

Ewa Poboży¹
Monika Filaber¹
Anna Koc¹
Juan F. Garcia-Reyes²

¹Department of Chemistry,
University of Warsaw, Warsaw,
Poland

²Analytical Chemistry Research
Group, Department of Physical
and Analytical Chemistry,
University of Jaén, Jaén, Spain

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Research Article

Application of capillary electrophoretic chips in protein profiling of plant extracts for identification of genetic modifications of maize

In this study, the chip gel electrophoresis with LIF detection was applied in protein profiling of fractionated and total extracts of maize standards. The sensitivity of such determinations can be enhanced by lyophilization of extracts or employing filtering and preconcentration with cutoff filters. Combinatorial peptide ligand library applied for sample processing prior to the electrophoretic analysis was, especially, an effective pretreatment step in the determination of low-abundance proteins. Several repeatable differences were observed for protein profiles between maize standards not containing the genetically modified organisms (GMOs) and those containing GMO, which can be potentially employed for identification of GMO in maize samples and foods of maize origin.

Keywords:

CGE / CE chip / Genetically modified organisms / Maize / Protein profiling

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1 Introduction

Determination of proteins in biological materials is one of the most difficult challenges of contemporary analytical chemistry for several reasons: the enormous amount of such analytes in natural materials, a very large span of their concentration level, and usually a very complex sample matrix. All these factors have stimulated a very intense progress of proteomics in the last decades with the use of continuously improved methods and instrumentation.

Miniaturization of instrumentation constitutes an important development trend in analytical chemistry in recent decades. Microfabricated devices, which are being developed for analysis of proteins, include different classes of measuring systems. Generally, all of them allow improving performance, increasing speed of determination, reducing costs, and reagent consumption; therefore, there is tremendous market potential for such devices [1–3]. The microdevices for application in proteomics have been especially intensively developed [4, 5], however, microfluidic devices are used in other fields of bioanalysis. In the course of further progress in this area, microfluidic systems are developed not only for electromigration measurements in liquid phase or gel in capillaries,

but also for flow-injection measurements by the integration of sample preparation procedures into microanalytical devices. In case of the chip-based immunoassay, the electrophoretic resolution is used to separate the products from the reactants of an immunological reaction [6]. Numerous designs of CE chips were reported for clinical and forensic analysis [7] as well as for food analysis [8, 9]. Although electrokinetically driven microfluidic devices are predominant, the pressure-driven flow microdevices or employing centrifugal pumping have been also developed. In CE chips, apart from the most common fluorescence detection, chemiluminescent and amperometric detections are used for protein determination. In separation of proteins, it was also demonstrated that chip separation can be shorter and more efficient than in standard setup [10]. An important development step is the design of more sophisticated microfluidic systems with both horizontal and vertical integration of parallel analysis lanes [11]. In case of protein analysis, a large attention is focused on coupling of CE chips with MS systems [12], but still the adsorption of proteins on the surface of CE chip channels is a major drawback of on-chip protein analysis [13, 14].

The Agilent 2100 Bioanalyzer, which was developed in collaboration with Caliper Technologies Co., is the first fully commercialized implementation of microfluidic technology [11]. The system can be used for fast analysis of proteins, DNA, RNA, cells, and recently it has also been employed for analysis of small molecules, such as amphetamines [15]. The separation method employed in these chips is CGE with the LIF detection. In recent years, about 20–30 research papers have been published annually on its different applications. An example of RNA determination can be, for instance, the evaluation of isolation methods and RNA integrity for

Correspondence: Dr. Ewa Poboży, Department of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland
E-mail: ewapob@chm.uw.edu.pl
Fax: +48-22-822 3532

Abbreviations: **Bt**, *Bacillus thuringiensis*; **CPLL**, combinatorial peptide ligand library; **Cry**, crystalline protein; **GM**, genetically modified; **GMO**, genetically modified organism

bacterial quantitation [16], while for DNA, it can be quantitative PCR analysis for fruit juice authentication [17].

Besides the determination of DNA and RNA, numerous applications of Agilent Bioanalyzer can be already found for the determination of proteins. There are three different chips designed for this purpose, which differ in the range of M_r of detected proteins and different sensitivity of detection. For example, applications can be indicated, for example, quality control of therapeutic proteins [18], determination of microalbuminuria, which is associated with nephropathy and cardiovascular disease [19], or impurity testing of antibiotics [20].

An increase in the cultivation of different genetically modified (GM) plants has been noted in recent two decades. Therefore, continuous improvement of analytical methods for the determination of transgenic material in foods is required. Moreover, the examination of biological and biomedical aspects of genetically modified organisms (GMOs) in foods [21] as well as the safety assessment [22] of GM foods are conducted. The most commonly used methods for analysis of GMO are the identification of the introduced DNA employing PCR techniques [23–25] and immunochemical determination of the encoded new proteins [26, 27].

In recent years, the main focus is on the use, for this purpose, of a protein or metabolite profiling, employing high-performance separation chromatographic and electromigration methods [28], and also MS-based analytical methodologies [29]. The chromatographic studies in this area, pioneered already in the beginning of 1990s, demonstrated measurable differences in the content of proteins between maize mutants [30, 31]. In the study concerning the proteome profile of the developing maize carried out using 2DE and 2D LC methods, 967 proteins were identified [32]. Then, in the LC/MS study of fractionated extraction of the maize standards, the presence of numerous proteins that can be considered as markers of GMO in plant material was demonstrated [33]. The electromigration methods in the format of the planar gel electrophoretic methods have found mainly their application in determination of amplified DNA chains. Combination of 2DE with LC/MS/MS may successfully be used for finding differences in proteome of transgenic plants, which was shown, for example, for soybean seeds [34] and maize [35]. However, in the latter work, it was shown that environment influence may affect protein expression more than gene manipulation. Recent years also brought several applications of CE with different detection for protein profiling in plant extracts or food products in order to find the difference in the content of proteins between conventional and transgenic maize [36–40].

Current analytical literature does not provide many examples of application of CE chips in the identification of GMO. Laboratory-made glass microchip with LIF detection was employed in DNA analysis of GM soybean [41]. The PCR products of both GM and non-GM maize were analyzed using a commercial CE system with LIF detection from Digital Bio Technology (Korea) [42]. Three alternative instruments, including the Agilent 2100 Bioanalyzer, were employed for the

detection of GMOs using PCR for food authenticity analysis [43]. These studies were carried out for samples of maize flour and soya. Comparison of Agilent 2100 Bioanalyzer with SDS gel CE to quantify relative amounts of 7S and 11S proteins from 20 soybean cultivars, so far, was the only application in the analysis of proteins in GMO [44].

The aim of reported studies was to examine the possibility of widening the arsenal of analytical methods for identification of GMO from routine PCR methods and immunoassays to CE methods of profiling proteins. This is because the altered expression of proteins is an evident attribute of genetic modification of living organisms. For this purpose, the chip format of CGE with commercially available instrumentation with LIF detection and optimized kits for analysis of proteins in different M_r ranges will be employed for the first time. Commercially available maize standards with different GMO content up to 5% (m/m) were used as samples for this stage of conducted studies. For proteins separation, different protein chips, as well as various methods of sample processing prior to the CE chip measurements, were used and compared.

2 Materials and methods

2.1 Materials and instrumentation

Maize GMO standards ERM BF412 Bt11 (maize with transgene originated from *Bacillus thuringiensis* [Bt]) with different GMO levels (0–5% m/m) were purchased from Fluka (Geel, Belgium). Reagents for extraction: Tris was obtained from Merck (Darmstadt, Germany); EDTA, urea, hydrochloric acid (HCl), NaCl, and KCl were obtained from POCh (Gliwice, Poland); and DTT and CHAPS were obtained from Sigma (St. Luis, MO, USA).

ProteoMiner[®] Protein Enrichment Kit (163–3006) was purchased from Bio-Rad Laboratories (Washington, DC, USA). The Protein 80 and 230 kits were purchased from Agilent Technologies (Mountain View, CA, USA). Amicon Ultra-0.5 Centrifugal filter units (cutoff filters 3 kDa) were purchased from Millipore (County Cork, Ireland).

All reagents used in this study were of analytical grade. MilliQ water (Millipore, Bedford, MA, USA) was used for solution preparation. For ProteoMiner[®] procedure, the proteomics grade water from Sigma was applied. For ultrasonication, bath sonicator Sonorex TK 52 (Bandelin, Germany) was applied. For sample preparation, Microcentrifuge MPW-55 Med. Instruments (Warsaw, Poland), rotator RL 2002 JWE Electronic, and thermo block JWE Electronic (Warsaw, Poland) were also used. The lyophilization of extracts was performed using GEA Lyophil LYOVAC GT 2-E instrument.

QuickStix Strips Envirologix (Portland, ME, USA) for Cry1Ab (where Cry is crystalline protein) protein immunoassay test was used for rapid determination of genetic modification of the maize. For quantification, the strips were analyzed with QuickScan (Portland, ME, USA) system.

2.2 Chip-based CE

The chip-based separations and detection of the protein samples were carried out using Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) in combination with the Protein 230 and Protein 80 kits. Each kit includes 25 microfluidic chips and the following reagents: sample buffer, sieving matrix, dye concentrate, and a protein sizing standard. Detection was based on LIF of a fluorescent dye that was added to the polymer and noncovalently bound to protein–SDS micelles. The electrophoretic analyses of the samples and the chips priming were performed as in the original protocol used for protein analysis. Each analysis of ten samples plus ladder lasted <30 min.

Protein extracts and extracts after ProteoMiner, lyophilization or cutoff filtration procedures, and Agilent Protein kit protocol were used as the samples.

2.3 Sample preparation

2.3.1 Extraction of plant material

For all the extractions, 100 mg of maize GMO standards and 1 mL of solutions were used. In fractionated extraction, albumins were extracted with water; globulins with 50 mM Tris buffer of pH 7.8 containing 50 mM KCl, 5 mM EDTA, or 0.5 M NaCl solution; and zeins were extracted with 6 M urea with 0.1–0.3 M DTT. At each stage, samples were shaken at room temperature for 1 h, ultrasonicated for 5 min, and then centrifuged at 10 000 rpm ($15\,000 \times g$) for 5 min. Next, the supernatants were treated with the protein assay reagents.

Total maize proteins were extracted from GMO maize standards and followed the procedure proposed by Boschetti et al. [45]. A total of 100 mg of maize standards were extracted with 1 mL of solution containing 50 mM Tris pH 7.4, 50 mM NaCl, and 2% (m/v) CHAPS. The mixtures were shaken at room temperature for 1 h, ultrasonicated for 5 min, and then centrifuged at 10 000 rpm ($15\,000 \times g$) for 5 min. The obtained extracts were used for ProteoMiner protocol and finally treated with the protein assay reagents.

2.3.2 ProteoMiner® procedure

Following the manufacturer instruction for use of a ProteoMiner small-capacity kit, the activation of column was carried out by twice rinsing the bed with 200 μ L wash buffer. After each rinsing, the column was rotated for 5 min and then centrifuged for 1 min at 2500 rpm ($1000 \times g$). Each sample was centrifuged for 10 min at 8500 rpm ($10\,000 \times g$) prior to transfer to the column. Then 400 μ L sample was placed in the column, which was rotated for 2 h and after that sample solution was centrifuged and removed from the column. This was followed by twice washing the column with wash buffer, 5 min rotation, and 1 min centrifugation at 2500 rpm

($1000 \times g$) after each washing. Then column was washed with 200 μ L of DI water (DNase and RNase free) with 1 min rotation. In order to wash out the retained analytes, a rehydrated elution reagent (20 μ L) was added to the column for 15 min with gentle vortexing during that period of time. Then, eluted sample solution was centrifuged for 1 min at 2500 rpm ($1000 \times g$). The elution step was repeated three times.

2.3.3 Sample lyophilization

For this sample-processing step, a fractionated extraction was performed simultaneously for six 100 mg samples. Then six portions of each fraction were combined and the obtained 6 mL solution was lyophilized in vacuum and the obtained solid was dissolved in 1 mL DI water.

2.3.4 Using cutoff filters

In this sample-processing step, the Amicon Ultra-0.5 centrifugal filters were placed in Eppendorf vials, then 500 μ L of extract of each fraction was placed separately, and it was followed by 30 min centrifugation at 9300 rpm ($14\,000 \times g$). Then, filter was transferred to a new vial and mounted upside down, and centrifuged for 2 min at 2500 rpm ($1000 \times g$). The obtained sample volume was about 100 μ L.

2.4 Application of immunoassay

The strips were dipped directly into the sample extract and the results—easily seen as bright, clear lines—were determined in 2–5 min without any special reagent preparation. Next, the strips were analyzed with QuickScan system to obtain quantitative results.

2.5 LC/MS separation

Proteins were separated using RP–HPLC with gradient elution. Mobile phases A and B were watered with 0.1% (v/v) formic acid and ACN, respectively. The chromatographic method held the initial mobile phase composition (20% B) constant for 20 min, followed by a linear gradient to 50% B at 30 min, and 97% B at 35 min and held constant for 5 min at 97% B. The flow rate used was 0.1 mL/min. The LC/MS measurements were carried out using LC–IT–TOFMS (Shimadzu) system and Aeris WidePore 3.6 μ m XB–C18 column (150 $\times$ 2.1 mm). The system was equipped with an electrospray interface operating in positive-ion mode, using the following operation parameters: interface 5 kV, nebulizing gas flow 1.5 L/min, curved desolvation line temperature 250°C. Mass spectra were recorded across the range 100–3000 Da.

3 Results and discussion

3.1 Profiling of fractionated protein extracts

The basic strategy of this study was to employ Agilent 2100 Bioanalyzer with different protein chips for separation of proteins obtained from plant material standards using the fractionated extraction and compare results of electrophoretic measurements for standards with different content of GMO. Based on vast available literature on this subject, one can expect either the presence of proteins of relatively low M_r values of few kilodalton in maize samples [33] and large proteins with masses up to 130 kDa, which corresponds to Cry1Ab protein, expected in Bt-modified material [46]. The conditions of fractionated extraction were optimized, first of all, from the point of view of the magnitude of recorded signals. The selection of different chips not only allows the performance of the measurements in different mass range but also affects the signal magnitude and obtained resolution. Each measurement was repeated three times in order to determine the precision of mass measurements and signal magnitude. The main purpose of measurements was to find out, in recorded electropherograms, some regular and repeatable differences in protein profiles between standards without and with different GMO content.

As it is widely described in the literature, albumins were extracted with water, but in case of globulins and zeins, different extracting solutions were compared using 80 kDa chip. In case of globulins, the application of Tris buffer containing KCl and EDTA, in comparison to 0.5 M NaCl solution, allows to obtain two- to threefold larger signals and also much larger number of separated signals, which gives

a richer protein profile. For extraction of zeins, it is necessary to use aqueous-mixed solutions with organic solvents, such as ethanol or ACN. The reported earlier comparison of different extracting solutions shows the best yield of extraction of α -zeins with 70% (v/v) ethanol, while for β - and γ -zeins for 6 M urea solution [47]. Due to limited stability of gel used in chip capillaries toward ethanol solution, 6 M urea solution containing 0.2 M DTT, which allows the breaking of disulfide bridges in protein structure, was used for the extraction.

Comparison of the extraction yield was carried out for the maize standard without GMO material. In optimized conditions for this maize standard, the comparison of electropherograms recorded for different protein chips is shown in Fig. 1. For both groups of proteins, the evidently larger signals were recorded for an 80 kDa chip. The masses of resolved proteins, both for standards without and with 5% (m/m) GMO and for two used chips, are listed in Table 1. The number of resolved electrophoretic peaks for both chips is similar, although many signals obtained for 230 kDa chip are not recorded on electropherogram for chip of 80 kDa. One can also note that there are certain differences between electropherograms recorded for both the maize standards. For 80 kDa chip signal, 6.0 kDa of albumin fraction is present for the standard containing 5% (m/m) GMO only. For 230 kDa chip signal, 18.4 kDa albumin fraction and also 163 kDa globulin fraction were recorded only for maize standard without GMO (Table 1). In earlier LC/MS studies [33], large signals for the maize standard with 5% (m/m) GMO were also observed at 5.2 and 6.0 kDa, however, not for albumin fractions, but for globulin and zein-like glutelins, respectively. Signal recorded at 17.7 kDa detected for the maize standard with GMO was found also in zein-like globulins. A significantly

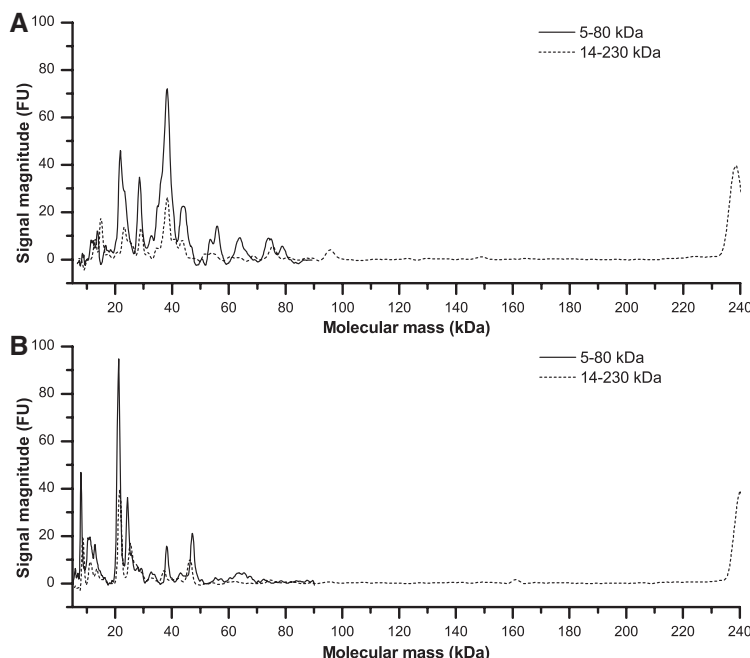


Figure 1. Electropherograms obtained for albumin (A) and globulin (B) fractions using different chips and maize standard without GMO.

Table 1. List of M_r (kDa) evaluated from electropherograms recorded for albumin and globulin fractions of maize standards with and without 5% (m/m) GMO content, using two different protein chips

0% GMO		5% GMO	
5–80 kDa	14–230 kDa	5–80 kDa	14–230 kDa
Albumins			
		6.0	
8.6		8.2	
12.5		12.5	
13.3		13.3	
14.9	14.0	14.8	14.4
	15.6		16.8
18.2	18.4	17.7	
19.9		20.7	
	21.7		22.2
23.2	23.9	23.1	24.4
	25.7		26.4
29.2	29.3	29.1	29.5
33.8	32.4	33.8	33.1
	35.8		36.5
39.3	39.6	39.0	40.1
	42.0		42.1
44.5	44.7	43.9	45.5
53.0		52.8	
55.0	55.1	55.0	55.6
61.7	61.9	61.2	62.6
	70.9		71.7
73.1		73.6	
78.9	79.4	78.8	79.9
	85.6		86.0
	93.4		93.6
	101.9		102.8
Globulins			
5.4		5.4	
6.1		6.0	
7.5		7.4	
11.1		10.8	
12.0		12.0	
14.2	14.4	14.0	14.4
22.8	22.4	22.8	22.2
25.6	25.8	25.6	25.7
30.3		30.1	
33.8	33.5	33.9	33.3
39.2	38.1	39.2	37.8
	43.1		43.0
47.5	47.1	47.4	46.9
	77.3		77.1
	163.0		

smaller number of well-resolved peaks were found for extracts of zeins. Because no qualitative and quantitative differences were found between non-GMO and 5% (m/m) GMO containing maize standards, the investigation of those fractions was discontinued at this stage. The additional argument for that was also much worse reproducibility of measurements with extracts of zeins.

Similarly, the comparison for the extracts of albumins and globulins using maize standards without and with 5% (m/m) GMO was conducted based on electropherograms recorded with the 80 kDa chip and high-sensitivity chip for the M_r range of 10–250 kDa. For albumin fraction, the magnitudes of signals recorded for both chips and number of resolved analytes are similar. Although for the high-sensitivity chip there were no signals of masses below 10 kDa, whereas some signals were recorded for 90 and 100 kDa. For this fraction, the only difference between standards without and with 5% (m/m) GMO with the high-sensitivity chip was the appearance of the signal at 27.9 kDa for standard without GMO. In the paper published earlier on proteomic analysis of GM maize [48], the presence of protein with M_r 29.1 kDa was reported, which was identified as endochitinase A and it was unique of non-GM maize hybrid flour. Signals for 28.4 kDa proteins detected only in the maize standard without GMO were also reported earlier [33], but only in fractions of zein-like glutelins and glutalins. They were attributed to 60S ribosomal protein L7–2 and peptide methionine sulfoxide reductase [32]. In the above-mentioned study [48], it was also shown that protein with M_r 32.3 kDa, which was identified as glucose and ribitol dehydrogenase homolog, was unique for Bt maize. This signal was not detected either in reported measurements or earlier published papers [33, 35]. In those measurements, no qualitative differences in number of recorded peaks were observed between maize standards without and with 5% (m/m) GMO.

3.2 Modification of sample processing prior to measurements with CE chip

During these studies, several different attempts were undertaken in order to improve the sensitivity of detection employing some additional off-line sample-processing steps prior to the electrophoretic measurements.

Following the procedure described in Experimental, the sample processing with cutoff filters was employed for extracts of albumins, globulins, and zeins for maize standards without and with 5% (m/m) GMO. Electrophoretic measurements were carried out with the 230 kDa chip. For all examined fractions, the application of the cutoff filtering results in three to five times increase of signal magnitude. The comparison of results obtained for albumins and globulins for maize standards without and with 5% (m/m) GMO, also in this case, does not show evident qualitative differences (18 signals recorded for albumins and 10 separated signals for globulins). Only in some cases, the reproducible differences between signal magnitudes, exceeding the bias of results attributed to the precision of measurements, were obtained.

Lyophilization of the analyzed extracts prior to measurements was the next additional sample-processing step. Compared to nonlyophilized samples, two- to threefold enhancement was observed for the signals in albumins and globulins fractions of maize standard without GMO. Then the results obtained for samples with lyophilization and cutoff 3 kDa

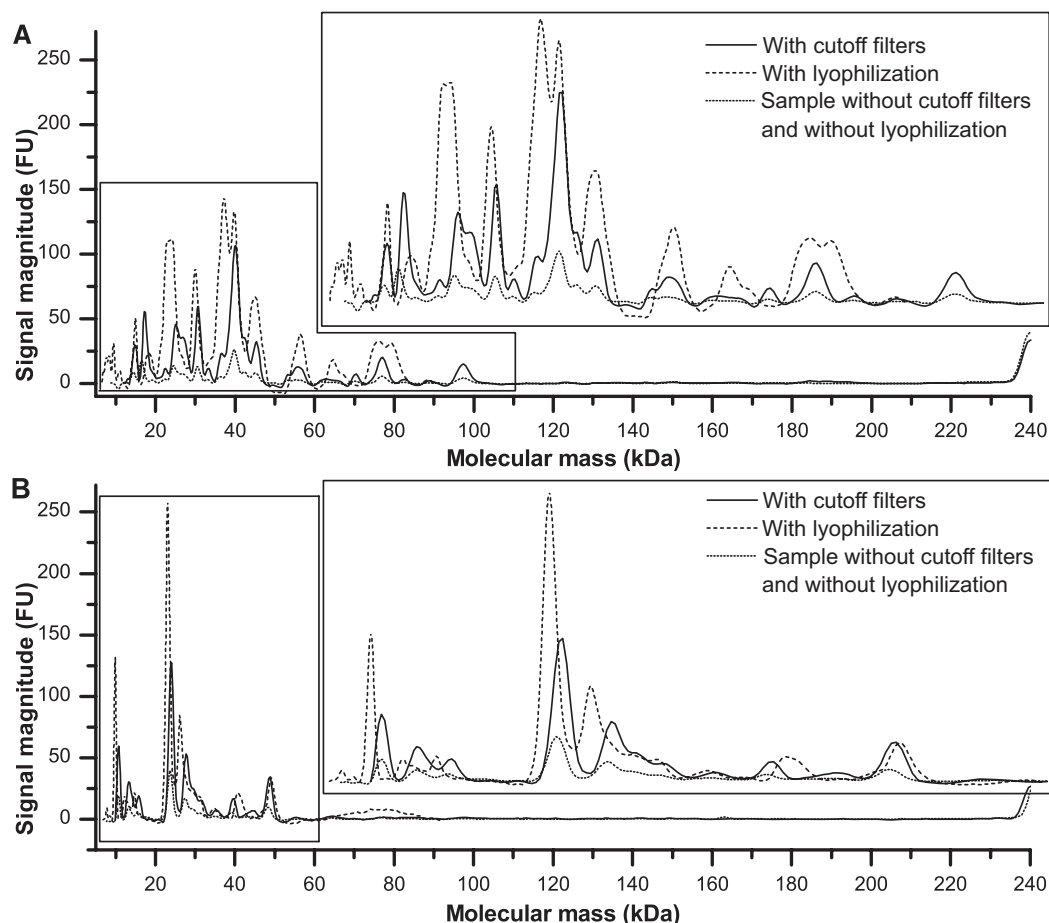


Figure 2. Comparison of effect of lyophilization and cutoff filtering on electropherograms obtained for albumin (A) and globulin (B) fractions for maize standard without GMO using the 230 kDa chip.

filtering were compared with the results for samples without preconcentration step. As it is shown in Fig. 2, larger signals for albumins and globulins were recorded using lyophilization, hence lyophilization was used as sample preparation in our further studies.

Using the optimized extraction conditions and lyophilization of extracts, the protein profiles of albumins and globulins were examined for maize standards with different content of GMO. Based on three repeated measurements, for each extract the precision of M_r measurements as well as signal magnitude measurements for two arbitrarily selected signals were evaluated. The obtained data about precision are shown in Table 2 and electropherograms in Figs. 3 and 4. These types of measurements were carried out parallel for both 80 and 230 kDa chips. The results obtained for both chips were very similar. The precision of mass measurements for separated proteins (usually with $RSD < 1\%$) is about ten times better than precision of signal magnitude measurements. A worse precision in the latter case can be attributed to a limited precision of the pipetting 4 μ L volume of analyzed extracts during the loading of chip. The precision of mass measurements is a function of precision of conducting all stages of measurements on a chip and obtaining results that can be

Table 2. Precision of electrophoretic determinations of selected albumins and globulins in extracts of maize standards

Fraction of extracted proteins	M_r of proteins taken for calculation	Type of used protein chip (kDa)	Range of RSD values (%) for M_r measurements	Range of RSD values (%) for signal magnitude
Albumins	23.1; 28.7	80	0.25–0.60	3.5–8.6
Albumins	14.6; 27.5	230	0–0.56	5.0–11.4
Globulins	8.7; 23.0	80	0–1.8	0.9–11.3
Globulins	11.4; 21.8	230	0–1.0	2.0–8.2

The RSD values are calculated for three repetitions of recording of selected proteins.

considered as satisfactory. The main purpose of this comparison was to search for differences in protein profiles, which might be employed for identification of GMO in analyzed maize samples and possibly also in products of their processing in food production. In the case of electropherograms recorded for albumins with both chips (Fig. 3), there were no signals found, which are present only in maize standard without GMO or with standards containing GMO, however,

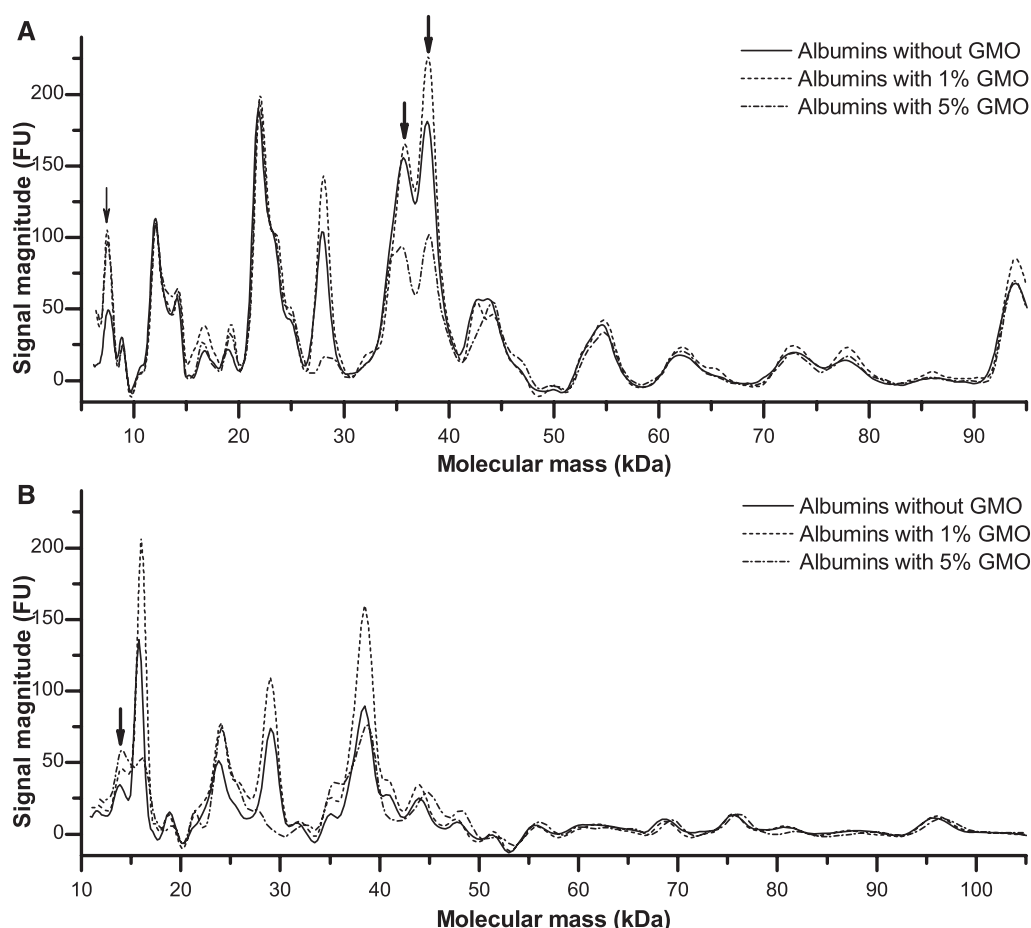


Figure 3. Comparison of electropherograms recorded for albumin fraction of maize standards without GMO and with 1 and 5% (m/m) GMO after lyophilization using 80 kDa (A) and 230 kDa (B) chips. Arrows show differences in signal magnitude for different mass standards.

several reproducible differences in the signal magnitude were found. For instance, for the 80 kDa chip a big difference in signal magnitude was recorded for signal 6.7 kDa, although a similar increase of signal magnitude was measured for both maize standards with 1 and 5% (m/m) GMO (Fig. 3A). Similar observation was made in evaluation of data shown in Table 1 for albumin extracts. Large differences are also observed for signals 36.0 and 37.5 kDa, but in this case there is no monotonous increase of signal magnitude with increase of GMO content. For the M_r range close to those masses, five different proteins were listed among the differentially expressed proteins between transgenic maize and isogenic control from 2DE measurements [35] namely protein disulfide isomerase (36.3 kDa), granule-bound starch synthase 1 (36.7 kDa), adenosine kinase 2 (35.7 kDa), cytosolic 3-phosphoglycerate kinase (36.8 kDa), and fructokinase-1 (35.5 kDa).

Distinct changes of albumin signals were also observed for measurements with 230 kDa chip (Fig. 3B), but in this case for protein 13.3 kDa, the signal changes were monotonous with increase of GMO content. In this case, three different proteins of similar M_r values were identified among the differentially expressed proteins in 2DE measurements [35], namely

late embryogenesis abundant protein EMB564 (12.2 kDa), globulin 2 (13.9 kDa), and thioredoxin h2 protein (13.3). It should be also admitted that in LC/MS measurements [33], a very strong signal at 14.8 kDa was observed in the maize standard with 5% (m/m) GMO and not in standard without GMO, while signal at 13.0 kDa was observed only in standard without GMO. For both used chips, there were 20 signals recorded with different degree of their resolution. The proteins of M_r in the range 6.0–6.5 kDa detected only in maize standards containing GMO were also detected in LC/MS measurements [33], however, they were found in fractions of zeins and glutelins, and not in albumins. The largest signal changes in albumin fraction were found for 15.0 kDa protein with 230 kDa chip, where for maize standard with 1% (m/m) GMO a 50% increase of signal magnitude was observed compared to maize standard without GMO, but for maize standard with 5% (m/m) GMO an evident drop of signal of about 75% was observed compared to standard without GMO was found. Those changes were not observed for 80 kDa chip.

The electropherogram with a smaller number of resolved signals was recorded for globulin fractions of the same maize

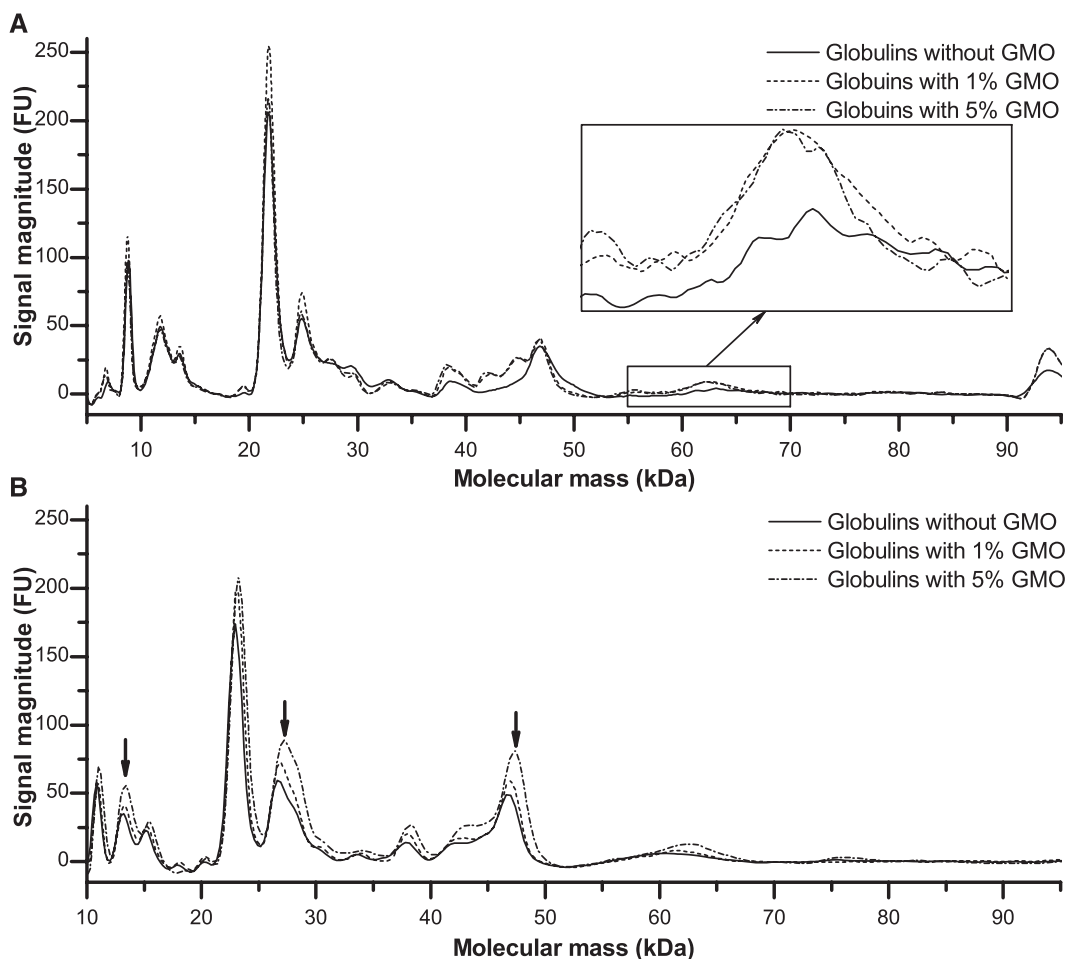


Figure 4. Comparison of electropherograms recorded for globulin fraction of maize standards without GMO and with 1 and 5% (m/m) GMO after lyophilization using 80 kDa (A) and 230 kDa (B) chips. Arrows show differences in signal magnitude for different mass standards.

standards (Fig. 4). With the 80 kDa chip, the signals occurring only in maize standards containing GMO were recorded at 42.0 and 44.0 kDa, however, practically they have the same magnitude for both maize standards with 1 and 5% (m/m) GMO. These masses were not observed in 2DE measurements [35], although they give evident signals in CE chip measurements. These proteins were not resolved with the 230 kDa chip (Fig. 4B). In this case, increase of some signals, compatible with the content of GMO, was observed. It concerns especially signals at 11.3, 25.3, and 46.0 kDa (marked by arrows). Remarks about signals close to 11.3 kDa were made already above. Proteins of masses 26.9 and 27.7 kDa were found only in maize standards with GMO using LC/MS, and also for extracts of globulins [33] and for M_r values in the range 24.9–26.1 kDa, five different proteins were listed among the differently expressed proteins obtained in 2DE measurements [35]. Triosephosphate isomerase of 27.0 kDa, detected in two mentioned works [33, 35], was also found as overexpressed protein in Bt maize with respect to wild-type one [48]. The protein 46.9 (guanosine diphosphate dissociation (GDP) inhibitor protein OsGDI1 [*Oryza sativa*]) was

found also using 2DE method [35]. All these signals marked by arrows in Fig. 4B can be practically considered as markers of the presence of GMO in analyzed sample with the use of reported measurements. The signal 63.0 kDa in Fig. 4A can be assigned to Cry1Ab protein, as this mass is close to active δ -endotoxin of 65 kDa [49].

3.3 Application of combinatorial peptide ligand library (CPLL)

In applied and above-reported methods of improvement of the detection limits of proteins in plant extracts, it is assumed that the preconcentration of proteins takes place in the similar extent for all proteins present in analyzed sample. It is known, however, that hundreds or even thousands of proteins occur in plant extracts in concentration levels that differ by several order of magnitudes. One can also expect that proteins expressed by slight genome modification may belong to low or very low abundance proteins, hence, it is so difficult to identify them in simple procedures reported so

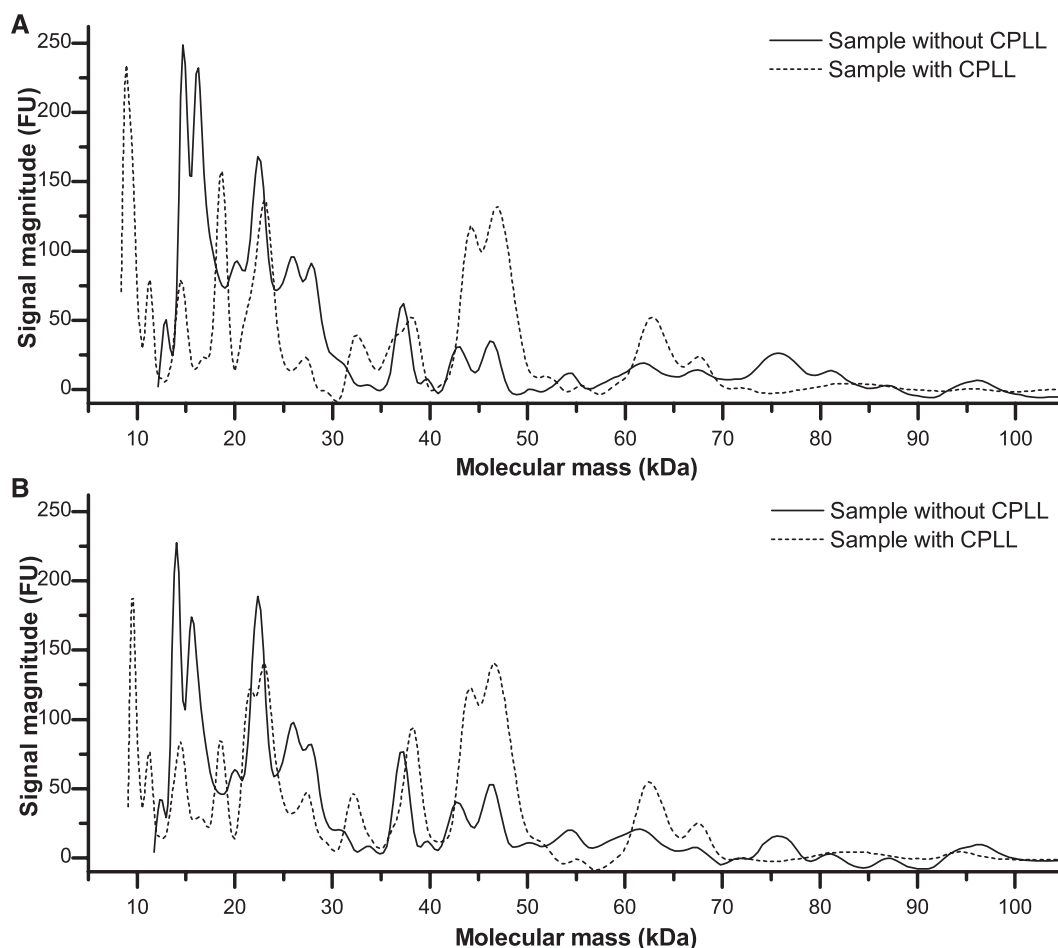


Figure 5. Effect of the application of CPLL as commercial ProteoMiner[®] kit on electropherograms recorded with the 230 kDa chips for total extracts of proteins from maize standards without (A) and with 5% (m/m) GMO (B).

far above. A helpful method in such situation for the target analysis of proteins can be immunoprecipitation of specific proteins developed for sensitive detection of proteins with the Agilent 2100 Bioanalyzer [50]. In this work, we employed a different strategy by using synthetic peptide library introduced already two decades ago [51]. It is widely used in modern proteomics as CPLL. As a result of the SPE of raw extract of the plant material, on the CPLL sorbent bed there are retained similar amounts of proteins that are present in raw extract at very different levels of concentration. Then, elution of the bed provides extract of proteins with much less biased level of concentration of proteins, so it reduces the concentration difference of proteins in biological liquids [52, 53]. Additionally, such a step allows the removing of numerous other interfering components of raw plant extracts, such as starches, polysaccharides, pigments, phenolic compounds, and secondary metabolites. Its application for capturing the “hidden proteome” [45] was already so widely recognized that it has been commercialized as the ProteoMiner[®] kit by Bio-Rad [54], which has already found several applications in plant proteomics, for example [45, 55].

In this work, a CPLL-based pretreatment step was used employing the procedure, which was reported earlier with commercial ProteoMiner[®] kit [45]. The maize standards were extracted with Tris buffer containing NaCl and CHAPS (see Experimental) and then obtained solutions were extracted using ProteoMiner beads. The eluted proteins were introduced into the CE chip. Figure 5 shows comparison of the electropherograms for rough extract from maize standards with extract after processing with CPLL. It is evident for both maize standards that proportion of recorded signals in whole range up to about 100 kDa has changed significantly. Numerous large signals recorded in measurement without application of CPLL are much smaller after CPLL treatment, and reverse situation was observed for many small signals. There are some cases where new signals were recorded after CPLL sample processing.

The results of application of that pretreatment step to the analysis of maize standard without and with 5% (m/m) GMO are illustrated by recorded electropherograms in Fig. 6 for 230 kDa chip. Four times repetition of measurements indicate their satisfactory precision (Fig. 6A and B).

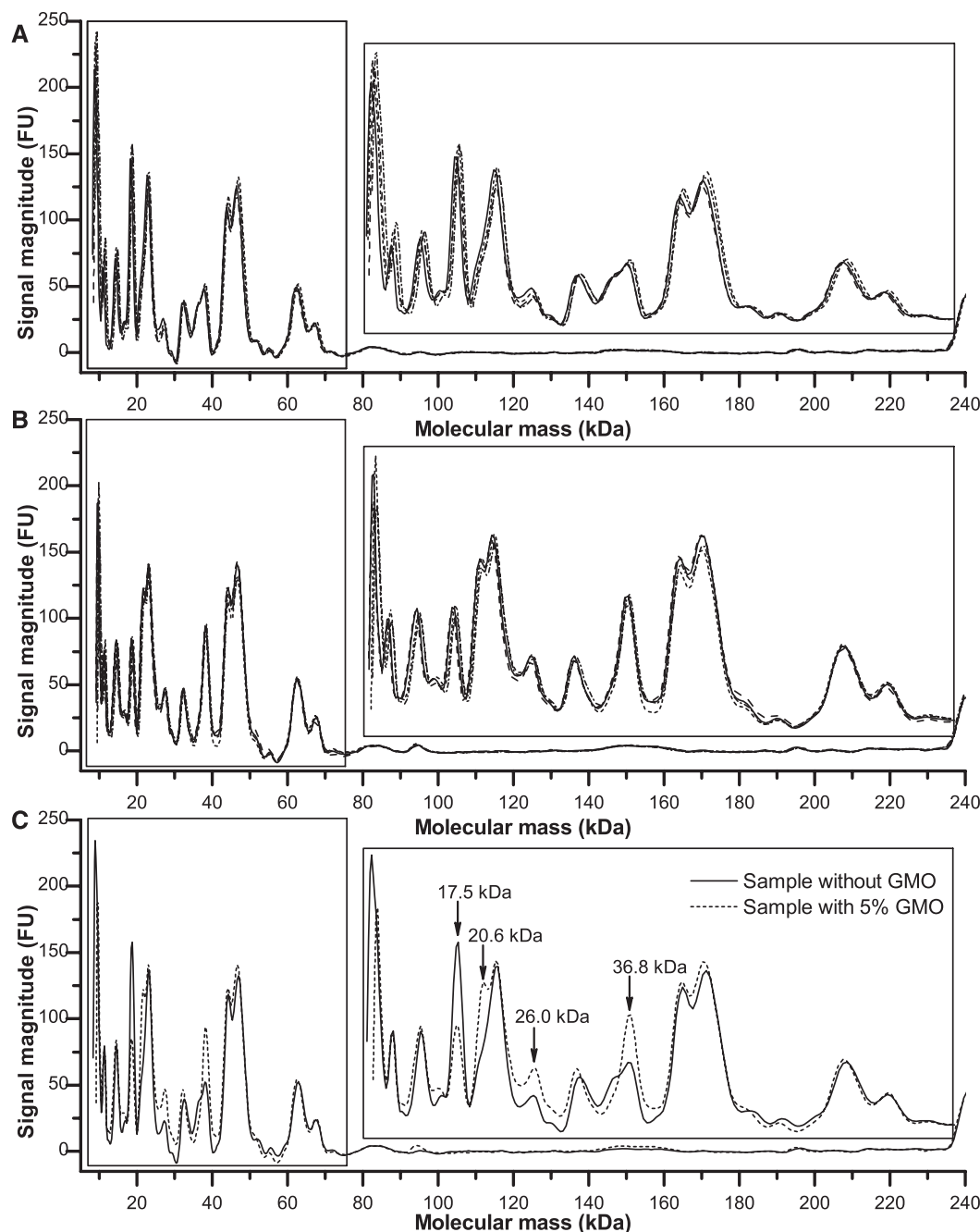


Figure 6. Electropherograms recorded in measurements with pretreatment of analyzed samples with ProteoMiner®. (A) Four repeated recordings for extract of maize standard without GMO, (B) four repeated recordings for extract of maize standard with 5% (m/m) GMO, (C) comparison of selected recordings for maize standard without and with 5% (m/m) GMO. Arrows indicate a difference observed between analyzed maize standards.

For signals at 13.8 and 17.5 kDa, the precision (RSD) of mass evaluation was from 0.3 to 1.0%, while for signal magnitude it was from 2.7 to 8.2%. The comparison of signals recorded for non-GMO and containing 5% (m/m) GMO standards (Fig. 6C) shows several reproducible differences. Evidently larger signal for the maize standard without than with 5% GMO was recorded at 17.5 kDa. In LC/MS

measurements signals 16.8 and 17.6 kDa detected in fractions of globulins and zein-line like glutelins, respectively, were not found in maize standard with 5% (m/m) GMO, but were detected only in standard without GMO [33]. Signals of similar masses 16.3 and 16.5 kDa assigned to glutathione peroxidase and cystathionine- β -synthase domain containing protein, respectively, were reported among the differently

expressed proteins in 2DE measurements [35]. Then signals significantly larger for maize standard with 5% (m/m) GMO than without GMO were detected at 26.0 and 36.8 kDa. These signals were already indicated in other measurements above and these proteins are listed among differently expressed proteins in 2DE measurements (globulin 2 26.1 kDa, triosephosphate isomerase 25.3 kDa, and ATP-dependent CLp protease 26.2 kDa, and also protein disulfide isomerase 36.3 kDa, granule-based starch synthase 1 36.7 kDa, adenosine kinase 35.7 and cytosolic 3-phosphoglycerate kinase 36.8 kDa) [35]. The signal recorded only for maize standard with 5% (m/m) and not detected in electropherograms of standard without GMO corresponded to M_r 20.6 kDa. Also, in this case one can find several proteins of similar M_r on the list of differently expressed proteins from 2DE measurements [35]. They were reported as dehydrin (20.0 kDa), globulin 2 (20 kDa), and vicilin-like embryo storage protein (20.2 kDa). From LC/MS measurements, the protein detected with closest masses 21.6 kDa were detected in fraction of zein-like glutelins only in maize standard with 5% (m/m) GMO and not detected in extract from standard without GMO.

3.4 Determination of Cry1Ab protein

The main purpose of genetic modification of maize with transgene originated from Bt is the obtaining of the expression of protoxin Cry1Ab in GM plant. Depending on the source of modified material, M_r values 131 or 91 kDa are assigned to this protein when it is a product of the expression of transgene MON 810 Cry1Ab [56]. Cry protoxins of Bt act as toxins in sensitive insect larval groups. As a result of different biochemical processes, a final toxic fragment of Cry1AB protoxin may possess a molecular size between 60 and 70 kDa. In another source [49], one can find that most commonly the protoxin Cry1Ab is considered as being either in the crystalline form or the nonactivated form at 135 kDa, while δ -endotoxin of 65 kDa is more soluble and active. As it was shown, for example, for Cry1Aa protein, the molecular mass evaluated by different methodologies can be in the range from 37.9 to 58.6 kDa [57]. Determinations of Cry1Ab reported in the literature are carried out practically only using immunoassays of different formats, both in plant materials [54, 58], water, and in soil extracts for tracking that protoxin

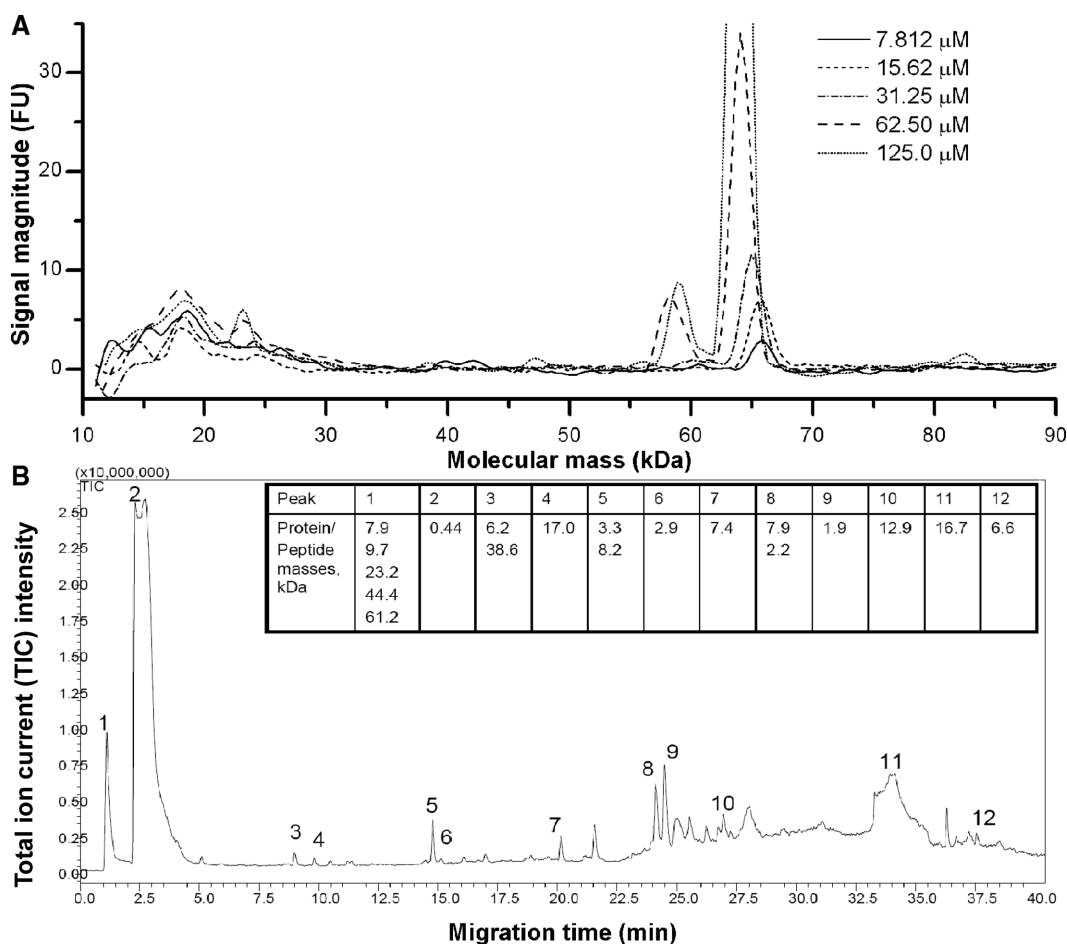


Figure 7. Electropherograms obtained with the 230 kDa chip for extract of proteins from standard preparation of Cry1Ab (A) and total ion current chromatogram for the solution of Cry1Ab preparation in 50% (v/v) glycerol using LC/MS (B).

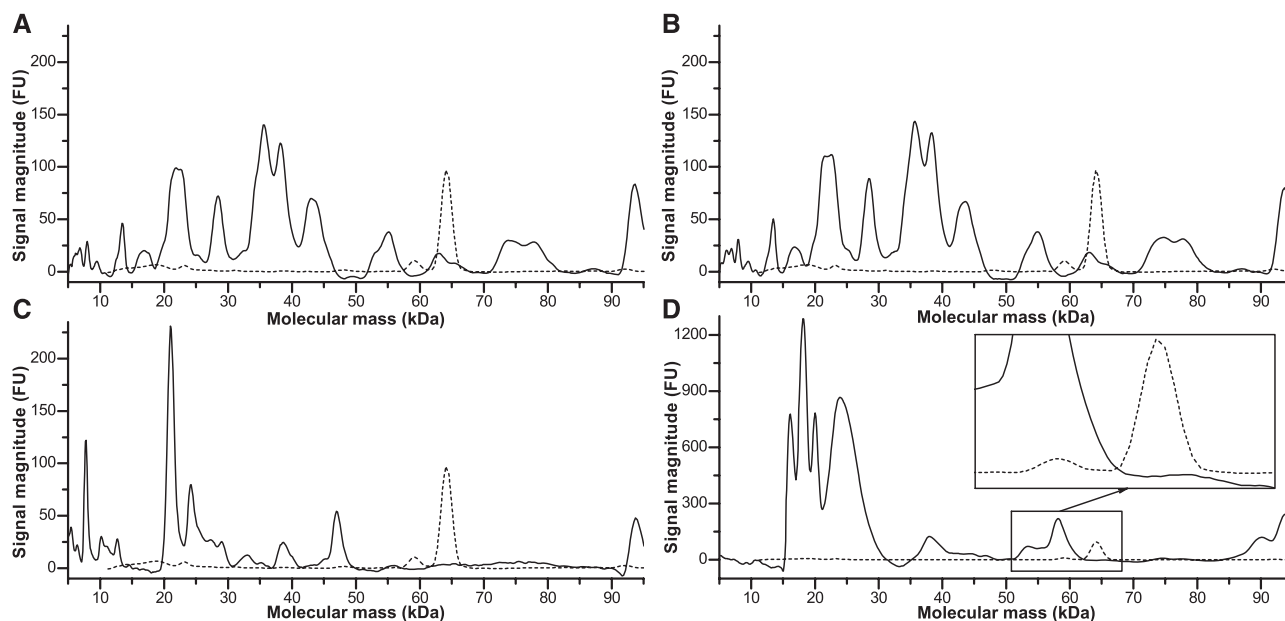


Figure 8. Comparison of electropherograms recorded with the 80 kDa chip for lyophilized extracts of maize standards with electropherograms of extract of Cry1Ab protein standard. (A) Extract of albumin fraction of standard with 5% (m/m) GMO, (B) extract albumin fraction of standard without GMO, (C) extract of globulin fraction of standard with 5% (m/m) GMO, (D) extract of zein fraction of standard with 5% (m/m) GMO.

in environment [49, 59], or in blood plasma of cows fed Bt maize [60].

As another attempt of finding a specific marker of the GMO presence in transgenic maize, a search was undertaken for Cry1Ab protein signal in electropherograms recorded with examined chips in maize standards. Additionally, the presence of GM materials in the tested maize standards was conformed to the commercial immunoassay kit. For CE measurements, Cry1Ab solution was used, which is employed as standard in immunoassay. The examination of the composition of the commercial Cry1Ab standard from Nuscana by chip electrophoretic measurement with 230 kDa chip shows the protein of 63.0 kDa mass being the main component and also smaller reproducible signal at 58.4 kDa, but also numerous smaller signals in the range from 10 to 25 kDa (Fig. 7A). This was also confirmed by LC/MS measurements, where, besides the large signal for 61.2 kDa, numerous other signals mostly in the range up to 23 kDa were recorded (Fig. 7B). A relatively small signal observed for protein 61.2 kDa can be attributed to the presence of glycerol in injected solution of Cry1Ab, which is used in order to reduce the aggregation of proteins, and it is also known that it significantly suppresses the protein signals in LC/MS. In 2DE measurements [35], the closest signal of both 63.0 and 58.4 kDa was obtained at 56.8 kDa, which was identified as vivin-like embryo storage protein.

A simple overlapping of the electropherogram of Cry1Ab standard recorded with 80 kDa chip, with the electropherogram recorded for extracts of maize standards is shown in Fig. 8. The electropherograms for maize standards were obtained for optimized extraction conditions and after lyophil-

ization of extracts. In case of albumins, the Cry1Ab signal at 63.0 kDa is close to one of signals recorded using CE chip, but that signal was detected practically of the same size in extracts from standards without and with 5% (m/m) GMO (Fig. 8A and B). In extract of globulins, there was no signal detected for mass close to the mass of Cry1Ab preparation (Fig. 8C), while for zein extract the observed signal at 59.4 is similar to 58.4 kDa of Cry1Ab (Fig. 8D), but it was also detected in both standards without and with 5% (m/m) GMO. As it was already mentioned above, signal at 60.5 kDa recorded for extracts of globulins with 80 kDa chip (Fig. 4A) was found only for extracts from maize standards with GMO, but it requires further identification.

4 Concluding remarks

The purpose of the analytical examination of transgenic plant material or foods produced from GM plants can be not only verifying the presence of GMO, but also determining the equivalence between plant material that was modified and one that was not.

A vast number of proteins in maize were identified [32] and already 20 years ago evident differences were reported between protein profiles of different genotypes [30]. Tens of differences were reported based on 2DE measurements between protein profiles in transgenic maize and respective isogenic controls, however, with conclusions indicating that environment plays the main influence on proteomic profiles of transgenic plants [35]. None of the proteins created as a result of inserting the gene (Bt) were identified so far, which

was attributed to their content below LOD values of employed method. However, there were a large number of proteins significantly modulated in transgenic lines compared to the parental ones. In earlier study, the number of detected differences was much smaller [48]. Our earlier research on LC/MS determination of low-molecular mass proteins in maize extracts showed a larger number of such differences [33]. Even though electrophoretic methods employing the CE chip reported in this study exhibit poorer resolution than 2DE or LC/MS, but several differences that can be employed for routine GMO identification were indicated. One of the possible markers can be protein 6.0 kDa in albumin extracts, which was confirmed by LC/MS as very strong signal obtained only for maize standard containing GMO. Similarly, the presence of triosephosphate isomerase (25.3 kDa) found in this study and confirmed in three other studies indicates this protein as a potential marker of GMO in maize extracts. Another potential marker based on CE chip analysis seems to be protein of M_r 20.6 kDa corresponding to several proteins detected by 2DE.

However, it seems that, similar to earlier publications, the search for protein Cry1Ab using CE chips in analyzed maize standards was not successful as yet. Therefore, further improvement of sample processing prior to the electrophoretic determination is necessary to obtain better sensitivity of determination.

The possibility of proteomic analysis application in the identification of GMO is the subject of increasing interest. Literature presents several examples of research in which there are not sizable differences between measured proteins in transgenic and control lines, for example [61, 62], nevertheless, there is an increasing number of reports showing measurable differences in proteome, for example [34, 35, 48]. Some differences of this sort were also shown in this study on protein profiling in maize extract with CE chips. This may potentially lead to the development of procedures of simplified detection of GMO for routine applications. However, it should be admitted that, simultaneously, several other instrumental alternatives are being developed for the detection and quantification of GMO [63]. They include, for example, different spectroscopic methods [64, 65] or genosensors with different signal transducers [66]. It also seems to be evident that the increasing role of GMO identification from technological and social point of views requires the appropriate reference systems for GM analysis to be developed.

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