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TITLE:

NUTRIENT COMPOSITION OF PROCESSED MEAL EXPRESSING LONG-CHAIN OMEGA-3 FROM FIELD-GROWN CANOLA DURING 2015.

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ABBREVIATIONS

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 tons
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 contains significant amounts of docosahexaenoic acid (DHA, 22:6-ω3) in the seed oil (DHA canola). The purpose of this report is to provide composition data comparing canola meal processed from DHA canola and its parental variety, AV Jade canola grain.

This report describes the evaluation of various nutritional characteristics and the test methodology utilized for processed fractions of meal crushed from grain of DHA canola (GMO) and the parental AV Jade (CMP). The analytes evaluated are the standard parameters by which many canola processed fractions are measured.

When the mean of the crude and hexane-extracted meals are compared for CMP and GMO, most values are within 10% of each other for most analytes. While some differences were above this 10% level, all were within the ranges usually observed in canola meal. Some differences were expected, specifically those reflected in the fatty acid profiles, which were intentionally modified. However, the amount of oil remaining in the meal is significantly reduced, especially after the solvent extraction process.

Mean values of tocopherols for the hexane-extracted meal from the CMP were quite different from the GMO, roughly one-half of the mean CMP values. Hexane-extraction greatly diminished the tocopherols for both CMP and GMO, and in most cases greater than 90% reduction was observed.

While the values for the remaining glucosinolates (glucoalyssin, progoitrin and 4-hydroxyglucobrassicin) did show differences between the CMP and GMO, the range of values overlapped and in every case, the highest value was linked to the CMP. Importantly, the sum of the means of these three glucosinolates for CMP crude meal is 15.55 µmol/g, for GMO crude meal is 16.04 µmol/g, for CMP hexane-extracted meal is 19.61 µmol/g and for GMO hexane-extracted mean is 18.05 µmol/g. All of these values are well below the limits included in the definition of canola (30 µmol/g)[1]. Finally, when all the glucosinolates values are combined, the levels remain below this same limit (mean range = 21.14 – 26.49 µmol/g).

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

As expected, the most striking result is the drastic reduction in fatty acids in hexane-extracted meals regardless of whether it is CMP or GMO-derived meals. In all cases the amount of fatty acids is less than 5% of that measured in crude meal. Thus, further comparisons of the fatty acid profile of hexane-extracted means for CMP and GMO is not meaningful.

Because DHA canola expresses seven fatty acid pathway enzymes, it is not surprising that the fatty acids profile is different when the CMP and GMO meals are compared. [redacted]

TITLE:

NUTRIENT COMPOSITION OF PROCESSED MEAL EXPRESSING LONG-CHAIN OMEGA-3 FROM FIELD-GROWN CANOLA 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 docosahexaenoic acid (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[2].

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 contains 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 and 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 including Lackl-Δ12D and Picpa-ω3D that introduce a double bond at the Δ12 and Δ15 positions, respectively; (2) algae fatty acid elongases including Pyrco-Δ6E and

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

Pyrco-Δ5E that add two carbons 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.

II. PURPOSE

This report describes the evaluation of various nutritional characteristics and the test methodology for the meal processed fraction from DHA canola and the parental AV Jade variety. The analytes evaluated are the standard parameters by which many canola meals 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 & METHODS

DHA canola (OECD ID NS-B5ØØ27-4) was harvested in 2015 at one location (1511_STH) in a major canola growing region of Australia to collect grain for processing into meal, commonly used for animal feeds. The parental variety, AV Jade, was harvested in 2015 from two locations near to the DHA canola site. The two AV Jade sites were 1508_DOU and 1509_GRN. Processed meal was prepared from DHA canola (GMO) and from AV Jade (CMP) for compositional analysis. The DHA canola grain was divided into two batches, which were crushed separately at CSIRO Agriculture and Food facility (Werribee, Vic, 3030, AUSTRALIA). The two AV Jade batches were crushed separately. The samples are listed below:

- Crude meal – GMO (DHA canola) Crush 1
- Crude meal – GMO (DHA canola) Crush 2
- Crude meal – CMP (AV Jade) Crush 1508_DOU
- Crude meal – CMP (AV Jade) Crush 1509_GRN
- Hexane-extracted meal – GMO (DHA canola) Crush 1
- Hexane-extracted meal – GMO (DHA canola) Crush 2
- Hexane-extracted meal – CMP (AV Jade) Crush 1508_DOU
- Hexane-extracted meal – CMP (AV Jade) Crush 1509_GRN

All canola meal samples were shipped at ambient temperature to the laboratory for analysis (Eurofins Nutritional Analysis Center).

A summary of each parameter, its method of analysis, appropriate units and the limits of quantitation (LOQ) are included in Table 1. When data points were at or below the LOQ, zero

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:

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

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

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

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

Table 1. Analyte Specifics for Canola Meal 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%
Neutral Detergent Fiber	MET-PR-008	%	0.3%
Amino Acids by Acid Hydrolysis	MET-LC-006	%	Serine, Glutamic Acid, Glycine, Alanine, Histidine, Total Lysine: 0.01% Aspartic Acid, Threonine, Valine, Isoleucine, Leucine: 0.02% Tyrosine: 0.04% Phenylalanine: 0.03% Arginine, 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*
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%
Phytosterols	MET-LI-034	%	0.002%*
Fatty Acid Profile	MET-LI-002/MET-LI-025	%	C16:0: 0.02% All others at 0.01%

* Listed LOQ applies to all analyte parameters.

IV. COMPOSITIONAL ANALYSIS FOR DHA CANOLA MEAL

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 nutritional elements of meal processed from the genetically modified organism, NS-B50027-4 (DHA canola; identified as GMO in reports), and comparator, AV Jade (parental variety; identified as non-GM isoline).

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

b. ANALYSIS OF PROXIMATES AND MINERALS IN DHA CANOLA MEAL

The levels of proximates and minerals were measured in meal samples of DHA canola (GMO), the parental line AV Jade (CMP) (Table 2). The test material, type of sample, % and average % of the two grain crushes are provided for each analyte.

When the mean of the crude and hexane-extracted meals are compared for CMP and GMO, most values are within 10% of each other for protein, ash, phosphorus and phytic acid. The crude meals are within 10% of each other for crude fiber, acid detergent fiber and neutral detergent fiber as were the carbohydrate values for hexane-extracted meal.

Differences of 13.4% were identified for the carbohydrate values for crude meal and 11.7% to 19% differences are observed for crude fiber, acid detergent fiber and neutral detergent fiber in hexane-extracted meals. Calcium is lower for the GMO, 16.9% and 25% for crude and hexane-extracted meals, respectively. [redacted]

Table 2. Proximates and Minerals in meal: DHA canola (GMO) and AV Jade (CMP); Crude and Hexane-extracted meals

Analyte	Test Material	Sample	%	Average of both crushes (%)
Crude Fat	CMP	Crude meal	16.95	21.44
			25.92	
		Hexane extracted meal	0.55	0.69
			0.83	
	[redacted]			
Protein	CMP	Crude meal	44.38	42.64
			40.89	
		Hexane extracted meal	55.76	54.59
			53.42	
	GMO	Crude meal	44.93	44.21
			43.49	
		Hexane extracted meal	52.67	53.04
			53.42	
Ash	CMP	Crude meal	4.86	5.04
			5.23	
		Hexane extracted meal	5.79	6.28
			6.76	
	GMO	Crude meal	5.13	5.03
			4.93	
		Hexane extracted meal	6.00	5.90
			5.80	
Carbohydrate	CMP	Crude meal	33.91	30.93
			27.95	
		Hexane extracted meal	37.90	39.22
			40.55	
	GMO	Crude meal	34.52	35.09
			35.66	
		Hexane extracted meal	41.14	40.64
			40.15	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Crude Fiber	CMP	Crude meal	9.02	8.59
			8.16	
		Hexane extracted meal	10.1	10.4
			10.6	
	GMO	Crude meal	8.74	8.74
			8.75	
		Hexane extracted meal	9.82	9.23
			8.65	
Acid Detergent Fiber	CMP	Crude meal	15.9	15.8
			15.6	
		Hexane extracted meal	19.7	21.1
			22.5	
	GMO	Crude meal	16.6	17.0
			17.4	
		Hexane extracted meal	21.6	18.2
			14.9	
Neutral Detergent Fiber	CMP	Crude meal	24.6	24.5
			24.4	
		Hexane extracted meal	32.2	31.6
			30.9	
	GMO	Crude meal	21.6	23.3
			25.0	
		Hexane extracted meal	26.7	28.3
			29.9	
Calcium	CMP	Crude meal	0.504	0.519
			0.534	
		Hexane extracted meal	0.605	0.648
			0.692	
	GMO	Crude meal	0.455	0.444
			0.434	
		Hexane extracted meal	0.530	0.520
			0.519	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Phosphorus	CMP	Crude meal	0.854	0.926
			0.998	
		Hexane extracted meal	1.02	1.14
			1.27	
	GMO	Crude meal	0.955	0.923
			0.891	
		Hexane extracted meal	1.12	1.10
			1.07	
Phytic Acid	CMP	Crude meal	2.2	2.5
			2.8	
		Hexane extracted meal	2.7	3.2
			3.6	
	GMO	Crude meal	2.6	2.5
			2.4	
		Hexane extracted meal	3.2	3.0
			2.8	

c. Analysis of Amino Acids in DHA Canola Meal

The levels of amino acids were measured in meal samples of DHA canola (GMO), the parental line AV Jade (CMP) (Table 3). The test material, type of sample, % and average % of the two grain crushes are provided for each analyte.

Comparison of the amino acid profile of the CMP and GMO for crude and hexane-extracted meal showed few differences and all were below 8%.

Table 3. Amino acids of meal: DHA canola (GMO) and AV Jade (CMP); Crude and Hexane-extracted meals

Analyte	Test Material	Sample	%	Average of both crushes (%)
Alanine	CMP	Crude meal	1.94	1.86
			1.77	
		Hexane extracted meal	2.35	2.33
			2.31	
	GMO	Crude meal	1.91	1.92
			1.93	
		Hexane extracted meal	2.34	2.35
			2.36	
Arginine	CMP	Crude meal	2.84	2.73
			2.62	
		Hexane extracted meal	3.47	3.42
			3.38	
	GMO	Crude meal	2.77	2.78
			2.80	
		Hexane extracted meal	3.39	3.40
			3.40	
Aspartic Acid	CMP	Crude meal	3.22	3.12
			3.01	
		Hexane extracted meal	3.93	3.90
			3.87	
	GMO	Crude meal	3.27	3.32
			3.37	
		Hexane extracted meal	4.02	3.94
			3.87	
Cystine	CMP	Crude meal	1.1	1.05
			1.0	
		Hexane extracted meal	1.4	1.35
			1.3	
	GMO	Crude meal	1.1	1.10
			1.1	
		Hexane extracted meal	1.3	1.30
			1.3	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Glutamic Acid	CMP	Crude meal	8.57	8.17
			7.77	
		Hexane extracted meal	10.4	10.25
			10.1	
	GMO	Crude meal	8.25	8.30
			8.36	
		Hexane extracted meal	10.1	10.15
			10.2	
Glycine	CMP	Crude meal	2.26	2.16
			2.06	
		Hexane extracted meal	2.74	2.70
			2.67	
	GMO	Crude meal	2.29	2.30
			2.31	
		Hexane extracted meal	2.80	2.81
			2.82	
Histidine	CMP	Crude meal	1.26	1.20
			1.14	
		Hexane extracted meal	1.53	1.50
			1.47	
	GMO	Crude meal	1.22	1.23
			1.24	
		Hexane extracted meal	1.49	1.50
			1.51	
Isoleucine	CMP	Crude meal	1.87	1.80
			1.72	
		Hexane extracted meal	2.27	2.24
			2.22	
	GMO	Crude meal	1.81	1.80
			1.80	
		Hexane extracted meal	2.21	2.22
			2.23	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Leucine	CMP	Crude meal	3.15	3.01
			2.87	
		Hexane extracted meal	3.82	3.78
			3.74	
	GMO	Crude meal	3.04	3.04
			3.05	
		Hexane extracted meal	3.70	3.72
			3.75	
Lysine	CMP	Crude meal	3.08	2.79
			2.50	
		Hexane extracted meal	3.37	3.40
			3.44	
	GMO	Crude meal	3.23	3.01
			2.79	
		Hexane extracted meal	3.42	3.57
			3.72	
Methionine	CMP	Crude meal	0.89	0.84
			0.80	
		Hexane extracted meal	1.1	1.05
			1.0	
	GMO	Crude meal	0.90	0.90
			0.89	
		Hexane extracted meal	1.0	1.0
			1.0	
Phenyalanine	CMP	Crude meal	1.81	1.74
			1.66	
		Hexane extracted meal	2.21	2.18
			2.15	
	GMO	Crude meal	1.74	1.74
			1.75	
		Hexane extracted meal	2.10	2.12
			2.13	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Proline	CMP	Crude meal	2.86	2.70
			2.55	
		Hexane extracted meal	3.48	3.42
			3.35	
	GMO	Crude meal	2.70	2.74
			2.77	
		Hexane extracted meal	3.29	3.30
			3.32	
Serine	CMP	Crude meal	1.85	1.76
			1.67	
		Hexane extracted meal	2.24	2.21
			2.18	
	GMO	Crude meal	1.81	1.83
			1.85	
		Hexane extracted meal	2.18	2.20
			2.21	
Threonine	CMP	Crude meal	1.85	1.78
			1.70	
		Hexane extracted meal	2.25	2.22
			2.20	
	GMO	Crude meal	1.84	1.86
			1.89	
		Hexane extracted meal	2.24	2.24
			2.25	
Tryosine	CMP	Crude meal	1.18	1.13
			1.08	
		Hexane extracted meal	1.41	1.38
			1.36	
	GMO	Crude meal	1.17	1.18
			1.18	
		Hexane extracted meal	1.41	1.41
			1.41	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Tryptophan	CMP	Crude meal	0.71	0.67
			0.63	
		Hexane extracted meal	0.87	0.86
			0.84	
	GMO	Crude meal	0.69	0.68
			0.67	
		Hexane extracted meal	0.82	0.83
			0.84	
Valine	CMP	Crude meal	2.36	2.26
			2.16	
		Hexane extracted meal	2.85	2.83
			2.81	
	GMO	Crude meal	2.30	2.29
			2.28	
		Hexane extracted meal	2.81	2.81
			2.81	

d. Analysis of Glucosinolates in DHA Canola Meal

The levels of glucosinolates were measured in meal samples of DHA canola (GMO), the parental line AV Jade (CMP) (Table 4). The test material, type of sample, and average % of the two grain crushes are provided for each analyte.

The following glucosinolates had values that are below 1 µmol/g for CMP and GMO meals: epi-progoitrin, glucoalyssin, glucobrassicanapin, glucobrassicin, gluconapoleiferin, gluconasturtiin and neoglucobrassicin. Values are not provided for glucoraphanin and glucoiberin, because they were <LOQ.

While the values for the remaining glucosinolates (glucoalyssin, progoitrin and 4-hydroxyglucobrassicin) did show differences between the CMP and GMO, the range of values overlapped and in every case, the highest value was linked to the CMP. Importantly, the sum of means of these three glucosinolates:

- CMP crude meal =15.55 µmol/g,

- GMO crude meal = 16.04 µmol/g
- CMP hexane-extracted meal = 19.61 µmol/g
- GMO hexane-extracted mean = 18.05 µmol/g.

All of these values are well below the limits included in the definition of canola (30 µmol/g)[3]. Finally, when all the glucosinolates values are combined, the levels remain below this same limit (mean range = 21.14 – 26.49 µmol/g).

Table 4. Glucosinolates of meal: DHA canola (GMO) and AV Jade (CMP); Crude and Hexane-extracted meals

Analyte	Test Material	Sample	µmol/g	Average of both crushes
Epi-progoitrin	CMP	Crude meal	0.1	0.15
			0.2	
		Hexane extracted meal	0.2	0.2
			0.2	
	GMO	Crude meal	0.2	0.2
			0.2	
		Hexane extracted meal	0.2	0.2
			0.2	
Glucoalyssin	CMP	Crude meal	0.43	0.54
			0.66	
		Hexane extracted meal	0.55	0.70
			0.85	
	GMO	Crude meal	0.63	0.60
			0.57	
		Hexane extracted meal	0.71	0.66
			0.62	

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

Analyte	Test Material	Sample	µmol/g	Average of both crushes
Glucobrassicanapin	CMP	Crude meal	0.59	0.70
			0.80	
		Hexane extracted meal	0.68	0.84
			1.00	
	GMO	Crude meal	0.57	0.58
			0.59	
		Hexane extracted meal	0.63	0.63
			0.63	
Glucobrassicin	CMP	Crude meal	0.38	0.37
			0.36	
		Hexane extracted meal	0.45	0.44
			0.44	
	GMO	Crude meal	0.42	0.42
			0.41	
		Hexane extracted meal	0.49	0.48
			0.47	
Gluconapin	CMP	Crude meal	3.75	4.05
			4.35	
		Hexane extracted meal	4.42	4.94
			5.46	
	GMO	Crude meal	3.70	3.58
			3.46	
		Hexane extracted meal	4.05	3.85
			3.75	
Gluconapoleiferin	CMP	Crude meal	0.06	0.08
			0.1	
		Hexane extracted meal	0.1	0.15
			0.2	
	GMO	Crude meal	0.1	0.1
			0.1	
		Hexane extracted meal	0.2	0.15
			0.1	

Analyte	Test Material	Sample	µmol/g	Average of both crushes
Gluconasturtiin	CMP	Crude meal	0.25	0.24
			0.24	
		Hexane extracted meal	0.29	0.31
			0.33	
	GMO	Crude meal	0.28	0.26
			0.24	
		Hexane extracted meal	0.33	0.30
			0.27	
Neoglucobrassicin	CMP	Crude meal	<LOQ	<LOQ
			<LOQ	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.07	0.08
			0.08	
		Hexane extracted meal	0.08	0.08
			0.09	
Progoitrin	CMP	Crude meal	6.10	7.89
			9.68	
		Hexane extracted meal	7.72	10.01
			12.3	
	GMO	Crude meal	8.98	8.78
			8.57	
		Hexane extracted meal	10.2	9.91
			9.62	
4-Hydroxyglucobrassicin	CMP	Crude meal	7.77	7.12
			6.47	
		Hexane extracted meal	9.42	8.90
			8.39	
	GMO	Crude meal	6.56	6.66
			6.76	
		Hexane extracted meal	7.44	7.48
			7.52	

e. Analysis of Organic Compounds in DHA Canola Meal

The levels of organic compounds were measured in meal samples of DHA canola (GMO), the parental line AV Jade (CMP) (Table 5). The test material, type of sample, mg/100g and average mg/100g of the two grain crushes are provided for each analyte.

The means for *p*-coumaric acid were very low, <LOQ for the GMO and 2-3X the LOQ for the CMP. All values for soluble tannins were <LOQ and are not represented herein. Mean values for ferulic acid were higher for the CMP, and the differences from GMO were 8.2% and 13.2% for crude and hexane-extracted meals, respectively. Mean values for sinapine were only 1.8% difference for crude meals of CMP and GMO, but 13.6% different for hexane-extracted meals. Again, the CMP mean values were greater than the GMO mean values.

Table 5. Organic compounds of meal: DHA canola (GMO) and AV Jade (CMP); Crude and Hexane-extracted meals

Analyte	Test Material	Sample	mg/100g	Average of crushes
Ferulic acid	CMP	Crude meal	179.2	173.0
			166.8	
		Hexane extracted meal	221.3	215.9
			210.5	
	GMO	Crude meal	160.8	159.9
			159.0	
		Hexane extracted meal	178.2	194.4
			210.5	
p-Coumaric acid	CMP	Crude meal	20.78	22.38
			23.97	
		Hexane extracted meal	25.79	28.79
			31.79	
	GMO	Crude meal	<LOQ	<LOQ
			<LOQ	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	

Analyte	Test Material	Sample	mg/100g	Average of crushes
Sinapine	CMP	Crude meal	1.842	1.767
			1.692	
		Hexane extracted meal	2.446	2.338
			2.231	
	GMO	Crude meal	1.735	1.736
			1.736	
		Hexane extracted meal	1.999	2.058
			2.117	

f. Analysis of Vitamins in DHA Canola Meal

The levels of vitamins were measured in meal samples of DHA canola (GMO), the parental line AV Jade (CMP) (Table 6). The test material, type of sample, mg/100g and average mg/100g of the two grain crushes are provided for each analyte.

Mean values for crude meals for both CMP and GMO were 11.8%, 12.7% and 2.6% for alpha, gamma and total tocopherols, respectively. However, the ranges overlapped and therefore these differences are not biologically significant. Mean values for the hexane-extracted meals from the CMP and GMO were quite different with the GMO, roughly one-half of the mean CMP values. Hexane-extraction greatly diminished the tocopherols for both CMP and GMO, and in most cases greater than a 90% reduction was observed. Both beta and delta tocopherol values were <LOQ.

Table 6. Vitamin E in meal: DHA canola (GMO) and AV Jade (CMP); Crude and Hexane-extracted meals

Analyte	Test Material	Sample	mg/100g	Average of crushes
Alpha Tocopherol Vitamin E	CMP	Crude meal	8.15	9.12
			10.1	
		Hexane extracted meal	1.38	1.48
			1.59	
	GMO	Crude meal	10.2	10.2
			10.1	
		Hexane extracted meal	0.817	0.780
			0.743	
Gamma Tocopherol Vitamin E	CMP	Crude meal	12.5	14.2
			15.9	
		Hexane extracted meal	0.888	0.922
			0.956	
	GMO	Crude meal	12.6	12.6
			12.7	
		Hexane extracted meal	0.459	0.454
			0.450	
Total Tocopherols Vitamin E	CMP	Crude meal	20.7	23.4
			26.0	
		Hexane extracted meal	2.27	2.40
			2.54	
	GMO	Crude meal	22.8	22.8
			22.9	
		Hexane extracted meal	1.28	1.24
			1.19	

g. Analysis of Fatty acids in DHA Canola Meal

The levels of fatty acids were measured in meal samples of DHA canola (GMO) and the parental line AV Jade (CMP) (Table 7). The test material, type of sample, mg/100g and average mg/100g of the two grain crushes are provided for each analyte.

As expected, the most striking result is the drastic reduction in fatty acids amounts in hexane-extracted meals regardless whether it is CMP or GMO meals. In all cases, the amount of fatty acids is less than 5% of that measured in crude oil. Thus, further comparisons of the fatty acid profile of hexane-extracted oil for CMP and GMO would not be meaningful.

[redacted]

Table 7. Fatty acids in meal: DHA canola (GMO) and AV Jade (CMP); Crude and Hexane-extracted meals

	Test Material	Sample	%	Average of both crushes
C14:0 Myristic acid	CMP	Crude meal	0.015	0.018
			0.020	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			

	Test Material	Sample	%	Average of both crushes
C16:0 Palmitic acid	CMP	Crude meal	0.742	0.864
			0.985	
		Hexane extracted meal	0.041	0.042
			0.043	
	[redacted]			
C16:1 n-7 Palmitoleic acid	CMP	Crude meal	0.052	0.056
			0.061	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C16:1 total	CMP	Crude meal	0.070	0.076
			0.081	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C18:0 Stearic acid	CMP	Crude meal	0.340	0.393
			0.446	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			

	Test Material	Sample	%	Average of both crushes
C18:1 n-7 Vaccenic acid	CMP	Crude meal	0.646	0.706
			0.766	
		Hexane extracted meal	0.086	0.080
			0.074	
	[redacted]			
C18:1 n-9 Oleic acid (OA)	CMP	Crude meal	8.754	10.114
			11.473	
		Hexane extracted meal	0.172	0.212
			0.253	
	[redacted]			
C18:1 total	CMP	Crude meal	9.404	10.82
			12.244	
		Hexane extracted meal	0.260	0.164
			0.068	
	[redacted]			
C18:2 n-6 Linoleic acid (LA)	CMP	Crude meal	3.126	3.638
			4.150	
		Hexane extracted meal	0.108	0.118
			0.128	
	[redacted]			

	Test Material	Sample	%	Average of both crushes
C18:2 total	CMP	Crude meal	3.141	3.656
			4.171	
		Hexane extracted meal	0.108	0.118
			0.128	
	[redacted]			
C18:3 n-3 Alpha Linolenic acid (ALA)	CMP	Crude meal	1.670	1.992
			2.313	
		Hexane extracted meal	0.034	0.042
			0.051	
	[redacted]			
C18:3 total	CMP	Crude meal	1.681	2.008
			2.336	
		Hexane extracted meal	0.034	0.042
			0.051	
	[redacted]			
C18:4 n-3 Stearidonic acid (SDA)	CMP	Crude meal	0.013	0.022
			0.031	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			

	Test Material	Sample	%	Average of both crushes
C20:0 Arachidic acid	CMP	Crude meal	0.076	0.088
			0.100	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C20:1 n-9 Gondoic acid	CMP	Crude meal	0.145	0.172
			0.198	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C20:2 n-6 Eicosadienoic acid	CMP	Crude meal	<LOQ	0.07
			0.014	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C20:4 n-3 Eicosatetraenoic acid (ETA)	CMP	Crude meal	<LOQ	0.007
			0.014	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			

	Test Material	Sample	%	Average of both crushes
C20:4 total	CMP	Crude meal	<LOQ	0.008
			0.015	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C22:0	CMP	Crude meal	0.032	0.037
			0.042	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C22:5 n-3 Docosapentiaenoic acid (DPA)	CMP	Crude meal	<LOQ	0.006
			0.012	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C22:5 total	CMP	Crude meal	<LOQ	0.008
			0.015	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			

	Test Material	Sample	%	Average of both crushes
C22:6 n-3 Docosahexaenoic acid (DHA)	CMP	Crude meal	0.028	0.058
			0.087	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
C24:0	CMP	Crude meal	0.018	0.022
			0.025	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
Total Trans Fatty Acids	CMP	Crude meal	<LOQ	0.010
			0.019	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	[redacted]			
FA Total	CMP	Crude meal	15.783	18.336
			20.889	
		Hexane extracted meal	0.474	0.530
			0.585	
	[redacted]			

V. CONCLUSIONS

This report describes the evaluation of various nutritional characteristics and the test methodology utilized for processed fractions of meal and oil crushed from grain of DHA canola (GMO) and the parental AV Jade (CMP). The analytes evaluated are the standard parameters by which many canola processed fractions are measured.

Compositional analysis of meal 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).

Most of the mean values of the crude and hexane-extracted meals are within 10% of each other for most analytes. While some differences were above this 10% level, all were within the ranges usually observed in canola meal. And some differences can be expected; specifically those reflected in the intentionally modified fatty acid profiles.

Tocopherols mean values for hexane-extracted GMO meal was roughly one-half of the mean CMP values. Hexane extraction greatly diminished the tocopherols for both CMP and GMO, and in most cases exhibited greater than a 90% reduction from crude meal.

While the values for the 3 main glucosinolates (glucoalyssin, progoitrin and 4-hydroxyglucobrassicin) did show differences between the CMP and GMO, the range of values overlapped and in every case, the highest value was linked to the CMP. Importantly, the sum of means of these three glucosinolates:

- CMP crude meal =15.55 µmol/g,
- GMO crude meal = 16.04 µmol/g
- CMP hexane-extracted meal = 19.61 µmol/g
- GMO hexane-extracted mean = 18.05 µmol/g.

All of these values are well below the limits included in the definition of canola (30 µmol/g)[4]. Finally, when all the glucosinolates values are combined, the levels remain below this same limit (mean range = 21.14 – 26.49 µmol/g).

As expected, the most striking result is the drastic reduction in fatty acid amounts in hexane-extracted meal, regardless of whether it is CMP or GMO meal. In all cases the amount of fatty

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

acids is less than 5% of that measured in crude meal. Thus further comparisons of the fatty acid profile of hexane-extracted meal for CMP and GMO would not be meaningful.

[redacted]

Because DHA canola expresses seven fatty acid pathway enzymes, it is not surprising that the fatty acids profile is different when the CMP and GMO meals are compared. [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 FiberSOP: **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 chromatographStandards 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