds were detected (Roessner et al. 2000). They included amino acids, other organic acids, sugars, sugar alcohols, and aromatic amines. Comparison of selected metabolite levels for three different mutants in relation to the wild-type plant showed in Fig. 8, indicates evident increased levels of disacchariudes such as maltose, isomaltose, and trehalose, which can be used as markers of genetic modifications.

For the purpose of total metabolite profiling, recent research shows that the application of CE-TOF-MS system was demonstrated for analysis of transgenic maize (Levandi et al. 2008) and soybean (Garcia-Villalba et al. 2008). In both cases, conventional plant materials were compared with transgenic samples. In the case of maize samples, 27 metabolites were detected and tentatively identified, mostly amino acids and small peptides. Example electropherograms recorded for wild and transgenic maize samples are showed in Fig. 9. By comparison of peaks area between

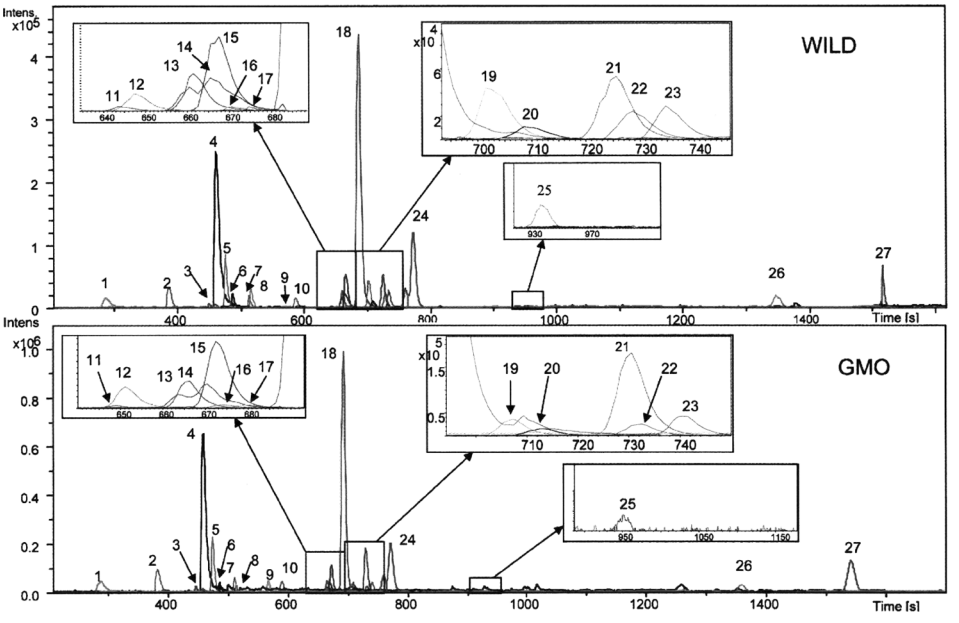

Figure 9. CE-TOF-MS extracted ion electropherograms of the 27 metabolites detected in PR33P66 and PR33P66 Bt maize. Experimental conditions: BGE composed of 5% formic acid at pH 1.90, total length of the capillary 80.0 cm; 50 μm i.d.; applied voltage 20 kV; volume injected 22 nL; temperature 30°C. Sheath liquid 2-propanol/water (50:50, v:v), at 0.24 mL/h, nebulizer pressure was 0.4 bar and dry gas conditions 4 L/min N2 at 180°C. MS scan range from 50 to 450 m/z (Levandi et al. 2008).

GMO and wild sample, the most pronounced differences were found for L-carnitine (peak 9 in Fig. 9) and stachydrine (L-proline-betaine) (peak 21 in Fig. 9), although in both cases very small signals were recorded. These two compounds are suggested by the authors as possible markers of GM maize. The same CE system was applied by the same research group to analysis of soybean extracts (Garcia-Villalba et al. 2008). In methanolic extracts of plant material 45 metabolites were detected and identified, including large numbers of amino acids, flavonoids, carboxylic acids, and peptides. For several detected analytes, some differences in their content between GM and wild-type samples were indicated, but the main qualitative difference was found for 4-hydroxy-L-threonine, which was not detected in GM samples.

CONCLUSIONS

This review presents possibilities of high performance separation methods for determination of genetic modification in food. All the described methods allowed for the determination of different kinds of changes, such as new metabolites or different proteins in bioengineered food. Until now, most PCR methods are official methods for controlling GM food, but it is quite possible that chromatographic and electrophoretic methods will become valuable instruments for identification of genetic modification.

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