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(54) Title: ANALYSIS OF PROTEINS

(57) Abstract: A method for determining the presence of genetically-modified plant-derived material in a product, the method comprising: providing a protein extract derived from the product; enriching the protein extract; digesting the protein extract using an enzyme; and detecting the presence or absence of at least one marker peptide resulting from the enzymatic digestion of a transgenic protein thereby determining whether the genetically modified plant derived material is present in the product.



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ANALYSIS OF PROTEINS

Field of the Invention

The present invention generally relates to the detection of genetically
5 modified plant-derived material in products. More specifically, the invention
relates to the identification of transgenic proteins present in plant-derived materials.

In particular, the present invention relates to a method for detecting the
presence of the transgenic protein 5-enolpyruvylshikimate-3-phosphate synthase
(EPSPS) in a sample of a plant-derived material, a marker peptide derived from
10 EPSPS and a method for isolating a marker peptide from EPSPS, use of the peptide
markers in the detection of EPSPS and a kit suitable for the detection, identification
and quantification of EPSPS in a plant-derived material.

Background to the Invention

15 The development of robust analytical techniques facilitating the detection,
identification and quantification of genetically-modified (GM) matter in plant-
derived materials to levels established by regulatory authorities is increasingly
desired.

Of particular interest is the development of techniques for the detection of
20 transgenic proteins in glyphosate-based herbicide resistant soyabean and maize
seeds. For example, resistance to glyphosate in Roundup Ready™ soybean is
conferred by introducing the 5-enolpyruvylshikimate-3-phosphate synthase
(EPSPS) gene from *Agrobacterium tumefaciens* CP4. EPSPS is involved in the
biosynthesis of aromatic amino acids and catalyzes the reaction of shikimate-3-
25 phosphate (S3P) and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate
(Figure 1). However, glyphosate inactivates this process by forming a complex with
EPSP-S3P.

Despite the benefits conferred from the improved traits of GM crops,
concerns about the potential environmental and human impact of these crops
30 persists. In Europe, for example, labelling is required for food products containing
GM materials where 0.9% by weight of an ingredient is derived from such
materials. Furthermore, screening for GM materials is important in other diverse

areas, such as quality control in GM crop development and the assessment of potential fraud as a result of mixed commodities. 0.9% by weight is currently the recognised lower limit for the detection of GM materials in a material available from known detection methods.

5 Analytical procedures known in the art may employ either polymerase chain reaction (PCR) or enzyme-linked immunoabsorbent assay (ELISA) for the detection of DNA and proteins, respectively. Quantitative competitive PCR as well as real-time quantitative PCR have been employed broadly for the detection of transgenic DNA. Whilst PCR methodologies are very sensitive, their reliability
10 depends on the integrity of the DNA, which can be degraded by heat, nuclease activity and low pH.

Protein immunoassays are utilized particularly in the food industry because of their ease-of-use, high sensitivity and capability to high-throughput. However, a suitable antibody is not always easily available, especially for new target proteins,
15 and the effect of food processing, heat, pH, etc., to the validity of the assay needs to be investigated in each case. Whilst these external parameters might also pose a problem for other types of protein analysis, the immunoassay methods additionally might suffer from non-specific binding and cross contamination.

In light of the problems associated with the detection techniques known in
20 the art, eg, PCR and ELISA, there is a clear need for further techniques for reliable detection, identification and quantification of proteins, eg, GM matter, in plant-derived materials.

Summary of the Invention

25 According to the invention there is provided a method for determining the presence of genetically-modified plant-derived material in a product, the method comprising:

- providing a protein extract derived from the product;
- enriching the protein extract;
- 30 - digesting the protein extract using an enzyme; and
- detecting the presence or absence of at least one marker peptide resulting from the enzymatic digestion of a transgenic protein

thereby determining whether the genetically modified plant derived material is present in the product.

The method according to the invention provides a practical, low-cost, accurate and robust proteomic approach to the detection of genetically-modified plant-derived material in a product. Furthermore, the method can be readily automated to provide an analytical approach for routine surveillance in the laboratory for the identification and detection of a transgenic protein (GM component) at levels established by regulatory authorities (i.e. < 0.9%). This is particularly the case when the product is a processed food product. The method can also be used for multiplex studies where a plurality of marker peptides or transgenic proteins can be analysed in the same analytical sample.

The invention also provides:

- a marker peptide for use in determining the presence of genetically-modified plant-derived material in a product, the marker peptide having a sequence selected from SEQ ID Nos. 1 to 18 and oxidised variants thereof;
 - a composition for use in determining the presence of genetically modified plant material in a product, the composition comprising two or more marker peptides of the invention;
 - a marker peptide:polymeric adsorbent complex, wherein the marker peptide has a sequence selected from SEQ ID NOs. 1 to 18 and oxidised variants thereof;
 - a method for isolating a marker peptide or composition of the invention comprising:
 - providing a protein extract derived from a plant-derived material comprising 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS);
 - enriching the protein extract; and
 - digesting the protein extract with an enzyme;
 - use of a marker peptide, composition or complex of the invention for detecting the presence of genetically modified plant-derived material in a product;
- and

- a kit for detecting the presence of a genetically modified plant-derived material in a product, the kit comprising:
 - a digestive enzyme;
 - an enzyme solubilisation reagent;
 - 5 an enzyme reaction buffer; and
 - at least one reference peptide marker or a computer-readable algorithm capable of elucidating a mass spectrometric signal of at least one reference peptide marker.

10 **Brief Description of the Figures**

Figure 1 shows a reaction scheme illustrating the inhibition of EPSP synthase by glyphosate. 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) catalyses the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. This step is inhibited by glyphosate, which is the active ingredient in Roundup ReadyTM herbicides.

Figure 2 shows the fractionation of GM soya proteins. Gel filtration fractions: (A) absorbance measurements at 280 nm (grey line) and 595 nm (black line) (B) SDS-PAGE. Anion exchange fractions: (C) absorbance measurements at 280 nm (grey line) and 595 nm (black line) (D) SDS-PAGE. Fractions in which high levels of CP4 EPSPS were detected with lateral immunostrips are indicated. Gel filtration fraction 12 was collected within the column void. Potential CP4 EPSPS SDS-PAGE bands at about 47 kDa were present in gel filtration fractions 27 and 29 (B) and anion exchange fractions 75, 80, 82 and 90 (D).

Figure 3 shows MALDI-TOF mass spectra of tryptic peptide maps of 47 kDa SDS-PAGE bands from 100% by weight GM soya. (A) Crude protein extract, (B) gel filtration fraction 27 and (C) anion exchange fraction 82. The detected CP4 EPSPS tryptic peptides in (B) and (C) are labeled by mass and residue number.

Figure 4 shows NanoLC-nanoESI-QTOF mass spectra of in-solution digested anion exchange fraction 92 from 50 % by weight GM soybean seeds. CP4 EPSPS biomarkers (A) $[M+2H]^{2+}$ at m/z 558.297 ($[M+H]^+$ at m/z 1115.605) and

(B) $[M+2H]^{2+}$ at m/z 779.933 were observed. Their characteristic MS/MS spectra were used as fingerprints for identification of CP4 EPSPS (C, D).

Figure 5 shows TOF MS ion extracted chromatograms (XIC) of combined anion exchange fractions 82 and 86 from non-GM and 0.9 % by weight GM soybean seeds. CP4 EPSPS tryptic peptides $[M+2H]^{2+}$ at m/z (A) 558.3, (B) 779.9 and (C) 881.9 were detected in 0.9 % by weight GM but not in non-GM soya. The y-axes from non-GM soybean XIC have been expanded approximately 5 to 20 times and demonstrate that there are no peaks at the expected retention time.

Figure 6 shows SDS-PAGE of various anion exchange fractions from 100% by weight GM maize. Potential CP4 EPSPS SDS-PAGE bands at about 47 kDa were present in fractions 79 and 81.

Figure 7 shows a MALDI-TOF mass spectrum of a 47 kDa SDS-PAGE band from anion exchange fraction 81 from 100% by weight GM maize. The detected CP4 EPSPS tryptic peptides are labeled by mass and residue number.

Figure 8 shows a graph of the ratio of the intensity of the molecular ions (m/z 558/561) in duplicate samples (Gel A and Gel B) of GM soya at concentrations of 5, 2, 0.9 and 0.5% following gel purification (as described herein) and AQUATM labelling where the gel bands were mixed with 400 fmol of the ¹³C labelled peptide L*AGGEDVADLR (L* = ¹³C). Trypsin digestions and LC/MS/MS analysis followed. Data points are from three replicates.

Figure 9 shows a comparison of experimental and theoretical ratios for EPSPS in GM soya using AQUATM labelling.

Detailed Description of the Invention

The invention provides a method for determining the presence of genetically-modified plant-derived material in a product, the method comprising:

- providing a protein extract derived from the product;
- enriching the protein extract;
- digesting the protein extract using an enzyme; and
- detecting the presence or absence of at least one marker peptide resulting from the enzymatic digestion of a transgenic protein

thereby determining whether the genetically modified plant derived material is present in the product.

Advantages according to the method of the present invention are that surprisingly the detection of GM materials present in a sample at amounts as low as 0.5% by weight of the sample are possible. In particular, the peptide with the sequence of SEQ ID No. 13 was detected in accordance with method of the present invention in 0.5% by weight genetically-modified soya. As previously indicated, 0.9% by weight is currently the recognised lower quantitative limit (and the European Union regulatory limit for foodstuffs) for the detection of GM materials in material available from methods known in the art, hence this is a significant improvement over known methods.

The genetically modified plant derived material is detected by determining the presence or absence of one or more peptide markers derived from one or more transgenic protein. Accordingly, the present invention also provides for the analysis of a plant-derived material to determine the presence of a transgenic protein, comprising the steps of:

- providing a protein extract derived from a plant-derived material potentially comprising a transgenic protein;
- enzymatic digestion of the protein extract suitable to produce a marker peptide; and
- detection of the presence of the marker peptide to determine the presence of a transgenic protein using at least one reference marker peptide.

The genetically modified plant-derived material may be from any plant. Typically, the plant is a crop plant such as, for example, soyabean, maize, corn, barley or wheat. In a preferred embodiment, the plant-derived material is from soyabean or maize.

The product is, in one preferred embodiment, a food matrix. The product employed according to the method of the invention may be maize or soyabean seed, typically genetically modified maize or soybean seed. The food matrix typically contains GM ingredients. The food matrix may be a processed foodstuff such as bread, cake, biscuit, confectionery, a processed meat product (eg, a

sausage), packaged dehydrated noodles and the like. It may also be a livestock feedstuff to be used in agriculture. Furthermore, the matrix may be derived from farm animal waste (eg, cow faeces). The wide variety of plant-derived materials that the method of the present invention can be applied to demonstrates its

5 versatility and usefulness in analysing all manner of materials ranging from protein isolates to raw ingredients to complex food matrices such as processed foods which are on the market. The method of the invention may also be applied to environmental surveillance studies, for example, as part of the laboratory analysis of farm animal waste.

10 The product may comprise up to 100% plant-derived material, such as from 0.5% to 95%, 0.9% to 90%, 1% to 85%, 5% to 80%, 10% to 70%, 20% to 60%, 30% to 40% or 50% plant-derived material. The product may contain plant-derived material from one, two or more different types of plant. The method may be used to detect transgenic proteins from each type of plant present in the product.

15 Preferably, the method of the invention includes the step of enrichment of the protein extract. Preferably, this takes place prior to enzymatic digestion.

 The enrichment of the transgenic protein from the protein extract may comprise the steps of gel filtration chromatography, anion exchange chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In one preferred embodiment gel filtration and SDS-PAGE are used to enrich the protein extract. Optionally anion exchange chromatography is also used prior to SDS-PAGE.

25 Enrichment may alternatively or additionally take place after enzymatic digestion. The peptides present after digestion may, for example, be enriched using reverse phase nano liquid chromatography.

 Enrichment is particularly important where the presence of the transgenic protein of interest is suppressed by the presence of other proteins which have a substantially higher abundance in the sample. This is of particular relevance for transgenic proteins present in GM soya, or other seeds or pulses, due to the abundant presence of major storage proteins. The above enrichment techniques for biomolecules (including proteins) are individually well known to the person skilled in the art.

Preferably, in the methods according to the invention the marker peptide is a transgenic marker peptide resulting from the enzymatic digestion of the transgenic protein to be detected.

Preferably, enzymatic digestion employed according to the method of the invention utilises an enzyme suitable for digesting the protein into an analysable peptide fragment. Suitable enzymes will be known to the person skilled in the art. Particularly preferably, trypsin, endoproteinase AspN and/or endoproteinase Glu-C are employed. Most preferably, trypsin is used for enzymatic digestion.

In one embodiment of the method of the invention, the transgenic protein being detected is 5-enolpyruvylshikimate-3-phosphate synthase (EPSP), preferably the transgenic protein CP4 EPSPS derived from *Agrobacterium tumefaciens* CP4.

Thus, in this embodiment, the marker peptide or peptides of the present invention are derived from 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).

The method of the invention may employ a marker peptide according to the invention. Therefore, in one embodiment, the method of the present invention employs at least one marker peptide having a sequence selected from SEQ ID NOs. 1 to 18 and including oxidised variants thereof.

The marker peptide may have a sequence selected from SEQ ID NOs 2, 3, 8, 13 and oxidised variants thereof. A marker peptide with the sequence of SEQ ID NO.13, including oxidised variants thereof, is preferred.

In one preferred embodiment, the detection of the marker peptide is by mass spectrometric analysis.

Mass spectrometric analytical methods employed according to the method of the invention include matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and/or nanoelectrospray ionisation quadrupole time-of-flight (nanoESI-QTOF) mass spectrometry and/or nano liquid chromatography nanoelectrospray ionisation quadrupole time-of-flight tandem mass spectrometry (nanoLC-nanoESI-QTOF MS/MS) and/or ion trap mass spectrometry.

The mass spectrometric techniques employed in the present invention available for the analysis of proteins in materials require only a minute amount of

sample, may be easily automated, and can provide highly detailed information in relation to samples of proteins including high mass accuracy.

In particular, MALDI-TOF MS is a soft ionization technique used in mass spectrometry, which ionizes biomolecules (eg, biopolymers like proteins, peptides
5 and sugars) which tend to lose their structural integrity when ionized by conventional ionization methods.

Electrospray ionization (ESI) mass spectrometry is a technique useful in producing ions from large biomolecules because it overcomes the propensity of these molecules to fragment when ionized. In ESI mass spectrometry,
10 quasimolecular ions are observed that are ionized by the addition of a proton to give $[M+H]^+$, or other cation (eg, a sodium ion) to give $[M+cation]^+$, or the removal of a proton $[M-H]^-$. In ESI mass spectrometry, multiply charged ions such as $[M+2H]^+$ may be frequently observed.

Quadrupole ion trap mass spectrometric techniques may also be employed
15 for mass spectrometric analysis according to the methods of the present invention.

Mass spectrometry can identify large and small molecules and assists in determining their molecular structure, providing a sensitive and versatile means for analysing complex biological mixtures including protein and peptide mixtures. Ions can be created by electron impact (EI), electrospray (ESI), or matrix-assisted laser
20 desorption (MALDI) ionization.

Quantitative mass spectrometric analysis may be used in a method of the invention to determine the amount of marker peptide present. This is preferably done by using a known amount of a reference marker peptide in a first sample, providing a second sample potentially comprising a quantity of the marker peptide,
25 subjecting the first and second samples to mass spectrometric analysis to produce a mass spectrometric signal, and making a quantitative comparative measurement of the intensity of the mass spectrometric signal produced by the first and second samples in order to determine the quantity of marker peptide present in the second sample and hence transgenic protein present in a plant-derived material.

30 To facilitate the quantification of the amounts of a transgenic protein present in a sample comprising a plant-derived material, amine-specific labeling reagents (eg, iTRAQTM reagents (Applied Biosystems)) and/or stable isotope

labeled peptides (eg, Protein-AQUA™) may be employed. These reagents and the methodologies for employing them as means for the quantitative protein analysis are known to the person skilled in the art. Preferably, the reference marker peptide is labeled.

5 Amine-specific labeling reagents may be employed for relative protein quantification using mass spectrometry. These reagents are a primary amine specific label that covalently binds to lysine side chains and the N-terminal group of a peptide. The labelled peptides are used as an internal standard when quantitatively analysing a sample containing a protein of interest. The sample to be
10 analysed may contain the labelled sample control and can be subjected to the purification, enzymatic digestion and mass spectrometric steps discussed hereinbefore. The quantity of the protein of interest present in the sample being tested may then be evaluated by a comparison of the ratio of the intensities (% abundance) of the molecular ion peak for the non-labelled peptide present in the
15 sample being tested (eg, LAGGEDVADLR derived from EPSPS) and the amine-specific labelled peptide standard which was added in a known amount to the sample prior to analysis.

 Stable isotope labeled (^{13}C and ^{15}N) peptides may be used as an internal standard with a single labelled amino acid per peptide. A specific example of a
20 labelled peptide used in accordance with the present invention is $\text{L}^*\text{AGGEDVADLR}$ ($\text{L}^* = ^{13}\text{C}$). Prior to enzymatic digestion of the protein extract, a labelled peptide (such as $\text{L}^*\text{AGGEDVADLR}$ for the detection of EPSPS) may be added to the sample to be analysed in a known quantity. The sample may then be subjected to the purification, enzymatic digestion and mass spectrometric steps
25 discussed hereinbefore. The labelled peptide may alternatively be added immediately prior to mass spectrometric analysis. The amount of the protein of interest present in the sample being tested could then be quantified by a comparison of the ratio of the intensities of the molecular ion peak for the non-labelled peptide present in the sample being tested (e.g. $\text{L}^*\text{AGGEDVADLR}$ for the detection of
30 EPSPS) and the ^{13}C -labelled peptide (e.g. $\text{L}^*\text{AGGEDVADLR}$ for the detection of EPSPS) added in a known amount to the sample prior to analysis.

Preferably the mass spectrometric analysis step of the method of the invention, comprises a step of elucidating a mass spectrometric signal from that of a known transgenic protein by means of computer analysis. This may comprise a step of providing a computer-readable algorithm able to elucidate a mass spectrometric signal resulting from a sample known to contain the transgenic protein (e.g. CP4 EPSPS). Such methods are known *inter alia* from WO 03/091695 A2 (pages 46 to 54).

When a sample is analysed by mass spectrometry, the signal from the mass spectrometer is transferred to a computer which contains a program which converts the signal into a computer-readable form. The software may include an algorithm which can identify whether a particular peak in the mass spectrum of the sample corresponds to a peptide marker of the invention. In some instances, such analysis may normally be done manually by observation with the eye. However, automated detection of peaks when multiple molecular ions are generated in the mass spectrometer can be a complex task. Hence, the provision of computer-aided analytical means as described can be of great benefit to the user in saving time and reducing the risk of error in the determination of results.

Further to this, the software can also include a code which distinguishes between a typical mass spectrometric signal characteristic of a known peptide marker or mixture of peptide markers present in a sample and a labeled peptide introduced (spiked) into the sample for quantitative analysis purposes.

MALDI-TOF mass spectrometry is a preferred form of mass spectrometry, particularly where the transgenic protein is CP4 EPSPS. In a further preferred embodiment, peptide sequence information is subsequently obtained by nanoLC-nanoESI-QTOF MS/MS.

MALDI-TOF mass spectrometry is a preferred method for the identification of a marker peptide with a sequence selected from SEQ ID NOs. 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16 and 17. NanoESI-QTOF is a preferred method for the identification of a marker peptide with a sequence selected from SEQ ID NOs. 1, 2, 3, 4, 5, 7, 8, 9, 11, 13, 15 and 18. NanoLC-nanoESI-QTOF MS/MS is a preferred method for the identification of a marker peptide with a sequence selected from SEQ ID NOs. 2, 3, 6, 8, 10, 12, 13, 15, 16 and 17.

In one embodiment, detection of the marker peptide according to the method of the invention comprises isolation of the marker peptide by a molecular imprinting technique using a polymeric adsorbent, wherein the method comprises:

- labelling the isolated marker peptide with a fluorophoric or
5 chromophoric molecular label; and
- spectroscopic detection of the presence of the marker peptide.

Molecular imprinting is a technique for creating molecular recognition sites in a polymeric material. The molecule of interest may act as a template for assembling polymerisable functional monomeric units (eg, acrylate monomers) in
10 non-covalent or weakly covalent interactions to form a complex with the template.

The monomeric units may then be polymerised, eg, by cross-linking the monomeric units, and the marker peptide template extracted to provide a polymeric substrate (polymeric solid-phase extraction (SPE) adsorbent) with a vacant recognition site corresponding to the extracted marker peptide. The polymeric
15 adsorbent can then act as a selective adsorbent for isolating the same marker peptide present in a sample being analysed which contains a mixture of peptides and/or other biomolecules including the marker peptide of interest. This provides an effective means for the selective enrichment of a transgenic protein or marker peptide derived from a transgenic protein present in a sample comprising an
20 abundance of different biomolecules.

For the purpose of the present invention, the identification and selection of suitable polymeric adsorbents for marker peptides according to the invention is preferably undertaken on pre-synthesised libraries of adsorbents or by a computational design approach. The computational design approach uses an
25 algorithm for determining likely interactions of a virtual library of polymeric adsorbents with a marker peptide of interest. Identification of new potential adsorbent can be achieved and synthesis of a polymeric adsorbent according to the general method outline above can subsequently be undertaken.

Marker peptides according to SEQ ID NOs 1 to 18 in quantities sufficient
30 for studies enabling identification and selection of suitable polymeric adsorbents may be prepared by protein synthesis routes known in the art.

In the methods of the invention, in a sample which is a crude digested protein extract, a marker peptide is able to complex (bind) in the recognition site of the polymeric adsorbent. Non-complementary molecules remain unbound.

5 Separation of a resultant polymeric adsorbent:marker peptide complex from the sample and physical separation (eg, by solvent elution) of the peptide from the adsorbent provides an efficient means for isolating a marker peptide from a sample containing numerous other peptides and/or biomolecules in abundance. Further purification of the marker peptide can be performed by HPLC or other suitable chromatographic technique(s).

10 The isolated marker peptide may then be labelled with a fluorophoric or chromophoric molecular label. Suitable fluorophoric or chromophoric labels include 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dansyl reagent) or Cascade Yellow succinimidyl ester. Other suitable fluorophoric or chromophoric labels are well known to the skilled person as are methodologies for attaching a
15 label to a peptide. Bonding of the molecular label to the marker peptide will typically be at the primary amine site at the N-terminus or the carboxylate site at the C-terminus of the peptide. As an alternative approach, the marker peptide may be labelled while still in a crude digested protein extract prior to isolation by a molecular imprinting technique.

20 Spectroscopic detection of the marker peptide may be undertaken using techniques such as ultra-violet/visible (UV/Visible) spectroscopy to detect chromophore-labelled species and fluorescence spectroscopy for detecting fluorophore-labelled species.

Many peptides (especially those of less than 10 amino acids) lack
25 chromophores, fluorophores or electrophores. Hence, their detection by UV absorption between about 205 to about 230 nm often relies on the presence of a peptide binding carbonyl group. If an aromatic side chain is present then detection may be possible at wavelengths of about 250 to about 280 nm.

However, many substances absorb in the UV wavelength range and it may
30 not be possible to distinguish the presence of a peptide of interest over solvent molecules, impurities, etc, contained in a sample, particularly at very low analyte concentrations. Therefore, the labelling of marker peptides with fluorophoric or

chromphoric labels which strongly absorb at ultra-violet and visible wavelengths can yield peptide derivatives which can be clearly identified in a test sample by their intense spectroscopic absorbance, even at low concentration (eg, by a pre-determined known absorbance wavelength). That is, derivatisation of the marker peptides by incorporating a fluorophoric or chromphoric molecular label improves selectivity and sensitivity of the analysis by increasing the ultraviolet/visible wavelength range of absorbance and absorbance signal intensity, thereby avoiding signals resulting from interference by other substances.

Preferably, the reference marker peptide used in the method of the invention also contains a fluorophoric or chromphoric label.

The acquisition of mass spectrometric, spectroscopic and chromatographic data may be used to confirm the nature of a marker peptide which has been labelled. Such data preferably include the determination of the extinction coefficient (ϵ) of the labelled peptide in solution in accordance with the Beer-Lambert law (Absorbance = ϵ x concentration of sample x path length of incidental light through the solution).

Preferably, spectroscopic detection of the marker peptide detects the amount of marker peptide present using quantitative analysis by UV/Visible spectroscopy or fluorescence spectroscopy. Preferably, this is by means of acquiring a spectrum of a sample solution containing a labelled marker peptide analyte in an unknown quantity and measuring the absorbance of the solution. The concentration of the labelled marker peptide in the solution can then be determined if the extinction coefficient and path length through the solution are known.

Preferably, the method of analysis according to the invention does not employ an enzyme-linked immunoabsorbent assay or other immunoassay-based analytical procedure for the detection of the marker peptide.

The invention provides a marker peptide for use in determining the presence of genetically modified plant-derived material in a product, the marker peptide having a sequence selected from:

SSGLSGTVR	(SEQ ID NO. 1);
SFMFGGLASGETR	(SEQ ID NO. 2);
ITGLLEGEDVINTGK	(SEQ ID NO. 3);

	AMQAMGAR	(SEQ ID NO. 4);
	VLMPRLR	(SEQ ID NO. 5);
	VLNPLREMGVQVK	(SEQ ID NO. 6);
	EMGVQVK	(SEQ ID NO. 7);
5	SEDGDRLPVTLR	(SEQ ID NO. 8);
	TPTPITYR	(SEQ ID NO. 9);
	TPTPITYRVPMASAVQVK	(SEQ ID NO. 10);
	VPMASAVQVK	(SEQ ID NO. 11);
	MLQGFGANLTVETDADGVR	(SEQ ID NO. 12);
10	LAGGEDVADLR	(SEQ ID NO. 13);
	GVTVPEDR	(SEQ ID NO. 14);
	ESDRLSAVANGK	(SEQ ID NO. 15);
	LNGVDCDEGETSLVVR	(SEQ ID NO. 16);
	GLGNASGAAVATHLDHR	(SEQ ID NO. 17);
15	IELSDK	(SEQ ID NO. 18)

and oxidised variants thereof.

A marker peptide of the invention may also be used to detect the presence of a transgenic protein in a plant-derived material.

The usefulness of peptides according to the invention could not have been predicted from theoretical studies of trypsin digests of translated nucleotide sequences encoding the EPSPS enzyme due to missed cleavages and oxidised methionine.

Furthermore, the marker peptides according to the invention are stable and retain their structural integrity while being subjected to a range of analytical procedures and chemical modifications making them effective candidates for the identification of EPSPS during the laboratory analysis of complex biomolecular matrices, eg, processed foodstuffs.

Preferably, the marker peptide according to the invention is a marker peptide having a sequence selected from SEQ ID NO. 2, 3, 8 and 13. Particularly preferably, the peptide marker is derived from 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) sourced from *Agrobacterium tumefaciens* CP4.

According to the invention, there is provided an oxidised variant of a marker peptide according to the invention. In particular, there is provided a marker peptide with a sequence selected from SEQ ID NOs 2, 6, 10 and 12, where there is oxidation of the peptide in accordance with the data of Table 1.

5 The invention also provides a composition comprising two or more marker peptides of the invention.

Preferred marker peptide mixtures, which are particularly useful in mass spectrometric analyses, are mixtures comprising at least one of:

- SEQ ID NOs. 2 and 3;
- 10 SEQ ID NOs. 2 and 8;
- SEQ ID NOs. 2 and 13;
- SEQ ID NOs. 3 and 8;
- SEQ ID NOs. 3 and 13;
- SEQ ID NOs. 8 and 13;
- 15 SEQ ID NOs. 2, 3 and 8;
- SEQ ID NOs. 2, 3 and 13;
- SEQ ID NOs. 2, 8 and 13;
- SEQ ID NOs. 3, 8 and 13;
- SEQ ID NOs. 2, 3, 8 and 13.

20 An additional useful peptide may result from a conservative modification to the amino acid sequences of a marker peptide according to the invention. A conservative modification refers to a change in an amino acid residue which does not alter the polarity or charge of the residue. Examples of conservative changes are well known to those skilled in the art, and include, for example, substitution of
25 one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine.

 Preferably, when determining the amino acid identity between two protein sequences conservative amino acid changes are considered to be identical amino
30 acids. Therefore, a conservative amino acid change in amino acid sequence will not affect the percentage amino acid identity between the two sequences. A

conservative modification will produce a peptide having functional and chemical characteristics similar to those of the master peptide.

The advantages of the invention manifest themselves in the overall stability of the marker peptides according to the invention as a reference in that the
5 corresponding peptide of the sample being analysed withstands the process steps of enzymatic digestion and mass spectrometric analysis and where necessary enrichment. This facilitates the preparation of a mass spectrometric pattern as a marker peptide reference indicative of an analysed protein, followed by mass spectrometric comparison with the sample being analysed. Hence, a marker peptide
10 according to the invention may be readily applied to a practical and easy-to-use kit for detecting, eg, a transgenic protein in a sample derived from a food matrix such as a food ingredient or a processed foodstuff.

Preferably, the marker peptide according to the invention will comprise a covalently bonded fluorophoric or chromophoric label or be labelled with an
15 isotopically-enriched substituent. Examples of suitable fluorophoric labels include dimethylaminonaphthalene-1-sulphonyl chloride (Dansyl reagent) or Cascade Yellow succinimidyl ester. Isotopic enrichment may be with ^2H , ^{13}C , ^{15}N or ^{18}O isotopes. ^{15}N enrichment is preferred.

According to the invention, there is further provided a marker peptide with a
20 sequence selected from SEQ ID NOs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18, including oxidised variants thereof, further comprising a covalently bonded fluorophoric or chromophoric molecular label, or isotopically-enriched label.

Preferably, the method of isolating the marker peptide according to the
25 invention will further comprise the step of isolating the marker peptide by a molecular imprinting technique using a polymeric adsorbent. More preferably, this will include forming a marker peptide:polymeric adsorbent complex and separating the marker peptide from the polymeric adsorbent by solvent elution.

According to the invention, there is also provided a marker
30 peptide:polymeric adsorbent complex, wherein the marker peptide has a sequence selected from SEQ ID no. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18, including oxidised variants thereof.

According to the invention, there is additionally provided the use of a peptide marker according to the invention or a marker peptide mixture according to the invention as a marker for detecting the presence of a transgenic protein extracted from a plant-derived material

5 According to the invention, there is also provided a method for isolating the marker peptides or marker peptide mixtures according to the invention comprising the steps of:

- providing a protein extract derived from a plant-derived material comprising 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS);
- 10 and
- enzymatic digestion of the protein extract.

According to the invention there is also provided a kit for detecting the presence of a transgenic protein extracted from a plant-derived material, comprising:

- 15 - a digestive enzyme;
- an enzyme solubilisation reagent;
- an enzyme reaction buffer; and
- a peptide marker as a reference comprising a marker peptide with a sequence selected from SEQ ID NOs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,
- 20 12, 13, 14, 15, 16, 17 and 18, including oxidised variants thereof.

According to the invention, there is provided a kit for the mass spectrometric detection and identification of a transgenic protein in a plant-derived material, comprising:

- a digestive enzyme;
- 25 - an enzyme solubilisation reagent;
- an enzyme reaction buffer; and
- a computer-readable algorithm capable of elucidating a mass spectrometric signal of a peptide marker reference with a sequence selected from SEQ ID NOs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,
- 30 14, 15, 16, 17 and 18, including oxidised variants, and any combination thereof.

Optionally, the kit according to the invention will include an amine-specific labelling reagent and/or a synthetic peptide and/or a stable isotope labelled peptide for the quantitative analysis of protein present in a sample. Examples of such reagents are the iTRAQTM and Protein-AQUATM reagents.

5 The kit according to the invention may also comprise solvents such as trifluoroacetic acid solution (0.1 to 1.0 %) and acetonitrile.

 The kit according to the invention may also comprise a matrix material for holding a sample to be analysed. Suitable matrix materials which apply to MALDI-TOF mass spectrometry include α -cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid and sinapinic acid. Other
10 suitable matrix material will be known to the skilled person.

 Examples of suitable enzyme solubilisation reagents include SDS, Triton X-100, Tween 20, Tween 40, Tween 60 and Tween 80, cholic acid and deoxycholate. Other suitable solubilisation reagents will be known to the skilled person.

15 Examples of enzyme reaction buffers include 100 mM Tris-HCl (pH 8.0), 500 mM tetraethylammonium bromide (TEAB) (pH 8.5) and 50mM ammonium bicarbonate (pH 8.0). Other suitable buffers will be known to the skilled person.

 The invention will now be illustrated with reference to the following examples which are not intended to limit the scope of the claimed invention.

20

EXAMPLE 1

Experimental procedure

25 Materials

 Tris(hydroxymethyl)methylamine (Tris-HCl), analytical grade acetone and ethanol, HPLC-grade acetonitrile (ACN), bromophenol blue and trifluoroacetic acid (TFA) for protein sequencing analysis were purchased from VWR (Poole, UK).

30 Sodium chloride (NaCl), SDS, DTT, iodoacetamide, EDTA, EGTA, N,N,N',N'-tetramethylethylenediamine (TEMED), glycerol, ammonium bicarbonate, ammonium persulfate, ProteosilverTM plus silver stain kit, HPLC-grade water,

endoproteinases Asp-N and Glu-C were from Sigma (Poole, UK). Bradford protein assay reagent was from Bio-Rad (Hemel Hempstead, UK) and formic acid (FA) super solvent purity from Romil (Cambridge, UK). Ammonium sulfate and MALDI grade 2,5-dihydroxybenzoic acid (2,5-DHB) were purchased from Fluka

5 BioChemica (Poole, UK).

Microcon centrifugal filter devices (MW cut off = 10,000 Da) and C₁₈ ZipTip pipette tips were from Millipore (Watford, UK). Ultrapure protogel and concentrated 10 X Tris/Glycine/SDS (electrophoresis grade) were from National Diagnostics (Hessle, UK). 0.45 µm Syringe filters were obtained from Fischer
10 Scientific (Loughborough, UK) and glass microfiber filters from Whatman (Brentford, UK). HiLoad 26/60 Superdex™ 75 gel filtration column and Q Sepharose resin were from Amersham Biosciences (Little Chalfont, UK).

Modified trypsin (sequencing grade) was purchased from Roche Diagnostics (Lewes, UK) and lateral flow immunostrips specific for CP4 EPSPS
15 were from Biofords Sarl (Evry, France). GM and non-GM soybean and maize seeds were obtained from Monsanto and distributed by Herbiseed (Twyford, UK).

Preparation of crude protein extracts from GM and non-GM seeds

20 GM and non-GM soybean and maize seeds were ground into a fine homogenous powder using a mechanical grinder. GM soybean at levels of 50 % and 0.9 % (w/w) was added to non-GM soybean powder. The soybean and maize mixtures were then mixed with 30 ml of cold acetone for 45 min and centrifuged at 4,000 g for 10 min at 4 °C. The acetone wash was repeated 4 times to remove any
25 lipid from the initial mixture. The soybean and maize samples were left to dry overnight at 4 °C under vacuum.

Extraction buffer (40 ml, 50 mM Tris-HCl pH 8, 5 mM DTT, 1 mM EDTA, 1 mM EGTA) was added to the dried soybean and maize pellet and mixed for 2.5 h at 4 °C. Each sample was then centrifuged at 4,000 g for 1 h at 4 °C, the pellet was
30 discarded, resulting in a soluble, dilapidated crude extract.

Immunodetection of CP4 EPSPS

Lateral flow strips were employed according to the manufacturer's recommendation to screen for the presence of CP4 EPSPS protein in the GM seeds. extracts and chromatographic fractions. A single line (control line) signified a negative result and the presence of two lines on the porous membrane indicated a positive sample.

Fractionation of soybean and maize proteins

10

60 % (w/v) Ammonium sulfate was slowly added to the crude extract and mixed for 2.5 h at 4 °C to precipitate proteins. The samples were then centrifuged at 100,000 g for 50 min, and the supernatants discarded. Each protein pellet was reconstituted with 20 ml of extraction buffer and filtered through a glass microfiber filter, followed by a 0.45 µm syringe filter.

The first chromatographic step in the purification procedure utilized a HiLoad 26/60 SuperdexTM 75 gel filtration column (selectivity range 30 to 70 kDa). An aliquot (13 ml) of the reconstituted protein pellet was loaded onto the column. 50 mM Tris-HCl pH 8, 2 mM DTT was used as the mobile phase, at a flow rate of 0.7 ml/min and 7 ml fractions were collected. The protein elution profile was monitored by measuring absorbance at 280 nm. In addition, a Bradford protein assay was performed on each gel filtration fraction according to manufacturer's instructions.

A strong anion exchange resin Q Sepharose in a column mode (10 x 2 cm) was used as the second chromatography step. Gel filtration fractions 25 to 29 (GM soya) or 22 to 25 (GM maize) were combined, loaded onto the anion exchange column and washed with 50 ml of 50 mM Tris-HCl pH 8, 2 mM DTT. The proteins were eluted using a linear gradient at a flow rate of 1 ml/min: mobile phase A (50 mM Tris-HCl pH 8, 2 mM DTT) and mobile phase B (50 mM Tris-HCl pH 8, 2 mM DTT, 400 mM NaCl). The protein elution profile was monitored by UV absorbance measurements as described above. Anion exchange fractions (1.1 ml) were desalted using Microcon filters (MW cut off = 10 kDa).

Gel filtration (1 ml aliquots) and anion exchange fractions were then lyophilized in a freeze dryer for two days and stored for further analysis.

SDS-PAGE and in-gel digestion

5

Lyophilized gel filtration and anion exchange fractions were reconstituted with 50 μ l and 20 μ l, respectively of 2 X treatment buffer (125 mM Tris-HCl, 4 % SDS, 20 % glycerol, 200 mM DTT, 0.02 % bromophenol blue, pH 6.8), then boiled for 3 min. Aliquots of 6 μ l (gel filtration fractions) and 10 μ l or 20 μ l from GM soya or maize (anion exchange fractions) were loaded onto a 10 % SDS-PAGE and a constant current of 15 mA was applied. The Sigma silver stain kit was employed to stain the gels according to the manufacturer's recommendations. Gel bands were excised at the expected molecular weight of CP4 EPSPS (47 kDa) and destained as described by the manufacturer.

15

The washed gel pieces were mixed with 25 μ l 10 mM DTT (in 25 mM ammonium bicarbonate, pH 8) and incubated at 56 °C for 1 h. The DTT solution was removed, 25 μ l 55 mM iodoacetamide (in 25 mM ammonium bicarbonate, pH 8) was added and then incubated at room temperature in the dark for 45 min. The gel pieces were washed three times for 20 min with 50 μ l 50 mM ammonium bicarbonate pH 8, and then dried three times with 50 μ l ACN for 15 min. The dried gel pieces were placed on ice for 10 min and 5 μ l (or as required to cover completely the gel) of 12.5 ng/ μ l trypsin (in 50 mM ammonium bicarbonate, pH 8) was added. They were left on ice for 30 min and subsequently 40 μ l 50 mM ammonium bicarbonate, pH 8 was added. In different experiments, aliquots of 25 ng/ μ l Glu-C (in 100 mM Tris-HCl, pH 7.8) or 20 ng/ μ l Asp-N (in 100 mM Tris-HCl, pH 8.5) were utilized. The samples were incubated at 37 °C overnight. The supernatant was then transferred into a clean Eppendorf tube. The gel pieces were mixed with 25 μ l ACN:0.1% TFA (50:50, v/v), sonicated for 15 min to elute the peptides and the supernatants were then combined. The solvent was evaporated in vacuo using a GyroVap GT (Howe, UK) and reconstituted with 7 μ l of HPLC-grade water. Alternatively, a 4 μ l aliquot was added to 0.9 % by weight GM soya fractions.

30

In-solution digestion

Aliquots (500 μ l) of gel filtration and anion exchange fractions were mixed
5 with 500 μ l of 50 mM Tris-HCl, pH 8, 8 M urea and 10 mM DTT for 4 h at 37 °C.
1 M iodoacetamide in ammonium bicarbonate, pH 8, was then added to a final
concentration of 100 mM. The samples were left in the dark at room temperature
for 45 min. Microcon filters (MW cut off = 10 kDa) were then employed to
concentrate the protein and exchange the buffer with 50 mM ammonium
10 bicarbonate, pH 8. Trypsin was added to create a protein-to-trypsin ratio of 1:100 to
1:1000 and left overnight at 37 °C. The digested peptides were again filtered
through Microcon filters.

MALDI-TOF mass spectrometry

15 Aliquots of in-solution (10 μ l) and in-gel (1 μ l) digested soybean samples
were acidified with 1 μ l of 1 % TFA (v/v) prior to desalting. Conditioning, loading
and washing of the ZipTips was according to the manufacturer's instructions.
Peptides were eluted from the ZipTips with 2 μ l of ACN:0.1 % TFA, (50:50 v/v)
20 directly onto a layer of 2,5-DHB crystals on a 600 μ m hydrophilic anchor
(AnchorChip600, Bruker Coventry, UK) formed from 0.7 μ l of a 5 mg/ml solution
of 2,5-DHB (ACN:0.1 % TFA, 50:50 v/v). MALDI-TOF spectra were acquired
with a Bruker MALDI-TOF Reflex III (Coventry, UK) operated in the positive
reflectron mode. Ions were generated by a nitrogen laser emitting at 337 nm. The
25 mass spectrometer was calibrated from m/z 500 to m/z 2500. Usually, the mass
spectra from 300 shots were averaged.

NanoLC-nanoESI- QTOF mass spectrometry

30 NanoESI MS and MS/MS experiments were performed on a QSTAR Pulsar
i (Applied Biosystems, Warrington, UK) hybrid quadrupole time-of-flight mass
spectrometer connected to a nanoLC system (LC Packings, Camberley, UK) using

a PepMap reverse phase C₁₈ column (15 cm x 75 µm i.d., 3 µm, 100 Å). The mobile phase consisted of solvent A (water:ACN 99:1 v/v in 0.1 % FA) and solvent B (water:ACN 5:95 v/v in 0.1 % FA). 5 µl of the in-gel (diluted 1 in 4 with 0.1 % FA) and in-solution digested mixtures were injected using an LC Packings FAMOS
5 autosampler and UltiMate LC pump. The peptides were desalted on a LC Packings C₁₈ trap column connected to a LC Packing Switchos micro column switching module. Peptide separation was achieved using linear gradient elution from 1 to 60 % B over 60 min then ramped to 99 % B in 1 min and held at 99 % B for 10 min. Subsequently it was returned to 1 % B in 1 min and allowed to reequilibrate in this
10 solution for 18 min. The flow rate was 200 nl/min. A Protana nanospray interface and 10 µm distal coated fused silica PicoTips (New Objective, Woburn, USA) were used for nanoESI. The instrument was automatically calibrated according to the manufacturer's instructions and collision energy was set automatically to produce optimum fragmentation of the precursor ion. Analyst QS 1.0 sp8 software from
15 Applied Biosystems was employed for data analysis.

Quadrupolar ion-trap mass spectrometry

Quadrupolar ion trap MS was carried out on a LCQ DECA from
20 Thermo Finnigan (Cambridge, United Kingdom).

10-20 µl samples were injected via TSP AS3000 autosampler, onto a reverse phase C18 LC column. The mobile phase comprised: A = water containing 0.5% formic acid; B = acetonitrile containing 0.5% formic acid and the gradient: 0-10 min 99:1, 30 min 50:50, 34 min 10:90, 40 min 10:90, 44 min 99:1, and 53 min
25 99:1. The flow rate was 0.2 ml/min, with a run time of 50 min. The ESI MS was set up as follows:

Tune method: pep-050820

Spray: 4.5 kV

30 Capillary temperature: 220

Capillary voltage: 15 V

Tube lens offset: 15 V

Sheath gas 70 (arbitrary units)

Auxiliary gas 10 (arb. units)

3 microscans

max ion inject time: 500 ms

5 AGC ON

Triple play MS/MS method m/z 200-1600

Default charge state 2

Default isolation width: 2

10 Normalized collision energy: 35.0% (arbitrary units)

Parent masses: 378.0, 755.3

For soya:

SIM: 555.3-565.3

15 MS/MS: 560.0, IW 8, NCE 35%.

Database search and protein identification

The peptide mass lists obtained by MALDI-TOF MS and nanoESI-QTOF
20 MS were submitted for database searching and compared to predicted sequences in
the viridiplantae category of the NCBI nr and SwissProt databases. Generally, the
mass tolerance was set to 100 ppm; up to one missed cleavage was allowed,
carbamidomethylation of Cys was considered as a fixed modification and
methionine oxidation as a variable modification. Analogously, nanoLC-nanoESI-
25 QTOF MS/MS spectra were submitted to MS/MS ion searches for protein
identification.

The results in accordance with the example are now discussed.

Enrichment of CP4 EPSs in GM soybean extracts

30

Protein extraction facilitated removal of lipids, carbohydrates and small
molecules from soybean seeds. The average protein content in crude extracts from two

different batches of GM soybean seeds analyzed in duplicate was 15.2 ± 1.2 mg/ml. Fractionation of the GM soybean proteome was necessary as the transgenic CP4 EPSPS could not be identified directly from crude protein extracts.

The protein elution profile from gel filtration chromatography showed that
5 higher molecular weight proteins or protein aggregates eluted in early fractions (15 to 20), as they were possibly outside the optimum fractionation range (column void volume 105 ml; Fig. 2A). Lateral flow immunostrips detected high levels of CP4 EPSPS in gel filtration fractions 25 to 29. However, SDS-PAGE of these and adjacent
10 fractions indicated that their protein contents were still very complex. Nonetheless, fractions 27 and 29 showed distinct bands at the expected molecular weight of CP4 EPSPS, at around 47 kDa (Fig. 2B). Approximately 80 to 90 % of the total protein content was removed when fractions 25 to 29 were combined for further purification. Protein elution profile from anion exchange chromatography was even over the majority of fractions and showed an elution maximum at higher salt concentration (Fig. 2C).
15 Immunostrips showed that most of CP4 EPSPS was eluted in fractions 75 to 90. SDS-PAGE of these fractions visualized intense bands at approximately 47 kDa implicating the potential presence of the GM protein (Fig. 2D).

Peptide mass mapping of CP4 EPSPS by MALDI-TOF MS

20

At the beginning of this investigation, the crude protein extract from 100 % by weight GM soya was analyzed by SDS-PAGE prior to gel filtration chromatography (data not shown). A tryptic mass map of the region of the gel at the expected molecular weight of CP4 EPSPS was obtained by MALDI-TOF MS
25 (Fig.3A). β -Conglycinin, an abundant seed storage protein, was identified with a total of twelve matched peptides. However, tryptic peptides from CP4 EPSPS were not observed, possibly because β -conglycinin suppressed the detection of the less abundant GM protein. In contrast, when a 47 kDa SDS-PAGE band of the gel filtration fraction 27 from 100 % by weight GM soybean was subjected to peptide
30 mass mapping, a different set of peptide signals was detected (Fig. 3B). Nine tryptic peptides enabled the identification of CP4 EPSPS from *Agrobacterium sp.* by searching the NCBI database. The amino acid sequence coverage was 26 %.

A CP4 EPSPS tryptic mass map was also obtained from a 47 kDa band of anion exchange fraction 82 from 100 % by weight GM soybean with eleven matched peptides (31 % sequence coverage; (Fig. 3C). Similarly, CP4 EPSPS was also identified by MALDI-TOF MS of digested 47 kDa gel bands from other gel filtration (e.g. 25 and 29; Fig. 2B) and other anion exchange fractions (e.g. 75, 77, 80, 85 and 95; Fig. 2D) from 100 % by weight GM soya. For 50 % by weight GM soya the same analytical strategy as presented above was successful.

Tryptic in-solution digestion of CP4 EPSPS-containing gel filtration and anion exchange fractions from 100 % by weight and 50 % by weight GM soya resulted in the identification of abundant soya proteins (e.g. glycinin, dehydrin, etc.) but not CP4 EPSPS.

In conclusion, gel filtration chromatography followed by SDS-PAGE and tryptic peptide mass mapping was necessary for the detection of CP4 EPSPS derived peptide markers as inferred from Fig. 3B and C.

Anion exchange chromatography provided further purification required for the analysis of lower levels of CP4 EPSPS as described below. Furthermore, CP4 EPSPS was identified after digestion of 47 kDa gel bands from anion exchange fractions with endoproteinases Glu-C and Asp-N. The combination of the analytical data of three proteolytic enzymes with different cleavage properties produced a more extensive overall sequence coverage of CP4 EPSPS (75 %; data not shown).

Identification of CP4 EPSPS with nanoLC-nanoESI-QTOF MS

Reverse phase nanoLC offers an additional separation step at the peptide level, and hence could facilitate the identification of the transgenic protein from more complex mixtures when coupled to a nanoESI-QTOF mass spectrometer. CP4 EPSPS marker peptides were mostly detected as doubly charged ions (i.e. $[M+2H]^{2+}$ at m/z 558.29, 679.36, 680.32, 779.92, 823.91, 881.93, 997.48, etc.) by nanoLC-nanoESI-QTOF MS. Subsequently, the parent ions were fragmented and the GM protein was identified by MS/MS ion searches. A comparison of CP4 EPSPS tryptic peptides from a 47 kDa gel band of anion exchange fraction 75 from

50 % by weight GM soya detected by MALDI-TOF MS and nanoESI-QTOF MS and MS/MS is presented in Table 1 (refer below).

NanoLC separation prior to QTOF MS of in-solution digested CP4 EPSPS containing anion exchange fraction from 50 % by weight GM soya showed CP4 EPSPS marker peptides, for example $[M+2H]^{2+}$ at m/z 558.29 (and its corresponding $[M+H]^+$ at m/z 1115.61) and $[M+2H]^{2+}$ at m/z 779.92 (Fig. 4A and B). A total of eight tryptic peptides were then fragmented and their MS/MS spectra (Fig. 4C and D) enabled the identification of the transgenic protein through database matching. Several other soya proteins were also identified such as glycinin G2 (4 peptides), G1 (7 peptides) and G4 (2 peptides) precursor, napin-type 2S albumin (7 peptides), stress-induced protein SAM22 (3 peptides) and maturation-associated protein MAT9 (2 peptides). In comparison, only napin-type 2S albumin but not CP4 EPSPS was identified when the same sample was analyzed by peptide mass mapping using MALDI-TOF MS.

Sample preparation strategies investigated for the detection of CP4 EPSPS are summarized in Table 2 (refer below) and comparable results were obtained from 100 % by weight and 50 % by weight GM soybean seeds. Tryptic mass maps from the in-gel digested CP4 EPSPS previously fractionated by gel filtration and/or gel filtration followed by anion exchange could be detected by MALDI and nanoESI. Generally, nanoLC-nanoESI-QTOF enabled the identification of CP4 EPSPS from more complex protein mixtures.

Identification of CP4 EPSPS from 0.9 % by weight GM soya

The current EU threshold level for labeling GM-containing products is $\leq$ 0.9 % by weight. Thus, the analytical procedures developed in this study were tested for their suitability to detect threshold amounts. Gel filtration and anion exchange chromatography followed by SDS-PAGE were used to fractionate proteins from 0.9 % by weight GM soybean seeds. Two anion exchange fractions (i.e. 83 and 84, 78 and 79, 82 and 86, etc.) were combined to increase the amount of CP4 EPSPS present on SDS-PAGE. MALDI TOF spectra of protein bands around 47 kDa from anion exchange combined fractions produced a tryptic peptide

map of CP4 EPSPS. The transgenic protein was also identified by nanoLC-nanoESI-QTOF MS/MS, as fingerprints from two CP4 EPSPS marker peptides $[M+2H]^{2+}$ at m/z 558.29 and 779.93 were obtained.

Non-GM soybean seeds were analyzed using an identical analytical approach and, as expected, CP4 EPSPS peptides were not observed. For comparison, selected masses (ion extract chromatogram TOF MS) from CP4 EPSPS tryptic peptides $[M+2H]^{2+}$ at m/z 558.29, 779.93 and 881.93 demonstrated the presence of these markers from a 47 kDa digested band of anion exchange combined fractions 82 and 86 from 0.9 % by weight GM soya. However, these CP4 EPSPS peptides were absent when the same combined fractions from non-GM soybean were analyzed (Fig. 5A-C).

GM maize: CP4 EPSPS enrichment and MS detection

CP4 EPSPS was also identified in 100 % by weight GM maize seeds employing the above strategies.

In analogy to GM soya, lateral flow immunostrips detected the transgenic protein in gel filtration fractions and the corresponding SDS-PAGE profiles were also highly complex (data not shown). However, compared to GM soya fewer SDS-PAGE bands were observed in the anion exchange fractions that contained bands at approximately 47 kDa for CP4 EPSPS (Fig. 6). An interesting observation was that despite identical sample preparation for both GM crops less intense CP4 EPSPS bands were obtained from the maize preparation used herein.

In analogy to GM soya, tryptic peptide maps of CP4 EPSPS were obtained from 47 kDa SDS-PAGE bands of gel filtration and anion exchange fractions from 100 % by weight GM maize by MALDI TOF MS that enabled its identification by database searching (Fig. 7). Maize globulin-2-precursor but not the transgenic protein was detected when a 47 kDa band from the crude protein extract was subjected to peptide mass mapping. CP4 EPSPS peptides from 100 % by weight GM maize were also detected by nanoLC-nanoESI QTOF MS/MS. The CP4 EPSPS peptide maps generated from GM maize and GM soya seeds were similar and generally the majority of marker peptides were found in both GM crops. Total

sequence coverage of CP4 EPSPS from GM maize was 71 % compared to 75 % from GM soya as reported above, using trypsin, endoproteinase Asp-N and Glu-C.

Example 2

5

Quantification of CP4 EPSPS from GM soya, maize and foods

CP4 EPSPS from GM soya

10 GM and non-GM soyabean seeds were ground into a fine homogenous powder using a mechanical grinder. GM soya at levels of 5, 2, 0.9 and 0.5 % (w/w) was added to non-GM soya powder. The seeds were delipidated using acetone washes. 7 g of the GM soya mixtures were then extracted using 40 ml of 50mM TEAB, pH 8.5 was employed instead of Tris-HCl.

15

Each soya extract was divided into four tubes that generally contained 6 ml of protein solution each. 6 volumes of cold acetone were slowly added to each tube and the crude extracts were mixed for 4 h at 4 °C to precipitate proteins. The samples were then centrifuged at 4,000 g for 15 min at 4 °C, and the supernatant
20 was discarded. Subsequently, protein pellets were reconstituted as follows: each protein pellet was mixed with 5 ml of 50 mM TEAB, pH 8.5, vortex mixed for 30 s, sonicated for 5 min and finally centrifuged at 4,000 g 15 min. The supernatants were transferred into a clean tube and the remaining protein pellet was subjected to the same procedure twice more. The four reconstituted pellets from each GM soya
25 preparation were combined in a single tube. Small insoluble pellets were still present in all reconstituted samples, which most likely contain hydrophobic and membrane proteins.

Fractionation of CP4 EPSPS by anion exchange chromatography was achieved as described above.

30

Sample preparation of soya for AQUA quantification of CP4 EPSPS

For each of the soya preparations (5, 2, 0.9 and 0.5 % GM), a 1 ml aliquot of the combined anion exchange fractions was desalted using Microcon filters (MW cut off = 10 kDa). The final protein concentration of all samples was estimated using Bradford assay. The solutions were then lyophilized in a freeze dryer for two days and stored until further analysis.

Protein separation was carried out using SDS-PAGE as described above. In these experiments the lyophilized samples were reconstituted with 50 µl of 2 X treatment buffer and 10 µl were loaded on the gel. Reduction, alkylation and in gel digestion of GM soya samples was performed as described above except that 400 fmol of a stable isotope labelled peptide was added along with trypsin. The synthetic peptide L*AGGEDVADLR ($L^* = {}^{13}\text{C}$) was selected as internal standard since LAGGEDVADLR was a particular intense CP4 EPSPS peptide detected by MS.

Extraction and quantification of CP4 EPSPS added to foods

7 g of various foods purchased from a local supermarket were deliberately contaminated with different levels of Roundup ReadyTM soya, i.e., soya and linseed bread contained 0.9 and 10 % GM, beef and tomato pot noodle 10 % GM and pork sausages 10 % GM. The non-contaminated counterpart of these foods were analysed in parallel. The same protocols as described above were applied to each sample, except that the lyophilized samples were reconstituted with 75 µl of 2 X treatment buffer and 7 µl were loaded on the gel. Additionally, quantification of CP4 EPSPS was accomplished by adding 400 fmol of the synthetic internal standard peptide L*AGGEDVADLR ($L^* = {}^{13}\text{C}$) alongside with trypsin before incubation overnight at 37 °C.

EPSPS Quantification using AQUA

In these studies, L*AGGEDVADLR with incorporated stable isotope ^{13}C on the N-terminal L was chosen as an internal standard to mimic the native LAGGEDVADLR peptide identified using various protein fractionation strategies combined with MS. Protein quantification was accomplished by two different MS
5 methods. Results are tabulated in Table 3.

Firstly, the mass spectrometer was set scan ions from m/z 400 to m/z 1400 and subsequently ions with intensity level above 10 counts were selected for fragmentation using an IDA experiments. The two target peptides LAGGEDVADLR and L*AGGEDVADLR at m/z 558.3 and 561.3 respectively,
10 were annotated in an inclusion list so that they had priority for fragmentation experiments. Data was processed by manual integration of the peaks in an extracted chromatogram for both monitored peptides (at m/z 558.3 and 561.3) and the selected window was ± 0.2 m/z . The peak area of the native peptide was divided by the peak area of the internal standard. Absolute quantification could be then
15 achieved by multiplying the obtained ratio by the absolute amount of the internal standard (400 fmol) (Refer Figure 8). Furthermore, native/internal standard ratios obtained in each sample could be compared against other GM soya preparations and evaluate against theoretical values, e.g. [5/0.9] % GM should be 5.56.

20 Example 3

Molecular imprinting analysis

GM soyabean seeds were ground into a fine homogenous powder in an
25 automated freezer mill (Glen Greston Ltd, Stanmore, UK). A 10.0 g sample was then delipidated by acetone washing (5 x 50 ml washings). The resulting protein precipitate was re-suspended in 100 ml of a 50 mM aqueous ammonium bicarbonate solution (pH 8). Trypsin was added to the suspension in a substantial excess to create a protein-to-trypsin ratio of $\sim 1:100$. The mixture was left
30 overnight at 37 °C to permit comprehensive digestion of the transgenic protein.

The digested suspension was filtered to separate particulate material from the suspension and the clear supernatant fraction transferred to a 250 ml flask

comprising 20 g of a fine polymeric adsorbent selective for LAGGEDVADLR. The polymeric adsorbent was assembled from methacrylic acid monomeric units from a LAGGEDVADLR template according to the methods generally referred to in Kandimalla VB, Ju H, *Anal Bioanal Chem* (2004) **380**, 587-605.

5 The polymeric adsorbent mixture was gently stirred at room temperature overnight to enable binding of LAGGEDVADLR present in the digested protein extract to the polymeric adsorbent. Polymeric adsorbent complexed to LAGGEDVADLR was filtered off without washing and dried *in vacuo* at room temperature. Elution of LAGGEDVADLR was undertaken by rigorous stirring of
10 the dried adsorbent:LAGGEDVADLR complex at 37 °C in 100 ml of methanol for 30 minutes. The de-complexed adsorbent was removed from the eluting solvent by filtration and washed with 2 x 50 ml aliquots of the acetonitrile. The supernatant fraction was reduced *in vacuo* to 100 ml and an excess (0.5 g) of dansyl reagent (Sigma-Aldrich) added with rigorous stirring. The solution was stirred for 2 hours
15 until labelling of LAGGEDVADLR was complete. To remove excess dansyl reagent, the solution underwent HPLC separation with a C18 column.

 The chromatographed sample of labelled LAGGEDVADLR-dansyl was reduced *in vacuo* at room temperature to 10 ml and a 1 ml aliquot subjected to UV/Visible spectrometry. From a known value for the extinction co-efficient of
20 LAGGEDVADLR-dansyl derived from synthetic LAGGEDVADLR and its characteristic absorbance wavelength, the concentration of GM protein (ie, EPSPS) present in the GM soyabeans of the example (ie, 10.0 g of starting material) could be easily extrapolated.

Table 1. Predicted and experimental CP4 EPSPS tryptic peptides.

Residue No.	[M+H] ⁺	Sequence	MALDI MS	NanoESI MS	NanoESI MS/MS
1-13	1354.701	MSHGASSRPATAR	-	-	-
14-14	147.113	K	-	-	-
15-23	863.458	SSGLSGTVR	-	+	-
24-28	529.298	IPGDK	-	-	-
29-33	599.326	SISHR	-	-	-
34-46	1359.636	SFMFGGLASGETR	+	+	+
34-46 oxid	1375.635	SFMFGGLASGETR	+	-	+
47-61	1558.832	ITGLLEGEDVINTGK	+	+	+
62-69	835.391	AMQAMGAR	+	+	-
70-71	288.203	IR	-	-	-
72-72	147.113	K	-	-	-
73-104	3243.538	EGDTWIIDGVGNGLLAPEAP DLFGNAATGCR	-	-	-
105-127	2450.216	LTMGLVGVDSTFIGDASL TK	-	-	-
128-132	616.335	RPMGR	-	-	-
133-138	711.451	VLMPLR	+	+	-
133-145	1482.846	VLNPLREMGVQVK	+	-	+
133-145 oxid	1498.845	VLNPLREMGVQVK	+	-	+
139-145	790.413	EMGVQVK	+	+	-
146-151	678.269	SEDGDR	-	-	-
146-157	1357.707	SEDGDRLPVTLR	+	+	+
152-157	698.456	LPVTLR	-	-	-
158-168	301.187	GPK	-	-	-
161-168	948.515	TPTPITYR	+	+	-
161-177	1860.00	TPTPITYRVPMASAQVK			+
161-177 oxid	1875.990	TPTPITYRVPMASAQVK			+
169-177	930.508	VPMASAQVK	+	+	

178-200	2367.332	SAVLLAGLNTPGITTVIEPIMTR	-	-	
201-205	629.289	DHTEK	-	-	-
206-224	1993.965	MLQGFGANLTVETDADGVR	+	-	+
206-224 oxid	2009.964	MLQGFGANLTVETDADGVR	+	-	+
225-227	389.251	TIR	-	-	-
228-231	474.267	LEGR	-	-	-
232-233	204.134	GK	-	-	-
234-274	4188.262	LTGQVIDVPGDPSSTAFPLVAA LLVPGSDVTILNVLMNPTR	-	-	-
275-294	2183.174	TGLLTLQEMGADIEVINPR	-	-	-
275-294 oxid	2199.1734	TGLLTLQEMGADIEVINPR		-	
295-305	1115.569	LAGGEDVADLR	+	+	+
306-307	274.187	VR	-	-	-
308-312	535.309	SSTLK	-	-	-
313-320	872.447	GVTVPEDR	+		-
321-351	3249.617	APSMIDEYPILAVAAFAEGAT VMNGLEELR	-	-	-
352-353	246.181	VK	-	-	-
354-357	506.221	ESDR	-	-	-
354-366	1359.676	ESDRLSAVANGLK	-	+	+
358-366	872.520	LSAVANGLK	-	-	-
367-382	1762.828	LNGVDCDEGETSLVVR	+	-	+
383-388	629.337	GRPDGK	-	-	-
389-405	1646.836	GLGNASGA AVATHLDHR	+	-	+
406-446	4320.102	IAMSFLVMGLVSENPTVDDA TMIATSFPEFMD LMAGLGAK	-	-	-
447-453	805.430	IELSDTK	-	+	-
454-455	161.092	AA	-	-	-

Predicted tryptic peptides from CP4 EPSPS are sorted by residue number. The experimental CP4 EPSPS matched peptides of a 47 kDa SDS-PAGE band of anion exchange fraction 75 from 50 % by weight GM soybean are also presented.

Tryptic peptides were identified by peptide mass fingerprinting (PMF) using MALDI and nanoESI and by MS/MS searches utilizing nanoESI. Matched peptides as a result of one missed cleavage and oxidation are also shown. "oxid" indicates peptide oxidation on methionine.

Table 2. Analytical approaches for the detection of CP4 EPSPS from 100 % by weight, 50 % by weight and 0.9 % by weight Roundup Ready™ soybean.

MALDI-TOF	Roundup Ready™ % GM soya (w/w)		
	100%	50%	0.9%
<i>Purification steps</i>	<i>CP4 EPSPS</i>		
GF→AE→SDS-PAGE	+	+	+
GF→ SDS-PAGE	+	+	ND
SDS-PAGE	-	-	ND
GF→AE	-	-	ND
GF	-	-	ND
Crude extract	ND	-	ND
NanoLC-nanoESI-QTOF	100%	50%	0.9%
<i>Purification steps</i>	<i>CP4 EPSPS</i>		
GF→AE→SDS-PAGE			
PMF	+	+	-
MS/MS	+	+	+
GF→ SDS-PAGE			
PMF	+	-	ND
MS/MS	+	+	ND
SDS-PAGE			
PMF	-	-	ND
MS/MS	+	+	ND
GF→AE			
PMF	-	-	ND
MS/MS	+	+	ND
GF			
PMF	-	-	ND
MS/MS	-	-	ND
Crude extract			
PMF	ND	-	ND
MS/MS	ND	-	ND

[001] Fractionation of CP4 EPSPS was carried out by gel filtration (GF), anion exchange chromatography (AE) and SDS-PAGE. MALDI-TOF MS and nanoLC-nanoESI-QTOF MS and MS/MS were employed for the detection of GM soya tryptic peptides. (+) indicates positive identification of CP4 EPSPS, (-) indicates no
5 identification of CP4 EPSPS and (ND) signifies that analysis was not performed.

Table 3. Ratios for EPSPS in GM soya using Protein-AQUA™ labelling.

	% GM soya	Theoretic	Gel A	Gel B	Control
5	5/2	2.50	1.98	2.02	2.39
	0.9/2	0.45	0.47	0.43	0.47
	0.5/2	0.25	0.28	0.16	0.30
10	5/0.9	5.56	4.19	4.72	5.11
	0.5/0.9	0.56	0.60	0.38	0.63
	0.5/2	0.25	0.28	0.16	0.30

15

[002] GM soya at concentrations of 5, 2, 0.9 and 0.5% were purified as described and gel bands mixed with 400 fmol of the ^{13}C labelled peptide L*AGGEDVADLR (L* = ^{13}C). Subsequent trypsin digestions and LC/MS/MS of two samples resulted in the results shown in Fig. 8, whilst the comparison between experimental and

20 theoretical levels is shown in Fig. 9.

CLAIMS

1. A method for determining the presence of genetically-modified plant-derived material in a product, the method comprising:
- 5 - providing a protein extract derived from the product;
- enriching the protein extract;
- digesting the protein extract using an enzyme; and
- detecting the presence or absence of at least one marker peptide resulting from the enzymatic digestion of a transgenic protein
- 10 thereby determining whether the genetically modified plant derived material is present in the product.
2. A method according to claim 1, wherein the enriching step is carried out prior to enzymatic digestion.
- 15 3. A method according to claim 1 or 2, wherein enrichment of the protein extract comprises gel filtration, anion exchange chromatography and/or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
- 20 4. A method according to any one of claims 1 to 3, wherein enrichment of the protein extract comprises gel filtration and SDS-PAGE.
5. A method according to any one of the preceding claims, wherein enrichment of the protein extract comprises gel filtration, anion exchange
- 25 chromatography and SDS-PAGE.
6. A method according to any one of the preceding claims, wherein the enriching step is carried out after enzymatic digestion or a further enrichment step is carried out after enzymatic digestion.
- 30 7. A method according to claim 6, wherein the enriching step comprises reverse phase nano liquid chromatography.

8. A method according to any one of the preceding claims, wherein the presence or absence of the at least one marker peptide is detected by mass spectrometric analysis.
- 5 9. A method according to claim 8, wherein the amount of marker peptide present is determined by quantitative mass spectrometric analysis.
- 10 10. A method according to claim 9, which further comprises:
- providing a sample containing a known quantity of a reference marker peptide;
 - 10 - subjecting the sample to mass spectrometric analysis to produce a mass spectrometric signal; and
 - 15 - comparing the mass spectrometric signal produced by the sample and the mass spectrometric signal produced by the digested protein extract to determine the quantity of marker peptide in the protein extract.
11. The method according to claim 10, wherein the reference marker peptide has a molecular label, the molecular label preferably being provided by an amine-specific labeling reagent, a synthetic peptide or a stable isotope-labeled peptide.
- 20 12. The method according to any one of claims 8 to 11, wherein the mass spectrometric analysis comprises elucidating a mass spectrometric signal of a known transgenic protein by means of computer analysis.
- 25 13. The method according to any one of claims 1 to 7, wherein the presence or absence of the at least one marker peptide comprises:
- isolating of the marker peptide by a molecular imprinting technique using a polymeric adsorbent;
 - 30 - labelling the isolated marker peptide with a fluorophoric or chromophoric molecular label; and

- detecting the presence or absence of the marker peptide by spectroscopy.
14. The method according to claim 13, further comprising:
- 5 - providing a reference marker peptide labelled with a fluorophoric or chromophoric molecular label and detecting the presence of the labelled reference marker peptide.
15. A method according to claim 13 or 14, wherein the amount of
- 10 marker peptide present is detected using quantitative analysis by UV/Visible spectroscopy or fluorescence spectroscopy.
16. A method according to any one of the preceding claims, wherein the transgenic protein is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).
- 15
17. A method according to claim 16, wherein the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is derived from *Agrobacterium tumefaciens* CP4.
- 20 18. The method according to claim 16 or 17, wherein the marker peptide has a sequence selected from SEQ ID NOs. 1 to 18 and oxidised variants thereof.
19. The method according to claim 18, wherein the marker peptide(s) has a sequence selected from SEQ ID NOs. 2, 3, 8 and 13 or any combinations
- 25 thereof.
20. The method according to any one of the preceding claims, wherein the product is a food matrix.
- 30 21. The method according to claim 20, wherein the food matrix is a processed foodstuff, an animal feedstuff or animal waste material.

22. The method according to any one of the preceding claims, wherein the plant-derived material is maize or soyabean.

23. A marker peptide for use in determining the presence of genetically modified plant-derived material in a product, the marker peptide having a sequence selected from SEQ ID NOS: 1 to 18 and oxidised variants thereof.

24. The marker peptide according to claim 23, wherein the marker peptide has a sequence selected from SEQ ID NOS. 2, 3, 8 and 13.

25. A composition for use in determining the presence of genetically modified plant-derived material in a product, the composition comprising two or more marker peptides as defined in claim 23.

26. A composition according to claim 25, which comprises at least:
SEQ ID NOS. 2 and 3;
SEQ ID NOS. 2 and 8;
SEQ ID NOS. 2 and 13;
SEQ ID NOS. 3 and 8;
SEQ ID NOS. 3 and 13;
SEQ ID NOS. 8 and 13;
SEQ ID NOS. 2, 3 and 8;
SEQ ID NOS. 2, 3 and 13;
SEQ ID NOS. 2, 8 and 13;
SEQ ID NOS. 3, 8 and 13; or
SEQ ID NOS. 2, 3, 8 and 13.

27. A marker peptide:polymeric adsorbent complex, wherein the marker peptide has a sequence selected from SEQ ID NOS. 1 to 18 and oxidised variants thereof.

28. A marker peptide according to claim 23 or 24, a composition according to claim 25 or 26 or a complex according to claim 27, wherein the marker peptide is covalently bonded to a fluorophoric or chromophoric molecular label, or an isotopically-enriched label.

5

29. The marker peptide, composition or complex according to claim 28, wherein the fluorophoric or chromophoric molecular label is 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dansyl reagent) or Cascade Yellow succinimidyl ester.

10

30. A method for isolating a marker peptide according to claim 23 or 24 or a composition according to 25 or 26 comprising the steps of:

- providing a protein extract derived from a plant-derived material comprising 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS);
- enriching the protein extract; and
- digesting the protein extract with an enzyme.

15

31. The method according to claim 30, further comprising the steps of forming a marker peptide:polymeric adsorbent complex and separating the marker peptide from the polymeric adsorbent by solvent elution.

20

32. The method according to claim 30 or 31, wherein said enriching is carried out prior to enzymatic digestion.

25

33. The method according to any one of claims 30 to 32, wherein the enrichment step comprises gel filtration, anion exchange chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

30

34. The method according to any one of claims 1 to 22 and 30 to 33 wherein the enzyme is trypsin, endoproteinase AspN and/or endoproteinase Glu-C.

35. Use of a peptide marker according to claims 23 or 24 or a composition according to claim 25 or 26 or a complex according to claim 27 for detecting the presence of a genetically modified in a plant-derived material in a product.

5

36. A kit for detecting the presence of a genetically modified plant-derived material in a product, the kit comprising:

- a digestive enzyme;
- an enzyme solubilisation reagent;
- 10 - an enzyme reaction buffer; and
- at least one reference peptide marker or a computer-readable algorithm capable of elucidating a mass spectrometric signal of at least one reference peptide marker.

15 37. A kit according to claim 36, wherein the at least one reference peptide marker is selected from SEQ ID NOS: 1 to 18 and oxidised variants thereof.

20 38. The kit according to claim 36 or 37, wherein the at least one reference marker peptide is derivatized by an amine-specific labeling reagent and/or a stable isotope labeled peptide and/or is labeled by a fluorophoric or chromphoric molecular label.

25 39. A kit according to claim 36 to 38, further comprising:

- a polymeric adsorbent selective for a peptide marker; and optionally
- a solvent suitable for eluting a peptide marker from a polymeric adsorbent.

30 40. A kit according to claim 36, wherein the algorithm is adapted for multivariate data analysis and pattern recognition of a mass spectrometric signal and wherein the mass spectrometric signal optionally relates to a plurality of molecular ions.

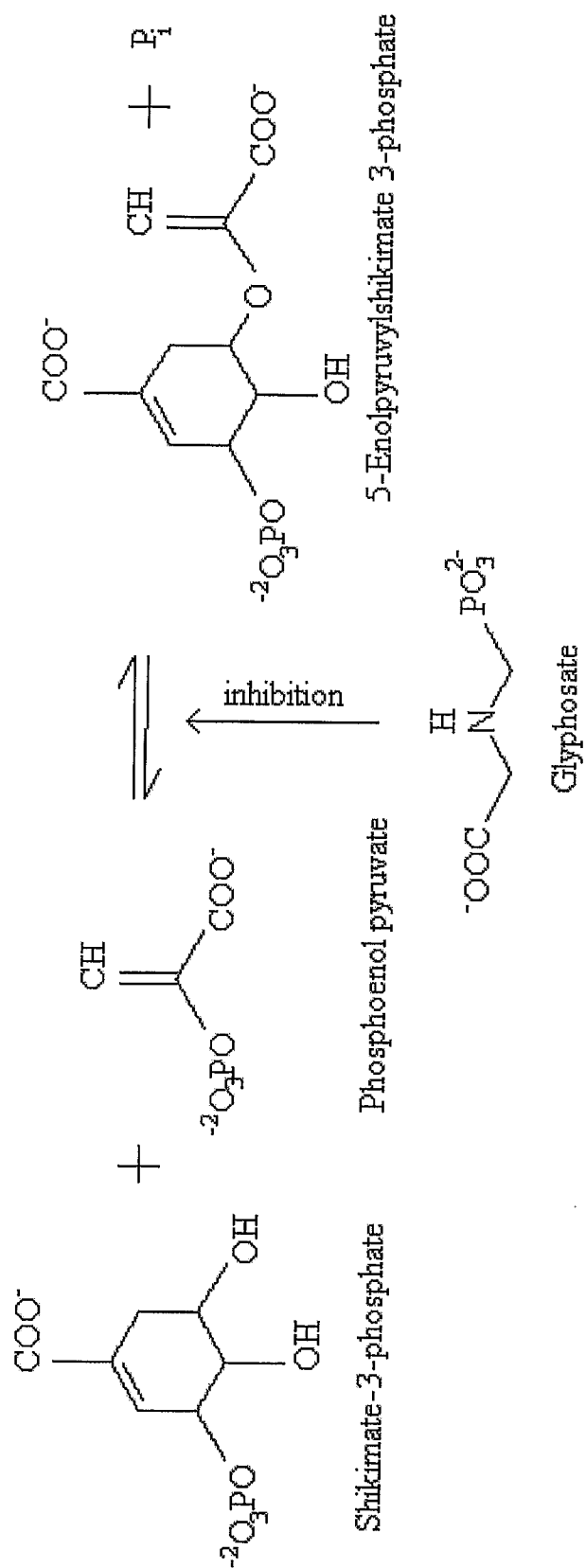


Fig. 1

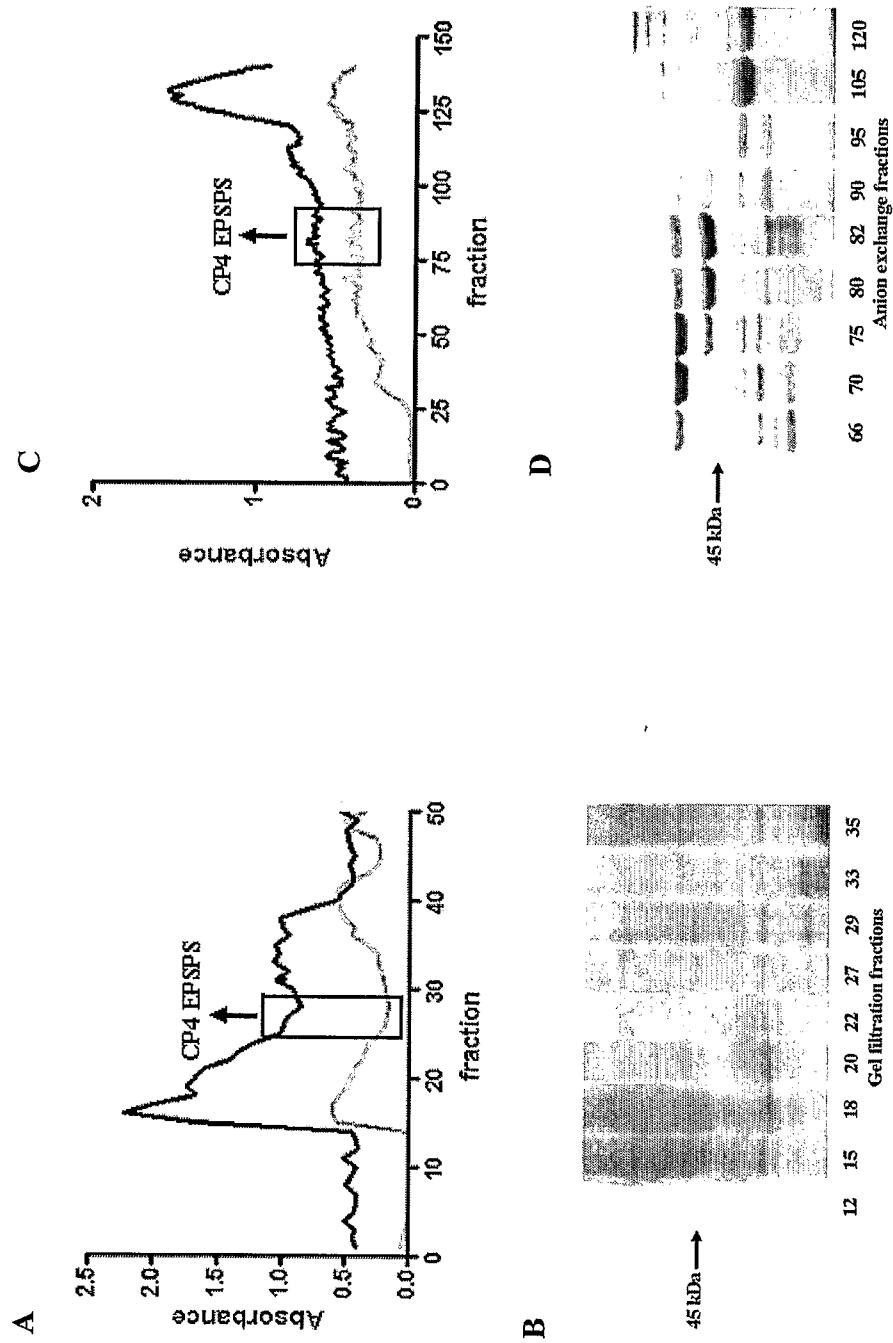


Fig. 2

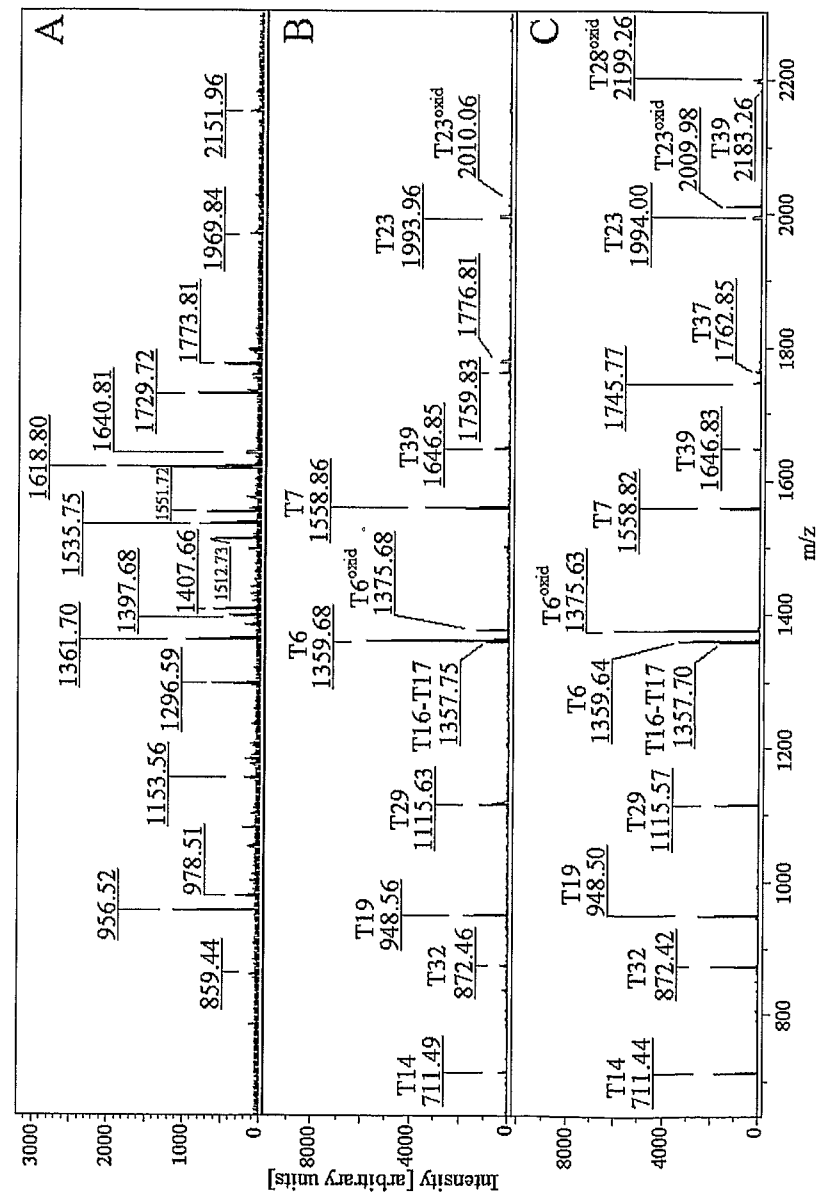
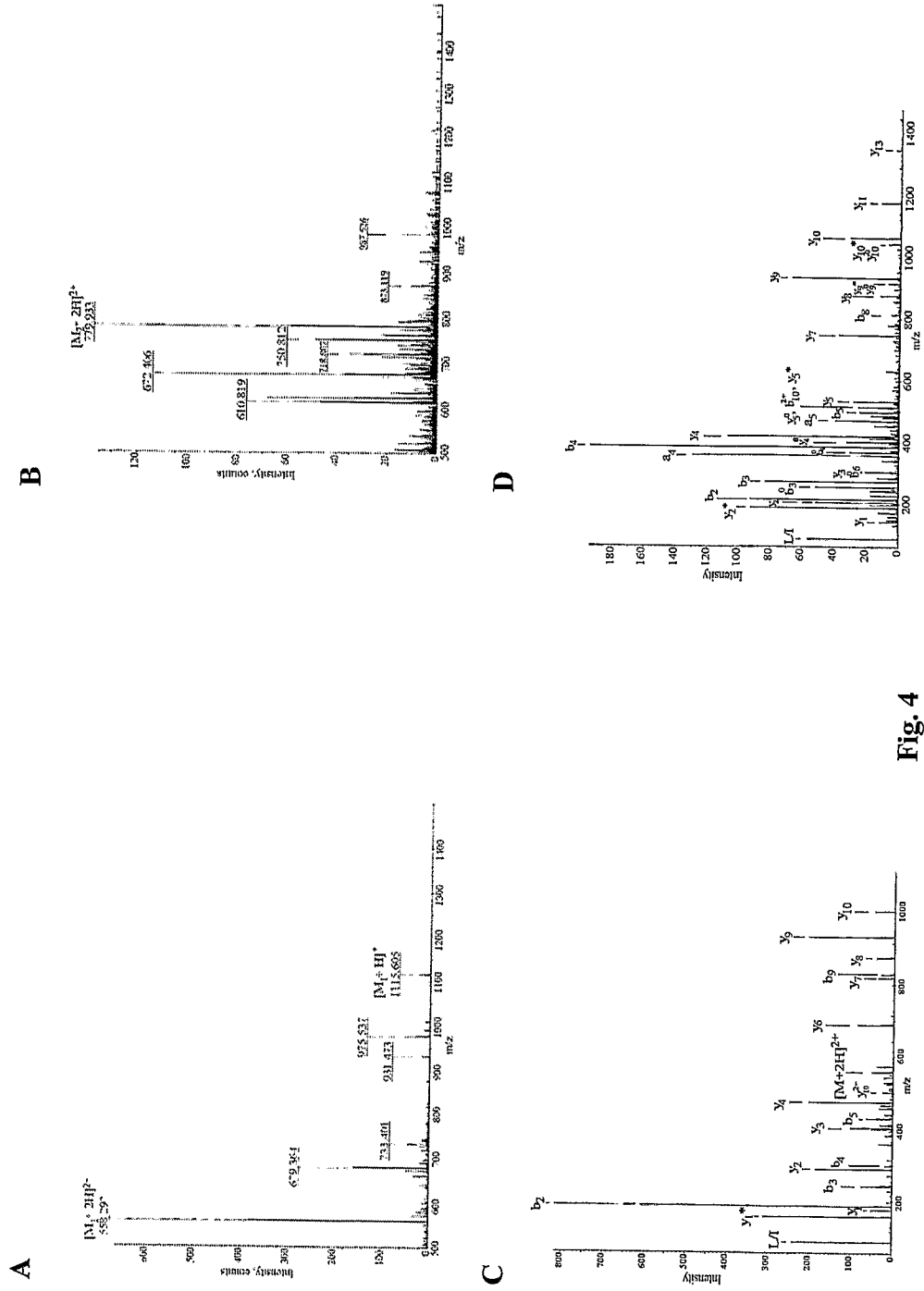
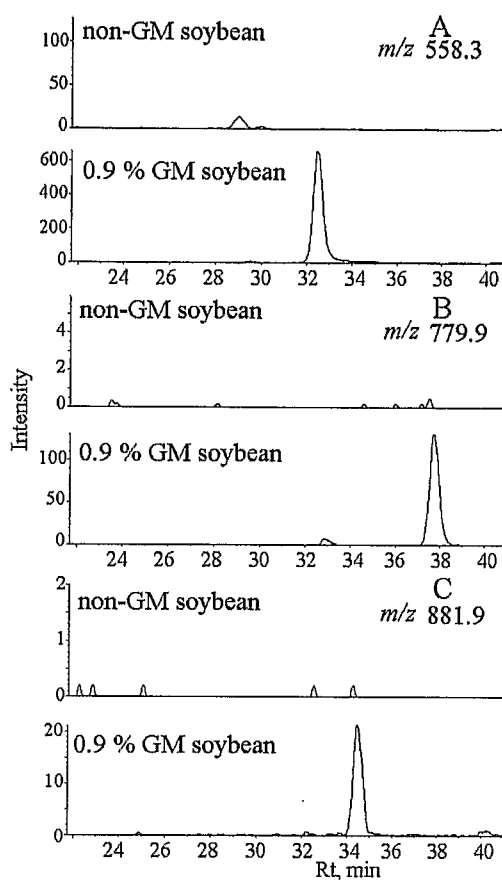


Fig. 3



**Fig. 5**

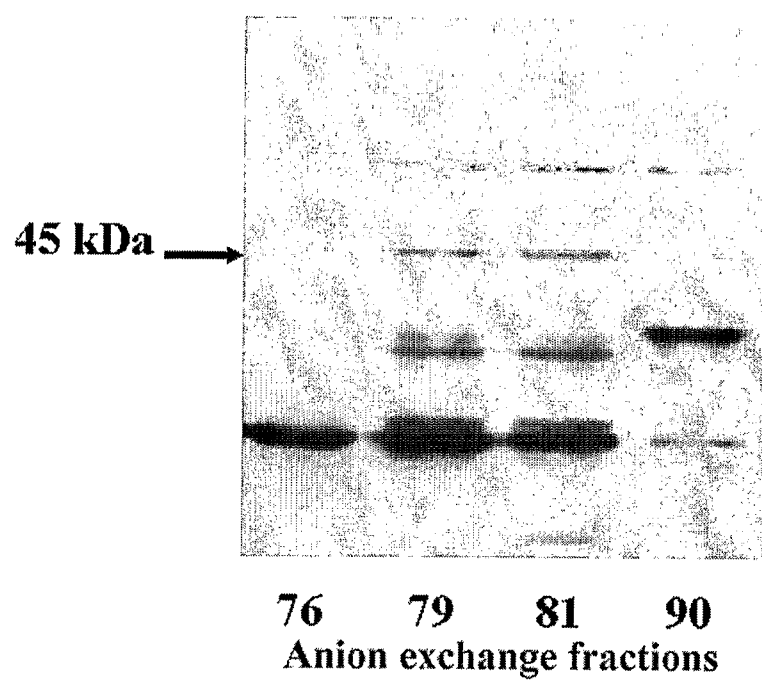


Fig. 6

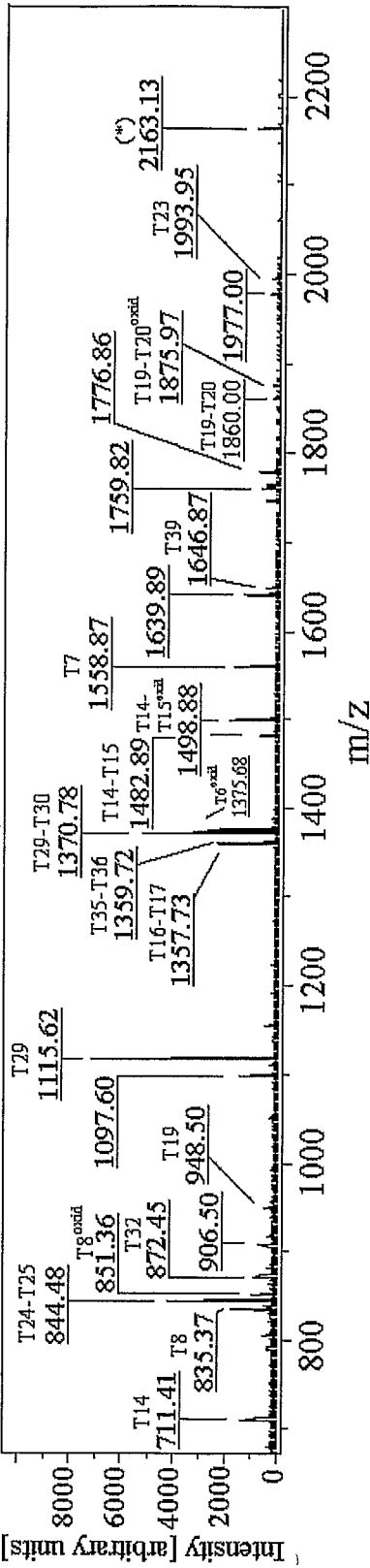


Fig. 7

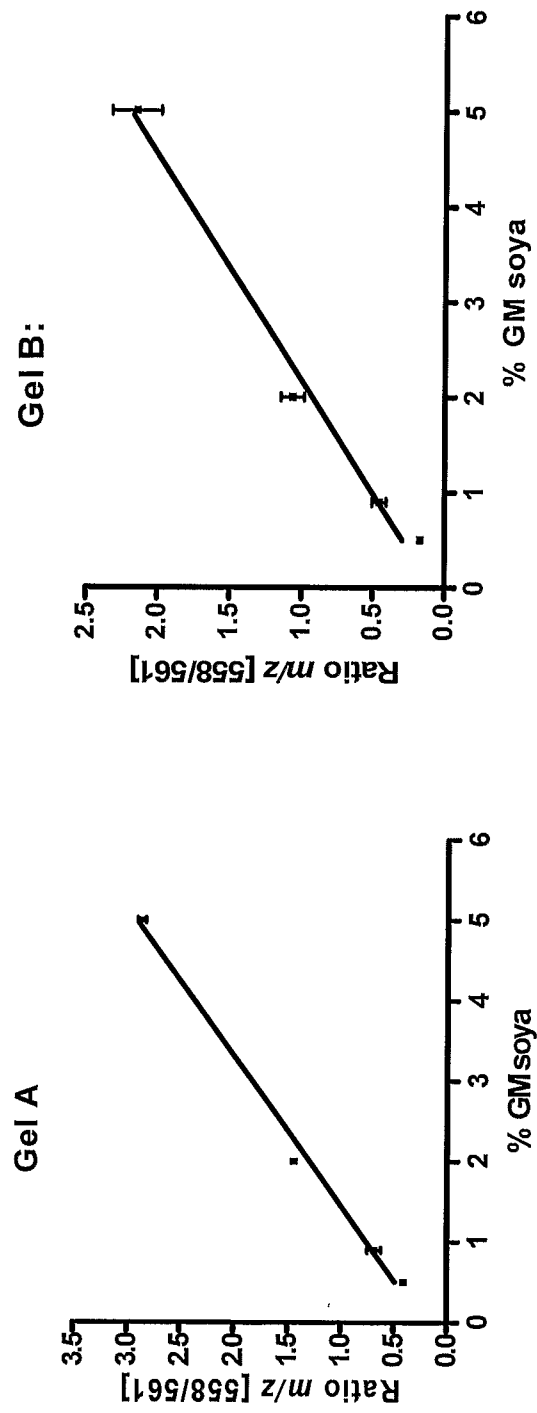


Fig. 8

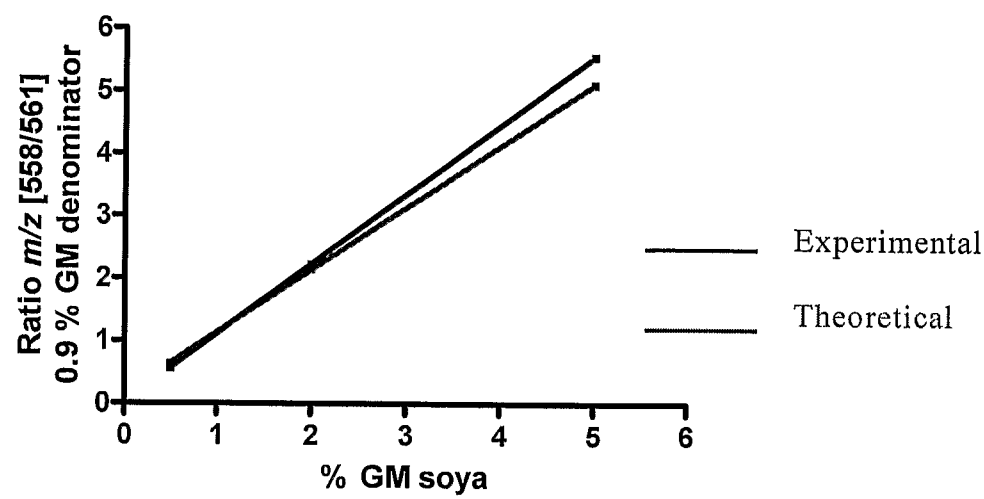


Fig. 9