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Title (version Nº) EXPURGATED	**Nutrient Composition of Harvested Canola expressing Long-Chain Omega-3 Field-grown in Australia during 2015**
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GLP	**Yes**
Confidentiality	**Yes**

TITLE:

NUTRIENT COMPOSITION OF HARVESTED CANOLA EXPRESSING LONG-CHAIN OMEGA-3 FIELD-GROWN IN AUSTRALIA DURING 2015

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ABBREVIATIONS

ADF	Acid Detergent Fiber
ALA	α-Linolenic acid, 18:3Δ9,12,15 (ω3)
CMP	Comparator (= parental variety)
CSIRO	Commonwealth Scientific and Industrial Research Organization
DHA	Docosahexaenoic acid, 22:6Δ4,7,10,13,16,19 (ω3)
DHA canola	Genetically modified canola, event NS-B50027-4
DPA	Docosapentaenoic acid, 22:5Δ7,10,13,16,19 (ω3)
DW	Dry weight
EPA	Eicosapentaenoic acid, 20:5Δ5,8,11,14,17 (ω3)
ETA	Eicosatetraenoic acid, 20:4Δ8,11,14,17 (ω3)
FA	Fatty acid
FW	Fresh weight
GLA	γ -linolenic acid, C18:3Δ6,9,12 (ω6)
GMO	Genetically modified organism (= DHA canola)
LA	Linoleic acid, 18:2Δ9,12 (ω6)
Lackl-Δ12D	Lachancea kluyveri Δ12-desaturase
LC-MS	Liquid chromatography-Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of Quantitation
Micpu-Δ6D	Micromonas pusilla Δ6-desaturase
MMT	Million metric ton
NDF	Neutral Detergent Fiber
OA	Oleic acid, 18:1Δ9
ω3 LC-PUFA	Omega-3 long-chain (≥C20) polyunsaturated fatty acids
OECD	Organisation for Economic Co-operation and Development
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
REF	Commercial canola references
SDA	Stearidonic acid, 18:4Δ6,9,12,15 (ω3)

EXECUTIVE SUMMARY

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4, which accumulates significant amounts of docosahexaenoic acid (DHA, 22 : 6ω3) in the seed oil (DHA canola).

This report describes the evaluation of various nutritional characteristics and the test methodology utilized for DHA canola (GMO), the parental AV Jade (CMP) variety along with several commercial canola varieties (REF). The analytes evaluated are the standard parameters by which many canola varieties are measured.

Samples were collected from field trials conducted in 2015 at eight locations in major canola growing regions of Australia for compositional analysis. Each trial was designed as a randomized complete block experiment consisting of five replicates (bloc) with the elite event and eight cultivars, which include the parental variety, AV Jade. Grain samples of 350-400g were collected and pooled from seedpods taken from the middle two rows of each plot and analyzed (Eurofins Nutritional Analysis Center). The methodology and statistical analyses are fully outlined within this report.

The results demonstrate that aside from the expected changes in the fatty acid profile, none of the compositional analytes showed any biologically significant differences between the CMP and GMO. While statistical differences in the calculated means were identified for several analytes, the calculated means are typically very close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. Therefore, it is unlikely that any of these differences indicate any biological significance.

Because DHA canola expresses seven fatty acid pathway enzymes, it is not surprising that many of the fatty acids are different from conventional canola, [redacted]

TITLE:

NUTRIENT COMPOSITION OF HARVESTED CANOLA EXPRESSING LONG-CHAIN OMEGA-3 FIELD-GROWN IN AUSTRALIA DURING 2015

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 DHA 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, 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 OA to DHA in a single pathway expression vector. The pathway is comprised of the Lackl-Δ12D (Watanabe et al. 2004), Picpa-ω3D (Zhang et al. 2008), Micpu-Δ6D (Petrie et al. 2010b), Pyrco-Δ6E (Petrie et al. 2010a), Pavsa-Δ5D (Zhou et al. 2007), Pyrco-Δ5E (Petrie et al. 2010a) and 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; Petrie et al. 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 Lackl-Δ12D and Picpa-ω3D that introduce a double bond at the Δ12 and Δ15 positions, respectively; (2) algae fatty acid

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

elongases Pyrco-Δ6E and Pyrco-Δ5E that add two carbons to the carboxyl end of fatty acids; and (3) algae front-end fatty acid desaturases Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D that introduce a double bond between an existing double bond and the carboxyl end of fatty acids.

II. PURPOSE

This report describes the evaluation of various nutritional characteristics and the test methodology utilized for DHA canola (GMO), the parental AV Jade (CMP) variety along with several commercial canola varieties (REF). The analytes evaluated are the standard parameters by which many canola varieties are measured and are specifically outlined in the revised OECD Consensus Document on Compositional considerations for new varieties of low erucic acid rapeseed (canola; *Brassica napus*) (OECD, 2011).

III. MATERIALS: SEED GENETICS, FIELD TRIAL CONDITIONS AND SAMPLING

DHA canola (OECD ID NS-B5ØØ27-4) was planted in fields in 2015 at eight locations in major canola growing regions of Australia to produce samples for compositional analysis. Each trial was designed as a randomized complete block experiment consisting of five replicates (bloc) with the elite event and eight cultivars, which include the parental variety, AV Jade.

The experimental sites were located across varying environments for soil type and rainfall and agronomic management practices.

Table 1. List of experimental trials, location and code names

Site Name	Location	Site synonym name
1506_NAR	NURRABIEL	NAR
1507_NARBL	NURRABIEL-BL	NARBL
1508_DOU	DOUGLAS	DOU
1509_GRN	GREEN LAKE	GRN
1510_TOO	TOOLONDO	TOO
1512_GYM	GYMBOWEN	GYM
1513_KAN	KANIVA	KAN
1514_ARA	ARARAT	ARA

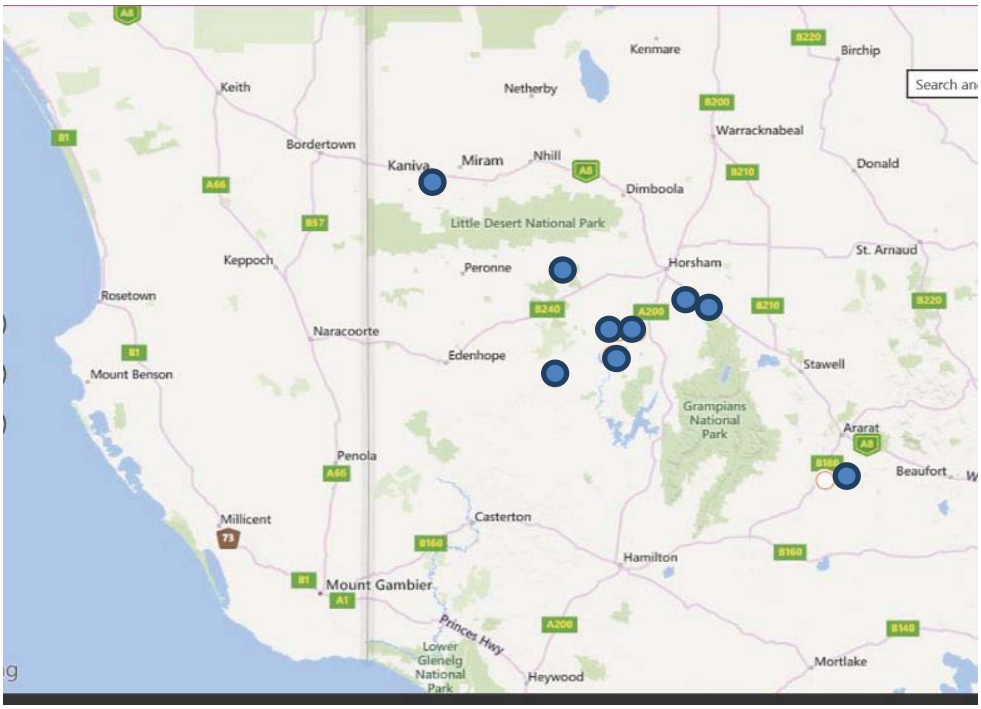


Figure 1. Map showing geographical locations of sites in Western Victoria.

The experimental sites were located across varying environments for soil type and rainfall and agronomic management practices. Rainfall and temperature were recorded at each location for the period from planting to harvest (See Report Nº 2016-018). Irrigation was occasionally used when available and required to supplement rainfall.

Grain samples of 350-400g were collected and pooled from seedpods taken from the middle two rows of each plot. Sampled grain was shipped at ambient temperature to the laboratory for analysis (Eurofins Nutritional Analysis Center).

IV. METHODS

A summary of each parameter, its method of analysis, appropriate units and the limits of quantitation (LOQ) are included in Table 2. Data means, standard deviations, ranges and p-values were determined for the compositional data. Data were analyzed using SAS v9.4 (SAS Institute, Cary NC) and ENAC on the rounded final results. The results of each analyte are summarized for each canola variety, namely Comparator, or AV Jade (CMP); DHA canola (GM), and commercial canola references (REF) including mean, minimum (min), maximum (max), and standard deviation (std. dev). A comparison was made of GMO and CMP on seeds across all sites with a linear mixed model with genotype as a fixed factor and site as a random factor. Across all sites and each analyte, the difference between GMO and CMP was estimated after conducting an ANOVA analysis using the site and line main effects and the site by line interaction. Single degree of freedom contrasts were used to test for differences between the experimental line and the parental line. P-values (≤0.05% indicates a significant difference) are not reported where the means were below the LOD, or where missing data made the effect non-testable. When data points were at or below the LOQ, the LOQ value was used to calculate the averages, standard deviations and data ranges. The range of determined values for each of the analytes for the reference lines is also reported.

Calculations of dry weights and fatty analysis were done as described below. Conversion from a fresh weight (FW) basis to dry weight (DW) basis:

$$\%DW = \%FW \times (100/(100-\text{moisture}))$$

Conversion from FW basis to a percent relative (Rel) basis for individual fatty acids (FA):

$$\%FA\ Rel = (\%FA / \%\text{total FA}) \times 100$$

For calculating percent relative fatty acids, where results were reported below the LOQ, the LOQ value was used for % FA.

Table 2. Analyte specifics for DHA canola compositional analysis

Parameter	Eurofins Method	Units	LOQ
Moisture	MET-PR-005	%	0.2%
Protein, Crude	MET-PR-002	%	0.1%
Fat, Crude	MET-LI-001	%	0.1%
Ash	MET-PR-004	%	0.4%
Carbohydrates, Calculated	OPS-024	%	N/A
Crude Fiber	MET-PR-003	%	0.2%
Acid Detergent Fiber	MET-PR-007	%	0.3%

Parameter	Eurofins Method	Units	LOQ
Neutral Detergent Fiber	MET-PR-008	%	0.3%
Amino Acids by Acid Hydrolysis	MET-LC-006	%	Aspartic Acid: 0.02% Threonine: 0.02% Serine: 0.01% Glutamic Acid: 0.01% Glycine: 0.01% Alanine: 0.01% Valine: 0.02% Isoleucine: 0.02% Leucine: 0.02% Tyrosine: 0.04% Phenylalanine: 0.03% Total Lysine: 0.01% Histidine: 0.01% Arginine: 0.05% Proline: 0.05%
Cystine & Methionine by Performic Acid Oxidation	MET-LC-005	%	Cystine: 0.01% Methionine: 0.01%
Tryptophan by Alkaline Hydrolysis	MET-LC-024	%	0.01%
Vitamin E (α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol)	MET-VT-009 MET-VT-030	mg/100g	0.1 mg/100g*
Vitamin K1	MET-VT-028	mg/100g	0.000625 mg/100g
Biotin	MET-VT-003	mg/100g	0.0037 mg/100g
Folic Acid	MET-VT-018	mg/100g	0.0033 mg/100g
Vitamin B3 – Niacin	MET-VT-005	mg/100g	0.22 mg/100g
Vitamin B5 – Panothenic Acid	MET-VT-007	mg/100g	0.055 mg/100g
Vitamin B6 – Pyridoxine	MET-VT-006	mg/100g	0.01 mg/100g
Vitamin B2 – Riboflavin	MET-VT-002	mg/100g	0.1 mg/100g
Vitamin B1 – Thiamin	MET-VT-019	mg/100g	0.011 mg/100g
Choline	MET-VT-031	mg/100g	1 mg/100g
Phenolic Acids	MET-LC-004	Sinapine (%) µg/g (ppm)	Sinapine: 0.05% Ferulic acid: 10 µg/g Coumaric acid: 10 µg/g
Glucosinolates	MET-LC-026	µmol/g	0.05 µmol/g*
Tannins – Soluble Condensed	MET-AN-012	%	0.05%
Phytic acid	MET-EL-011	%	0.14%
Calcium	MET-EL-002/MET-EL-	%	0.004%
Phosphorus	MET-EL-002/MET-EL-	%	0.004%

Parameter	Eurofins Method	Units	LOQ
Magnesium	MET-EL-002/MET-EL-	%	0.001%
Potassium	MET-EL-002/MET-EL-	%	0.004%
Sodium	MET-EL-002/MET-EL-	%	0.002%
Iron	MET-EL-002/MET-EL-	%	0.0002%
Zinc	MET-EL-002/MET-EL-	%	0.001%
Copper	MET-EL-002/MET-EL-	%	0.0001%
Manganese	MET-EL-002/MET-EL-	%	0.00005%
Sulfur	MET-EL-009	%	0.02%
Molybdenum	MET-EL-002/MET-EL-	%	0.00012%
Chloride	MET-CM-018	%	0.06%
Phytosterols	MET-LI-034	%	0.002%*
Fatty Acid Profile	MET-LI-002/MET-LI-025	%	C16:0: 0.02% All others at 0.01%

V. COMPOSITIONAL ANALYSIS FOR DHA CANOLA

a. OVERVIEW OF ANALYSIS

Detailed compositional analysis was conducted in accordance with the revised OECD Consensus Document on Compositional considerations for new varieties of low erucic acid rapeseed (canola; *Brassica napus*) (OECD 2011). This analysis was conducted to investigate the difference between genetically modified organism, B0050-027-18-20 (DHA canola; identified as GMO in reports), and comparator, AV Jade (parental variety; identified as CMP) and the equivalence between DHA canola and the reference variety, the remainder of genotypes (identified as REF).

Compositional analysis of grain samples included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, ash, carbohydrates, fatty acids (FA), amino acids, vitamins, minerals, phytosterols and key anti-nutrients. All compositional analyses were conducted at Eurofins Nutritional Analysis Center (Des Moines, IA).

b. ANALYSIS OF PROXIMATES IN CANOLA GRAIN

The levels of proximates were measured in grain samples of DHA canola (GMO), the parental line AV Jade (CMP) and seven other commercial reference varieties (REF) (Table 3). The test material, number of samples, standard deviation and ranges are provided for each analyte. P-values are provided for the comparison of the GMO and CMP.

No statistically significant differences were identified for acid detergent fiber, crude fiber, neutral detergent fiber and protein. While statistical differences in the calculated means were identified for ash, carbohydrates and crude fat, the calculated means are very close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. Therefore, it is unlikely that these differences indicate any biological significance.

Table 3. Proximate analysis of DHA canola (GMO), AV Jade (CMP) and Commercial References (REF)

Analyte	Test Material	Nº	Mean (% DW)	Std Dev	Range	P-value
Acid Detergent Fiber	CMP	39	11.2	1.4	8.7-14.6	0.4709
	GMO	40	11.4	1.4	9.6-16.6	
	REF	280	11.5	1.5	8.6-16.5	NA
Ash	CMP	39	3.7	0.5	2.9-4.5	<0.0001
	GMO	40	3.8	0.4	3.1-4.6	
	REF	280	3.5	0.4	2.7-4.5	NA
Carbohydrates	CMP	39	33.0	2.3	27.0-37.0	<0.0001
	GMO	40	35.4	2.0	31.4-38.4	
	REF	280	34.8	2.5	27.3-42.3	NA
Crude Fat	CMP	39	33.2	2.9	27.8-39.5	<0.0001
	GMO	40	30.5	2.7	25.8-35.9	
	REF	280	32.8	2.9	25.5-42.1	NA
Crude Fiber	CMP	39	14.9	1.9	10.1-17.5	0.5936
	GMO	40	14.7	2.0	11.3-17.9	
	REF	280	15.8	2.2	10.9-22.6	NA
Neutral Detergent Fiber	CMP	39	15.6	1.6	12.6-18.8	0.9676
	GMO	40	15.6	1.1	13.6-18.1	
	REF	280	15.7	1.7	12.1-21.8	NA
Protein	CMP	39	30.1	1.2	26.9-32.2	0.3797
	GMO	40	30.4	1.2	27.5-32.5	
	REF	280	28.8	1.6	23.5-32.1	NA

Nº = number of samples analyzed; Std Dev = Standard Deviation

c. Analysis of Amino Acids and Sterols

The levels of amino acids and sterols were measured in grain samples of DHA canola (GMO), the parental line AV Jade (CMP) and seven other commercial reference varieties (REF) (Table 4). The test material, number of samples, standard deviation and ranges are provided for each analyte. P-values are provided for the comparison of the GMO and CMP.

No statistically significant differences were identified for the following amino acids (highlighted in blue Table 4): arginine, cystine, glutamic acid, histidine, isoleucine, leucine, phenyalanine, serine, tryptophan, valine. While statistical differences in the calculated means were identified for alanine, aspartic acid, glycine, lysine, methionine, proline, threonine and tyrosine, the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. For alanine, aspartic acid, glycine, lysine, threonine and tyrosine the levels were slightly higher in DHA canola than the parental and reference varieties, but this does not raise any nutritional concerns. Therefore, it is unlikely that these differences indicate any biological significance.

No statistically significant differences were identified for the following glucosinolates (highlighted in yellow Table 4): epi-progoitrin, glucoalyssin, glucobrassicanapin, gluconapin, gluconapoleiferin, gluconasturtin, neoglucobrassicin, progoitrin, 4-hydroxyglucobrassicin. While statistical differences in the calculated means were identified for glucobrassicin, the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. Furthermore, when the glucosinolates are combined, the totals are 3.126, 1.891 and 4.534 µmol/g for the CMP, GMO and REF, respectively, well below the limits for the definition of canola (30 µmol/g)[2]. Therefore, it is unlikely that these differences indicate any biological significance.

No statistically significant differences were identified for the following phytosterols (highlighted in green Table 4): cholesterol and sitostanol. While statistical differences in the calculated means were identified for brassicasterol, campesterol, clerosterol, delta-5-avenasterol, delta-7-avenasterol, sitosterol, stigmasterol, 24-methylene cholesterol, and total phytosterols the calculated means are close numerically and the standard deviation and

[2] https://www.gipsa.usda.gov/fgis/standards/810canola.pdf and http://www.canolacouncil.org/oil-and-meal/what-is-canola/

ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. It should also be noted that for eight of the thirteen phytosterols the values were very low and close to the LOQ; cholesterol, clerosterol, delta-7-avenasterol, sitostanol, stigmasterol, 24-methylene cholesterol, delta-5 24-stigmastadienol, delta-7 stigmastenol. Taken together, it is unlikely that these differences indicate any biological significance.

Finally, no statistically significant differences were identified for the following organic compounds: ferulic acid and soluble tannins. While statistical differences in the calculated means were identified for sinapine and *p*-coumaric acid, the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. It should also be noted that the levels of soluble tannins is close to the LOQ and the ranges of *p*-coumaric acid for all three test groups, CMP, GMO and REF, are quite large (0.000-30.510). Taken together, it is unlikely that these differences indicate any biological significance.

Table 4. Amino acid and sterol analysis of DHA canola (GMO), AV Jade (CMP) and Commercial References (REF).
Units for amino acids (% DW), glucosinolates (µmol/g), phytosterols (µg/g), organic compounds (% DW).

Analyte	Test Material	Nº	Mean	Std Dev	Range	p-value
Alanine	CMP	39	1.239	0.049	1.130-1.360	0.0053
	GMO	40	1.268	0.046	1.160-1.350	
	REF	278	1.190	0.064	0.999-1.340	NA
Arginine	CMP	39	1.923	0.092	1.700-2.090	0.8397
	GMO	40	1.919	0.087	1.720-2.060	
	REF	278	1.823	0.115	1.480-2.090	NA
Aspartic Acid	CMP	39	2.164	0.106	1.920-2.350	<0.0001
	GMO	40	2.282	0.097	2.070-2.440	
	REF	278	2.080	0.134	1.680-2.420	NA
Brassicasterol	CMP	39	0.112	0.005	0.097-0.120	<0.0001
	GMO	40	0.052	0.004	0.045-0.066	
	REF	280	0.118	0.022	0.045-0.170	NA
Campesterol	CMP	39	0.287	0.010	0.268-0.310	<0.0001
	GMO	40	0.385	0.018	0.352-0.425	
	REF	280	0.317	0.043	0.226-0.397	NA

Analyte	Test Material	Nº	Mean	Std Dev	Range	p-value
Cholesterol	CMP	39	0.002	0.002	0.000-0.006	0.7472
	GMO	40	0.002	0.003	0.000-0.020	
	REF	280	0.003	0.004	0.000-0.050	NA
Clerosterol	CMP	39	0.006	0.000	0.005-0.007	0.0009
	GMO	40	0.006	0.000	0.006-0.007	
	REF	280	0.005	0.000	0.004-0.006	NA
Cystine	CMP	39	0.754	0.037	0.680-0.840	0.1520
	GMO	40	0.743	0.038	0.630-0.820	
	REF	278	0.734	0.044	0.580-0.820	NA
Delta_5_avenasterol	CMP	39	0.036	0.006	0.026-0.046	<0.0001
	GMO	40	0.044	0.008	0.030-0.064	
	REF	280	0.018	0.005	0.008-0.037	NA
Delta_7_avenasterol	CMP	39	0.002	0.001	0.000-0.003	<0.0001
	GMO	40	0.004	0.000	0.003-0.005	
	REF	280	0.002	0.001	0.000-0.004	NA
Epi progoitrin	CMP	39	0.094	0.052	0.000-0.200	0.9182
	GMO	40	0.096	0.053	0.000-0.200	
	REF	278	0.071	0.066	0.000-0.300	NA
Ferulic Acid	CMP	39	137.238	23.680	101.10-184.60	0.1768
	GMO	40	130.084	20.960	98.72-171.70	
	REF	278	139.527	24.031	88.91-217.50	NA
Glucoalyssin	CMP	39	0.349	0.147	0.062-0.730	0.7523
	GMO	40	0.363	0.150	0.130-0.670	
	REF	278	0.347	0.286	0.000-1.800	NA
Glucobrassicanapin	CMP	39	0.311	0.155	0.073-0.680	0.0905
	GMO	40	0.250	0.121	0.061-0.530	
	REF	278	0.251	0.204	0.000-1.300	NA
Glucobrassicin	CMP	39	0.205	0.055	0.000-0.280	<0.0001
	GMO	40	0.282	0.073	0.081-0.420	
	REF	278	0.241	0.088	0.090-0.550	NA
Gluconapin	CMP	39	2.166	0.723	0.664-3.650	0.2947
	GMO	40	1.972	0.681	0.627-3.510	
	REF	278	1.770	1.087	0.417-6.390	NA

Analyte	Test Material	Nº	Mean	Std Dev	Range	p-value
Gluconapoleiferin	CMP	39	0.048	0.047	0.000-0.200	0.3315
	GMO	40	0.037	0.047	0.000-0.200	
	REF	278	0.051	0.063	0.000-0.300	NA
Gluconasturtin	CMP	39	0.094	0.052	0.000-0.180	0.1092
	GMO	40	0.135	0.084	0.000-0.380	
	REF	278	0.151	0.127	0.000-0.520	NA
Glutamic Acid	CMP	39	5.681	0.258	5.090-6.210	0.1426
	GMO	40	5.599	0.269	4.930-6.030	
	REF	278	5.408	0.353	4.360-6.170	NA
Glycine	CMP	39	1.519	0.062	1.380-1.660	<0.0001
	GMO	40	1.584	0.061	1.440-1.690	
	REF	278	1.471	0.081	1.240-1.660	NA
Histidine	CMP	39	0.843	0.032	0.774-0.910	0.8145
	GMO	40	0.845	0.036	0.755-0.900	
	REF	278	0.810	0.044	0.677-0.922	NA
Isoleucine	CMP	39	1.218	0.052	1.080-1.320	0.9692
	GMO	40	1.218	0.048	1.100-1.290	
	REF	278	1.159	0.069	0.931-1.310	NA
Leucine	CMP	39	2.129	0.092	1.890-2.300	0.6732
	GMO	40	2.120	0.086	1.920-2.280	
	REF	278	2.019	0.124	1.660-2.300	NA
Lysine	CMP	39	1.890	0.107	1.670-2.130	0.0171
	GMO	40	1.948	0.129	1.730-2.240	
	REF	278	1.833	0.123	1.490-2.140	NA
Methionine	CMP	39	0.611	0.023	0.570-0.660	0.0197
	GMO	40	0.623	0.027	0.560-0.660	
	REF	278	0.592	0.031	0.490-0.670	NA
Neoglucobrassicin	CMP	39	0.000	0.000	0.000-0.000	0.0512
	GMO	40	0.004	0.017	0.000-0.080	
	REF	278	0.001	0.007	0.000-0.080	NA
Phenyalanine	CMP	39	1.217	0.054	1.080-1.320	0.1830
	GMO	40	1.202	0.046	1.100-1.290	
	REF	278	1.154	0.069	0.949-1.310	NA

Analyte	Test Material	Nº	Mean	Std Dev	Range	p-value
Progoitrin	CMP	39	4.914	1.895	0.933-8.680	0.9660
	GMO	40	4.936	1.874	1.590-9.120	
	REF	278	4.422	2.606	0.838-17.000	NA
Proline	CMP	39	1.925	0.086	1.700-2.110	0.0010
	GMO	40	1.865	0.091	1.670-2.090	
	REF	278	1.832	0.110	1.460-2.050	NA
Serine	CMP	39	1.279	0.050	1.150-1.370	0.2342
	GMO	40	1.292	0.051	1.180-1.390	
	REF	278	1.216	0.068	1.020-1.380	NA
Sinapine	CMP	39	1.264	0.078	1.089-1.415	0.0002
	GMO	40	1.191	0.070	1.031-1.330	
	REF	278	1.167	0.095	0.876-1.463	NA
Sitostanol	CMP	39	0.000	0.000	0.000-0.003	0.3099
	GMO	40	0.000	0.001	0.000-0.003	
	REF	280	0.000	0.000	0.000-0.004	NA
Sitosterol	CMP	39	0.551	0.028	0.501-0.616	<0.0001
	GMO	40	0.579	0.036	0.512-0.650	
	REF	280	0.477	0.032	0.346-0.580	NA
Stigmasterol	CMP	39	0.003	0.000	0.002-0.004	<0.0001
	GMO	40	0.000	0.001	0.000-0.006	
	REF	280	0.002	0.001	0.000-0.005	NA
Tannins Soluble Condensed	CMP	39	0.000	0.000	0.000-0.000	0.9908
	GMO	40	0.000	0.000	0.000-0.000	
	REF	278	0.002	0.012	0.000-0.100	NA
Threonine	CMP	39	1.280	0.044	1.170-1.380	<0.0001
	GMO	40	1.318	0.045	1.220-1.400	
	REF	278	1.231	0.060	1.040-1.360	NA
Tryosine	CMP	39	0.789	0.035	0.702-0.854	<0.0001
	GMO	40	0.817	0.029	0.756-0.878	
	REF	278	0.756	0.041	0.644-0.839	NA
Tryptophan	CMP	39	0.456	0.020	0.410-0.500	0.4308
	GMO	40	0.453	0.021	0.400-0.500	
	REF	278	0.432	0.026	0.340-0.490	NA

Analyte	Test Material	Nº	Mean	Std Dev	Range	p-value
Valine	CMP	39	1.562	0.063	1.400-1.690	0.7330
	GMO	40	1.566	0.068	1.420-1.680	
	REF	278	1.492	0.088	1.160-1.650	NA
24-Methylene cholesterol	CMP	39	0.013	0.005	0.008-0.020	0.0013
	GMO	40	0.011	0.004	0.007-0.020	
	REF	280	0.008	0.003	0.003-0.020	NA
4-Hydroxyglucobrassicin	CMP	39	3.938	0.769	1.360-5.220	0.6366
	GMO	40	3.849	0.964	1.120-5.730	
	REF	278	3.465	0.785	0.000-5.540	NA
Delta-5 24-Stigmastadienol	CMP	39	0.007	0.001	0.006-0.008	<0.0001
	GMO	40	0.009	0.001	0.008-0.010	
	REF	280	0.005	0.001	0.003-0.009	NA
Delta-7 stigmastenol	CMP	39	0.000	0.001	0.000-0.002	<0.0001
	GMO	40	0.003	0.001	0.000-0.005	
	REF	280	0.002	0.002	0.000-0.006	NA
p-Coumaric Acid	CMP	39	18.700	5.281	0.000-30.510	<0.0001
	GMO	40	0.273	1.728	0.000-10.930	
	REF	278	3.965	6.965	0.000-26.650	NA
Total Phytosterols	CMP	39	1.025	0.040	0.966-1.118	<0.0001
	GMO	40	1.106	0.061	1.013-1.249	
	REF	280	0.965	0.059	0.702-1.097	NA

d. Analysis of Minerals

The levels of minerals were measured in grain samples of DHA canola (GMO), the parental line AV Jade (CMP) and seven other commercial reference varieties (REF) (Table 5). The test material, number of samples, standard deviation and ranges are provided for each analyte. P-values are provided for the comparison of the GMO and CMP.

No statistically significant differences were identified for the following minerals: copper, magnesium, manganese, phosphorus, phytic acid, sodium and sulfur. While statistical differences in the calculated means were identified for calcium, iron, potassium and zinc, the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. It should also be noted that for four of eleven of these analytes the values were very low and close to the LOQ: copper, manganese, sodium, zinc. Therefore, it is unlikely that these differences indicate any biological significance.

Molybdenum and chloride mineral results were below the LOQ, with the exception of three chloride data points that had dry weight basis values of 0.065%, which are equivalent to the LOQ on a dry weight basis. These analytes were excluded from the statistical analyses.

Finally, due to limited sample availability no vitamin or mineral analyses were performed on some samples, therefore these samples were excluded in the statistical analysis of those analytes. The Nº column reflects which sample numbers were reduced, but did not have any impact on the overall analyses of minerals and vitamins.

Table 5. Minerals of DHA canola (GMO), AV Jade (CMP) and Commercial References (REF).

Analyte	Test Material	Nº	Mean (% DW)	Std Dev	Range	p-value
Calcium	CMP	39	0.356	0.061	0.223-0.464	<0.0001
	GMO	40	0.312	0.048	0.206-0.395	
	REF	278	0.345	0.056	0.204-.0488	NA
Copper	CMP	39	0.000	0.000	0.000-0.000	0.1285
	GMO	40	0.000	0.000	0.000-0.002	
	REF	278	0.000	0.000	0.000-0.002	NA
Iron	CMP	39	0.005	0.001	0.004-0.007	<0.0001

Analyte	Test Material	Nº	Mean (% DW)	Std Dev	Range	p-value
	GMO	40	0.007	0.001	0.005-0.009	
	REF	278	0.006	0.001	0.004-0.008	NA
Magnesium	CMP	39	0.308	0.021	0.261-0.351	0.9910
	GMO	40	0.308	0.021	0.262-0.351	
	REF	278	0.307	0.022	0.246-0.370	NA
Manganese	CMP	39	0.003	0.001	0.002-0.004	0.7774
	GMO	40	0.003	0.001	0.002-0.005	
	REF	278	0.003	0.001	0.002-0.004	NA
Phosphorus	CMP	39	0.655	0.122	0.418-0.882	0.0639
	GMO	40	0.669	0.123	0.437-0.886	
	REF	278	0.579	0.117	0.365-0.869	NA
Phytic Acid	CMP	39	1.918	0.433	1.100-2.700	0.7961
	GMO	40	1.895	0.440	1.100-2.700	
	REF	278	1.612	0.406	0.840-2.500	NA
Potassium	CMP	39	0.666	0.093	0.485-0.866	<0.0001
	GMO	40	0.782	0.082	0.621-0.968	
	REF	278	0.700	0.076	0.532-0.915	NA
Sodium	CMP	39	0.002	0.001	0.000-0.005	0.9508
	GMO	40	0.003	0.002	0.000-0.007	
	REF	278	0.004	0.002	0.000-0.010	NA
Sulfur	CMP	39	0.512	0.031	0.440-0.570	0.7002
	GMO	40	0.510	0.033	0.430-0.580	
	REF	278	0.488	0.042	0.380-0.650	NA
Zinc	CMP	39	0.004	0.001	0.003-0.006	0.0026
	GMO	40	0.005	0.001	0.003-0.006	
	REF	278	0.004	0.001	0.003-0.006	NA

e. Analysis of Vitamins

The levels of vitamins were measured in grain samples of DHA canola (GMO), the parental line AV Jade (CMP) and seven other commercial reference varieties (REF) (Table 6). The test material, number of samples, standard deviation and ranges are provided for each analyte. P-values are provided for the comparison of the GMO and CMP.

No statistically significant differences were identified for the following vitamins: delta-tocopherol, folic acid and gamma-tocopherol. While statistical differences in the calculated means were identified for alpha-tocopherol, beta-tocopherol, biotin, niacin, pantothenic acid, pyridoxine, riboflavin, thiamin, total tocopherols and vitamin K, the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. It should also be noted that for half of these analytes the values were very low and close to the LOQ: beta-tocopherol, biotin, folic acid, pantothenic acid, pyridoxine, riboflavin and vitamin K. Therefore, it is unlikely that these differences indicate any biological significance.

Finally, due to limited sample availability no vitamin or mineral analyses were performed on some samples, therefore these samples were excluded in the statistical analysis of those analytes. The Nº column reflects which sample numbers were reduced, which did not have any impact on the overall analyses of minerals and vitamins.

Table 6. Vitamins of DHA canola (GMO), AV Jade (CMP) and Commercial References (REF).

Analyte	Test Material	Nº	Mean (% DW)	Std Dev	Range	p-value
Alpha Tocopherol Vitamin E	CMP	39	11.9	6.6	9.2-51.7	<0.0001
	GMO	40	15.7	5.8	12.4-49.7	
	REF	278	15.4	2.5	10.9-31.3	NA
Beta Tocopherol Vitamin E	CMP	39	0.1	0.1	0.0-0.5	0.0021
	GMO	40	0.1	0.1	0.0-0.2	
	REF	278	0.2	0.1	0.0-0.6	NA
Biotin	CMP	34	0.1	0.0	0.0-0.1	<0.0001
	GMO	40	0.1	0.0	0.1-0.1	
	REF	267	0.1	0.0	0.0-0.1	NA
Choline	CMP	34	262.7	21.6	220.5-312.3	0.0249
	GMO	40	276.0	23.3	229.1-328.4	

Analyte	Test Material	Nº	Mean (% DW)	Std Dev	Range	p-value
	REF	267	283.7	30.8	195.4-381.3	NA
Delta Tocopherol Vitamin E	CMP	39	0.5	0.5	0.2-3.4	0.2990
	GMO	40	0.3	0.1	0.1-0.6	
	REF	278	0.4	0.8	0.0-13.5	NA
Folic Acid	CMP	34	0.1	0.0	0.1-0.2	0.9802
	GMO	40	0.1	0.0	0.0-0.2	
	REF	267	0.1	0.1	0.0-0.6	NA
Gamma Tocopherol Vitamin E	CMP	39	21.2	1.8	17.7-25.0	0.0648
	GMO	40	22.8	1.9	17.8-26.2	
	REF	278	20.5	4.2	10.2-72.2	NA
Niacin Vitamin B3	CMP	34	9.7	1.0	7.9-11.5	<0.0001
	GMO	40	15.1	1.9	10.6-18.9	
	REF	267	12.7	1.5	8.4-16.8	NA
Panothenic Acid Vitamin B5	CMP	34	0.5	0.1	0.2-0.8	<0.0001
	GMO	40	0.6	0.1	0.3-0.8	
	REF	267	0.4	0.1	0.2-0.8	NA
Pyridoxine Vitamin B6	CMP	34	0.5	0.1	0.4-0.7	<0.0001
	GMO	40	0.9	0.1	0.6-1.1	
	REF	267	0.7	0.1	0.4-1.0	NA
Riboflavin Vitamin B2	CMP	34	0.3	0.1	0.3-0.6	0.0241
	GMO	40	0.3	0.0	0.3-0.4	
	REF	267	0.3	0.0	0.2-0.6	NA
Thiamin Vitamin B1	CMP	34	1.3	0.2	0.8-1.7	0.0023
	GMO	40	1.5	0.2	1.1-2.0	
	REF	267	1.4	0.3	0.2-2.3	NA
Total Tocopherols Vitamin E	CMP	39	33.7	7.2	28.6-75.1	0.0001
	GMO	40	38.9	5.9	31.2-71.0	
	REF	278	36.5	5.8	24.5-96.9	NA
Vitamin K1	CMP	34	0.0	0.0	0.0-0.1	0.0205
	GMO	40	0.1	0.0	0.0-0.1	
	REF	267	0.0	0.0	0.0-0.1	NA

f. Analysis of Fatty Acids

The levels of fatty acids were measured in grain samples of DHA canola (GMO), the parental line AV Jade (CMP) and seven other commercial reference varieties (REF) (Table 7). The test material, number of samples, standard deviation and ranges are provided for each analyte. P-values are provided for the comparison of the GMO and CMP.

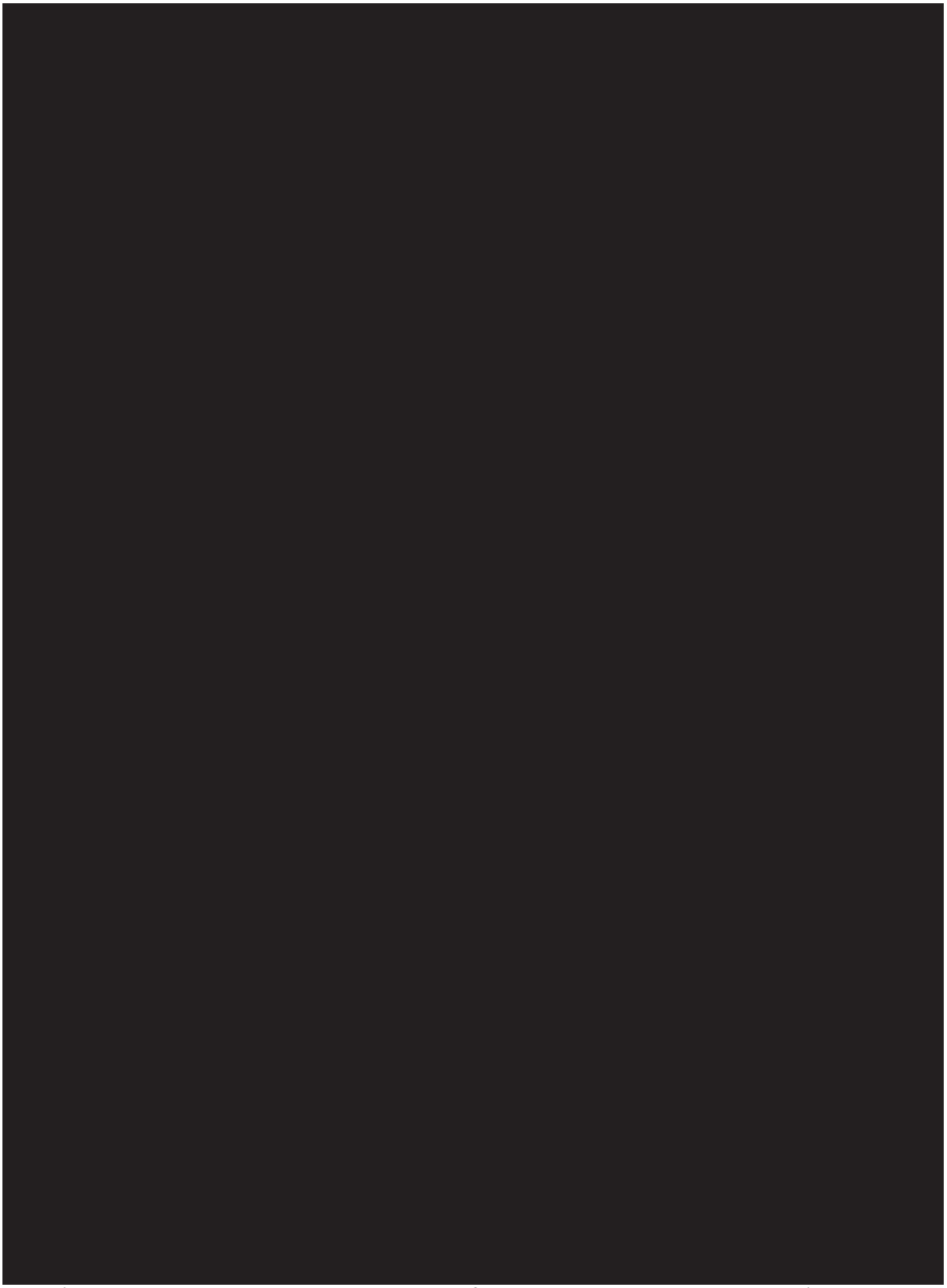
Because DHA canola expresses seven fatty acid pathway enzymes, it is not surprising that many of the fatty acids are different from conventional canola, [redacted]

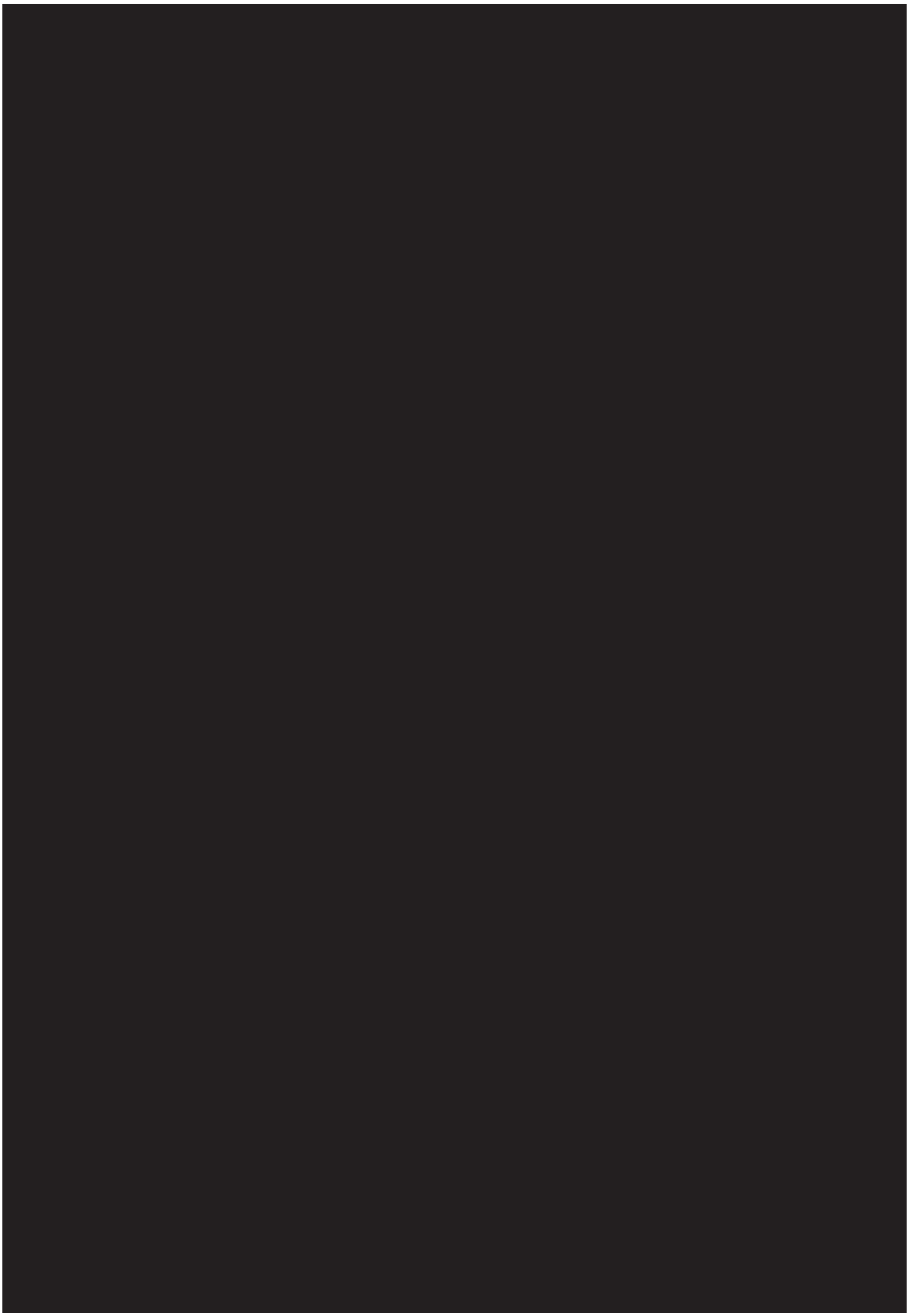
Please also note, the range observed for C22:6 n-3 for the REF varieties indicates a max value of 7.76, which is unusual given these are conventional canola lines. However, when the data is reviewed in detail, a single replication of a single site gave this high value. If this value is removed from the analysis, the other 39 values ranged from 0.0-0.338%.

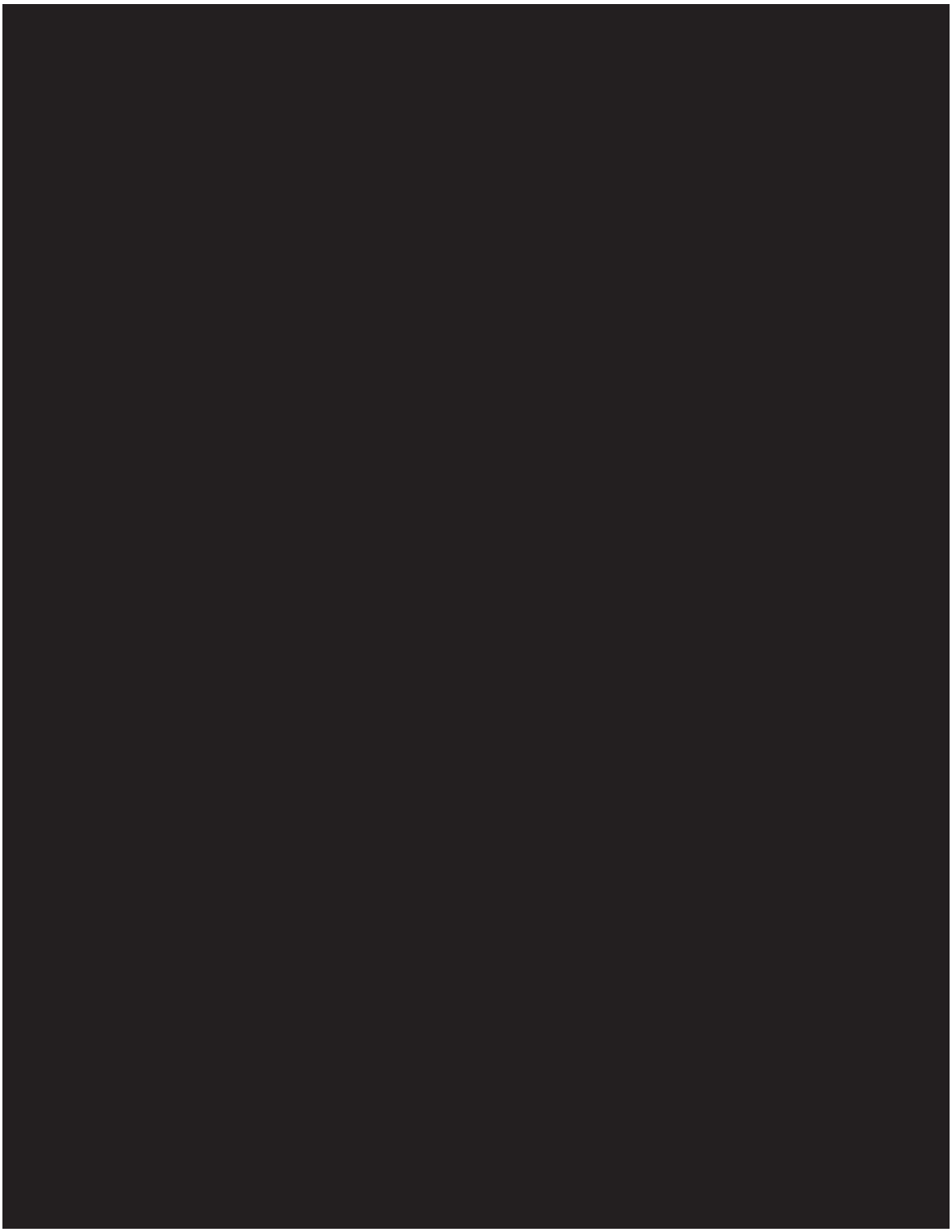
It should also be noted that for half of these analytes the values were very low and close to the LOQ (highlighted in blue in Table 7). [redacted] These analytes were included in the statistical analyses despite the limited quantifiable results. A value of 0 was used for results below the LOQ. [redacted]

No statistically significant differences were identified for the following fatty acids: [redacted] While statistical differences in the calculated means were identified for [redacted] the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. Therefore, it is unlikely that these differences indicate any biological significance.

Table 7. Fatty Acids of DHA canola (GMO), AV Jade (CMP) and Commercial References (REF).







VI. CONCLUSIONS

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4, which accumulates significant amounts of docosahexaenoic acid (DHA, 22:6ω3) in the seed oil (DHA canola).

This report describes the evaluation of various nutritional characteristics and the test methodology utilized for DHA canola (GMO), the parental AV Jade (CMP) variety along with several commercial canola varieties (REF). The analytes evaluated are the standard parameters by which many canola varieties are measured.

Samples were collected from field trials conducted in 2015 at eight locations in major canola growing regions of Australia for compositional analysis. Each trial was designed as a randomized complete block experiment consisting of five replicates (bloc) with the elite event and eight cultivars, which include the parental variety, AV Jade. Grain samples of 350-400g were collected and pooled from seedpods taken from the middle two rows of each plot and analyzed (Eurofins Nutritional Analysis Center). The methodology and statistical analyses are fully outlined within this report.

The results demonstrate that aside from the expected changes in the fatty acid profile, none of the compositional analytes showed any biologically significant differences between the CMP and GMO. While statistical differences in the calculated means were identified for several analytes, the calculated means are typically very close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. Therefore, it is unlikely that any of these differences indicate any biological significance.

Because DHA canola expresses seven fatty acid pathway enzymes, it is not surprising that many of the fatty acids are different from conventional canola, [redacted]

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VIII. APPENDICES

APPENDIX A: ANALYTICAL METHOD SUMMARIES AND REFERENCE STANDARDS

Moisture **SOP: MET-PR-005**

Summary: Samples were dried in an oven at 130°C for two hours, removed from oven, cooled in a desiccator and re-weighed. Moisture loss was calculated as the difference between the initial and dried weight.

References:

- AOCS Ba 2a-38

Reference Standard: n/a

Crude Fat **SOP: MET-LI-001**

Summary: Samples were weighed, placed in a soxhlet extraction tube and attached to a condenser. Samples were extracted for 5 hours using diethyl ether, dried in a forced draft oven for 30 minutes, cooled to room temperature and weighed. Fat was then calculated as a percentage of the sample.

References:

- AOAC 920.39

Reference Standard: n/a

Crude Protein **SOP: MET-PR-002**

Summary: Samples were entered into the combustion chamber of a protein analyzer, in which the gas from the combustion was analyzed for nitrogen content and calculated to protein. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25.

References:

- AOAC 992.15
- AOCS Ba 4e-93
- AOAC 990.03

Reference Standard:

- LECO EDTA, 9.56 ± 0.04 % Nitrogen, LECO Corporation, Lot#: 1061

Ash **SOP: MET-PR-004**

Summary: Samples were weighed into a dry crucible, ashed in a muffle furnace at 600 °C, and then the weight of the ash determined.

References:

- AOCS 942.05

Reference Standard: n/a

Carbohydrates **SOP: OPS-024**

Summary: Carbohydrates were calculated as the difference between 100 – (moisture + protein + fat + ash).

References:

- 21CFR101.9
- USDA Handbook No. 74

Reference Standard: n/a

Crude Fiber **SOP: MET-PR-003**

Summary: 2 grams of sample is weighed and a fat extraction performed by placing the sample in a soxhlet extraction tube, which is attached to a condenser for a minimum of 1 hour. The sample is then digested using acid (sulfuric acid solution) and base (sodium hydroxide solution) and filtered. The sample is then dried at 130°C for a minimum of 1 hour. The weight of the residue minus the ash from the residue determines the crude fiber.

References:

- AOCS Ba 6-84
- AOAC 962.09

Reference Standard: n/a

Neutral Detergent Fiber **SOP: MET-PR-008**

Summary: Sample was digested with neutral detergent. The weight of the fiber residue determined the NDF result, which consisted predominantly of hemicellulose, cellulose and lignin.

References:

- Ankom Technologies: NDF for Ankom 2000 Fiber Analyzer

Reference Standard: n/a

Acid Detergent Fiber **SOP: MET-PR-007**

Summary: Sample was digested with acid detergent. The weight of the residue minus the ash from the residue determined the ADF result, which consists predominantly of cellulose and lignin.

References:

- ANKOM Technology Method 10-21-05

Reference Standard: n/a

Amino Acids by Acid Hydrolysis **SOP: MET-LC-006**

Summary: Samples were hydrolyzed in 6 N HCl at 110°C for 24 hr. Quantification was performed via ion exchange chromatography with a post-column ninhydrin reaction and UV/Vis detection.

Reference:

- AOAC 982.30, modified

Reference Standard:

DL-Norleucine, Sigma, Purity 100%, Lot#: 020M5303V

21 Amino Acids + Cystine

L-alanine, Sigma, Purity 100.1%, Batch#: BCBN6412V
L-arginine monohydrochloride, Sigma, Purity 100.0%, Batch#: 1361811 L-aspartic acid, Sigma, Purity 99.8%, Lot#: BCBN3442V
L-cystine, Sigma, Purity 100.7%, Lot#: BCBP1335V L-glutamic acid, Sigma, Purity 100.2%, Lot#: 1423805 Glycine, Fluka, Purity 100.0%, Lot#: 1119375
L-histidine monohydrochloride monohydrate, Sigma, Purity 99.8%, Lot#: BCBP4059V L-isoleucine, Fluka, Purity 100.0%, Lot#: 1423806
L-leucine, Sigma, Purity 99.7%, Lot#: BCBN3570V
L-lysine monohydrochloride, Fluka, Purity 99.6%, Lot#: BCBN9886V L-methionine, Fluka, Purity 100.1%, Lot#: 1423807
L-phenylalanine, Sigma, Purity 100.0%, Lot#: BCBM2088V L-proline, Sigma, Purity 100.0%, Lot#: BCBJ3904V
L-serine, Fluka, Purity 99.9%, Lot#: 1336081
L-threonine, Fluka, Purity 99.8%, Lot#: BCBD4901V L-tyrosine, Sigma, Purity 99.7%, Lot#: BCBP6351V L-valine, Fluka, Purity 100.0%, Lot#: BCBM0163V
Trans-4-hydroxy-l-proline, Sigma, Purity 100.0%, Lot#: BCBL2666V

Amino Acids by Performic Acid Oxidation **SOP: MET-LC-005**

Summary: Cystine and cysteine were first converted to cysteic acid and methionine to methionine sulfone by performic acid oxidation. The sample was then hydrolyzed to release the cysteic acid and methionine sulfone from the protein. Quantification was performed via ion exchange chromatography with OPA (o-phthalaldehyde) post-column reaction and detection was done using a fluorescence detector.

Reference:

- AOAC 994.12,

modified

Reference Standard:

DL-Norleucine, Sigma, Purity 100%, Lot#: 020M5303V
DL-Norleucine, Alfa Aesar, Purity 98.6%, Lot #: A10791
L-Cysteic acid, ACROS, Purity 99%, Lot 340819
L-Methionine sulfone, ACROS, Purity 98%, Lot 336201

Tryptophan by Alkaline Hydrolysis **SOP: MET-LC-024**

Summary: Samples were subjected to an alkaline digestion with lithium hydroxide at 110°C for 22hr. Quantification was performed via reverse-phase chromatography with UV/Vis detection.

Reference:

- AOAC 988.15, modified

Reference Standard:

5-Methyl-DL-tryptophan, ACROS, Purity 98%, Lot#: A0275990, A0344741
L-Tryptophan, Sigma, Purity 99.9%, Lot#: BCBP2408V

Vitamin E (α-tocopherol) **SOP: MET-VT-009/MET-VT-030**

Summary: The samples were saponified with ethanolic KOH in the presence of an antioxidant (ascorbic acid). The mixture was extracted with a petroleum ether/ethyl acetate solution. The combined organic phases were washed with water and dried over sodium sulfate. The solvent was exchanged to isooctane before injection on an HPLC equipped with a silica column and fluorescence detector.

Reference:

- AOAC 971.30 with HPLC quantification, mod.

Reference Standard:

(±)-α-Tocopherol, Sigma, Purity ≥96% Batch # MKBV6129V, MKBS2473V
Rac-beta-Tocopherol; 5,8-Dimethyltocol, Matreya, Purity >98%, Lot#: 24337, 24276
(+)-γ-Tocopherol, Sigma, Purity 98%, Lot#: SLBP1332V
(+)-δ-Tocopherol, Acros, Purity >=94%, Lot#: A0083534

Vitamin K1 **SOP: MET-VT-028**

Summary: Vitamin K was extracted from samples using dimethyl sulfoxide and hexane. The extracts were cleaned using a SPE cartridge. Vitamin K was eluted by methylene chloride, dried under a stream of nitrogen, and reconstituted in 2-propanol, and then analyzed on the HPLC with fluorescence detection.

Reference:

- AOAC 999.15, mod.

Reference Standard:

Vitamin K1, Sigma, Lot#: MKBS6018V
Vitamin K2, Sigma, Lot#: SLBM4864V

Biotin **SOP: MET-VT-003**

Summary: The samples were autoclaved for 120 minutes, cool to room temperature prior to pH adjustments, and then filtered to the retain step. Biotin concentrations must be within the standard curve range, so dilutions may be necessary. Each tube is inoculated, except the uninoculated tube then they are placed in an incubator for 16-20 hours. Samples are analyzed with a spectrophotometer set at 640 nm.

Reference:

Biotin, *Methods of Vitamin Assay*, 3rd ed., Interscience Publishers, 1966, chap. 12 Reference Standard: Biotin Batch# BCBN0180V

Total Choline **SOP: MET-VT-031**

Summary: The samples were incubated for 15-18 hours and then allowed to reach room temperature prior to pH adjustments and filtration. After adding ACN (acetonitrile) and MgO (magnesium oxide) the samples were vortexed, shaken, and centrifuged. An aliquot from each sample was analyzed on the HPLC.

Reference:

- AOAC 999.14, mod.

Reference Standard:

Choline bitartrate, 97% Lot# A0334400

Folic Acid **SOP: MET-VT-018**

Summary: The samples were autoclaved and then filtered prior to addition of chicken pancreas and Creon enzyme; then allowed to incubate for 16-17 hours. Samples are autoclaved again and then diluted as necessary to be within the standard curve. Samples are inoculated, except the un-inoculated tube and then incubated for 16-20 hours. Samples are analyzed with a spectrophotometer set at 600 nm.

Reference:

- AOAC 992.05, mod.

Reference Standard:

Folic Acid ≥97% Batch# SLBN1618V

Vitamin B3 - Niacin **SOP: MET-VT-005**

Summary: Vitamin B3 was extracted using 1 N H2SO4, autoclaved, cooled to room temperature, and then pH adjusted. Samples were diluted for the final concentration of Niacin to be within the standard curve and inoculated, except the un-inoculated tube and incubated for 16-24 hours. Samples are analyzed with a spectrophotometer set at 600 nm.

Reference:

- AOAC 944.13, mod.

Reference Standard:

Niocotinic Acid >= 99.5% Batch # BCBP0239V

Vitamin B5 – Pantothenic Acid **SOP: MET-VT-007**

Summary: Vitamin B5 was extracted using NaOAc/HOAc buffer , autoclaved, cooled to room temperature, and then pH adjusted. Samples were diluted for the final concentration of pantothenic acid within the standard curve and the inoculated, except the uninoculated tube, and incubated for 16-20 hours. Samples are analyzed with a spectrophotometer set at 600 nm.

Reference:

- AOAC 945.74, mod.

Reference Standard:

Calcium Pantothenate Batch# SLBF6179V

Vitamin B6 – Pyridoxine **SOP: MET-VT-026**

Summary: Vitamin B6 was extracted using 0.05 M sodium acetate, 1M glyoxylic acid, ferrous sulfate, and acid phosphatase and incubated for 14 to 18 hours. Samples were then filtered and an aliquot from each sample was analyzed on the HPLC.

Reference:

- J. AOAC, 88, 30-37 (2005)

Reference Standard:

Pyridoxine hydrochloride ≥ 98% Batch # SLBM7795V

Pyridoxine hydrochloride ≥ 98% Product # P9755

Vitamin B2 – Riboflavin **SOP: MET-VT-002**

Summary: Vitamin B2 was extracted using 0.1 N HCl and then autoclaved. Samples were pH adjusted, filtered, and then pH adjusted again. Acetic acid, 4% KMnO4, and 3% H2O2 were added and excess oxygen was expelled. Samples analyzed using a fluorometer.

Reference:

- AOAC 970.65, mod.

Reference Standard:

Riboflavin 98% Lot# A0353185

Vitamin B1 - Thiamin **SOP: MET-VT-019**

Summary: Vitamin B1 was extracted from samples using hydrochloric acid and sodium acetate then samples were incubated for 14-18 hours. Samples were diluted and filtered prior to being filtered with a resin bed then eluted with KCl. Samples analyzed using a fluorometer.

Reference:

- AOAC 942.23, mod.

Reference Standard:

Thiamine HCl Lot# 152817

Sulfur **SOP: MET-EL-009**

Summary: Summary: The digest was analyzed by Inductively Coupled Plasma Optical Emission Spectrophotometry against a standard curve of NIST traceable standards to determine the mineral content.

Reference:

- T.T. Nham. *Analysis of soil extracts using the Varian 725-ES,* Varian ICP-OES Application Note No. 34
- R. Jurgensen, J.C. Hart, L.L. Farrow. *Sulfur limits of detection and spectral interference corrections for DWPF sludge matrices by inductively coupled plasma emission spectrometry,* WSRC-TR-2004-0090,
- Z.A. Grosser, L.J. Davidowski, P. Wee. *The analysis of biodiesel for inorganic contaminants, including sulfur, by ICP-OES,* Application note, PerkinElmer 2009

Reference Standard:

- ICP Custom Solution, Inorganic Ventures, Lot#: J2-S02028
  Sulfur (S), Certified Value: 1001 ± 3 µg/mL

Chloride Soluble **SOP: MET-CM-018**

Summary: Samples are charred on a hot plate and then placed in a muffle oven at 550°C muffle oven for 1 to 2 hours. Samples are acidified by adding 1:3 nitric acid solution and then placed on the autosampler where the samples are titrated by adding silver nitrate until the potentiometric end point is reached.

Reference:

- AOAC 971.27, mod.
- AOAC 2016.03

Reference Standard:

Sodium Chloride, Purity: 99.8%, Lot# 153848

Phenolic Acids **SOP: MET-LC-004**

Summary: Samples are saponified and extracted in basic conditions in MeOH/water, and the extracts are acidified and analyzed using LC/UV.

Reference:

- J. Atric. Food Chem, 30 (1982) 1098

Reference Standard:

2-hydroxycinnamic acid (o-Coumaric acid), Sigma, Purity 99.7%, Batch#: STBF4129V
p-Coumaric acid, Sigma, Purity 99.6%, Batch#: BCBN8568V
Trans-Ferulic acid, Sigma, Purity 99.8%, Batch#: BCBM6076V
Sinapic acid, Sigma, Purity 99.6%/99%, Lot# BCBM8241V/Batch# SLBN7067V

Glucosinolates **SOP: MET-LC-026**

Summary: Ground samples, together with internal standard sinigrin, are extracted with hot methanol (70% v/v in water). The anionic glucosinolates are then loaded onto ion-exchange column. After treatment by sulfatase, the desulfoglucosinolates are eluted by water and quantitated by reverse-phase UPLC and UV detection

Reference:

- ISO 9167-1:1992

Reference Standard:

(-)-Sinigrin hydrate, Sigma, Purity 100.0%/99.8%/100.0%, Batch #: BCBR8070V/BCBP6528V/BCBQ6052V

Tannins – Soluble Condensed **SOP: MET-AN-012**

Summary: Samples were weighed into filter paper, placed in a soxhlet extraction tube and attached to a condenser. Samples were defatted for 5 hours using diethyl ether, and evaporated overnight in a fume hood. Condensed tannin molecules react with vanillin to form a red adduct whose absorbance is determined at 500nm. The sample absorbance is then compared to a standard curve that is generated from the vanillin reaction with catechin standard.

Reference:

- J. Agric. Food Chem. 1978. 26, 1214

Reference Standard:

Vanillin, Sigma, Purity 100.0%, Lot#: MKBV7916V
(+)-Catechin Hydrate, Sigma, Purity 99.6%, Batch#: WXBC0787V

Phytic Acid **SOP: MET-EL-011**

Summary: Sample aliquot was extracted with Na2SO4 solution for a minimum of 3 hours, phytic acid (phytate) was precipitated with FeCl3, the precipitant ashed, and the phosphorus content in the precipitate was determined by ICP-OES method. The phosphorus content was expressed in phytic acid equivalents.

Reference:

- Analytical Biochemistry 77: 536-539 (1977)

Reference Standard:

- ICP Custom Solution, Inorganic Ventures, Lot#: J2-MEB568043
  Phosphorus (P), Certified Value: 2000 ± 10 µg/mL
- 10000 µg/mL Yttrium, Inorganic Ventures, Purity 99.9995%, Lot#: J2-Y02023
- 10000 µg/mL Gallium, Inorganic Ventures, Purity 100%, Lot#: J2-GA01121

Molybdenum **SOP: MET-EL-002/MET-EL-004**

Summary: Sample was digested after dry ashing, then analyzed by AAS.

References:

- AOAC 965.17, modified
- AOAC 986.08, modified

Reference Standard:

Product Code: Single Analyte Custom Grade Solution 99.9942% Lot # H2-MO02073

Sample Preparation for ICP and AAS Analysis **SOP: MET-EL-002**

Summary: Sample was digested after dry ashing.

References:

- AOAC 965.17, modified
- AOAC 985.01, modified

Reference Standard: N/A

Elemental Analysis by ICP **SOP: MET-EL-003**

Summary: The digest was analyzed by Inductively Coupled Plasma Optical Emission Spectrophotometry against a standard curve of NIST traceable standards to determine the mineral content.

Reference:

- AOAC 965.17, modified
- AOAC 985.01, modified

Reference Standards:

- ICP Custom Solution, Inorganic Ventures, Lot#: J2-MEB568043, J2-S02028
  Calcium (Ca), Certified Value: 2000 ± 9 µg/mL
  Phosphorus (P), Certified Value: 2000 ± 10 µg/mL Magnesium (Mg), Certified Value: 500.0 ± 2.3 µg/mL Potassium (K), Certified Value: 2000 ± 9 µg/mL Sodium (Na), Certified Value: 1000 ± 4 µg/mL
  Iron (Fe), Certified Value: 100.0 ± 0.5 µg/mL Zinc (Zn), Certified Value: 500.0 ± 2.4 µg/mL
- ICP Custom Solution, Inorganic Ventures, Lot#: J2-MEB583102
  Copper (Cu), Certified Value: 2000 ± 9 µg/mL
  Manganese (Mn), Certified Value: 199.9 ± 0.9 µg/mL
- 10000 µg/mL Yttrium, Inorganic Ventures, Purity 99.9995%, Lot#: J2-Y02023
- 10000 µg/mL Gallium, Inorganic Ventures, Purity 100%, Lot#: J2-GA01121

Fatty Acids **SOP: MET-LI-025, MET-LI-011**

Summary: Fat was extracted from samples using pet ether. The extracted fat was then reacted with boron-trifluoride/methanol reagent to convert fatty acids present in any form into their methyl ester forms. These were then extracted into hexane, and injected onto a capillary column gas chromatograph. Standards of known composition were used to identify the fatty acids present, and the amount of each individual fatty acid was reported as a percentage of the total sample weight.

Reference:

- AOCS Ce 2-66
- AOCS Ce 1-62

Reference Standard:

GLC 85, Nu Chek Prep, Lot#: M12-A
C4:0 Methyl Butyrate, Purity of 99.8% C6:0 Methyl Hexanoate, Purity of 99.7% C8:0 Methyl Octanoate, Purity of 99.7% C10:0 Methyl Decanoate, Purity of 99.7%
C11:0 Methyl Undecanoate, Purity of 99.8% C12:0 Methyl Laurate, Purity of 99.9% C13:0 Methyl Tridecanoate, Purity of 99.7% C14:0 Methyl Myristate, Purity of 99.8% C14:1 Methyl Myristoleate, Purity of 99.6%
C15:0 Methyl Pentadecanoate, Purity of 99.6% C15:1 Methyl 10-Pentadecenoate, Purity of 99.5% C16:0 Methyl Palmitate, Purity of 99.9%
C16:1 Methyl Palmitoleate, Purity of 99.6% C17:0 Methyl Heptadecanoate, Purity of 99.7%
C17:1 Methyl 10-Heptadecenoate, Purity of 99.6% C18:0 Methyl Stearate, Purity of 99.9%
C18:1 Methyl Oleate, Purity of 99.8% C18:1T Methyl Elaidate, Purity of 99.7% C18:2 Methyl Linoleate, Purity of 99.8% C18:3 Methyl Linolenate, Purity of 99.7%
C18:3 Methyl Gamma Linolenate, Purity of 99.6% C20:0 Methyl Arachidate, Purity of 99.7%
C20:1 Methyl 11-Eicosadienoate, Purity of 99.6% C20:2 Methyl 11-14 Eicosadienoate, Purity of 99.6% C22:0 Methyl Behenate, Purity of 99.8%
C22:1 Methyl Erucate, Purity of 99.7%
C20:3 Methyl 11-14-17 Eicosatrienoate, Purity of 99.5% C20:3 Methyl Homogamma Linolenate, Purity of 99.5% C20:4 Methyl Arachidonate, Purity of 99.5%
C24:1 Methyl Nervonate, Purity of 99.6% C22:2 Methyl Docosadienoate, Purity of 99.4%
C22:6 Methyl Docosahexaenoate, Purity of 99.4%
1,2,3-Tritridecanoylgycerol, NU-CHEK-PREP, Purity >99%, Lot #: T-135-M6-Z

Phytosterols **SOP: MET-LI-034**

Summary: Fat was extracted from the samples using petroleum ether. The extracted fat was saponified. The saponified extract was washed on to a neutral alumina solid phase extraction cartridge. The unsaponifiable material including the sterols was eluted using diethyl ether. The sterol fraction of the unsaponifiable material was isolated using a normal phase high pressure liquid chromatograph. The sterols were then derivatized to silyl esters using chlorotrimethylsilane and injected onto a capillary column gas chromatograph. Identification of sterols was performed using an internal quality control sample of known

sterol composition. Quantification of sterols was performed using the response relative to the response of the cholestanol internal standard.

Reference:

- ISO 12228; AOCS Ch. 6-91

Reference Standard:

Cholesterol, Sigma, Purity 99.1% Lot#: SLBM9596V

5α-Cholestan-3β-ol, Sigma, Purity 99%, Batch#: SLBK1161V