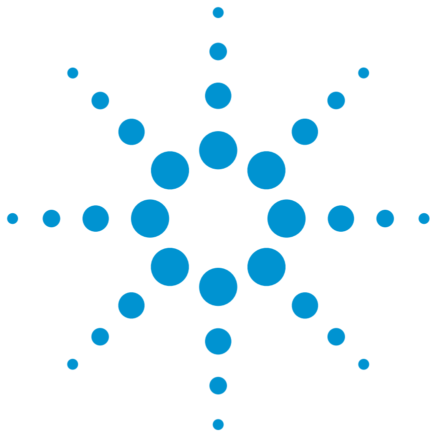

Screening of Glyphosate-Resistant Genetically Modified Soy Bean and Corn by Ultra-high Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry

Application Note

Food

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Abstract

Food derived from genetically modified (GM) crop species is required to be properly labeled in many countries to protect the freedom of choice for consumers. This makes it essential to screen the food of GM origins with reliable approaches. This application note describes an LC/MS/MS-based method as a complementary approach to the conventionally used DNA assay to detect the specific peptides derived from a commonly introduced gene, the glyphosate-resistant CP4 EPSP synthase gene, in soybean and corn samples. Briefly, the total protein was extracted from the samples, followed with SDS-PAGE separation to enrich the specific proteins and to remove the abundant interference proteins. The obtained protein gel band was then digested by trypsin, extracted into solution by solvent, enriched by vacuum drying, and analyzed by LC/MS/MS under multiple reaction monitoring (MRM) mode. Four specific peptides with eight MRM transitions were selected as the markers for monitoring the target proteins. Analysis of the known GM soybean species and the negative control demonstrated that all the peptides with the MRM transitions were selective, which were only observed in the GM species. The analytical method was further validated using other traits of soybean and corn species. The results demonstrated that the developed approach is selective, sensitive, and can be used to qualitatively screen food derived from glyphosate-tolerant GM species. The strategy can also be extended for detection of other exogenous or endogenous target proteins in food matrixes.

Introduction

Genetically modified (GM) crops including soy bean, corn, sugar beet, rapeseed, tomato, and so forth have been cultivated in many countries [1]. These plants are altered by conferring resistances to common pests, or by introducing tolerance to selected herbicides for better weed control [2]. However, debate on health or environmental concerns for food of GM origin remains unresolved. Nonetheless, the general public has the right to be aware of food with GM origin. Therefore, proper food labeling of GM or non-GM origin is required or highly recommended in some countries [3,4]. This makes it essential for monitoring agencies to routinely screen the food products of GM origin in both domestic and cross-board trading markets.

Conventional DNA-based techniques such as quantitative real-time fluorescence PCR have been the primary methods used to screen food of GM origins. This technique detects the specific sequence derived from the introduced genes [5], but may encounter cross-contamination or degradation of DNA under some harsh manufacturing processes. Alternatively, detecting the downstream encoding products of transferred genes (proteins) mainly through specific protein-based techniques such as ELISA, chemiluminescence immunoassay, and so forth [6,7], has also been reported widely. Their high dependence upon the availability of specific antibodies makes these methods cost-ineffective. With the maturing of the proteomics methodology, screening a particular protein through selective detection of specific peptides using the LC/MS/MS method has attracted much attention recently [8]. It is possible to detect the particular exogenous proteins encoded by the transferred genes in GM food through LC/MS/MS techniques. Among the commercially available GM plants, glyphosate-resistant species account for 90 % of the total [9]. It contains the protein of CP4 EPSP synthase gene, which can express the CP4 EPSP Synthase. This protein has a high tolerance to the widely used herbicide glyphosate, which controls the growth of weeds with the least interruption to crop growth [2]. Using glyphosate-resistant GM crops as a model, this application note attempts to develop a method to detect the CP4 EPSP protein for reliable screening of GM food, which could serve as a complimentary approach to the conventionally used DNA-based methods.

Experimental

Materials and reagents

Trifluoacetic acid (TFA), acetonitrile, and acetone were all HPLC grade and purchased from Merck (Germany). Dithiothreitol and iodoacetic acid were analytical grade and purchased from Sigma-Aldrich (US). Deionized water was produced in the lab using a Milli-Q water purification system. Sequence-grade modified trypsin was obtained from Promega (US).

Extraction and preseparation method

The soybean or corn sample was ground into powder, and 0.10 g of the powder was then subjected to acetone precipitation to obtain the protein pellet. The pellet was vacuum-dried and redissovled in SDS loading buffer, followed by SDS-PAGE separation. The target protein gel band in SDS-PAGE was:

1. Cut out and decolorized in a water/acetonitrile solution (v/v = 1/1) containing 50 mM ammonium bicarbonate
2. Reduced by 10 mM dithiothreitol under 60 °C for 1 hour
3. Sequentially alkylated by 50 mM iodoacetic acid for 45 minutes
4. Digested overnight with trypsin digestion at 37 °C with an enzyme-to-protein ratio of (1:40)

The resultant peptides were:

1. Extracted by sonication in 0.1 % formic acid/60 % acetonitrile aqueous solution for 30 minutes
2. Vacuum-dried to a pellet
3. Redissolved in a mixed solution containing acetonitrile, formic acid, and water at a volume ratio of 5:0.1:95
4. Analyzed by LC/MS/MS in MRM mode

Detailed LC/MS Conditions

Table 1. Instrument Conditions

LC conditions	
Instrument	Agilent 1260 Infinity LC System with built-in degasser
Autosampler	Agilent 1260 Infinity Autosampler
Column temperature compartment	Agilent 1260 Infinity Thermostatted Column Compartment
Column	Agilent ZORBAX SB-C18, 300 Å pore size, 1.8 µm particle size, 2.1 × 150 mm (id ×L)
Column temperature	30 °C
Mobile phase	Aqueous solution containing 0.1 % formic acid and 5 % acetonitrile.
	Aqueous solution containing 0.1 % formic acid and 95 % acetonitrile.
Flow rate	0.2 mL/min
Injection volume	5.0 µL
Post time	5 minutes
Gradient elution profile	0–5 minutes: hold 0 %B;
	5–25 minutes: 0 to 45 %B;
	25–30 minutes: 45 to 80 %B;
	30–35 minutes: hold 80 %B;
	35–40 minutes: 80 to 0 %B ramp down

ESI-MS/MS conditions	
Instrument	Agilent 6460 Triple Quadrupole LC/MS system with an Agilent Jet Stream electrospray ionization source
Ionization mode	Positive
Drying gas temperature	350 °C
Drying gas flow rate	10 L/min
Nebulizer gas pressure	45 psi
Sheath gas temperature	250 °C
Sheath gas flow rate	11 L/min
Capillary voltage	4,000 V
Nozzle voltage	500 V
Scanning mode	Multiple Reaction Monitoring

Results and Discussion

Optimized UHPLC separation and MS/MS detection

The EPSP synthase gene, with its ability to tolerate as high as millimolar concentrations of glyphosate [2], has been introduced to crops for better weed control. EPSP Synthase has an MW of 47.5 kDa. Table 2 shows the protein sequence [10]. Using trypsin digestion, it is possible that up to 40 digestion sites can be found in the protein (Table 2).

Table 2. Sequence of CP4 EPSP Synthase (Q9R4E4), Its Theoretical Trypsin Digestion Sites (red underlined), and the Specific Peptides (blue colored) Selected for MRM Measurement

10	20	30	40	50
MSHGASSRPA	TARK**SSGLSG**	**TVR**IPGDKSI	SHRSFMFGGL	ASGETR**ITGL**
60	70	80	90	100
LEGEDVINTG	**K**AMQAMGARI	RKEGDTWIID	GVGNGGLLAP	EAPLDFGNAA
110	120	130	140	150
TGCRLTMGLV	GVYDFDSTFI	GDASLTKRPM	GRVLNPLREM	GVQVKSEDGD
160	170	180	190	200
RLPVTLRGPK	**TPTPITYR**VP	MASAQVKSAV	LLAGLNTPGI	TTVIEPIMTR
210	220	230	240	250
DHTEKMLQGF	GANLTVETDA	DGVRTIRLEG	RGKLTGQVID	VPGDPSSTAF
260	270	280	290	300
PLVAALLVPG	SDVTILNVLM	NPTRTGLILT	LQEMGADIEV	INPR**LAGGED**
310	320	330	340	350
VADLRVRSST	LKGVTVPEDR	APSMIDEYPI	LAVAAAFAEG	ATVMNGLEEL
360	370	380	390	400
RVKESDRLSA	VANGLKLNGV	DCDEGETSLV	VRGRPDGKGL	GNASGAAVAT
410	420	430	440	450
HLDHRIAMSF	LVMGLVSENP	VTVDDATMIA	TSFPEFMDLM	AGLGAKIELS
DTKAA				

With a known GM soybean (GM_SB) as the model system, we did an initial survey by direct trypsin digestion of the total soybean proteins to find the specific peptides for target monitoring. However, due to the presence of many abundant interference proteins in soybean, peptides related to the target protein were not observed. To remove the abundant interference proteins, preseparation of the total soybean proteins through SDS-PAGE was conducted. As shown in lane 2 of Figure 1, with the loaded amount of total protein at 32 µg, a trace amount of target protein with MW of approximately 47.5 KDa can be observed, although in very low abundance (red square labeled region). The percentage of the target protein over the total protein was approximately 0.1 % based on the intensity of the gel band. Similar bands were also observed for another soybean GM species, MON 87705, but slightly fainter for the non-GM species (control), A3325 (Figure 1). By cutting the specific bands for digestion, the major abundant proteins were removed efficiently, and the peptides related to CP4 EPSP Synthase were detected using LC/MS/MS. Particularly, the four peptides labeled in blue in Table 2 were clearly detected. Using the peptide optimizer provided in MassHunter software, the MRM transitions for the specific peptides were then optimized to achieve the desirable analytical sensitivity, and the corresponding parameters are shown in Table 3.

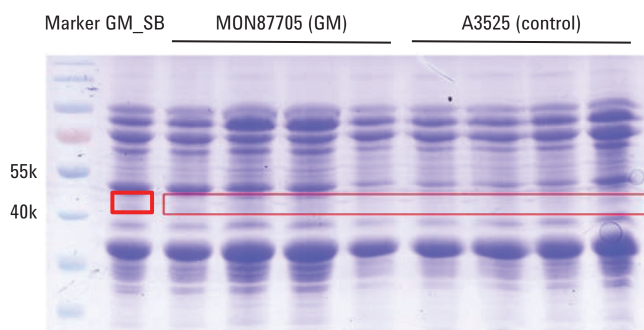

Figure 1. Separation of soybean proteins by SDS-PAGE. The red rectangle shows the protein band with theoretical MW of 47.5 kDa.

Table 3. MRM Parameters for Measurement of Specific Peptides Selected for CP4 EPSP Synthase

Peptide	**t_R/min**	**MW/Da**	**Parent *m/z***	**Parent ion**	**Frag. *m/z***	**Frag. ion**	**Frag./V**	**CE/V**
ITGLLEGEDVINTGK	20.41	1557.7	779.9	$[M+2H]^{2+}$	419.3	y_4	125	30
ITGLLEGEDVINTGK	20.41	1557.7	779.9	$[M+2H]^{2+}$	932.6	y_9	130	30
ITGLLEGEDVINTGK	20.41	1557.7	779.9	$[M+2H]^{2+}$	1061.5	y_{10}	125	25
ITGLLEGEDVINTGK	20.41	1557.7	779.9	$[M+2H]^{2+}$	1174.5	y_{11}	125	30
LAGGEDVADLR	16.19	1114.5	558.3	$[M+2H]^{2+}$	288.2	y_2	130	15
LAGGEDVADLR	16.19	1114.5	558.3	$[M+2H]^{2+}$	931.5	y_9	125	25
TPTPITYR	15.18	947.5	474.8	$[M+2H]^{2+}$	649.4	y_4	130	15
SSGLSGTVR	6.33	862.4	432.2	$[M+2H]^{2+}$	689.4	y_4	125	15

Method performance

The analytical method was initially tested using one known glyphosate-tolerant soybean species, MON87705, as the positive control, and using one non-GM soybean species, A3525, as the negative control. Both species were previously validated using a DNA-based method. With an initial total protein extract of 100 µg loaded into the gel, the target protein gel band was subjected to trypsin digestion, followed by UHPLC/MS/MS analysis. As shown in Figure 2, all eight MRM transitions related to the selected four peptides exhibit intense signals for MON87705 (GM species, trace b), while there is no MRM transition showing a clear signal for A3525 (non-GM species, trace a).

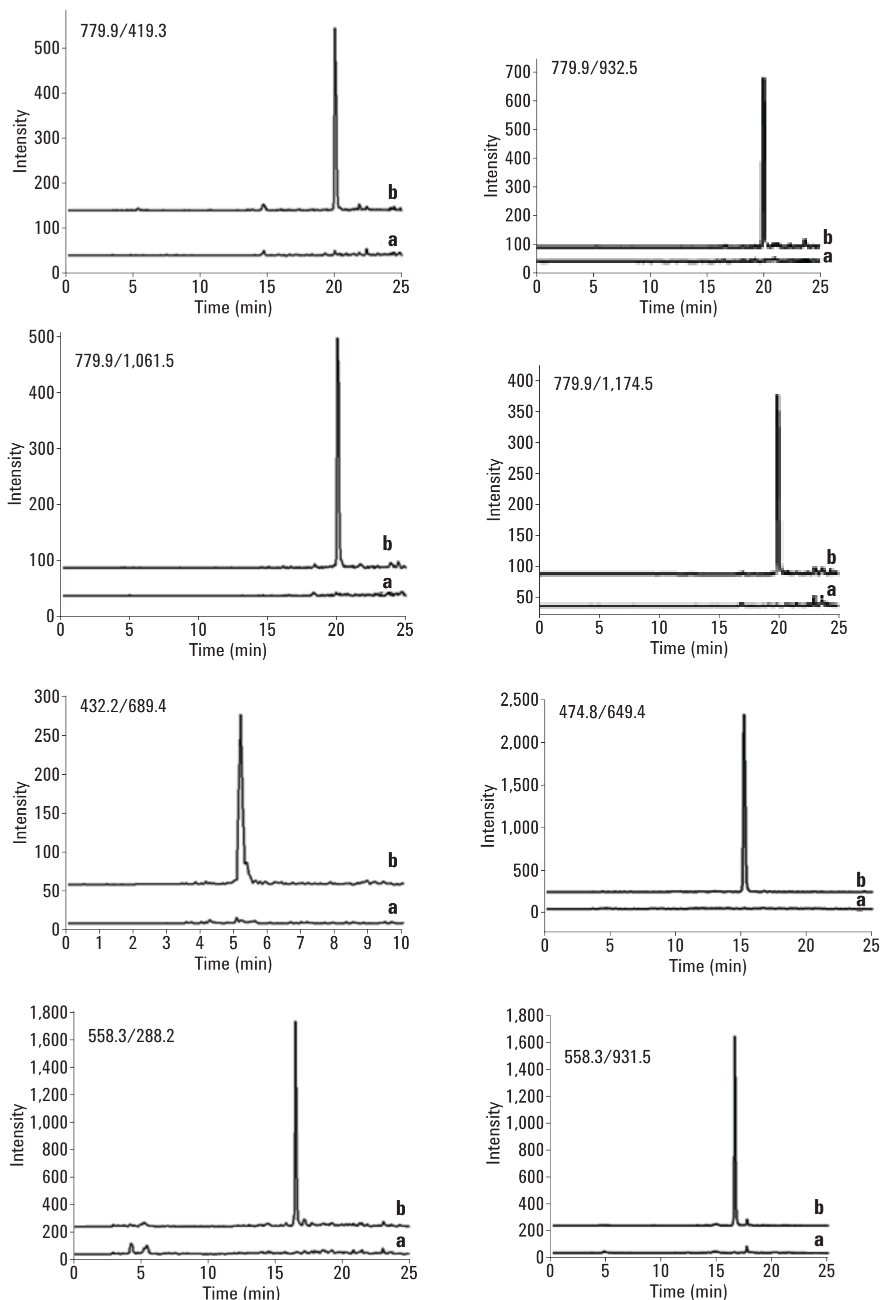

Figure 2. Extracted eight selected MRM chromatograms showing no signal for non-GM soybean A3525 (a) and high intensity for GM soybean MON 87705 (b), both of which were previously validated by a DNA-based method.

The signal-to-noise ratio for these MRM chromatograms ranged from 91 to 356, indicating that the method can screen the glyphosate-tolerant soybean at a 10-fold lower level. With the estimated 0.1 % of target protein in the total protein extract based on Figure 1, it was estimated that the lowest detection limit for the target protein was 0.01 µg. With the total proteins extracted from 0.1 g of raw soybean sample, the estimated lowest detection limit for the target protein was 0.1 µg/g raw sample.

Extended application to other soybean species and corn species

We extended the developed method to screen another type of GM soybean, 40-3-2, and one type of corn, NK603, both of which were previously screened and confirmed using a DNA method. As shown in Figure 3, for 0.1 g of raw samples, all four peptides with eight MRM transitions were clearly observed, indicating that both samples were derived from glyphosate-resistant plant species. This was consistent with the DNA screening result. The relative intensity of species NK603 to 40-3-2 for the same pair of ions ranges from 1/2 or 1/3, suggesting that NK603 contains a lower amount of EPSP Synthase in its seeds.

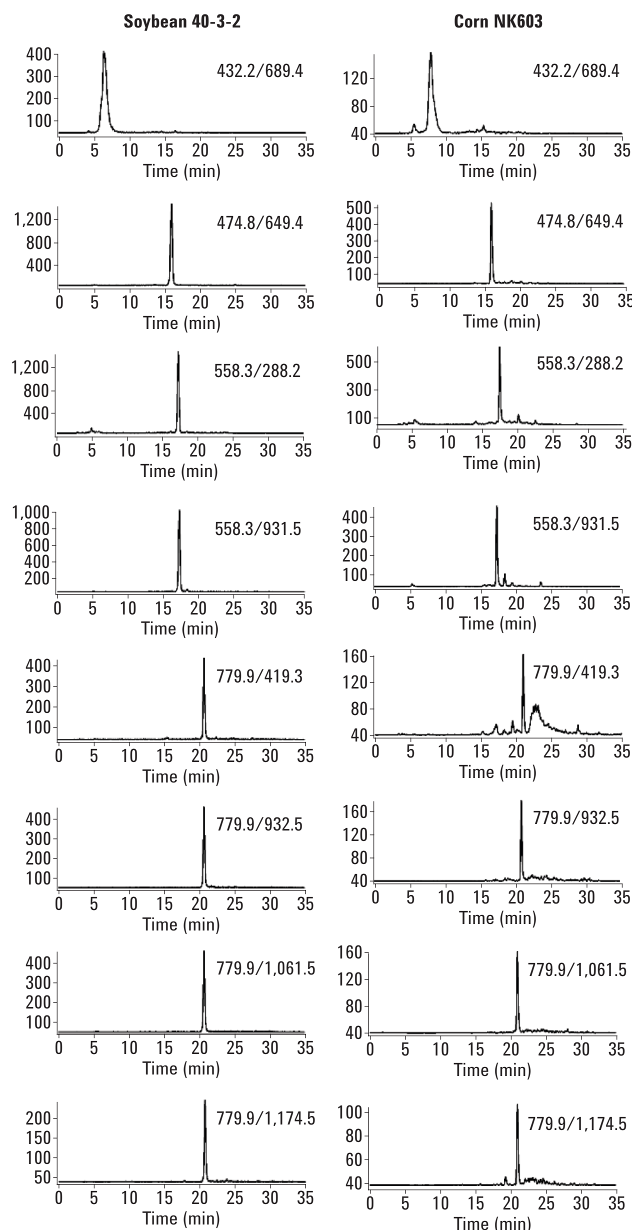

Figure. 3. Extracted MRM chromatograms demonstrating both soybean 40-3-2 (left) and corn NK603 (right) are glyphosate-resistant GM species.

Random sample screening

Genetically modified plant species, particularly soybean and corn, have not been approved for commercial cultivation within China. A random collection of soybean and corn samples from local supermarkets were analyzed using the developed method, and no glyphosate-tolerant soybean species were detected. The typical MRM chromatograms for soybeans collected from local markets is shown in Figure 4. There were no clearly observed peaks corresponding to the four peptides, specifically for the detection of glyphosate-tolerant species, even viewed at expanded scale within the retention time windows (dashed rectangles in Figure 4). This indicates that the collected sample is not a glyphosate-tolerant GM species.

Conclusion

An SDS-PAGE preseparation followed by UHPLC Triple Quadrupole multiple reaction monitoring method has been successfully developed for detecting trace amounts of CP4 EPSP Synthase in samples as small as 0.1 g of soybean or corn. The method has been validated using known GM species, including soybeans MON 87705 and 40-3-2, and corn NK603, with soybean A3525 as a non-GM control. A survey of the collected soybean and corn samples from the local market did not find glyphosate-tolerant species. The lowest detection limit for the target protein was estimated as 0.1 µg/g raw sample. By introducing an internal standard peptide, it is possible to quantitate the amount of the transgenic protein accurately and sensitively in the food samples. The developed analytical method can be applied to screen other glyphosate-tolerant GM plant species. The strategy has the potential to be extended to the detection of other exogenous or endogenous proteins in food matrixes.

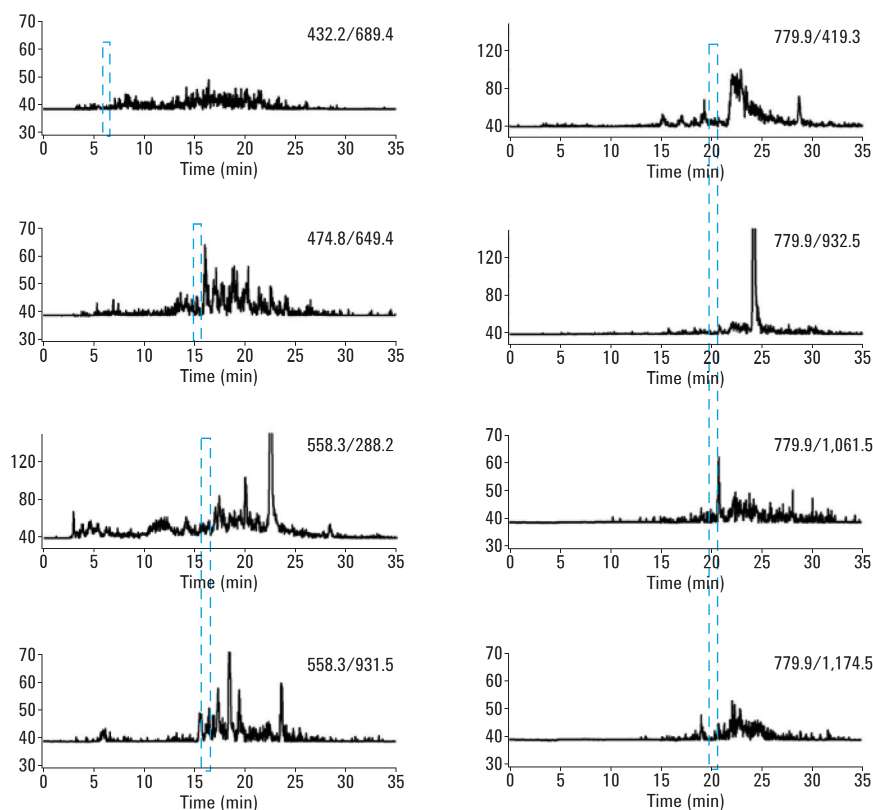

Figure. 4. Eight extracted selected MRM chromatograms showing no signal for any peptides related to CP4 ESPS Synthase in the typical sample collected from a local superstore. The dashed rectangles indicate the retention time windows for the specific peptides.

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