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TITLE:
Protein Expression of DHA Biosynthesis Pathway Enzymes in Canola

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

6500 QTRAP	AB SCIEX 6500 QTRAP LC-MS/MS system
AAA	Amino acid analysis
ALA	α-Linolenic acid, $18:3^{\Delta 9,12,15}$ (ω3)
CE	Collision energy
DHA	Docosahexaenoic acid, $22:6^{\Delta 4,7,10,13,16,19}$ (ω3)
DHA canola	Genetically modified canola, event NS-B50027-4
DPA	Docosapentaenoic acid, $22:5^{\Delta 7,10,13,16,19}$ (ω3)
DTT	Dithiothreitol
EPA	Eicosapentaenoic acid, $20:5^{\Delta 5,8,11,14,17}$ (ω3)
ETA	Eicosatetraenoic acid, $20:4^{\Delta 8,11,14,17}$ (ω3)
FA	Formic acid
FASP	Filter-assisted sample preparation
FDR	False discovery rate
HPLC	High performance liquid chromatography
IAM	Iodoacetamide
IS	Internal standard
kDa	Kilo dalton
LA	Linoleic acid, $18:2^{\Delta 9,12}$ (ω6)
Lackl-Δ12D	*Lachancea kluyveri* Δ12-desaturase
LC-MS	Liquid chromatography-Mass Spectrometry
LLOQ	Lower limit of quantification (LLOQ)
LOD	Limit of detection
Micpu-Δ6D	*Micromonas pusilla* Δ6-desaturase
MMT	Million metric tons
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut-off
m/z	Mass-to-charge ratio
OA	Oleic acid, $18:1^{\Delta 9}$
ω3 LC-PUFA	Omega-3 long-chain (≥C20) polyunsaturated fatty acids
PAT	Phosphinothricin-N-acetyltransferase
Pavsa-Δ4D	*Pavlova salina* Δ4-desaturase
Pavsa-Δ5D	*Pavlova salina* Δ5-desaturase
Picpa-ω3D	*Pichia pastoris* Δ15-/ω3-desaturase
Pyrco-Δ5E	*Pyramimonas cordata* Δ5-elongase
Pyrco-Δ6E	*Pyramimonas cordata* Δ6-elongase

Q1	Quadrupole 1 (referring to the analysis of the precursor ion)
Q3	Quadrupole 3 (referring to the analysis of the fragment ion)
RT	Retention time (min)
SD	Standard deviation
SDA	Stearidonic acid, $18{:}4^{\Delta 6,9,12,15}$ (ω3)
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
S/N	Signal to noise ratio
UA buffer	8 M urea, 0.1 M Tris-HCl, pH 8.5
ULOQ	Upper limit of quantification

EXECUTIVE SUMMARY

The DHA biosynthesis pathway is comprised of seven desaturase and elongase enzymes that are expressed in the DHA canola event NS-B50027-4 (OECD identifier). The concentrations of each of these enzymes in the pathway were quantified during the life cycle of DHA canola within different tissues. Samples were taken from two field trial sites and included DHA canola and wild type (WT) canola planted at the same sites with the DHA canola. The quantification was achieved by highly sensitive LC-MRM-MS.

The results of this study demonstrated that the enzymatic proteins that drive the production of DHA using seed-specific promoters were only detected in developing seed and mature seed at low levels (20-740 ng/mg total protein), while none of the DHA biosynthesis pathway enzymes were detected in the non-seed tissues of the transgenic canola, irrespective of the sampling time or the tissues tested. As expected the pathway enzymes were also absent in wild type canola.

I. INTRODUCTION

The omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA) eicosapentaenoic acid (EPA, 20:5ω3), docosapentaenoic acid (DPA, 22:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are widely recognised for their beneficial roles in human health, particularly those related to cardiovascular and inflammatory health. EPA, DPA and DHA are primarily sourced from wild-caught fish oils and algal oils, with algae being the primary producer in the marine food web. These sources are under pressure due to increasing demand for ω3 LC-PUFA by aquaculture, as well as nutraceutical and pharmaceutical applications. Additional sources of these fatty acids can be produced by engineering land-based oilseed crops to convert native fatty acids to marine-type ω3 LC-PUFA, which are then accumulated in seed oil. Canola is a commonly grown oilseed with 67 million metric tons (MMT) of rapeseed produced globally in 2015/16[1].

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4 (DHA canola), which accumulates significant amounts of DHA in the seed oil. In this DHA canola, seven fatty acid desaturases and elongases were introduced to convert oleic acid (OA) to DHA in a single pathway expression vector. The pathway consists of the *Lachancea*

[1] http://www.ers.usda.gov/data-products/oil-crops-yearbook/oil-crops-yearbook/#World Supply and Use of Oilseeds and Oilseed Products

kluyveri Δ12-desaturase (Lackl-Δ12D, Watanabe et al. 2004), *Pichia pastoris* Δ15-/ω3-desaturase (Picpa-ω3D, Zhang et al. 2008), *Micromonas pusilla* Δ6-desaturase (Micpu-Δ6D, Petrie et al. 2010b), *Pyramimonas cordata* Δ6-elongase (Pyrco-Δ6E, Petrie et al. 2010a), *Pavlova salina* Δ5-desaturase (Pavsa-Δ5D, Zhou et al. 2007), *P. cordata* Δ5-elongase (Pyrco-Δ5E, Petrie et al. 2010a) and *P. salina* Δ4-desaturase (Pavsa-Δ4D, Zhou et al. 2007). The functionalities and activities of these enzymes have been demonstrated in different heterologous expression systems (see Report Nºs 2016-005, 2016-006, 2016-007, 2016-008, 2016-009, 2016-010, 2016-011) and in transgenic Arabidopsis or Camelina seeds (Petrie et al. 2012, 2014). Based on the sequence similarity and functionality, these seven proteins can be classified into three groups, (1) yeast acyl-CoA type fatty acid desaturases including Lackl-Δ12D and Picpa-ω3D (Figure 1, blue) that introduces a double bond at the Δ12 and Δ15 positions, respectively; (2) algae fatty acid elongases including Pyrco-Δ6E and Pyrco-Δ5E (Figure 1, purple) that add a carbon to the carboxyl end of fatty acids; and (3) algae front-end fatty acid desaturases that introduce a double bond between an existing double bond and the carboxyl end of fatty acids (Zhou et al. 2007) including Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D (Figure 1, green).

The safety assessment of the DHA canola includes characterization of the introduced proteins and confirmation of their function and chemical properties. The levels of transgenic proteins expressed in plant tissue are among the characterisation aspects for safety assessment and are assessed in this report.

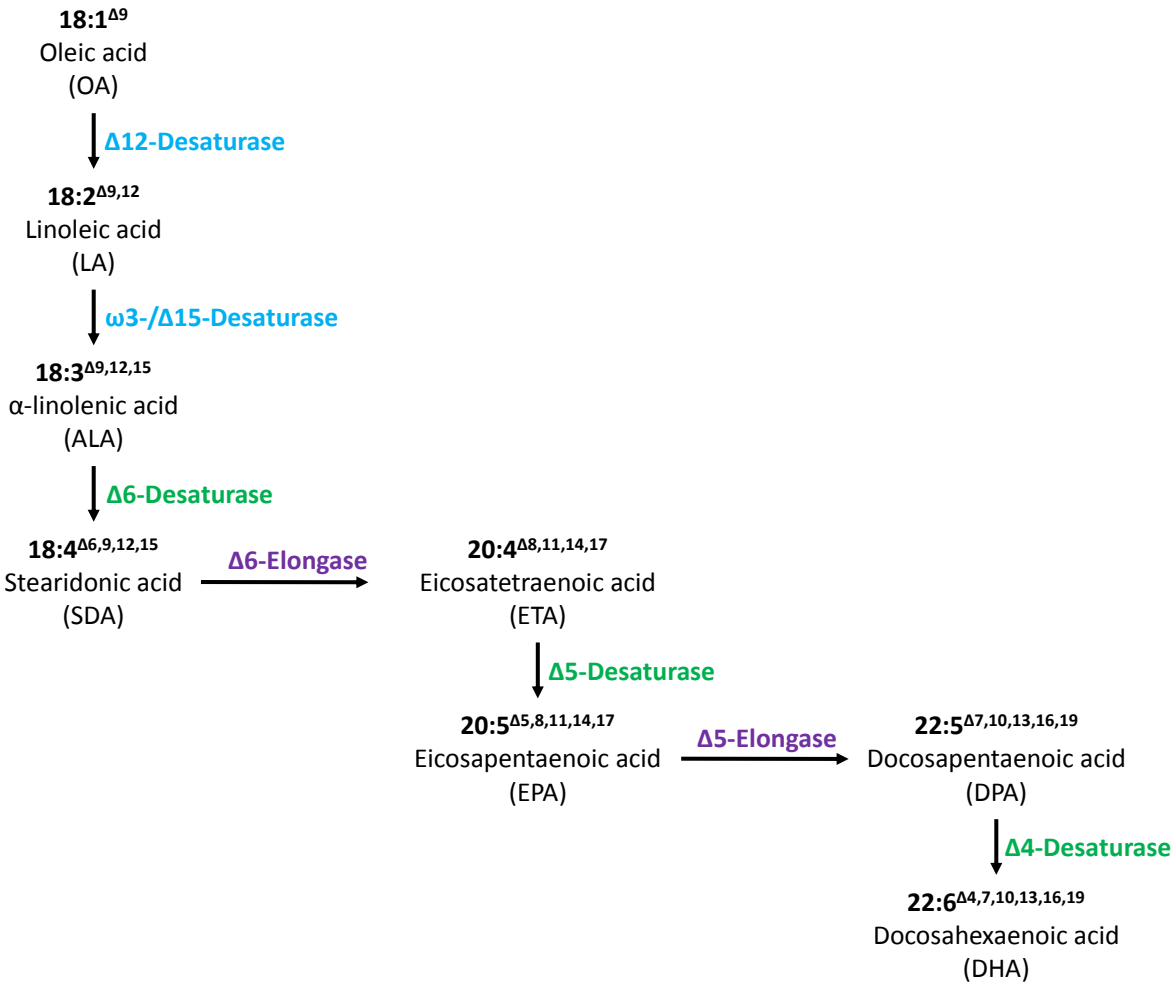


Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4.

Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into three classes: two fatty acid desaturases from yeast in blue; two elongases from microalgae in purple; and three front-end desaturases from microalgae in green (see text for detail).

II. PURPOSE

The purpose of this study was to quantify the amount of transgenic proteins expressed from engineered ω3 LC-PUFA synthesis pathway in DHA canola that accumulates significant amounts of DHA in the seed oil. Although the engineered DHA synthesis pathway genes were under the control of seed-specific promoters, other tissues in addition to seed were also assessed. In the absence of functioning antibodies against these membrane integral proteins as typically used for traditional Western blot analysis that would allow for protein quantitation, an alternative approach using high sensitivity LC-MRM-MS quantification was developed to quantify the target proteins.

This particular report focuses on the quantification of all seven of the DHA biosynthesis pathway enzymes as shown in Figure 1.

III. MATERIALS

A. TARGET PROTEINS

All seven of the DHA biosynthesis pathway enzymes that are expressed in DHA canola were the targets for quantification of protein content in various tissues of transgenic canola across the growing season.

B. COLLECTION OF CANOLA SAMPLES

Wild type (WT, AV Jade) and DHA canola were planted at two field trial sites (Site 1506 & Site 1508, see Report Nº 2016-018) in 2015 at Horsham (Victoria, Australia). The tissues that were sampled for both WT and transgenic plants at each site are listed in Table 1. The sampling times represent specific growth stages of canola allowing for various tissue types, including leaves, roots, pods and reproductive tissues. Samples were harvested at the following developmental stages, as described by Lancashire et al. (1991).

Table 1. Canola tissues sampled at different stages

Growth stage	Timing	Tissue sampled	Replicate
BBCH15	5 True leaves	3 Whole plants	3
BBCH35	3 Visibly extended internodes	1 Whole plant	3
BBCH65	50% Full flowering	All flowers from 1 plant	3
		All roots from 1 plant	3
		All leftover from 1 plant	3
BBCH79	Developing seed	All pods from 1 plant	3
BBCH90	Senescence	All grain from 1 plant	3

The tissues harvested were maintained on wet ice during transit, and then transferred into -80ºC freezer until processing.

C. SYNTHESIS OF PEPTIDES

The selected peptides were synthesized at Creative Proteomics (Shirley NY, USA) at 99% purity. The amount of synthesized peptides was determined by high sensitivity amino acid analysis (AAA) at the Australian Proteomics Analysis Facility (Sydney, Australia).

IV. METHODS

A. PRINCIPLE OF PROTEIN QUANTIFICATION BY LC-MRM-MS

Protein quantification by multiple reaction monitoring (MRM), using a triple quadrupole mass spectrometer, is an approach that has been successfully applied to clinical laboratory studies (Rauh 2012; Gillette & Carr 2013). Analysis of proteins by MRM is based on detection of peptides derived from proteolytic digestion of the target protein, typically by trypsin. The measurement of the peptides in a complex sample matrix is achieved by adding a known concentration of an isotope-labelled peptide isomer as an internal standard (IS) to the sample before analysis. The labelled peptide isomer (typically referred to as "heavy") contains an amino acid labelled with the stable isotopes ^{15}N and/or ^{13}C, resulting in a mass increase compared to that of the native peptide isomer (typically referred to as "light"). The heavy and light peptides when subjected to chromatographic separation, show identical elution profiles allowing the detection of the analytes (light peptides) in the matrix

background. When subjecting the peptides to MS/MS under conditions of collision-induced dissociation, the light and heavy peptides also undergo an identical fragmentation mechanism providing an additional level of quality control in confirming the peptide identity.

Calibration curves were generated wherein the analyte concentration was varied and a defined amount of IS was spiked into the standards. The response of the mass spectrometer is the integrated peak area for each MRM transition. The top three MRM transitions were selected and summed (for both analyte and IS). The ratio of the (summed analyte peak area)/(summed IS peak area) was plotted against the known analyte concentration. The endogenous peptide response (as defined above) was determined and the concentration was interpolated from the calibration curve, thus allowing the quantification of the peptide as femtomoles per 100 µg total protein. This value was converted to a nanogram equivalent per mg total protein based on the molecular mass of each protein.

B. Sample Preparation for LC-MS Method Development

Protein extracts from a variety of sources including total protein extracts from canola, recombinant proteins expressed in either yeast, bacterial or baculovirus expression systems were used. Furthermore, the proteins were either provided in-solution or as excised gel slices. The solutions were subjected to filter-assisted sample preparation (FASP) as described in Colgrave et al. (2014). The gel bands were digested as described in Byrne et al. (2012).

C. Preliminary LC-MS Analysis

Proteolytically digested protein were analysed as described previously (Colgrave et al, 2014) with chromatographic separation (2%/min linear gradient from 2-40% acetonitrile) using a nano HPLC system (Shimadzu Scientific, Rydalmere, Australia) directly coupled to a 5600 TripleTOF MS. ProteinPilot™ 4.0 software (AB SCIEX) with the Paragon Algorithm (Shilov et al. 2007) was used for protein identification. Tandem mass spectrometry data was searched against *in silico* tryptic digests of a custom-built database comprising the transgenic proteins. The search parameters were defined as: iodoacetamide modified for cysteine alkylation and trypsin as the digestion enzyme.

D. Selection of Peptides for Quantification

The total protein extracts from DHA canola seed or from recombinant proteins expressed in either yeast, bacterial or baculovirus expression systems were first analysed by non-targeted LC-MS for detection of the tryptic peptides generated for each target protein (DHA biosynthesis pathway desaturase or elongase). The total protein from *Nicotiana benthamiana*

leaf with transiently expressed Phosphinothricin-N-acetyltransferase (PAT) was also used for detection of the tryptic peptides of PAT protein. After searching all generated data against the custom protein database, two peptides were selected from each target protein as proxies to be used for quantification. The selection of peptides was based on the criteria: good MS response (high intensity), absence of amino acids within the peptide sequence that are likely to be modified (for example, oxidation of methionine) or miscleaved (presence of dibasic residues at either terminus), specific/unique to the target protein and of a size amenable to LC-MS (~6-20 amino acids in length). For each selected peptide, both the endogenous (light) peptides and ^{15}N and ^{13}C labelled (heavy) peptides were synthesized.

E. Quality Control of Synthesized Peptides

The purity of synthesized peptides was analysed by LC-MS. Dilutions equivalent to ~5 pmol/µL were prepared in aqueous solution (1% formic acid, FA) and analysed by LC-ESI-MS/MS. Any peptides showing significant contamination including the presence of the truncated, modified and/or synthesis by-products were excluded from further analysis. The amount of synthesized peptides were determined by high sensitivity amino acid analysis (AAA) at Australian Proteomics Analysis Facility (Sydney, Australia). All samples were analysed in duplicate. The calculated amount of amino acid (µg/mL) is based on the amino acid residue mass in the protein (molecular weight minus H_2O). Using the determined concentrations, stock solutions were prepared at 100 pmol/µL.

F. LC-MRM-MS Quantification

A series of standards (n=4 replicates) comprising a double blank (no analyte, no IS), a blank (IS only) and 17 standards containing a known, but varied amount (0.08 to 5,000 femtomoles) of each peptide and 1 pmol of the IS peptide (WEGEPI*SK) were analysed by LC-MRM-MS. The data were acquired using the Analyst 1.6.3 software (AB SCIEX) on a 6500 QTRAP LC-MS/MS system (AB SCIEX). The data were imported into MultiQuant v3.0 and the peak areas for each of five monitored MRM transitions were integrated. The peak area of the top three MRM transitions (quantifiers) were summed and the remaining two MRM transitions were used as qualifiers (allowing confirmation of peptide identification by assessment of retention time (RT) and the order of intensity of the MRM transitions). Using the preliminary data, the best performing peptide per protein was selected as the proxy for each enzyme based on criteria such as chromatographic performance (good peak shape), intensity in MS, free from interference (as assessed in sample matrix). It was initially planned to use the light and heavy peptide pairs for absolute quantification, however, the contamination of the heavy peptides during synthesis with <1% of the light peptide partner precluded their use. Instead the heavy peptides were spiked into pooled (n=6) WT or DHA canola samples of each tissue and these served as reference standards for determining the

correct retention time (RT, min) and MRM transition order. The heavy peptide WEGEPI*SK derived from Δ4D was selected as an alternate IS based on its high MS response, reproducible detection and excellent chromatographic performance (elution at 3.2 min with ~1.6 s peak width at half-maximum).

G. TOTAL PROTEIN EXTRACTION FROM CANOLA

The collected samples previously stored in a -80ºC freezer were first ground with mortar and pestle into a fine powder with liquid nitrogen. All samples were maintained frozen on dry ice during the process. To avoid cross contamination, WT samples were processed first, then transgenic samples, in the order of BBCH15, BBCH35, BBCH65 root, BBCH65 other parts, BBCH65 flower, BBCH79, BBCH90. Total protein was extracted from multiple aliquots of 100 mg in 2 mL plastic tubes in order to obtain more than 1 mg of total protein.

The tubes with samples were filled with 1 mL of 10% TCA in acetone and vortexed and sonicated at frequency of 25% amplitude for 20 s using a Branson digital probe sonicator (St. Louis, MO, USA). Samples were centrifuged at 16,000 x *g* for 3 min at 4°C. The supernatant was removed by careful decanting. The pellet was resuspended in 1 mL of 0.1 M ammonium acetate in 80% methanol, mixed by vortexing and centrifuged at 16,000 x *g* for 3 min at 4°C. The supernatant was discarded by careful decanting. The pellet was resuspended in 1 mL 80% acetone, vortexed until the pellet was fully dispersed, and centrifuged at 16,000 x *g* for 3 min at 4°C. The supernatant was discarded, and the pellet was air dried to remove the residual acetone.

The air-dried pellet was re-suspended in 0.6 mL of UltraPure buffer-saturated phenol (Invitrogen, catalogue #15513-039, Carlsbad CA, USA) and 0.6 mL freshly prepared SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl pH 8.8, 0.1 M DTT), mixed thoroughly and incubated for 5 min at room temperature. The samples were centrifuged at 16,000 x *g* for 5 min at room temperature. The upper phenol phase was transferred to a new 2 mL tube, and 1 mL of 0.1 M ammonium acetate in 80% methanol was added. The proteins were precipitated at -20°C overnight. Samples were centrifuged at 16,000 x *g* for 5 min at 4°C. The supernatant was carefully discarded, and the pellet was first washed with 100% methanol then with 80% acetone. The proteins were pelleted by centrifuging at 16,000 x *g* for 5 min at 4°C. The final protein pellet was left to air dry.

H. WESTERN BLOT

Aliquots of 20 µg total proteins from whole canola plant or developing seed were loaded onto sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analysed

by Western blot using rabbit anti-PAT antibody (Sigma) at a 1:1000 dilution. The total protein from *N. benthamiana* leaf with transiently expressed PAT was used as control of Western blot.

I. CANOLA PROTEIN DIGESTION

The extracted proteins from different tissues were dissolved in UA buffer (8 M urea, 0.1 M Tris-HCl, pH 8.5). Protein estimations were performed using the Bio-Rad microtiter Bradford protein assay (California, USA). The instructions provided by the reagent manufacturer (version: Lit 33 Rev C) were followed. Samples were diluted in water over three dilutions in duplicate and measurements were made at 595 nm using a SpectraMax Plus. Bovine serum albumin (BSA) standard was used in the linear range 0.05 mg/mL to approximately 0.5 mg/mL. The BSA standard concentration was determined by high sensitivity AAA at Australian Proteomics Analysis Facility (Sydney, Australia). Blank-corrected standard curves were run in duplicate. Linear regression was used to fit the standard curve.

Protein samples were stored at -80ºC prior to processing. Protein was subjected to filter-assisted sample preparation (FASP) wherein the protein extract (250 µg) in UA buffer was applied to a 10 kDa molecular weight cut-off (MWCO) filter (Millipore, Australia) and diluted to 200 µL with UA buffer before centrifugation (20,800 x *g*, 15 min). The protein on the filter was washed with two 200 µL volumes of UA buffer with centrifugation (20,800 x *g*, 15 min). To reduce the protein on the filter, dithiothreitol (100 mM, 100 µL) was added and the solution incubated at room temperature for 50 min with shaking. The filter was washed with two 200 µL volumes of UA buffer with centrifugation (20,800 x *g*, 15 min). To alkylate the cysteine residues, iodoacetamide (50 mM, 100 uL) was applied to the protein on the filter with incubation for 20 min at room temperature in the dark. The filter was washed with two 200 µL volumes of UA buffer with centrifugation (20,800 x *g*, 15 min). The buffer was exchanged using 50 mM ammonium bicarbonate (pH 8.0) by two consecutive wash/centrifugation steps. Sequencing grade porcine trypsin (Promega, Alexandria, Australia) was added (25 µg, 0.125 µg/µL in 200 µL of 50 mM ammonium bicarbonate, 1 mM $CaCl_2$) to the protein on the 10 kDa filters and incubated for 16 h at 37°C in a wet chamber. The filters were transferred to fresh centrifuge tubes and the filtrate (digested peptides) was collected following centrifugation (20,800 x *g*, 10 min). The filters were washed with 200 µL of 100 mM ammonium bicarbonate and the filtrates were combined and lyophilised. The resultant peptides were resuspended in 62.5 µL of 1% formic acid (FA) containing 0.04 pmol/µL of the IS peptide WEGEPI*SK and 25 µL (equivalent to ~100 µg of total protein and 1 pmol of IS) and was analysed by LC-MS/MS.

J. LC-MRM-MS Quantification of Canola Proteins

The extracted and digested protein samples representing five growth stages (seven samples) from two sites, comprising both WT and DHA canola (n=3 replicates, total 84 samples) containing the spiked IS were analysed by LC-MRM-MS alongside aqueous peptide standards. An aliquot (25 µL) of aqueous standard or canola peptide extract was chromatographically separated on a Shimadzu Nexera UHPLC and analyzed on a 6500 QTRAP mass spectrometer (AB SCIEX, Foster City, USA) as described previously (Colgrave et al. 2014). Quantification was achieved using scheduled MRM scanning experiments using a 120 s detection window for each MRM transition and a 0.3 s cycle time. Peaks were integrated using MultiQuant v3.0 (AB SCIEX) wherein all three transitions were required to co-elute at the same retention time (RT, min) with a signal-to-noise (S/N)>3 for detection and a S/N>5 for quantification. The graphs showing the calibration curves for the synthetic peptides were generated in Graphpad Prism v6. The sum of the peak area for the top three MRM interference-free transitions for each targeted light peptide was compared to sum of MRM peak area of the IS peptide to generate a MRM response ratio. The amount of each target peptide (as femtomoles per 100 µg total protein) was determined by interpolation from the appropriate calibration curve. The amount of protein detected in these samples was then calculated based on the protein molecular mass, by conversion to a nanogram equivalent per mg total protein.

V. Results and Discussion

A. Selection of Peptides for Quantification

Using protein extracts from a variety of sources including total protein extracts from canola or recombinant proteins expressed in either yeast, bacterial or baculovirus expression systems, the peptides liberated after tryptic digestion were assessed. The protein sequences are given in Figure 2 wherein fully tryptic peptides potentially useful as peptide markers are underlined. For each protein, the score, protein sequence coverage (at 95% confidence) and number of detected peptides is given. Peptides were excluded as markers if they contained methionine (M) which is commonly modified by oxidation, contained adjacent dibasic sites (KK, KR, RK, RR) which result in missed cleavage and hence variability in digestion efficiency. Peptides were selected ideally to be of a size amenable to LC-MS/MS analysis (6-20 amino acids in length). Peptides giving the highest signal intensity and that were consistently detected (in multiple digests) were selected for peptide synthesis for protein quantification.

(A) Picpa-ω3D: Score = 49.7, 43.9% coverage, 39 peptides

MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAIPKHCYERSLVKSMSYVVR
DIVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVM
VPYFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLATNA
TGQPYPGVSKFFKSHYWPSSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVF
VTFLQHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVSRIPFYHAREATECIKKVMGE
HYRHTDENMWVSLWKTWRSCQFVENHDGVYMFRNCNNVGVKPKDT

(B) Lackl-Δ12D: Score 16.5, 28.6% coverage, 13 peptides

MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERRLATSLYYVFRDIFCML
TTGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMV
PYFSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYLFVNV
TGQPYPDVPSWKWNHFWLTSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVF
ITFLQHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYCSRIPFYNARPASEAIKKVMG
KHYRSSDENMWKSLWKSFRSCQYVDGDNGVLMFRNINNCGVGAAEK

(C) Micpu-Δ6D: Score = 67.6, Coverage = 66.7%, 48 peptides

MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVRVEDVEYDVTNFKHPGGSV
IFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPAEIKRSESEDAPMLEDFARWRAELERDGFFKPSITHV
AYRLLELLATFALGTALMYAGYPIIASVVYGAFFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGE
MWNQMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLHP
RQVLRKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFAHFSTSHTHLPVVPSDKHLSW
VNYAVDHTVDIDPSRGYVNWLMGYLNCQVIHHLFPDMPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFS
NLDKVGQHYYVNGKAEKAH

(D) Pyrco-d6E: Score 8.3, 22.9% coverage, 6 peptides

MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPLILSLLAYFAIVGSGLVYR
KVFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLVYEAYVNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYE
FMDTFIMLLKGNVNQVSFLHVYHHGSISGIWWMITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAVLPKDEKTK
RKYLWWGRYLTQMQMFQFFMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK

(E) Pavsa-Δ5D: Score 27.3, 34.6% coverage, 17 peptides

MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQVGTDATDAYKQFHVRSAK
ADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAG
IAMLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHDVDLDTLPL
VAFHERIAAKVKSPAMKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAANY
GAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCSPSWWCDWWMSYLNYQIEHHL
YPSMPQFRHPKIAPRVKQLFEKHGLHYDVRGYFEAMADTFANLDNVAHAPEKKMQ

(F) Pyrco-d5E: Score 25.6, 51.3% coverage, 28 peptides

MASIAIPAALAGTLGYVTYNVANPDIPASEKVPAYFMQVEYWGPTIGTIGYLLFIYFGKRIMQNRSQPFGLKNA
MLVYNFYQTFFNSYCIYLFVTSHRAQGLKVWGNIPDMTANSWGISQVIWLHYNNKYVELLDTFFMVMRKKFDQL
SFLHIYHHTLLIWSWFVVMKLEPVGDCYFGSSVNTFVHVIMYSYYGLAALGVNCFWKKYITQIQMLQFCICASH
SIYTAYVQNTAFWLPYLQLWVMVNMFVLFANFYRKRYKSKGAKKQ

(G) Pavsa-d4D: Score = 61.4, 82.1% coverage, 51 peptides

MPPSAAKQMGASTGVHAGVTDSSAFTRKDVADRPDLTIVGDSVYDAKAFRSEHPGGAHFVSLFGGRDATEAFME
YHRRAWPKSRMSRFHVGSLASTEEPVAADEGYLQLCARIAKMVPSVSSGFAPASYWVKAGLILGSAIALEAYML

Figure 2. Protein sequences of the enzymes of the DHA biosynthetic pathway as characterized by LC-MS/MS after tryptic digestion.
Using a variety of protein sources, the DHA biosynthetic enzymes were characterized allowing selection of peptides as biomarkers of each protein for quantification. Green, peptides identified with >95% confidence; yellow, peptides identified with 50-95% confidence; red, peptides identified with <50% confidence; grey, not detected. Solid and dashed underline are used to distinguish adjacent fully tryptic peptides (6-20 amino acids in length).

Based on the preliminary results from both the quality control assessment and determination of linearity of response for the synthetic peptides, the peptide with optimal performance characteristics (high signal intensity, good chromatographic properties) for each protein was selected as the protein proxy for quantification. The final selected peptides for the DHA synthesis pathway enzymes are shown in Table 2.

Table 2. The peptides used for protein quantification of the DHA biosynthesis pathway enzymes.

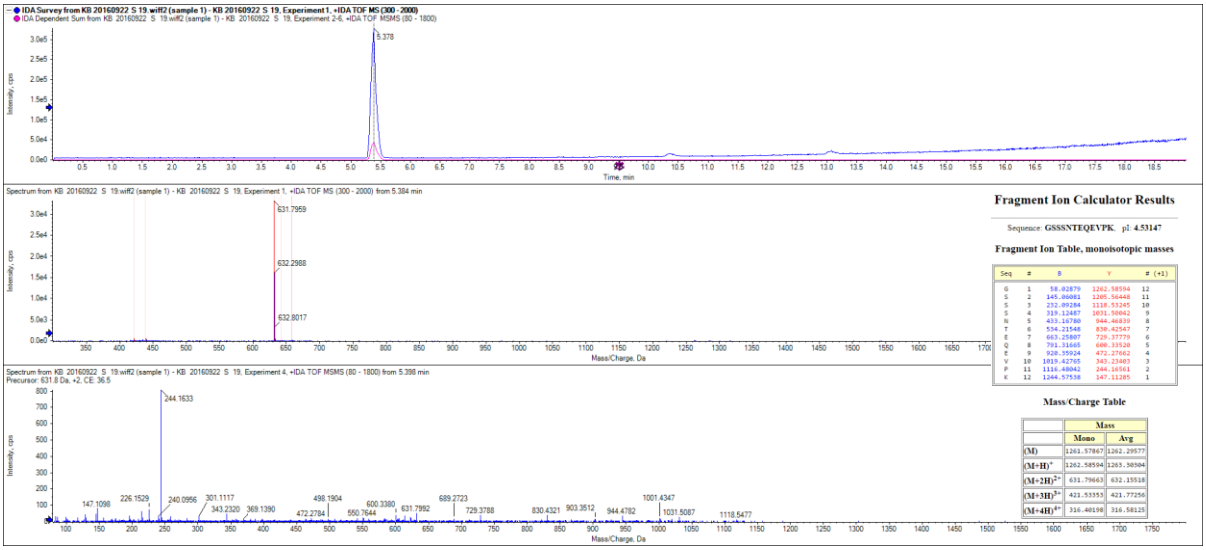
Protein	Light Peptide Sequence	MW Light Peptide (Da)	Heavy Peptide Sequence	MW Heavy Peptide (Da)
Lackl-Δ12D	GSSSNTEQEVPK	1261.58	GSSSNTEQEV*PK	1267.58
Picpa-ω3D	IPFYHAR	902.48	IPFYHA*R	906.48
Micpu-Δ6D	DASTAPVDLK	1015.52	DASTAPVDL*K	1022.52
Pyrco-Δ6E	GQDPFLLK	916.50	GQDPFLL*K	923.50
Pavsa-Δ5D	AYDVTNFVK	1055.53	AYDVTNFV*K	1061.53
Pyrco-Δ5E	SQPFGLK	775.42	SQPFGL*K	782.42
Pavsa-Δ4D	LAPLVK	639.43	LAPLV*K	645.44
IS	NA	NA	WEGEPI*SK	951.46

The amino acid residues followed by asterisks indicated the stable isotope ^{15}N and/or ^{13}C labelled in this residue. The heavy peptides were used as reference standards for determining the correct retention time and fragmentation pattern. NA=Not Applicable.

The purity of synthesized peptides was analysed by LC-MS, and the results are shown below (Figures 3-11). In each case, the top panel shows the total ion chromatogram (TIC) for the light (A) and heavy peptide (B) in blue (MS) and pink (MS/MS). The second panel shows the determined *m/z* values for the light (A) and heavy peptide (B). The bottom panel shows the

MS/MS spectrum revealing the correct peptide sequence where the theoretical *m/z* values are shown in the inset.

(A) QC: Light GSSSNTEQEVPK



(B) QC: Heavy GSSSNTEQEV*PK

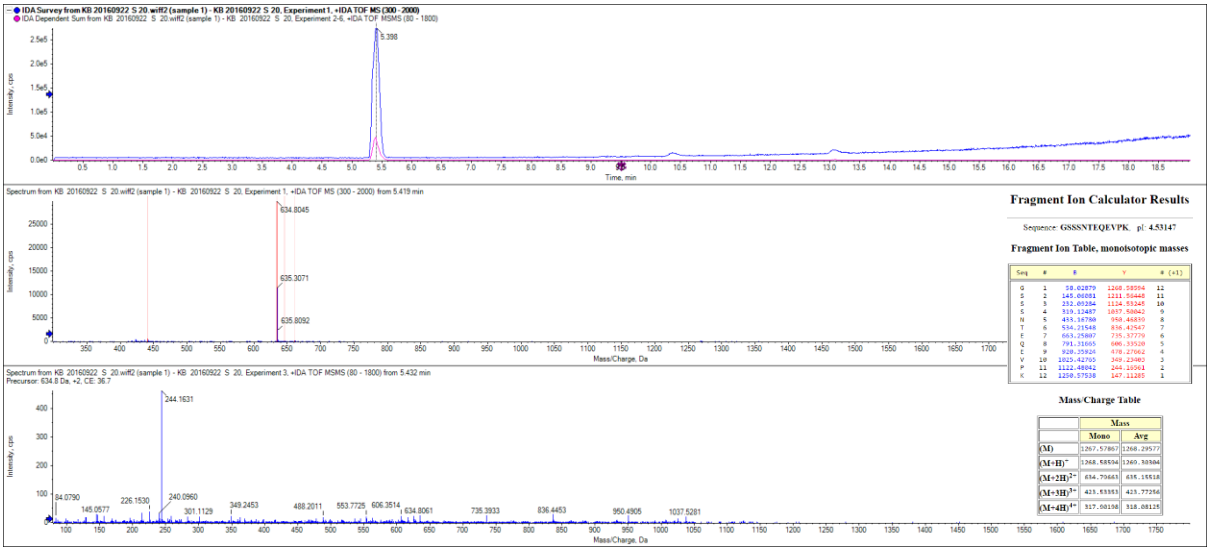
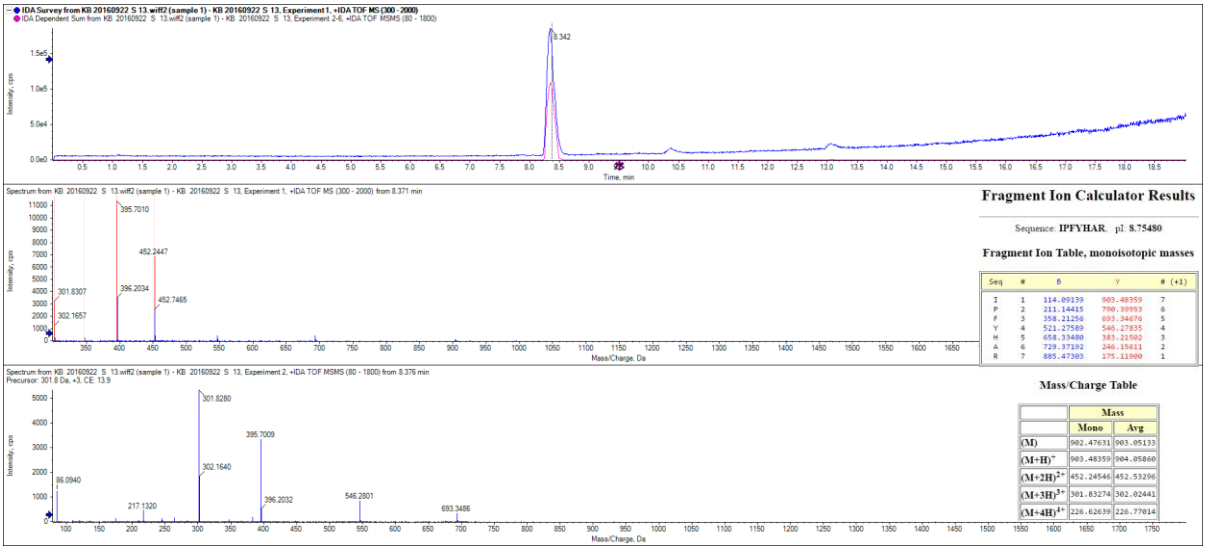


Figure 3. Quality control of Lackl-Δ12D peptide.
(A) LC-ESI-MS/MS TIC shows the Lackl-Δ12D peptide GSSSNTEQEVPK at 5.38 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 5.38 min of 631.79^{2+} is shown (second panel) and matches the theoretical *m/z* value 631.79^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset). **(**B) LC-ESI-MS/MS TIC shows the GSSSNTEQEV*PK peptide at 5.39 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 5.38 min of 634.80^{2+} is shown (second panel) and matches the theoretical *m/z* value 634.80^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light IPFYHAR



(B) QC: Heavy IPFYHA*R

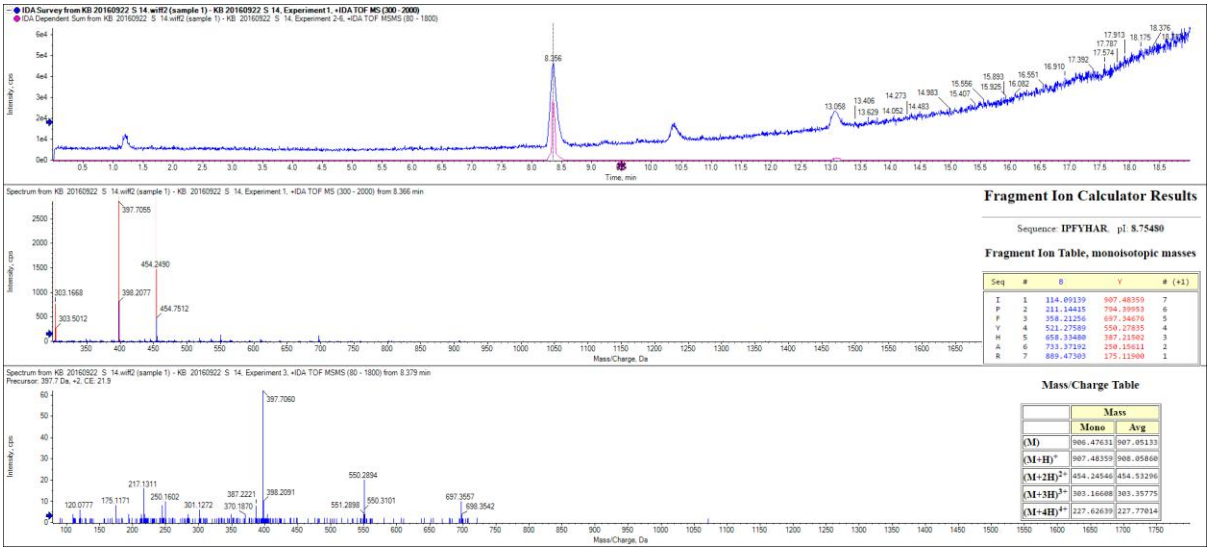
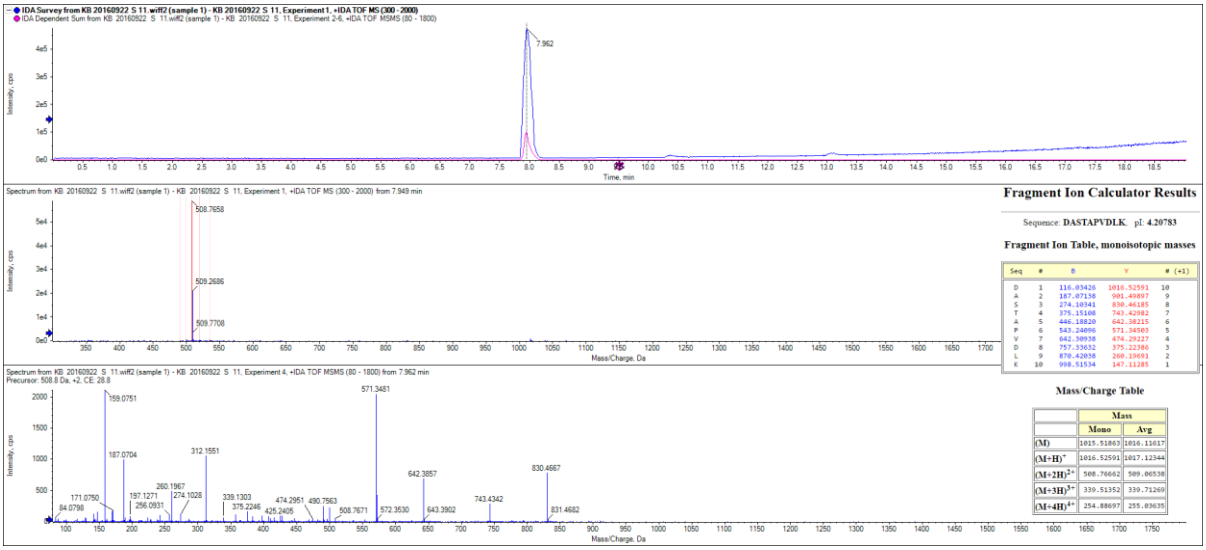


Figure 4. Quality control of Picpa-ω3D peptide.
(A) LC-ESI-MS/MS TIC shows the Picpa-ω3D peptide IPFYHAR at 8.34 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 8.34 min of 452.24^{2+} is shown (second panel) and matches the theoretical *m/z* value 452.24^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC shows the peptide IPFYHA*R at 8.36 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 8.36 min of 454.25^{2+} is shown (second panel) and matches the theoretical *m/z* value 454.25^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light DASTAPVDLK



(B) QC: Heavy DASTAPVDL*K

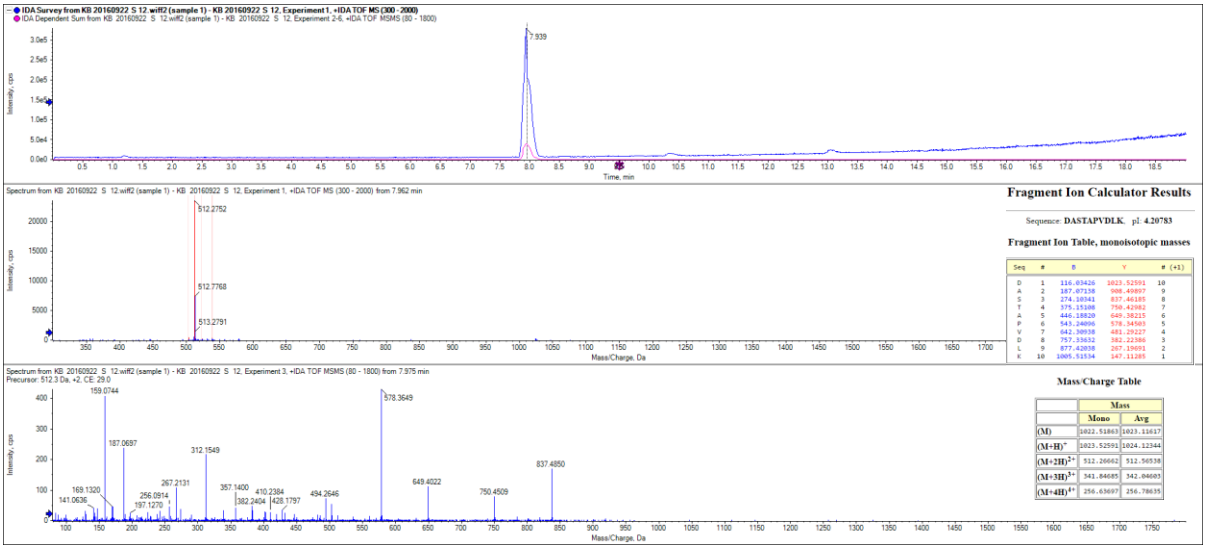
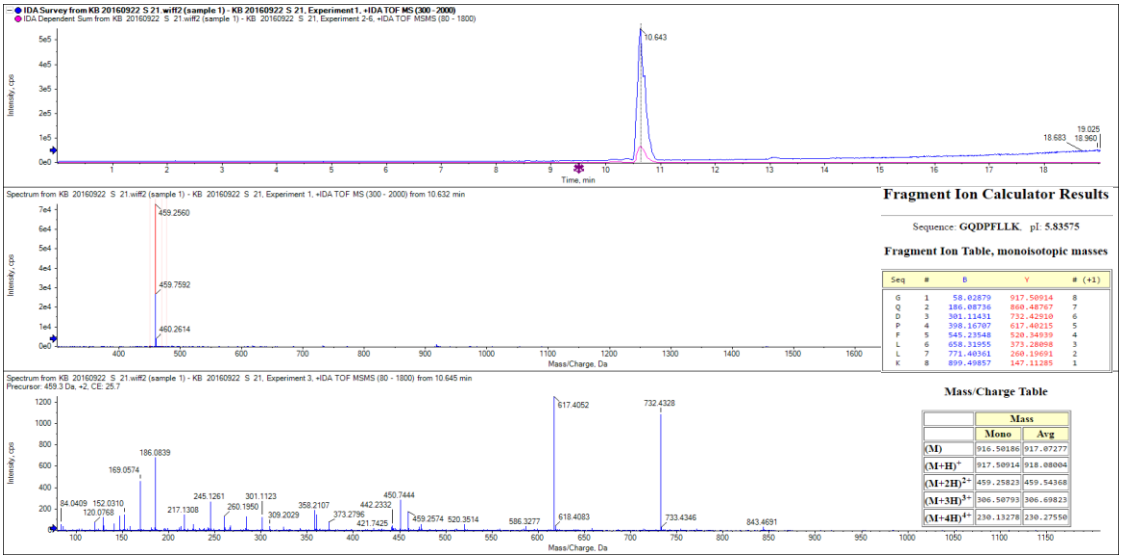


Figure 5. Quality control of Micpu-Δ6D peptide.
(A) LC-ESI-MS/MS TIC shows the Micpu-Δ6D peptide DASTAPVDLK at 7.96 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 7.96 min of 508.76^{2+} is shown (second panel) and matches the theoretical *m/z* value 508.76^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC shows the peptide DASTAPVDL*K at 7.94 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 7.94 min of 512.27^{2+} is shown (second panel) and matches the theoretical *m/z* value 512.27^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light GQDPFLLK



(B) QC: Heavy GQDPFLL*K

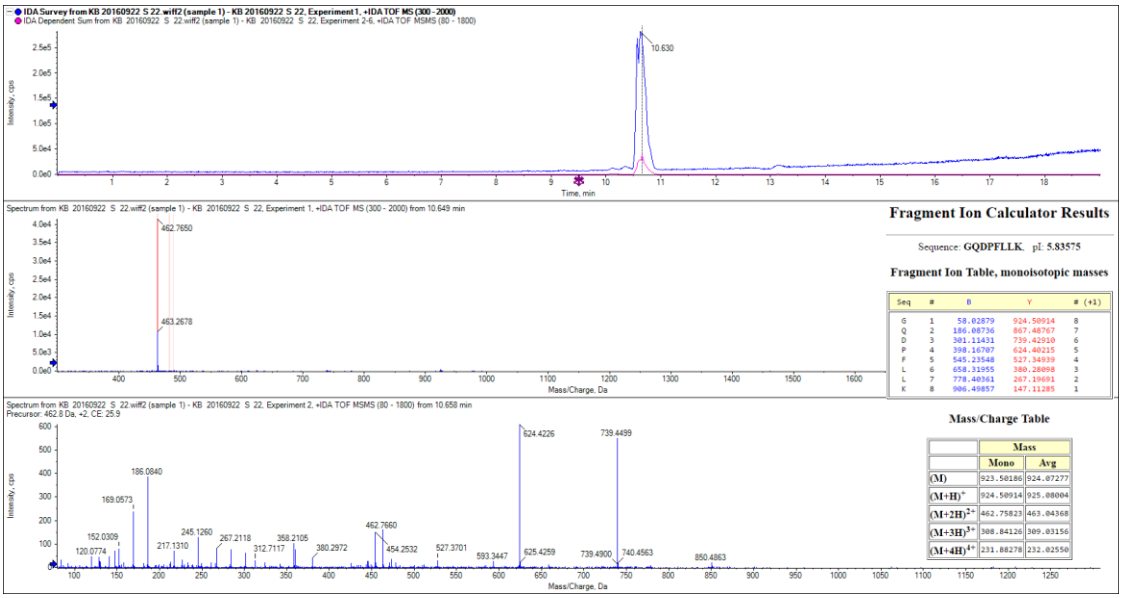
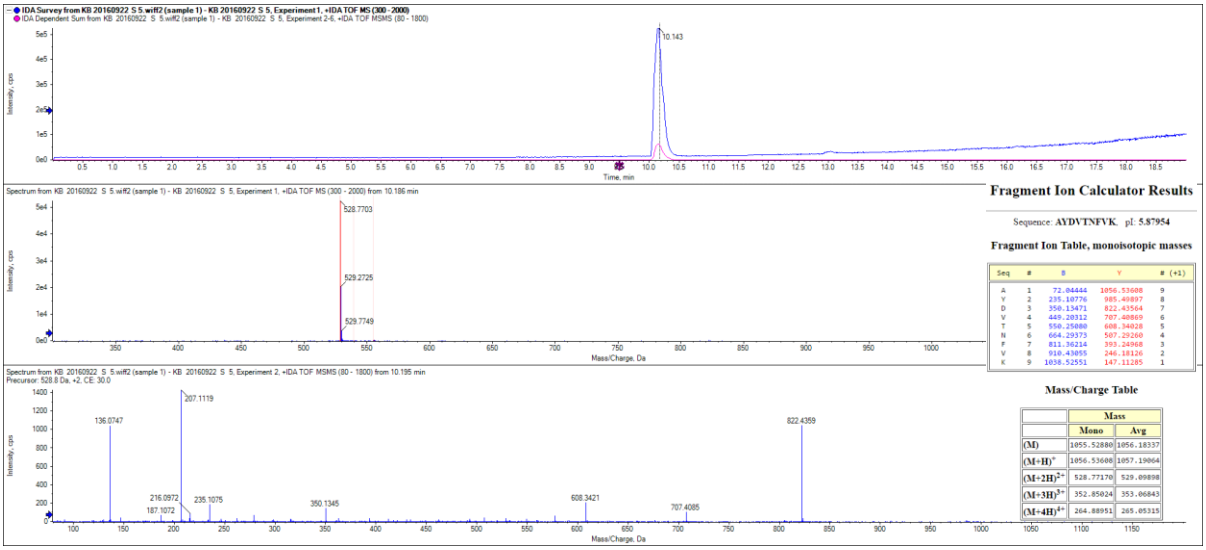


Figure 6. Quality control of Pyrco-Δ6E peptide.
(A) LC-ESI-MS/MS TIC shows the Pyrco-Δ6E peptide GQDPFLLK at 10.64 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 10.64 min of 459.26^{2+} is shown (second panel) and matches the theoretical *m/z* value of 459.26^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC shows the peptide GQDPFLL*K at 10.63 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 10.64 min of 462.76^{2+} is shown (second panel) and matches the theoretical *m/z* value of 462.76^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light AYDVTNFVK



(B) QC: Heavy AYDVTNFV*K

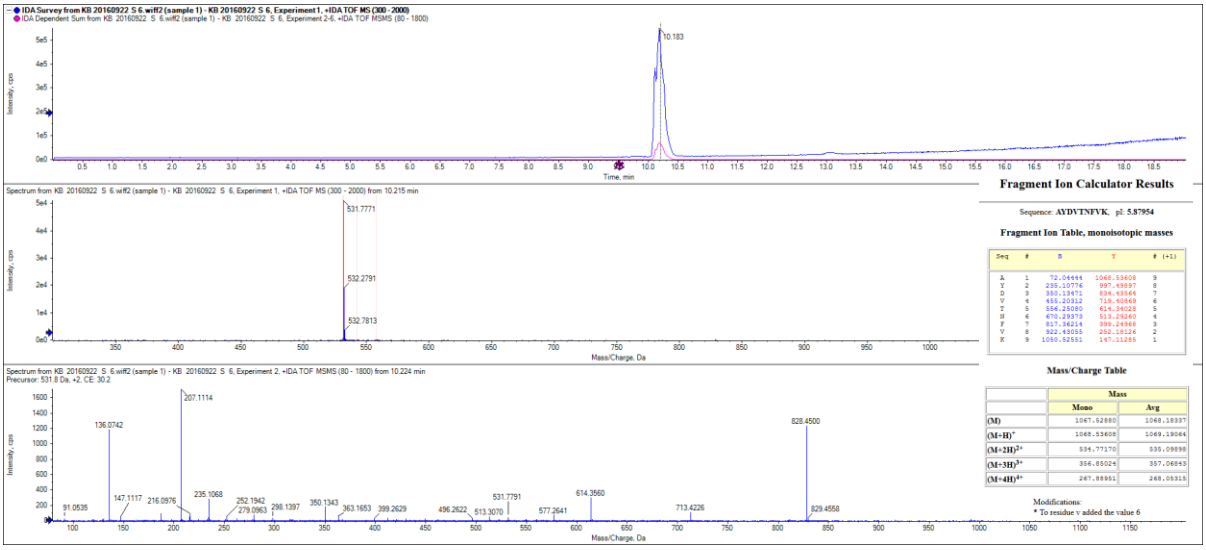
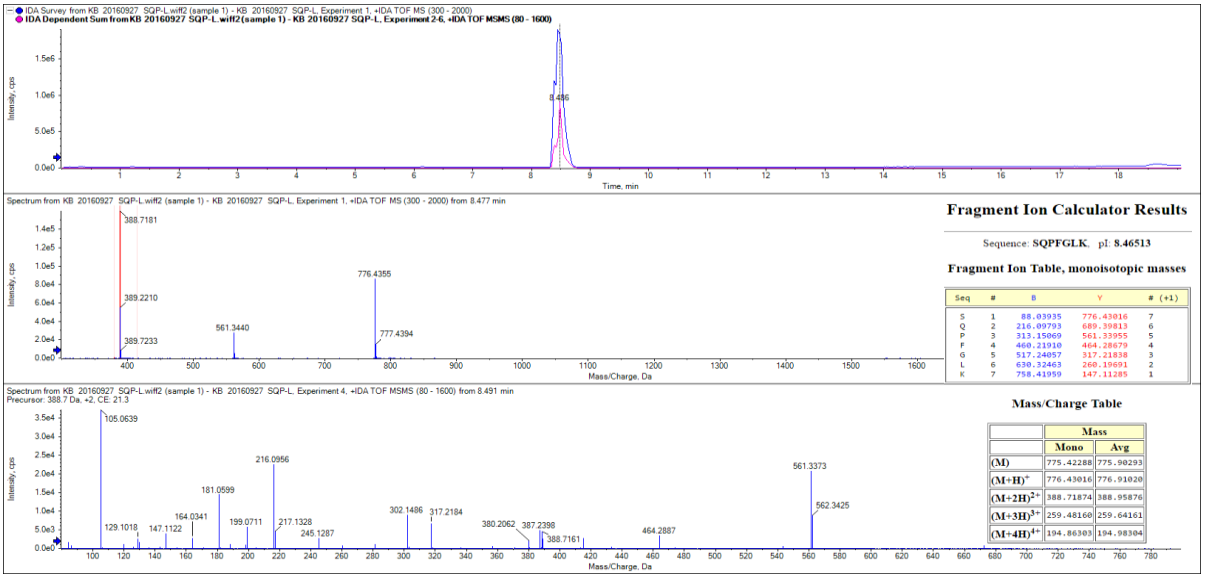


Figure 7. Quality control of Pavsa-Δ5D peptide.
(A) LC-ESI-MS/MS TIC shows the Pavsa-Δ5D peptide AYDVTNFVK at 10.14 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 10.14 min of 528.77^{2+} is shown (second panel) and matches the theoretical *m/z* value 528.77^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC shows the peptide AYDVTNFV*K at 10.18 min (top panel). No other significant peaks were detected. The determined *m/z* value for the peak at 10.18 min of 531.77^{2+} is shown (second panel) and matches the theoretical *m/z* value 531.77^{2+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light SQPFGLK



(B) QC: Heavy SQPFGL*K

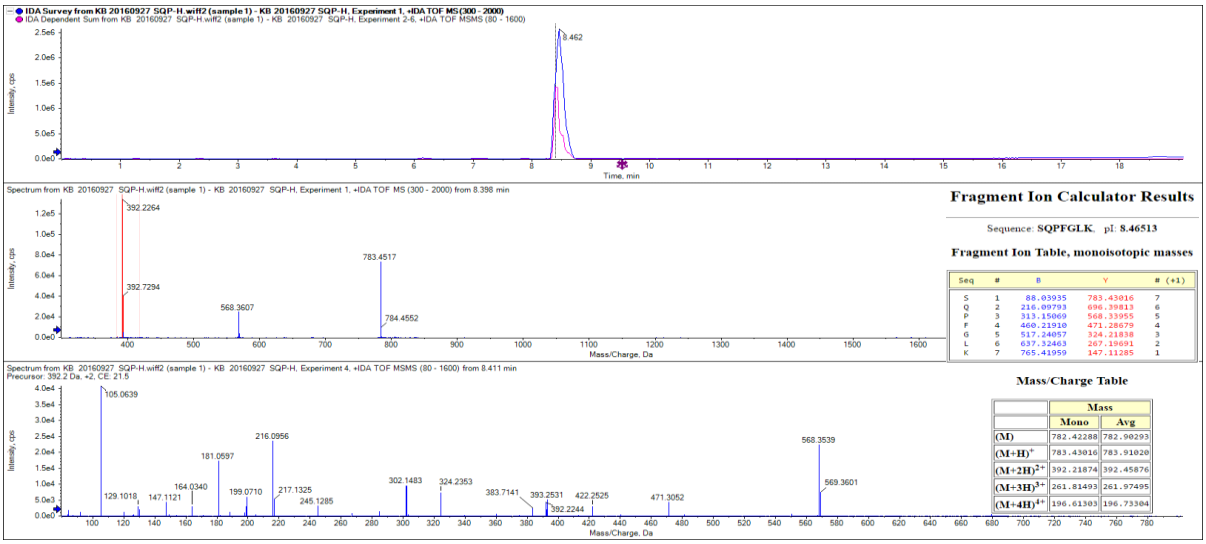
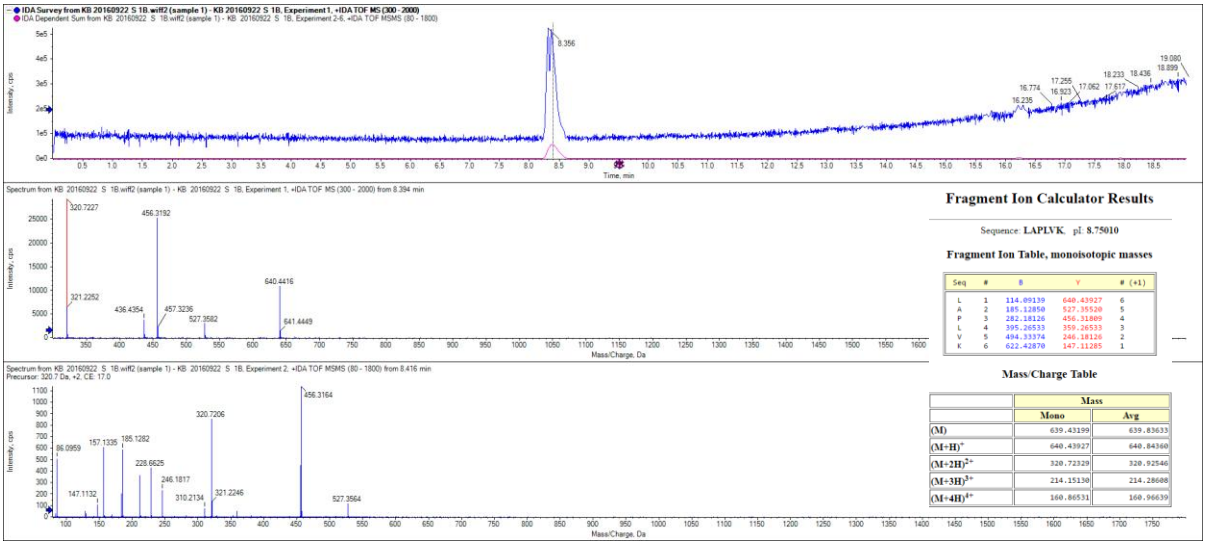


Figure 8. Quality control of Pyrco-Δ5E peptide.
(A) LC-ESI-MS/MS TIC shows the Pyrco-Δ5E peptide SQPFGLK at 8.44 min (top panel). No other significant peaks were detected. The determined m/z values for the peak at 8.44 min: 388.72^{2+} and 776.43^{1+} are shown (second panel) and match the theoretical *m/z* values: 388.72^{2+} and 776.43^{1+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC shows the peptide SQPFGL*K at 8.46 min (top panel). No other significant peaks were detected. The determined *m/z* values for the peak at 8.46 min: 392.22^{2+} and 783.45^{1+} are shown (second panel) and match the theoretical *m/z* values: 392.22^{2+} and 783.43^{1+}. The MS/MS spectrum revealing the correct peptide sequence (third panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light LAPLVK



(B) QC: Heavy LAPLV*K

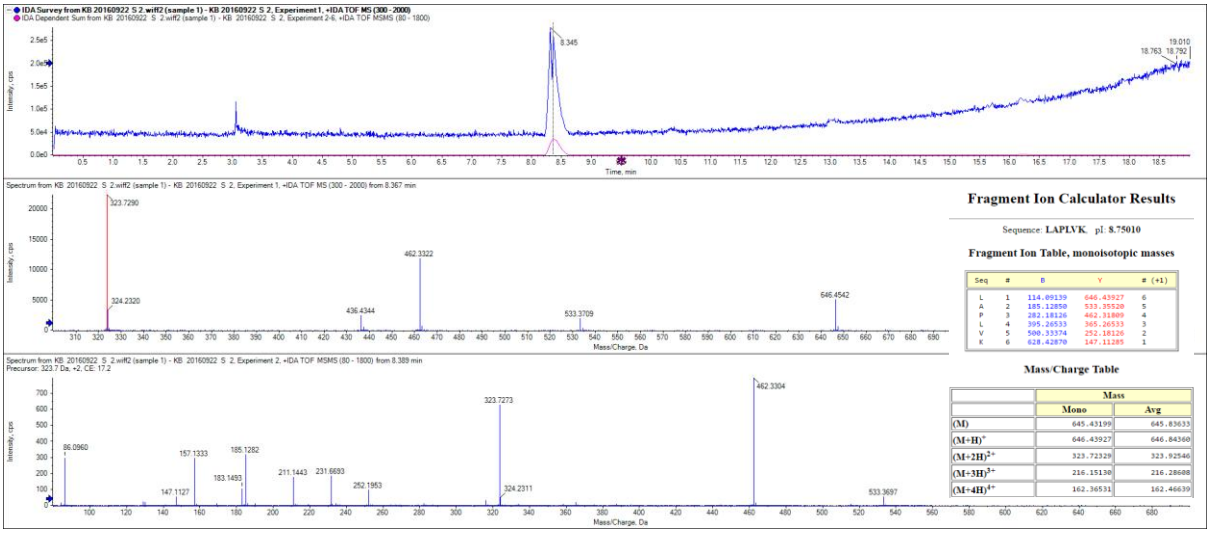
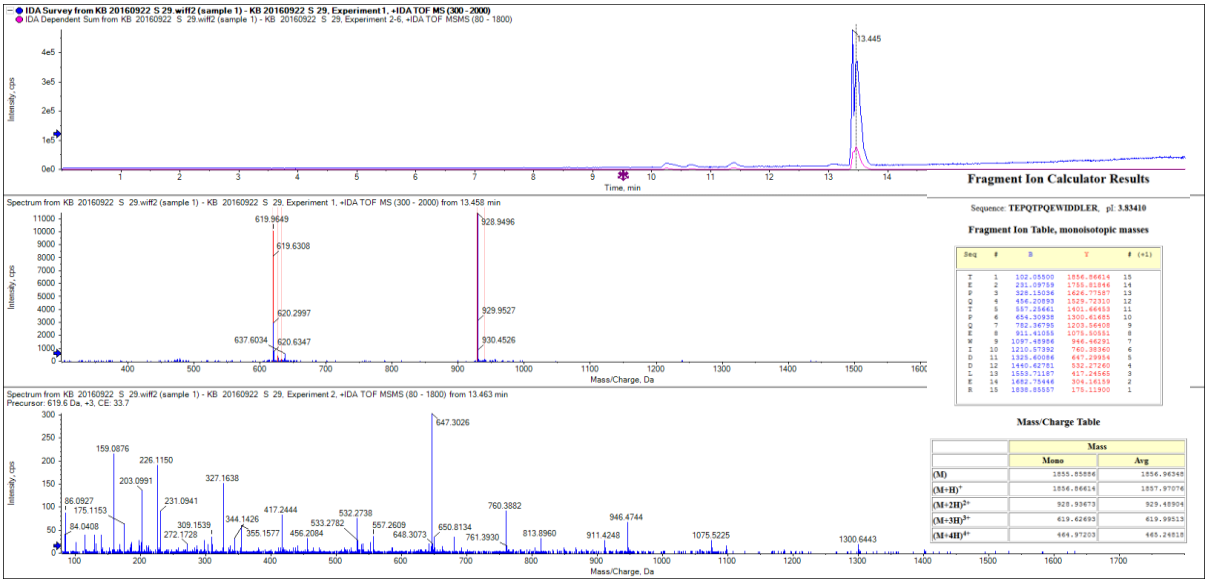


Figure 9. Quality control of Pava-Δ4D peptide.

(A) LC-ESI-MS/MS TIC show the Pavsa-Δ4D peptide LAPLVK at 8.36 min (top panel). No other significant other peaks were detected. The determined *m/z* values for the peak at 8.36 min: 320.72^{2+} and 640.44^{1+} are shown (second panel) and match the theoretical *m/z* values: 320.72^{2+} and 639.44^{1+} (representing ≤1 ppm mass error respectively). The MS/MS spectrum revealing the correct peptide sequence (bottom panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC show the LAPLV*K peptide at 8.35 min (top panel). No other significant peaks were detected. The determined *m/z* values for the peak at 8.35 min: 323.73^{2+} and 646.45^{1+} are shown (second panel) and match the theoretical *m/z* values: 323.73^{2+} and 645.45^{1+} (representing ≤1 ppm mass error respectively). The MS/MS spectrum revealing the correct peptide sequence (bottom panel) and the theoretical *m/z* values are shown (inset).

(A) QC: Light TEPQTPQEWIDDLER



(B) QC: Heavy TEPQTPQEWIDDL*ER

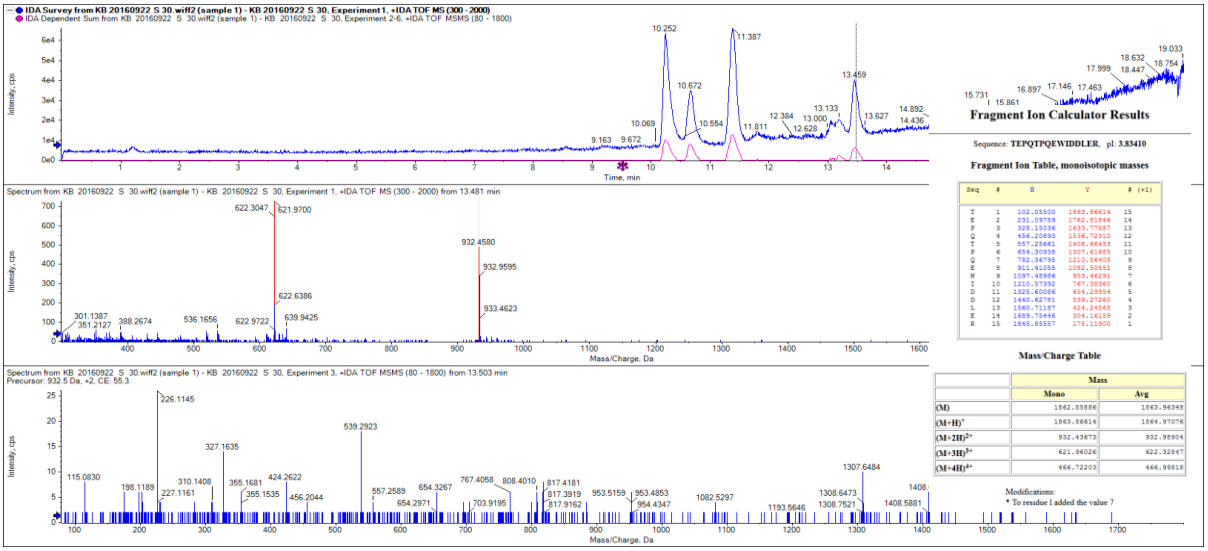


Figure 10. Quality control of PAT peptide.

(A) LC-ESI-MS/MS TIC show the PAT peptide TEPQTPQEWIDDLER at 13.45 min (top panel). The determined *m/z* values for the peak at 13.45 min: 619.63^{3+} and 928.95^{2+} are shown (second panel) and match the theoretical *m/z* values: 619.63^{3+} and 928.94^{2+}. The MS/MS spectrum revealing the correct peptide sequence (bottom panel) and the theoretical *m/z* values are shown (inset). **(B)** LC-ESI-MS/MS TIC show the TEPQTPQEWIDDL*ER peptide at 13.46 min (top panel). Three peaks were detected at 10.25, 10.67 and 11.39 min that were determined to be due to artefactual modification of the Trp residue. The determined *m/z* values for the peak at 13.46 min: 621.97^{3+} and 932.46^{2+} are shown (second panel) and match the theoretical *m/z* values: 621.96^{3+} and 932.44^{2+}. The MS/MS spectrum revealing the correct peptide sequence (bottom panel) and the theoretical *m/z* values are shown (inset).

QC: Heavy WEGEPI*SK (IS)

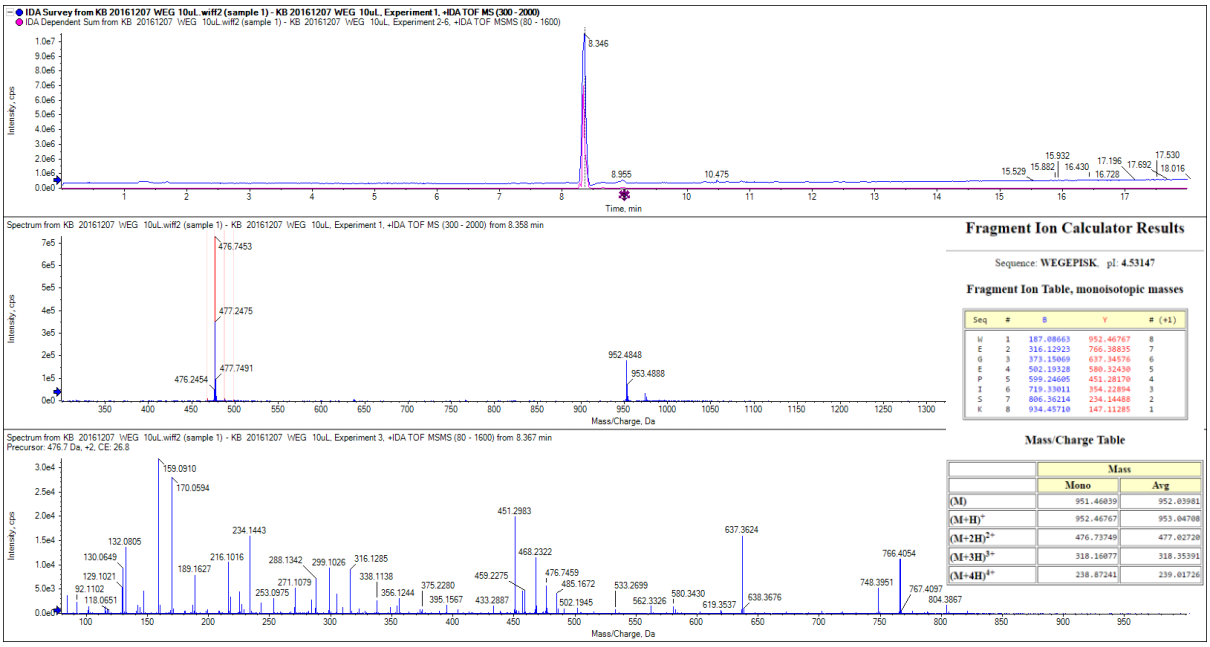


Figure 11. Quality control of IS peptide.
LC-ESI-MS/MS TIC showing the WEGEPI*SK IS peptide at 8.35 min (top panel). No other significant peaks were detected. The determined *m/z* values for the peak at 8.35 min: 476.75^{2+} and 952.48^{3+} are shown (second panel) and match the theoretical *m/z* values: 476.74^{2+} and 952.47^{1+} (representing ≤0.01 ppm mass error respectively). The MS/MS spectrum revealing the correct peptide sequence (bottom panel) and the theoretical *m/z* values are shown (inset).

The concentrations of the synthetic peptides were determined by high sensitivity amino acid analysis and results were expressed as averages of duplicate measurements (Tables 3-11). The calculated amount of amino acid (µg/mL) is based on the amino acid residue mass in the protein (molecular weight minus H_2O). Using the determined concentrations, stock solutions were prepared at 100 pmol/µL.

Table 3. Amino Acid Analysis: GSSSNTEQEVPK

Amino acid [a]	GSSSNTEQEVPK MW 1261.58 Da				GSSSNTEQEV*PK MW 1267.58 Da			
	Amount (µg/ml)	**nmol/ml**	**Exp. mole %**	**Theor. mole %**	**Amount (µg/ml)**	**nmol/ml**	**Exp. mole %**	**Theor. mole %**
Ser	14.07	161.6	22.7	25.0	15.96	183.3	22.7	25.0
Gly	3.49	61.2	8.6	8.3	3.99	70.0	8.7	8.3
Asp	7.06	61.3	8.6	8.3	7.99	69.4	8.6	8.3
Glu	23.69	183.4	25.7	25.0	26.91	208.4	25.8	25.0
Thr	5.81	57.5	8.1	8.3	6.59	65.2	8.1	8.3
Pro	5.97	61.5	8.6	8.3	6.75	69.5	8.6	8.3
Lys	7.87	61.4	8.6	8.3	8.84	69.0	8.5	8.3
Val	6.44	65.0	9.1	8.3	7.30	73.7	9.1	8.3
Total	74.40	712.9	100.0	100.0	84.33	808.4	100.0	100.0

a. Ser, serine; Gly, glycine; Asp, aspartic acid; Glu, glutamic acid; Thr, threonine; Pro, proline; Lys, lysine; Val, valine.

Table 4. Amino Acid Analysis: IPFYHAR

Amino acid [a]	IPFYHAR MW 902.48 Da				IPFYHA*R MW 906.48 Da			
	Amount (µg/ml)	**nmol/ml**	**Exp. mole %**	**Theor. mole %**	**Amount (µg/ml)**	**nmol/ml**	**Exp. mole %**	**Theor. mole %**
His	9.66	70.5	14.2	14.3	2.38	17.4	14.3	14.3
Arg	11.47	73.4	14.8	14.3	2.82	18.1	14.9	14.3
Ala	4.80	67.6	13.6	14.3	1.19	16.8	13.8	14.3
Pro	6.83	70.3	14.2	14.3	1.65	17.0	14.0	14.3
Tyr	11.58	71.0	14.3	14.3	2.83	17.3	14.3	14.3
Ile	8.00	70.7	14.3	14.3	1.93	17.1	14.1	14.3
Phe	10.60	72.0	14.5	14.3	2.58	17.5	14.5	14.3
Total	62.95	495.5	100.0	100.0	15.39	121.2	100.0	100.0

a. His, histidine; Arg, argininine; Ala, alanine; Pro, proline; Tyr, tyrosine; Ile, isoleucine; Phe, phenylalanine.

Table 5. Amino Acid Analysis: DASTAPVDLK

Amino acid [a]	DASTAPVDLK MW 1015.51 Da				DASTAPVDL*K MW 1022.52 Da			
	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %
Ser	5.47	62.9	8.9	10.0	3.44	39.5	9.0	10.0
Asp	16.50	143.4	20.4	20.0	10.47	91.0	20.6	20.0
Thr	6.86	67.9	9.7	10.0	4.30	42.6	9.7	10.0
Ala	9.73	136.9	19.5	20.0	6.08	85.6	19.4	20.0
Pro	6.92	71.3	10.1	10.0	4.34	44.7	10.2	10.0
Lys	9.22	72.0	10.2	10.0	5.66	44.2	10.0	10.0
Val	7.64	77.1	11.0	10.0	4.78	48.2	10.9	10.0
Leu	8.09	71.5	10.2	10.0	5.08	44.9	10.2	10.0
Total	70.45	702.9	100.0	100.0	44.16	440.6	100.0	100.0

a. Ser, serine; Asp, aspartic acid; Thr, threonine; Ala, alanine; Pro, proline; Lys, lysine; Val, valine; Leu, leucine.

Table 6. Amino Acid Analysis: GQDPFLLK

Amino acid [a]	GQDPFLLK MW 916.50 Da				GQDPFLL*K MW 923.5 Da			
	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %
Gly	4.34	76.2	12.6	12.5	2.70	47.3	12.4	12.5
Asp	8.75	76.0	12.6	12.5	5.53	48.0	12.6	12.5
Glu	9.84	76.2	12.6	12.5	6.11	47.3	12.4	12.5
Pro	7.33	75.4	12.5	12.5	4.62	47.6	12.5	12.5
Lys	9.58	74.7	12.3	12.5	6.15	48.0	12.6	12.5
Leu	17.01	150.3	24.8	25.0	10.79	95.3	25.0	25.0
Phe	11.22	76.2	12.6	12.5	7.04	47.8	12.5	12.5
Total	68.06	605.0	100.0	100.0	42.94	381.4	100.0	100.0

a. Gly, glycine; Asp, aspartic acid; Glu, glutamic acid; Pro, proline; Lys, lysine; Leu, leucine; Phe, phenylalanine.

Table 7. Amino Acid Analysis: AYDVTNFVK

Amino acid [a]	AYDVTNFVK MW 1055.52 Da				AYDVTNFV*K MW 1061.52 Da			
	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %
Asp	15.38	133.6	22.3	22.2	15.02	130.5	22.3	22.2
Thr	6.32	62.6	10.5	11.1	6.18	61.2	10.4	11.1
Ala	4.48	63.0	10.5	11.1	4.36	61.4	10.5	11.1
Lys	8.41	65.6	11.0	11.1	8.24	64.3	11.0	11.1
Tyr	10.66	65.3	10.9	11.1	10.62	65.1	11.1	11.1
Val	13.92	140.4	23.5	22.2	13.59	137.1	23.4	22.2
Phe	9.97	67.7	11.3	11.1	9.77	66.4	11.3	11.1
Total	69.14	598.3	100.0	100	67.78	585.8	100.0	100

a. Asp, aspartic acid; Thr, threonine; Ala, alanine; Lys, lysine; Tyr, tyrosine; Val, valine; Phe, phenylalanine.

Table 8. Amino Acid Analysis: SQPFGLK

Amino acid [a]	SQPFGLK MW 775.42 Da				SQPFGL*K MW 782.42 Da			
	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %
Ser	8.1	93	13.0	14.3	10.4	119	13.1	14.3
Gly	6.0	106	14.7	14.3	7.6	134	14.7	14.3
Glu	13.4	104	14.4	14.3	16.9	131	14.4	14.3
Pro	10.1	104	14.5	14.3	12.8	132	14.5	14.3
Lys	13.2	103	14.3	14.3	16.6	129	14.2	14.3
Leu	11.7	104	14.4	14.3	14.8	131	14.4	14.3
Phe	15.4	105	14.6	14.3	19.7	134	14.7	14.3
Total	78.0	718	100.0	100.0	98.8	909	100.0	100.0

a. Ser, serine; Glu, glutamic acid; Pro, proline; Lys, lysine; Leu, leucine; Phe, phenylalanine.

Table 9. Amino Acid Analysis: LAPLVK

Amino acid [a]	LAPLVK MW 639.43 Da				LAPLV*K MW 645.43 Da			
	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %
Ala	8.13	114.3	16.0	16.7	4.69	66.0	15.8	16.7
Pro	11.63	119.7	16.8	16.7	6.71	69.1	16.6	16.7
Lys	15.19	118.5	16.6	16.7	8.78	68.5	16.5	16.7
Val	12.37	124.7	17.5	16.7	7.45	75.2	18.1	16.7
Leu	26.76	236.5	33.1	33.3	15.58	137.7	33.1	33.3
Total	74.07	713.8	100.0	100.0	43.21	416.4	100.0	100.0

a. Ala, alanine; Pro, proline; Lys, lysine; Val, valine; Leu, leucine.

Table 10. Amino Acid Analysis: TEPQTPQEWIDDLER

Amino acid [a]	TEPQTPQEWIDDLER MW 1855.86 Da				TEPQTPQEWIDDL*ER MW 1862.86 Da			
	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %	Amount (µg/ml)	nmol/ml	Exp. mole %	Theor. mole %
Arg	6.12	39.2	7.3	7.1	4.90	31.4	7.5	7.1
Asp	9.43	82.0	15.3	14.3	6.98	60.6	14.5	14.3
Glu	25.1	194.4	36.4	35.7	19.35	149.9	35.8	35.7
Thr	7.12	70.4	13.2	14.3	5.75	56.9	13.6	14.3
Pro	7.15	73.7	13.8	14.3	5.74	59.1	14.1	14.3
Ile	4.16	36.8	6.9	7.1	3.32	29.4	7.0	7.1
Leu	4.25	37.6	7.0	7.1	3.50	31.0	7.4	7.1
Total	63.35	534.0	100.0	100.0	49.55	418.3	100.0	100.0

a. Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Thr, threonine; Pro, proline; Ile, isoleucine; Leu, leucine.

Table 11. Amino Acid Analysis: WEGEPISK Internal Standard

Amino acid [a]	WEGEPISK MW 944.46 Da				WEGEPI*SK MW 951.46 Da			
	Amount (µg/ml)	**nmol/ml**	**Exp. mole %**	**Theor. mole %**	**Amount (µg/ml)**	**nmol/ml**	**Exp. mole %**	**Theor. mole %**
Ser	6.6	76	12.8	14.3	6.8	78	13.0	14.3
Gly	4.9	86	14.4	14.3	5.0	88	14.8	14.3
Glu	22.1	171	28.8	28.6	22.1	171	28.7	28.6
Pro	8.4	87	14.6	14.3	8.4	87	14.5	14.3
Lys	11.3	88	14.8	14.3	10.9	85	14.2	14.3
Ile	9.8	87	14.6	14.3	9.9	88	14.7	14.3
Total	63.2	595	100.0	100.0	63.1	596	100.0	100.0

a. Ser, serine; Gly, glycine; Glu, glutamic acid; Pro, proline; Lys, lysine; Ile, isoleucine.

B. Development of Quantitative LC-MRM-MS Method

Using the data collected from the tryptic digest of the enzymes of the DHA biosynthetic pathway, the peptide mass and hence precursor mass-to-charge (*m/z*) ratio was determined. Subsequently five fragment ions were selected that were representative of the target peptide. Together the Q1 *m/z* and Q3 *m/z* are termed the MRM transition and these are given in Table 12 for the light peptides and Table 13 for the heavy peptides. As noted above, unfortunately, due to low level contamination of the heavy peptides during purification following synthesis, the heavy peptides could not be utilized as partner peptides for quantification, but were utilized as reference standards for qualitative assessment.

Table 12. MRM transitions of light peptides (analytes).

Protein	Peptide	RT (min)[a]	Q1 *m/z* [a]	z [a]	Q3 *m/z* [a]	Fragment	CE [a]
Δ12D	GSSSNTEQEVPK	1.62	631.797	2+	729.380	y6+	32.0
					944.468	y8+	28.0
					1019.428	b10+	28.0
ω3D	IPFYHAR	3.56	452.245	2+	546.278	y4+	27.2
					693.347	y5+	27.2
					395.703	y6++	23.2
Δ6D	DASTAPVDLK	3.63	508.767	2+	571.345	y5+	23.9
					642.382	y6+	23.9
					743.430	y7+	21.9
Δ6E	GQDPFLLK	5.07	459.258	2+	260.197	y2+	28.5
					617.402	y5+	21.5
					732.429	y6+	19.5
Δ5D	AYDVTNFVK	4.85	528.772	2+	608.34	y5+	22.9
					707.409	y6+	22.9
					822.436	y7+	22.9
Δ5E	SQPFGLK	3.84	388.719	2+	216.098	b2+	18.9
					317.218	y3+	26.9
					561.34	y5+	18.9
Δ4D	LAPLVK	3.72	320.723	2+	185.128	b2+	14.0
					246.181	y2+	22.0
					456.318	y4+	14.0
PAT	TEPQTPQEWIDDLER	6.60	619.627	3+	647.299	y5+	29.7
			619.627	3+	650.812	y10++	27.7
			928.937	2+	1300.617	y10+	48.5

a. RT, retention time (min); Q1 *m/z*, precursor ion mass-to-charge ratio; z, charge state; Q3 *m/z*, fragment ion *m/z*; CE, collision energy in V.

b. Collision energy settings were also optimized for all targeted transitions by analysing 2 µL of each peptide chromatographically separated on a Shimadzu Nexera UHPLC and analyzed on a 6500 QTRAP mass spectrometer (AB SCIEX, Foster City, USA) as described previously (Colgrave et al. 2014)

Table 13. MRM transitions of heavy peptides (reference standards) including IS peptide.

Protein	Peptide	RT (min)[a]	Q1 *m/z* [a]	z [a]	Q3 *m/z* [a]	Fragment	CE [a]
Δ12D	GSSSNTEQEV*PK	1.62	634.79	2+	735.380	y6+	32.0
					950.470	y8+	28.0
					1025.420	b10+	28.0
ω3D	IPFYHA*R	3.56	454.245	2+	550.278	y4+	27.3
					697.346	y5+	27.3
					397.703	y6++	23.3
Δ6D	DASTAPVDL*K	3.63	512.266	2+	578.345	y5+	23.9
					649.382	y6+	23.9
					750.429	y7+	21.9
Δ6E	GQDPFLL*K	5.07	462.758	2+	267.196	y2+	27.7
					624.402	y5+	21.7
					739.429	y6+	19.7
Δ5D	AYDVTNFV*K	4.85	531.771	2+	614.340	y5+	22.9
					713.408	y6+	22.9
					828.435	y7+	22.9
Δ5E	SQPFGL*K	3.84	392.218	2+	216.098	b2+	24.9
					324.218	y3+	26.9
					568.339	y5+	18.9
Δ4D	LAPLV*K	3.72	323.723	2+	185.128	b2+	14.0
					252.181	y2+	22.0
					462.318	y4+	14.0
PAT	TEPQTPQEWIDDL*ER	6.60	619.627	3+	654.299	y5+	25.9
			619.627	3+	654.312	y10++	29.9
			932.436	2+	1307.617	y10+	46.7
IS	WEGEPI*SK	3.26	476.737	2+	451.281	y4+	28.0
					637.346	y6+	24.0
					766.388	y7+	26.0

a. RT, retention time (min); Q1 m/z, precursor ion mass-to-charge ratio (m/z); z, charge state; Q3 m/z, fragment ion m/z; CE, collision energy in V.

b. Collision energy settings were also optimized for all targeted transitions by analyzing 2 µL of each peptide were chromatographically separated on a Shimadzu Nexera UHPLC and analyzed on a 6500 QTRAP mass spectrometer (AB SCIEX, Foster City, USA) as described previously (Colgrave et al. 2014)

C. VALIDATION OF PROTEIN QUANTIFICATION BY LC-MRM-MS

The MS response (peak area) of the light peptides (analytes) were measured and plotted relative to the amount of peptide loaded onto the LC-MS system. All peptides gave a linear response over the range 0 to 1,250 femtomoles, with the exception of the Pavsa-Δ4D peptide LAPLV*K, for which the linear range extended to 2,500 femtomoles as shown in Figure 12. The analytical parameters for quantification of canola peptides wherein limit of detection (LOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) are given in femtomoles and listed in Table 14.

Table 14. Analytical parameters for quantification of canola peptides (femtamoles).

Protein	Peptide Sequence	LOD	LLOQ	ULOQ	m	b	R^2
Lackl-Δ12D	GSSSNTEQEVPK	0.31	0.31	1,250	7.088e-5	-0.0004562	0.9980
Picpa-ω3D	IPFYHAR	0.61	1.22	1,250	0.0003545	-0.003352	0.9974
Micpu-Δ6D	DASTAPVDLK	0.08	0.15	1,250	0.006326	-0.002506	0.9989
Pyrco-Δ6E	GQDPFLLK	0.08	0.31	1,250	0.001400	-0.007894	0.9989
Pavsa-Δ5D	AYDVTNFVK	0.15	0.15	1,250	0.001385	-0.008230	0.9988
Pyrco-Δ5E	SQPFGLK	0.08	0.15	1,250	0.002457	-0.002724	0.9996
Pavsa-Δ4D	LAPLVK	0.08	0.31	2,500	0.001461	-0.02667	0.9958

LOD, limit of detection; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; m, slope or gradient; b, y-intercept; R^2, linear regression. Units in femtomoles loaded on-column.

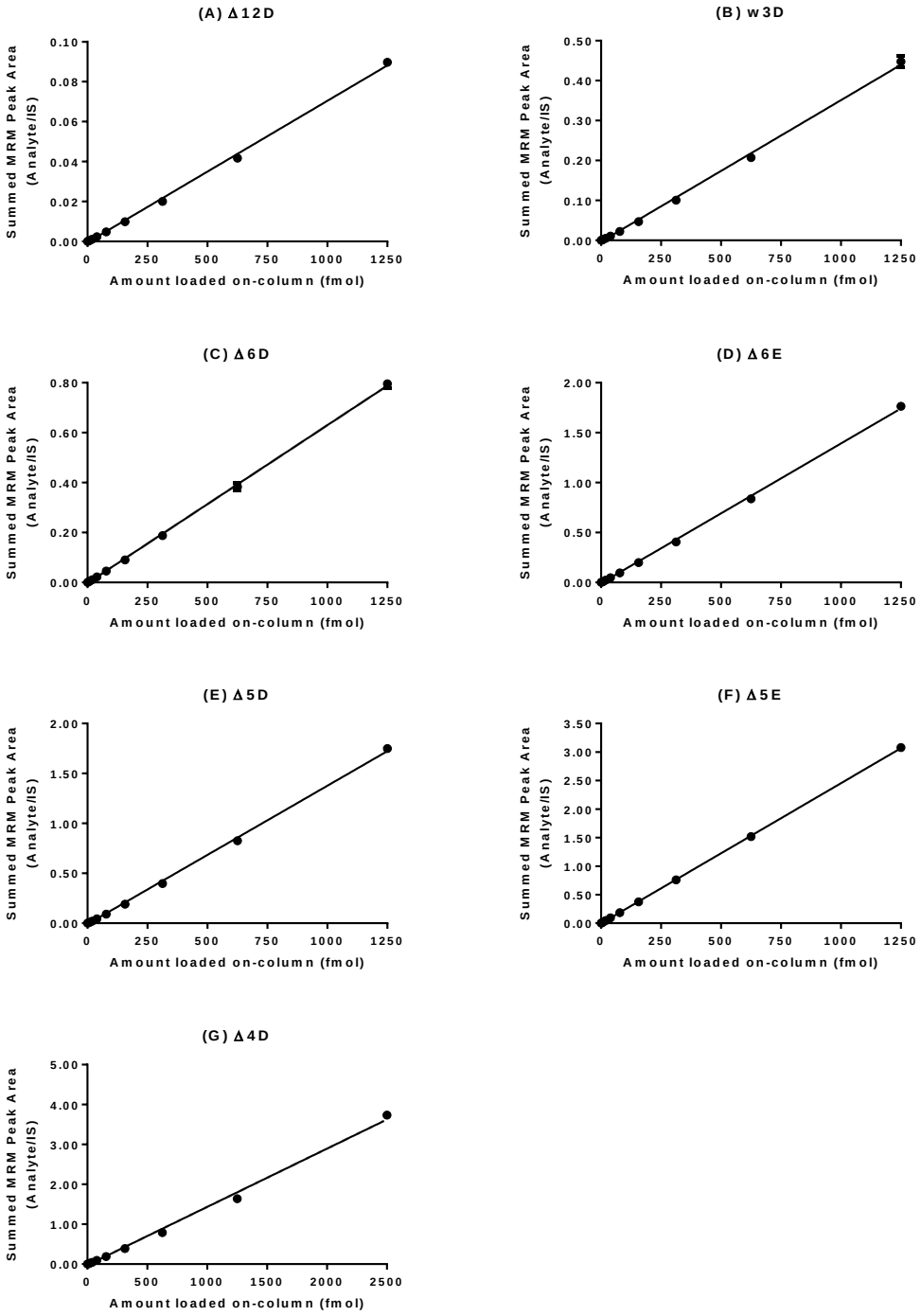


Figure 12. Calibration curve for quantifier peptides for each of the enzymes from the DHA biosynthetic pathway.
(A) Lackl-Δ12D; (B) Picpa-ω3D; (C) Micpu-Δ6D; (D) Pyrco-Δ6E; (E) Pavsa-Δ5D; (F) Pyrco-Δ5-E; (G) Pavsa-Δ4D.

D. LEVELS OF THE DHA BIOSYNTHESIS PATHWAY ENZYMES IN DHA CANOLA

A range of 2.38~7.40 mg or 2.54~4.92 mg total protein was obtained from 400 mg of developing or mature seeds, respectively. LC-MRM-MS quantification confirmed that none of the targeted peptides were detected in 250 µg of total protein extracts from WT canola, including all seven sampling points at five growth stages, from two field trial sites. Moreover, none of peptides were detected in total protein extracts in the non-seed tissues of DHA canola, from the seven sampling points at five growth stages, from two field trial sites (Table 15). However, all seven peptides representing the DHA biosynthesis pathway enzymes were detected in developing and/or mature seeds of DHA canola, and were quantified as shown in Table 16. The Pyrco-Δ5E and Pyrco-Δ6E proteins revealed the lowest protein abundance in the DHA canola (ranging from 64-90 femtomoles). The Pyrco-Δ5E was below the limit of detection in developing seeds, while the Pyrco-Δ6E protein was below the limit of detection in mature seeds. The Pavsa-Δ4D was the protein that was detected with the highest abundance of the seven enzymes, with up to 1,500 femtomoles in mature seeds. Based on the molecular mass of each protein, the level of each transgenic protein was determined (on a per mg protein basis, Table 17). The lowest protein was 20 ng of the Pyrco-Δ5E per mg total protein, and highest Pavsa-Δ4D was 740 ng per mg total proteins. All the detected peptides were confirmed as shown in Figures 13-19.

Table 15. Detection of peptides of transgene proteins in canola plant parts.

Protein	Peptide Sequence	Whole plant BBCH15	Whole plant BBCH35	Root BBCH65	Flower BBCH65	Other BBCH65	Develop. Seed BBCH79	Mature Seed BBCH90
Lackl-Δ12D	GSSSNTEQEVPK	ND	ND	ND	ND	ND	✓	✓
Picpa-ω3D	IPFYHAR	ND	ND	ND	ND	ND	✓	✓
Micpu-Δ6D	DASTAPVDLK	ND	ND	ND	ND	ND	✓	✓
Pyrco-Δ6E	GQDPFLLK	ND	ND	ND	ND	ND	✓	ND
Pavsa-Δ5D	AYDVTNFVK	ND	ND	ND	ND	ND	✓	✓
Pyrco-Δ5E	SQPFGLK	ND	ND	ND	ND	ND	ND	✓
Pavsa-Δ4D	LAPLVK	ND	ND	ND	ND	ND	✓	✓

Table 16. Quantification of transgenic proteins in developing and mature seed of DHA canola.

Protein	Peptide Sequence	Developing seed (BBCH79)		Mature seed (BBCH90)	
		Site 1506	Site 1508	Site 1506	Site 1508
Lackl-Δ12D	GSSSNTEQEVPK	507.1 ± 14.1	461.6 ± 149.5	441.0 ± 89.6	551.0 ± 87.3
Picpa-ω3D	IPFYHAR	351.1 ± 51.9	352.0 ± 148.7	469.3 ± 189.1	551.2 ± 55.1
Micpu-Δ6D	DASTAPVDLK	166.0 ± 28.7	257.1 ± 57.3	85.6 ± 7.5	80.9 ± 14.9
Pyrco-Δ6E	GQDPFLLK	79.0 ± 5.3	89.9 ± 19.6	ND	ND
Pavsa-Δ5D	AYDVTNFVK	131.6 ± 34.1	136.4 ± 65.7	129.2 ± 31.6	155.5 ± 41.6
Pyrco-Δ5E	SQPFGLK	ND	ND	64.1 ± 38.7	89.7 ± 15.7
Pavsa-Δ4D	LAPLVK	974.6 ± 296.6	888.7 ± 629.1	1500 ± 408.7	1470 ± 313.7

The amount of peptide detected is reported in units of femtomole/100 µg total protein, as mean ± SD, n=3. ND, not detected.

Table 17. Quantification of transgenic proteins in developing and mature seed of DHA canola.

Protein	MW (Da)	Developing Seed (BBCH79)		Mature Seed (BBCH90)	
		Site 1506	Site 1508	Site 1506	Site 1508
Lackl-Δ12D	48,158	244.2 ± 6.8	222.3 ± 72.0	212.4 ± 43.2	265.4 ± 42.0
Picpa-ω3D	47,760	167.7 ± 24.8	168.1 ± 71.0	224.1 ± 90.3	263.3 ± 26.3
Micpu-Δ6D	52,935	87.9 ± 15.2	136.1 ± 30.3	45.3 ± 4.0	42.8 ± 7.9
Pyrco-Δ6E	33,078	26.1 ± 1.8	29.7 ± 6.5	ND	ND
Pavsa-Δ5D	48,215	63.4 ± 16.4	65.8 ± 31.7	62.3 ± 15.2	75.0 ± 20.0
Pyrco-Δ5E	31,268	ND	ND	20.0 ± 12.1	28.0 ± 4.9
Pavsa-Δ4D	49,307	480.5 ± 146.2	438.2 ± 310.2	739.5 ± 201.5	724.7 ± 154.7

Units are ng of transgene protein per mg total protein extracted.

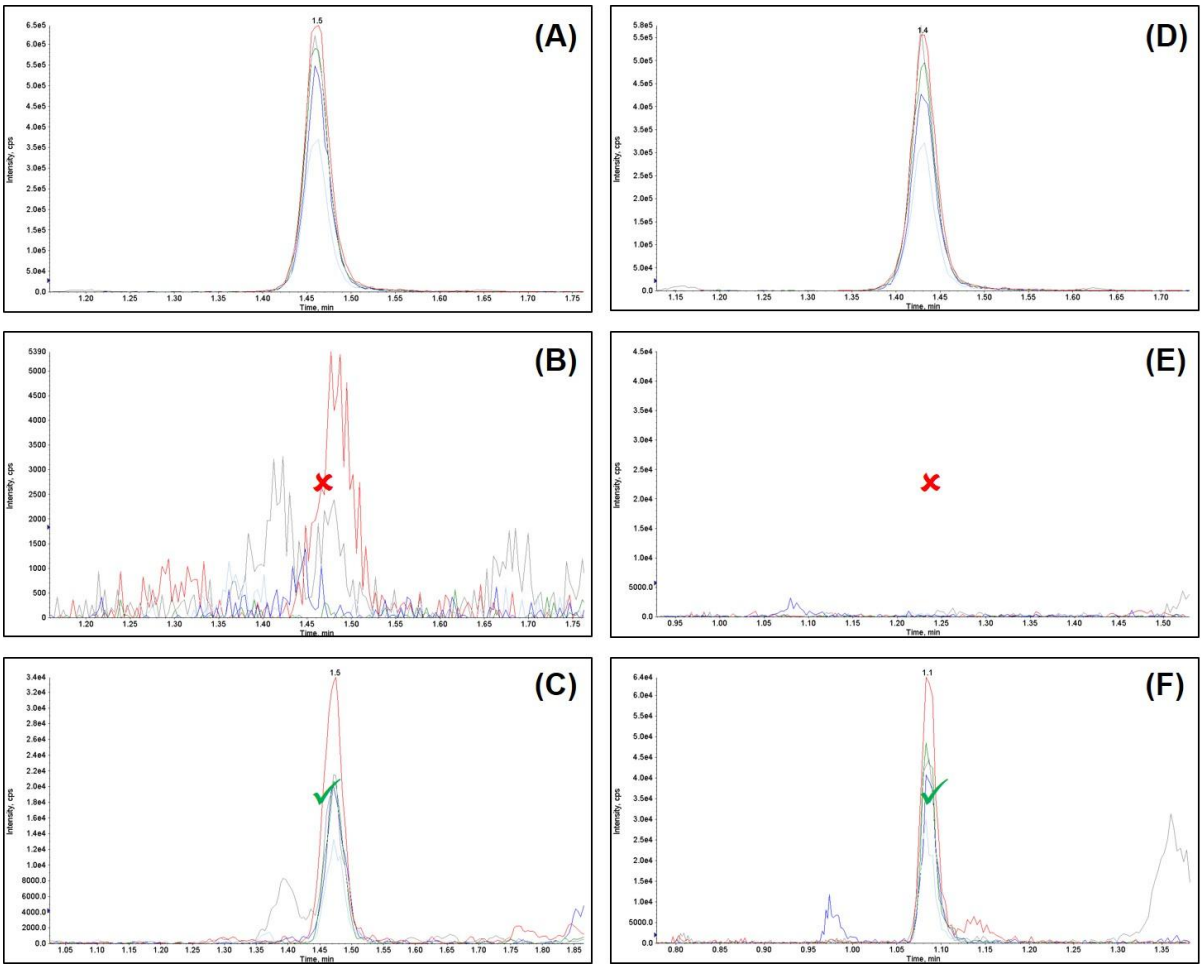


Figure 13. Detection of Lackl-Δ12D peptide GSSSNTEQEVPK in canola.
(A) Heavy labelled reference standard GSSSNTEQEVP*K spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo protein from DHA canola; (D) heavy labelled reference standard GSSSNTEQEVP*K spiked into mature seed protein background from WT canola (2 pmol on-column); (E) mature seed protein from WT canola; (F) mature seed protein from DHA canola.

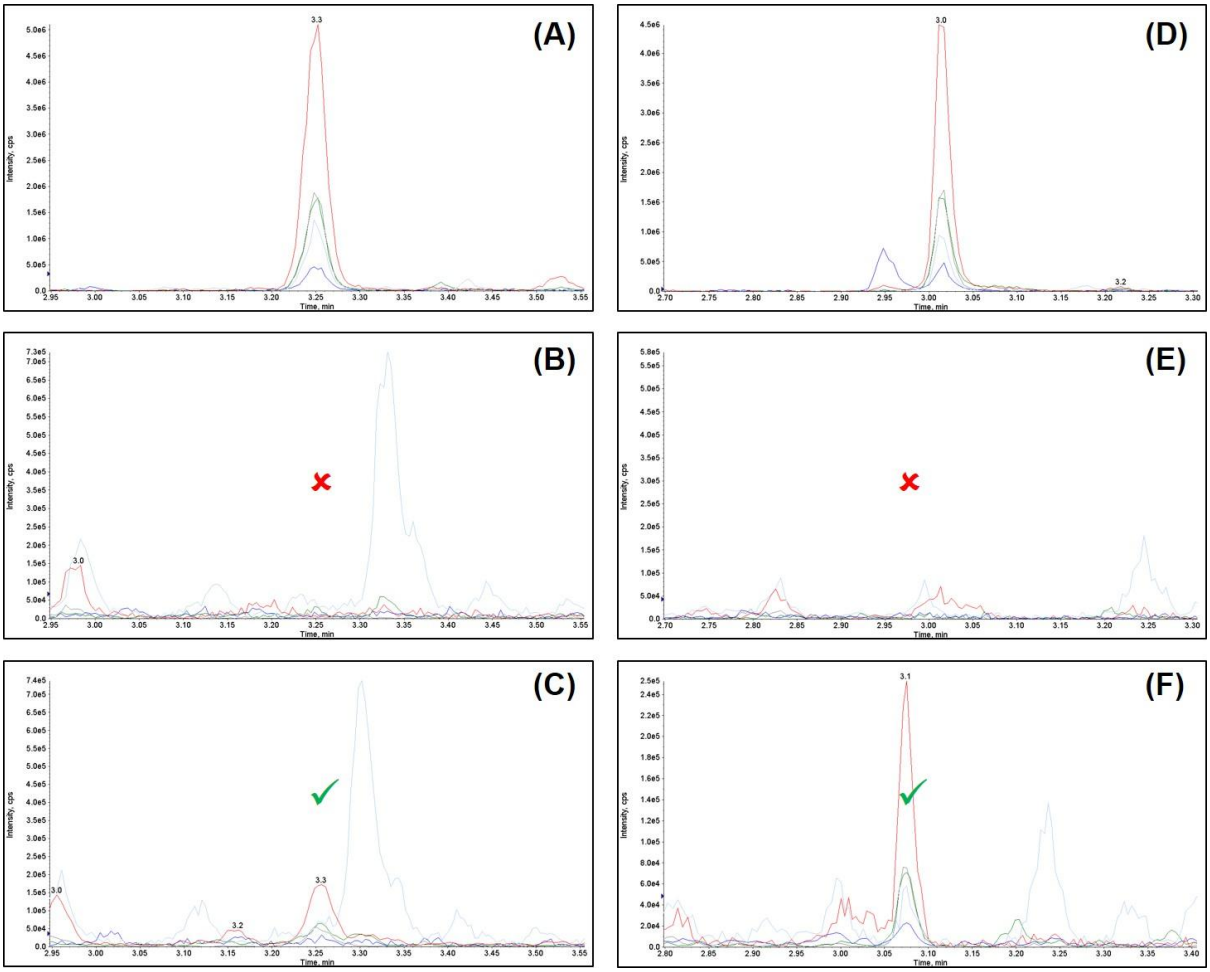


Figure 14. Detection of Picpa-ω3D peptide IPFYHAR in canola.
(A) Heavy labelled reference standard IPFYHA*R spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo protein from DHA canola; (D) heavy labelled reference standard IPFYHA*R spiked into mature seed protein background from WT canola (2 pmol on-column); (E) mature seed protein from WT canola; (F) mature seed from DHA canola.

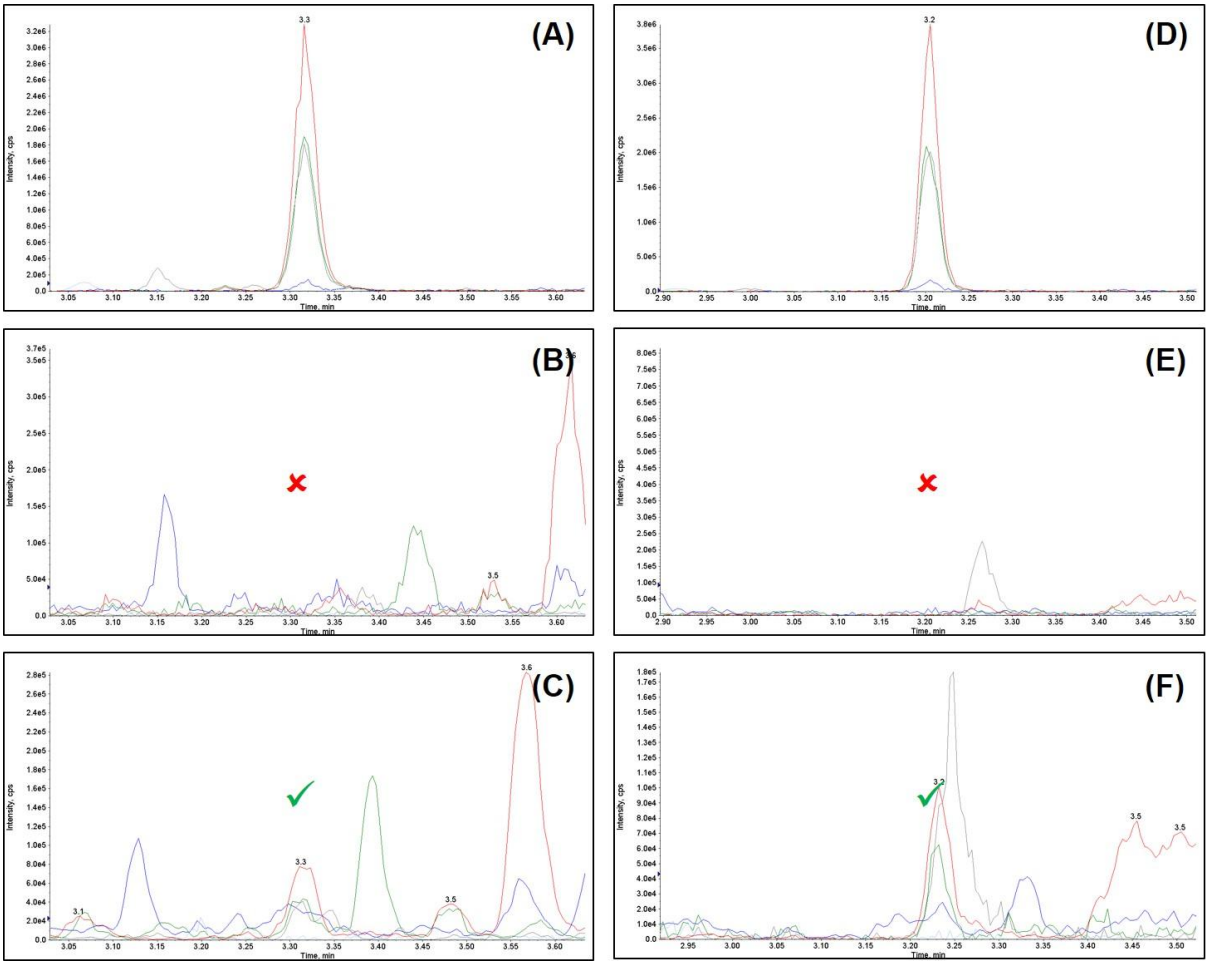


Figure 15. Detection of Micpu-Δ6D peptide DASTAPVDLK in canola.
(A) Heavy labelled reference standard DASTAPVDL*K spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo protein from DHA canola; (D) heavy labelled reference standard DASTAPVDL*K spiked into mature seed protein background from WT canola (2 pmol on-column); (E) mature seed protein from WT canola; (F) mature seed protein from DHA canola.

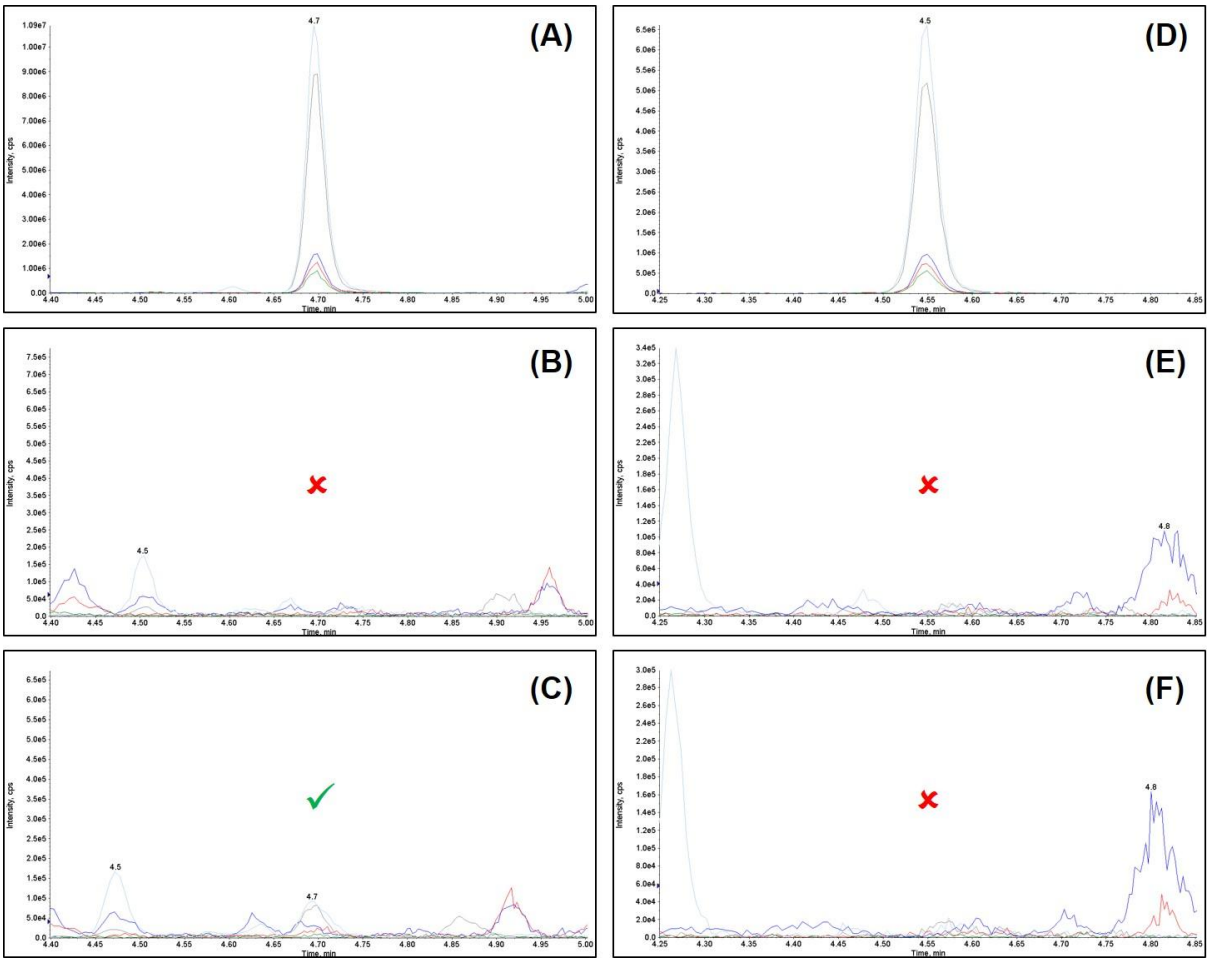


Figure 16. Detection of Pyrco-Δ6E peptide GQDPFLLK in canola.
(A) Heavy labelled reference standard GQDPFLL*K spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo from DHA canola; (D) heavy labelled reference standard GQDPFLL*K spiked into mature seed background from WT canola (2 pmol on-column); (E) mature seed from WT canola; (F) mature seed from DHA canola.

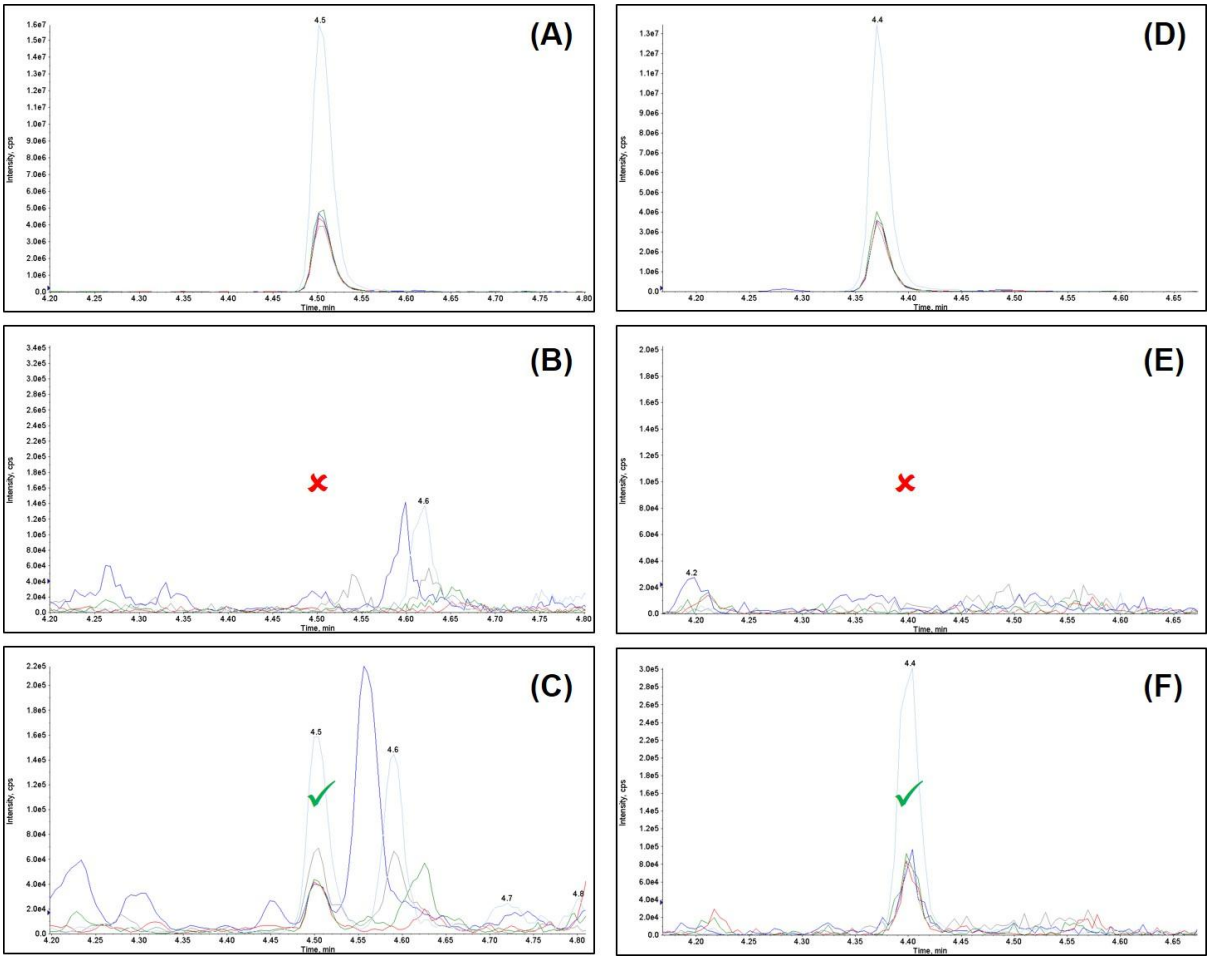


Figure 17. Detection of Pavsa-Δ5D peptide AYDVTNFVK in canola.
(A) Heavy labelled reference standard AYDVTNFV*K spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo protein from DHA canola; (D) heavy labelled reference standard AYDVTNFV*K spiked into mature seed protein background from WT canola (2 pmol on-column); (E) mature seed protein from WT canola; (F) mature seed protein from DHA canola.

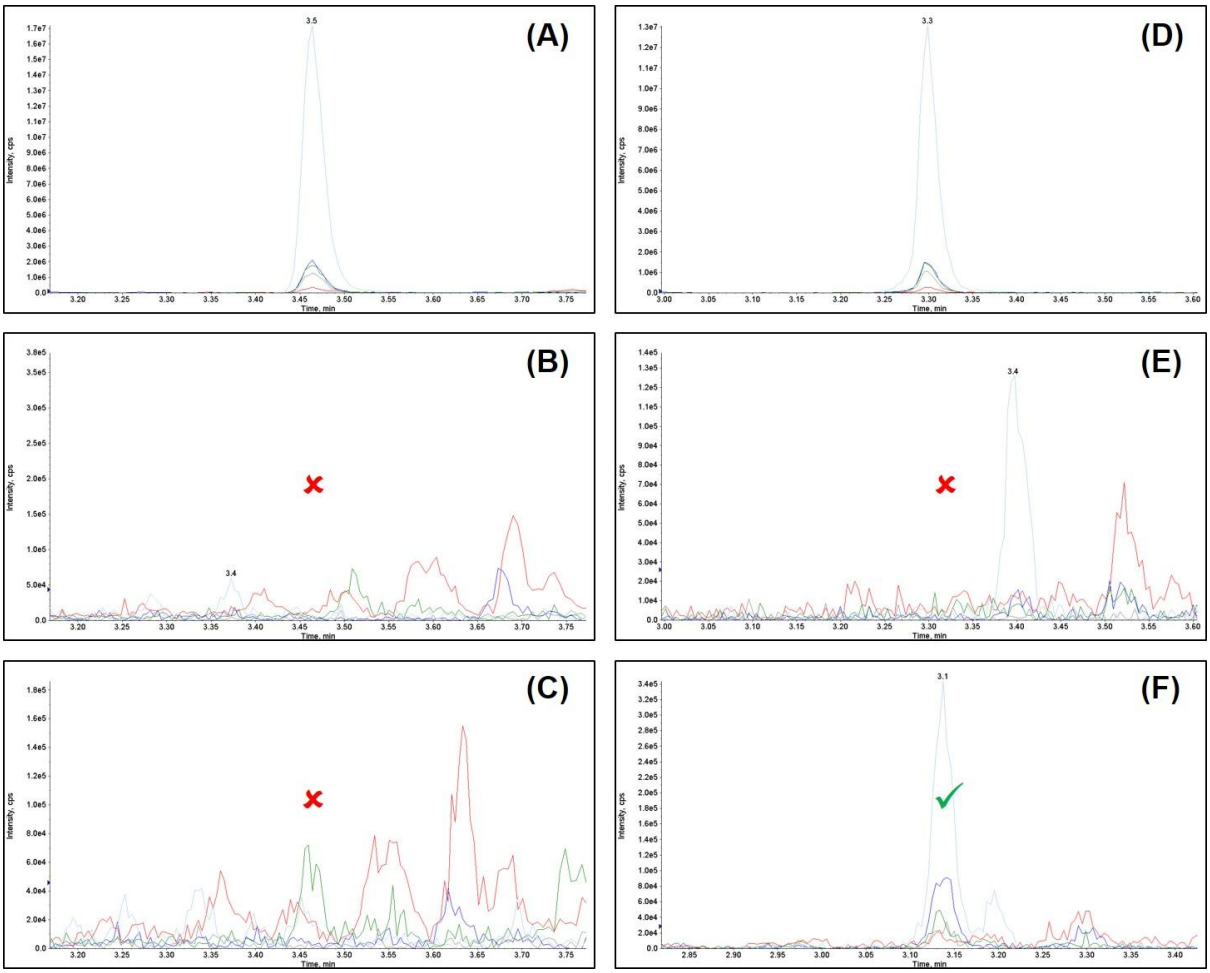


Figure 18. Detection of Pyrco-Δ5E peptide SQPFGLK in canola.
(A) Heavy labelled reference standard SQPFGL*K spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo protein from DHA canola; (D) heavy labelled reference standard SQPFGL*K spiked into mature seed protein background from WT canola (2 pmol on-column); (E) mature seed protein from WT canola; (F) mature seed protein from DHA canola.

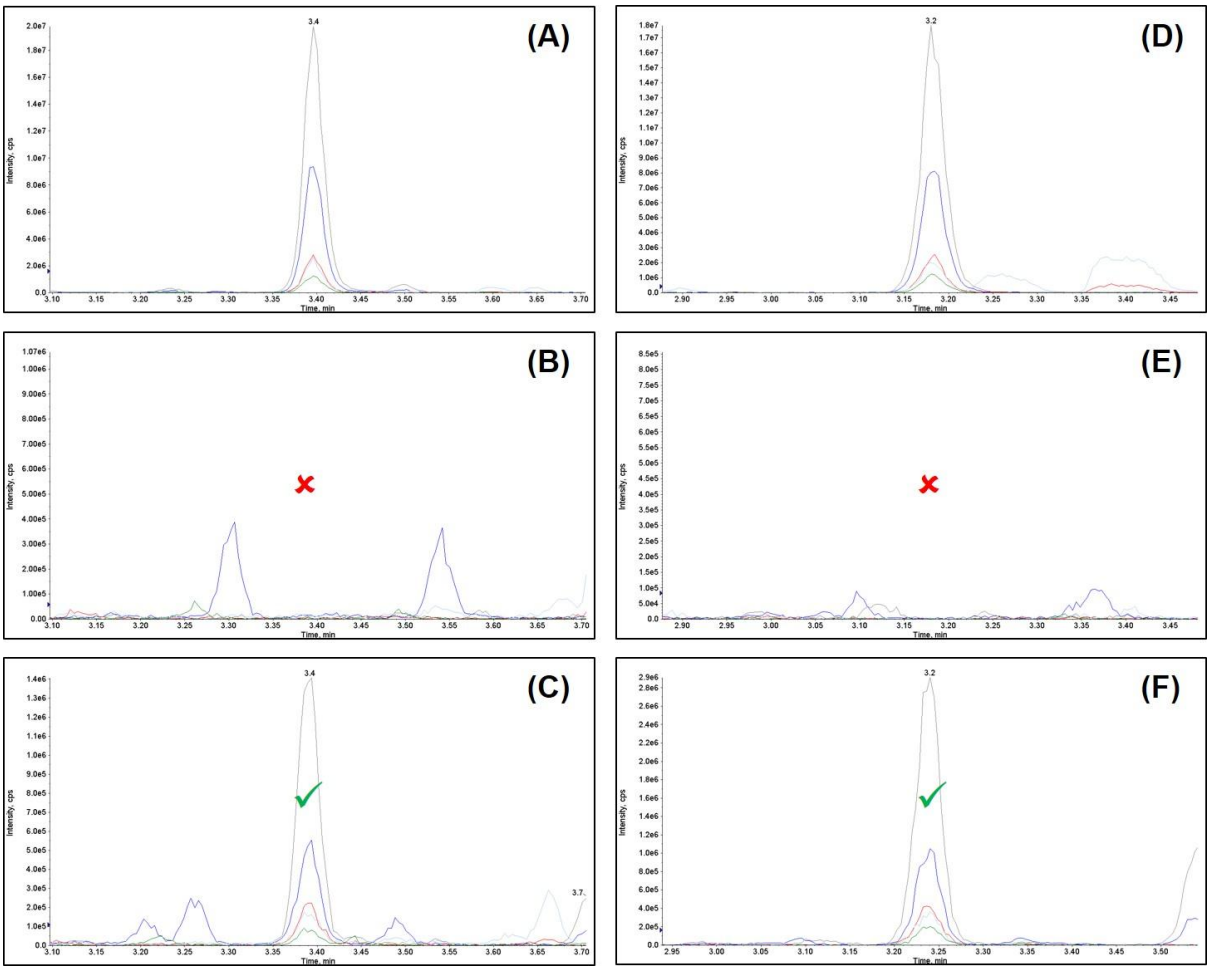


Figure 19. Detection of Pavsa-Δ4D peptide LAPLVK in canola.
(A) Heavy labelled reference standard LAPLV*K spiked into developing embryo protein background from WT canola (2 pmol on-column); (B) developing embryo protein from WT canola; (C) developing embryo protein from DHA canola; (D) heavy labelled reference standard LAPLV*K spiked into mature seed protein background from WT canola (2 pmol on-column); (E) mature seed protein from WT canola; (F) mature seed protein from DHA canola.

The seed-specific expression of the DHA biosynthesis pathway enzymes was confirmed by examining a range of plant tissues. There was no detection of the target peptides in the non-seed tissues of DHA canola. The Pavsa-Δ4D protein was the most abundant among the seven transgene products detected in developing seed or mature seed, thus was chosen as the representative protein as depicted in Figures 20-24. There was no detection of the Pavsa-Δ4D peptide LAPLVK in BBCH15 whole plant, BBCH35 whole plant, BBCH65 root, BBCH65 flower or the other tissues of BBCH65.

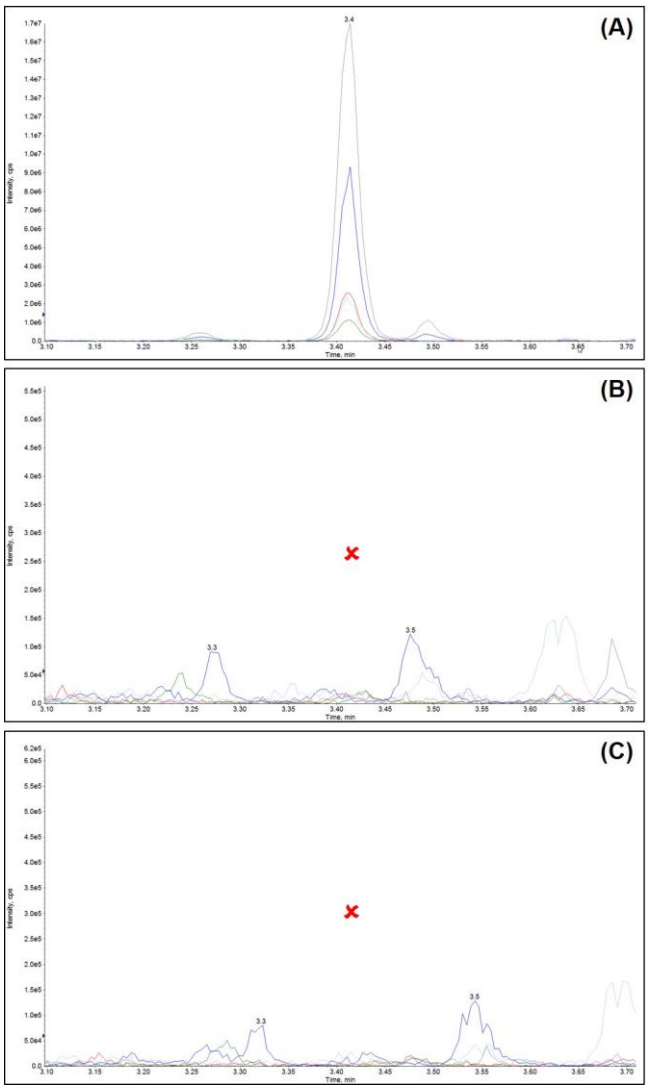


Figure 20. Pavsa-Δ4D was not detected in canola BBCH15 whole plant.
The Pavsa-Δ4D was most abundant of the seven transgene products detected in seed, but undetected canola BBCH15 whole plant. (A) Heavy labeled reference standard spiked into WT canola protein; (B) WT canola protein; (C) DHA canola protein.

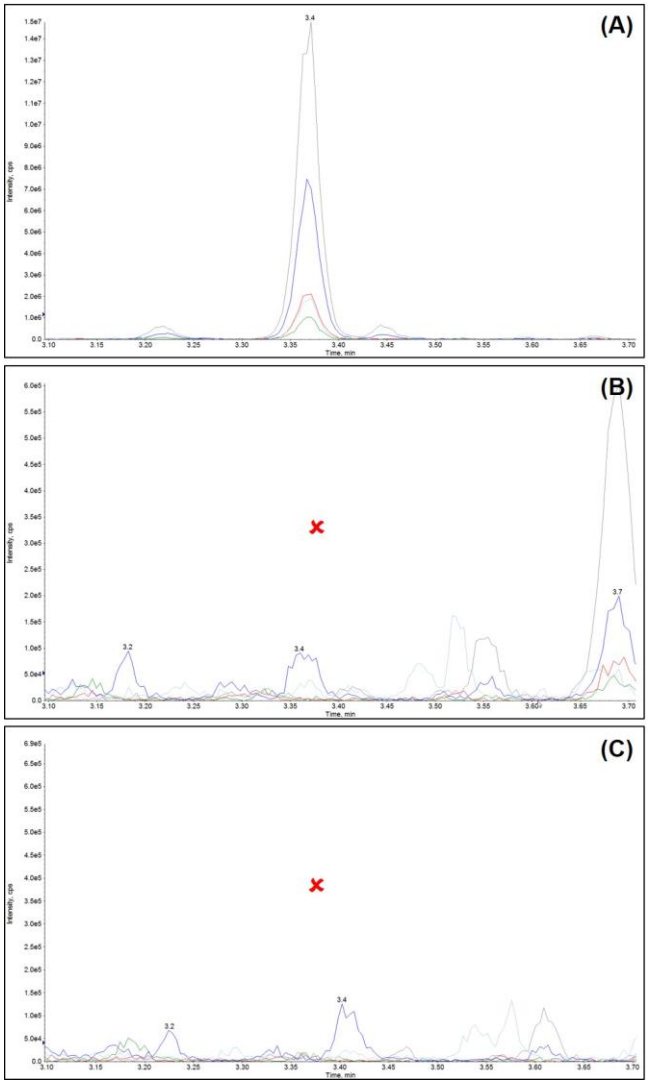


Figure 21. Pavsa-Δ4D was not detected in canola BBCH35 whole plant.
The Pavsa-Δ4D was most abundant of the seven transgene products detected in seed, but undetected canola BBCH35 whole plant. (A) Heavy labeled reference standard spiked into WT canola protein; (B) WT canola protein; (C) DHA canola protein.

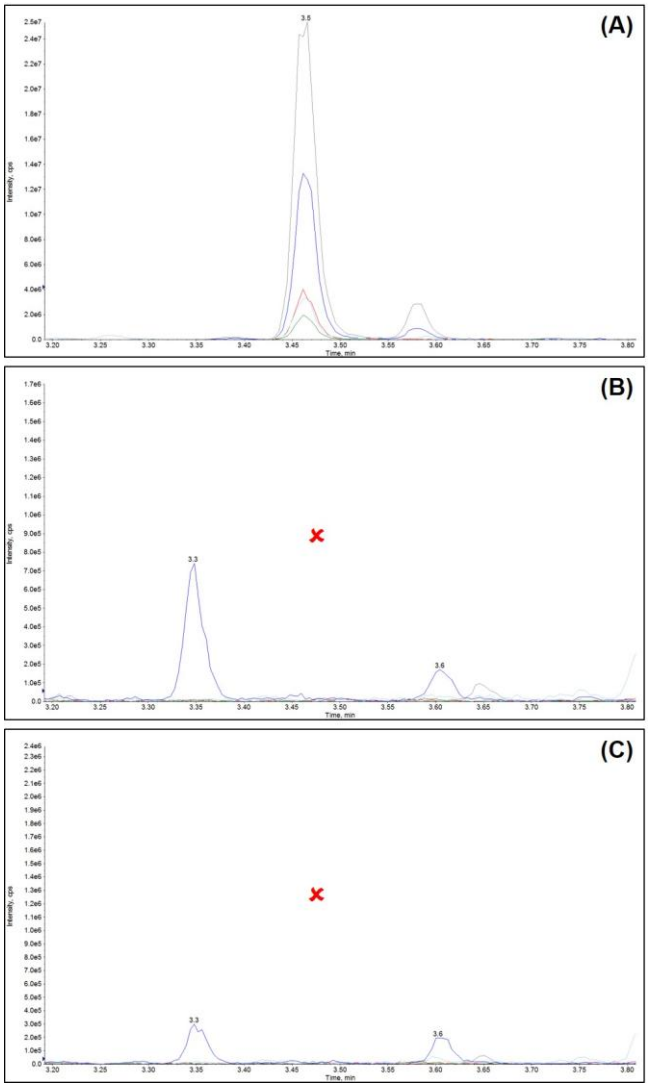


Figure 22. Pavsa-Δ4D was not detected in canola BBCH65 root.
The Pavsa-Δ4D was most abundant of the seven transgene products detected in seed, but undetected canola BBCH65 root. (A) Heavy labeled reference standard spiked into WT canola protein; (B) WT canola protein; (C) DHA canola protein.

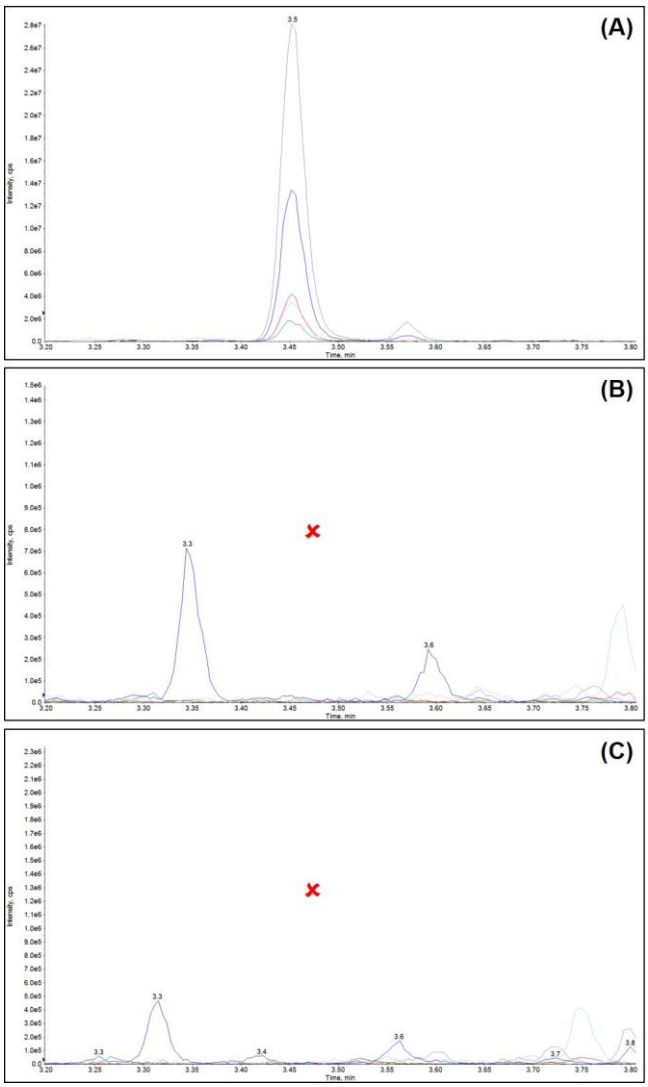


Figure 23. Pavsa-Δ4D was not detected in canola BBCH65 flower.
The Pavsa-Δ4D was most abundant of the seven transgene products detected in seed, but undetected canola BBCH65 flower. (A) Heavy labeled reference standard spiked into WT canola protein; (B) WT canola protein; (C) DHA canola protein.

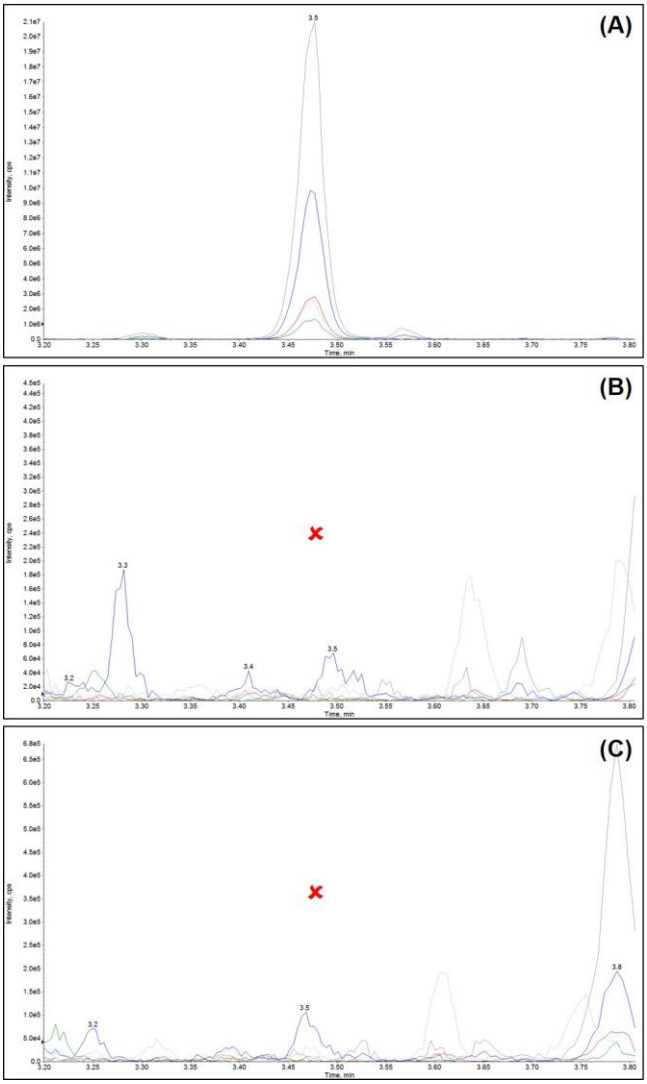


Figure 24. Pavsa-Δ4D was not detected in canola BBCH65 other tissues.
The Pavsa-Δ4D was most abundant of the seven transgene products detected in seed, but undetected canola BBCH65 other tissues. (A) Heavy labeled reference standard spiked into WT canola protein; (B) WT canola protein; (C) DHA canola protein.

E. DETECTION OF SELECTION MARKER PROTEIN IN DHA CANOLA

Low level expression (below the limit of detection) of the selection marker *Streptomyces viridochromogenes* phosphinothricin-N-acetyltransferase (PAT) gene was confirmed in DHA canola, as shown in Figure 25 wherein a trace amount of PAT protein was detected in all tested tissues of DHA canola. The highest signal intensity was detected for the whole plant at stages BBCH15 and BBCH35 (Figure 25D-E). The low expression of PAT in DHA canola was also supported by Western blot analysis with anti-PAT antibody (Figure 26). The transiently-expressed PAT protein was detected in total protein of *N. benthmiana* leaf at an

expected size of about 20 kDa, but not in WT *N. benthmiana* leaf total protein. No obvious specific band was detected in DHA canola BBCH15 whole plant, BBCH35 whole plant, and BBCH79 developing seed, suggesting the PAT expression level was below the Western blot analysis limit.

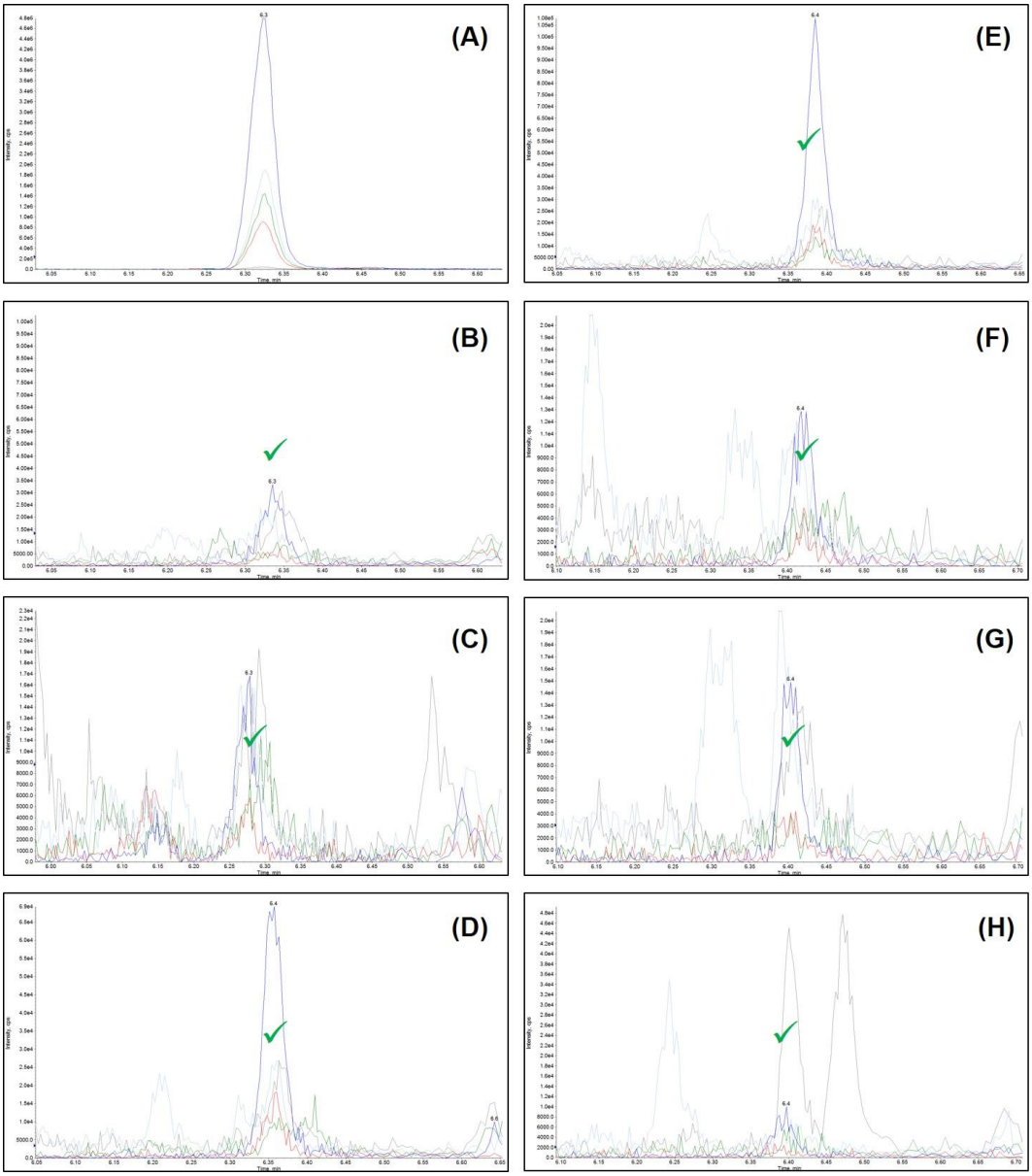


Figure 25. Expression of PAT in canola seeds.
(A) Heavy labelled reference standard TEPQTPQEWIDDL*ER spiked into developing embryo background from WT canola (2 pmol on-column). Detection of trace levels of PAT in DHA canola plant parts: (B) developing seed; (C) mature seed; (D) whole plant (BBCH15); (E) whole plant (BBCH35); (F) root (BBCH65); (G) flower (BBCH65); and other tissue (BBCH65).

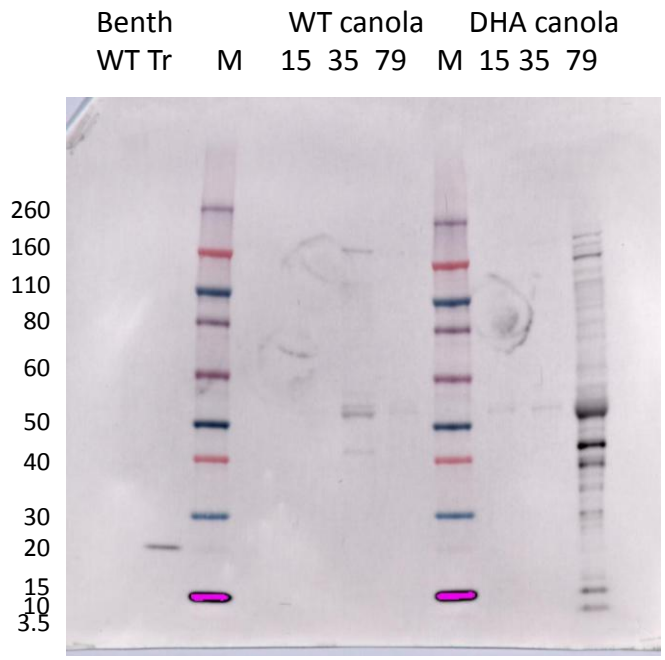


Figure 26. Western blot analysis of PAT in canola.
Left part, total proteins from *N. benthamiana* leaf (Benth): WT, wild type untreated; Tr, transient expression of PAT for 5 days. Middle part, total proteins from wild type (WT) canola. Right part, total protein from DHA canola. M, molecular weight marker in kDa indicated to the left of the gel. Numbers 15, 35 and 79 represents canola materials of BBCH15 (whole plant), BBCH35 (whole plant) and BBCH79 (developing seed). To each lane 20 µg of protein was loaded, and blotted with anti-PAT antibody (Sigma) at a 1:1000 dilution.

VI. CONCLUSION

The protein content was detected and quantified in DHA canola for all seven enzymes in the DHA fatty acid biosynthetic pathway. The results of this study demonstrated that the enzymatic proteins that drive the production of DHA using seed-specific promoters were only detected in developing seed and mature seed at low levels (20-740 ng/mg total protein), while none of the DHA pathway enzymes were detected in other tissues of transgenic canola regardless of the sampling time, or any tissues tested in WT canola.

A range of 5.95 to 18.0 mg total protein per gram of developing or mature seeds was obtained in this study for each of the different enzyme levels. Considering the lowest amount obtained (5.95 mg total protein per gram of seed), the lowest protein level among the 7

transgenic proteins (20.0 ng Pyrco-Δ5E per mg total protein), then at least 16.8 kg of DHA canola seeds would be required in order to obtain at least 2 mg of this transgenic protein.

The enzymes in the DHA fatty acid biosynthetic pathway represent a negligible portion of the total protein present in the DHA canola developing seed and mature seed, which indicate that it is highly unlikely that any of these pathway enzymes will pose any safety concern.

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