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The development of digestibility coefficient database and the subsequent establishment of NIRS prediction equations for the digestibility of energy, protein and amino acids of rapeseed meal in pigs and broilers chickens

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CONTENTS

1. Abstract

The aim of the study was to establish the variation in chemical composition and amino acid concentration of rapeseed meal (RSM) and develop near infrared reflectance spectroscopy (NIRS) equations to predict ileal and total tract digestibility of RSM for pigs and poultry. Ninety two samples of RSM (representative of the range in crude protein (CP) and neutral detergent fibre (NDF) content of commercially available RSM) were collected over a two-year period, scanned by two NIRS instruments and transferred to AFBI for wet chemistry analysis (CP, NDF and amino acids) and for formulation into pig and broiler diets. NIRS 1 was based at Aunir and was a NIRSystems 6500 spectrophotometer (Foss). NIRS 2 was based at QUB and was an Antaris II FT-NIR instrument (Thermo Fisher Scientific). Pig and broiler diets contained 500g/kg RSM as the only protein source and energy was supplied in the form of maize starch and dextrose. Nitrogen-free diets were formulated to enable measurement of basal endogenous losses of amino acids. For pigs, the diets were offered to eight batches of 12 post value T caecum cannulated pigs (Landrace x Large White) in a partially balanced, eight period changeover design. For broilers, diets were offered to 15 batches of 64 male broilers (Ross 308) over 15 experimental trials. Each batch contained two controls (the N-free diet and RSM3) in order to compare results between batches. Therefore, there were eight diets offered to eight birds in each batch. Apparent ileal digestibility (AID) of dry matter (DM) and amino acids for pigs and broilers was determined as was standardised ileal digestibility (SID) of amino acids using the basal endogenous losses to calculate. Total tract digestibility of DM, energy and NDF were also determined for pigs and broilers.

There was wide variation in CP, NDF and amino acid content of RSM available for use in animal feed in the UK indicating the need for a means to accurately predict the nutritive value of RSM when offered to pigs and broilers. NIRS was found to accurately predict CP, NDF and lysine content of RSM and has the potential to predict the content of other amino acids. Digestibility co-efficients (AID, SID and total tract) were also highly variable with variation in some parameters over 70%, again highlighting the requirement for an alternative to average “book” values within formulation packages. A large database of digestibility values (AID, SID and total tract) for RSM has been established. NIRS has the potential to predict AID and SID of some amino acids (particularly lysine in pigs and threonine in broilers) with ratio to prediction (RPD) values being greater than >1.5. However, it must be noted that the RPD values were generally <1.5 and, as such, suggest that the NIRS prediction calibration is unsatisfactory. Future work should focus on those particular amino acids where NIRS prediction of digestibility resulted in RPD values of greater than 1.5. These NIRS prediction equations could be further developed by increasing the dataset. Measured basal endogenous losses were successfully determined and were in line with published values which will provide a useful basis for future research on different feed ingredients.

2. Introduction

The inclusion of rapeseed meal (RSM) in diets for broilers and pigs has received much attention as RSM is viewed as an attractive home-grown partial alternative to soyabean meal for both species. Several workers have conducted inclusion rate trials with varying conclusions and it is difficult to compare the studies due to a wide range of inclusion rates and "quality" of RSM used. For example, Szterk *et al.* (1997) reported that up to 100 g/kg of RSM could be included in diets for finishing broilers, whereas Richter *et al.* (1996) found that diets containing as little as 50 g/kg reduced performance. In addition, broiler and pig diets are often formulated based on total amino acid content of ingredients. However, a number of workers have observed improved animal responses when diets are formulated based on digestible amino acid content (e.g. Fernandez *et al*., 1995; Perttila *et al.*, 2002; Eklund *et al.*, 2012, Landero *et al.*, 2011 and 2012; Seneviratne *et al.*, 2010 and 2011). There is a dearth of information available on the variability and extent of digestible amino acid content of RSM and formulations are based on average "book" values for digestible amino acid content. There is often wide variation in these book values and they have been derived under different methodologies, including prediction from total amino acid content. Ravindran *et al.* (2005) highlighted the need for a database of apparent ileal amino acid digestibility coefficients to ensure accurate formulation and subsequent optimal performance. A large-scale database of nutrient digestibility coefficients (including amino acid digestibility coefficients) in RSM has not been developed. Furthermore, due to the variability in quality of available RSM there is a need for an accurate means to predict the digestibility of a given RSM sample to ensure accurate ration formulation. Near infrared reflectance spectroscopy (NIRS) is widely used and trusted in the animal feed industry to predict the chemical composition of many raw materials. Initiated in 1964 for moisture content in grain, it has been used for rapid analysis of mainly moisture, protein and fat content of a wide variety of agricultural and food products (Davies and Grant, 1987; Gunasekaran and Irudayaraj, 2001). With respect to oilseed rape, it has been used extensively to assess oil and protein contents (Zhang *et al.*, 2012) but it has yet to be applied comprehensively to assign nutritional quality of RSM, with only limited studies by Chen *et al.* (2011) on RSM and amino acid content and by Petsico *et al.* (2010) who used it to determine protein, oil and glucosinolates contents of whole oilseed rape. Given the ability of NIRS to accurately predict the chemical composition of RSM and its success with regard to wheat quality for broilers and silage for ruminants (Ball *et al.,* 2016 and Park *et al.*, 1998), it is possible that NIRS could be used to establish an accurate prediction test of the nutritional value of RSM for pigs and poultry.

This report presents the results from part of a major study which aimed to:

1. Establish the variation in chemical quality, glucosinolate level and amino acid concentration of RSM and develop NIRS equations to predict energy, crude protein and amino acid digestibility (lysine, methionine and threonine) of RSM for pigs and poultry.

2. Establish a database of digestibility coefficients of RSM for the development of NIRS equations above.

3. Materials and methods

3.1. Screening, selection and analysis of RSM samples

A selection matrix was developed based on the CP and NDF content of 517 RSM samples which were processed by Cargill UK between May 2012 and May 2013. The proximate analysis profile of these samples as determined by in-house NIRS prediction equations is presented in Table 1. Using the results from Table 1, the selection matrix was formed to collect 90 samples representative of the range in CP and NDF content of commercially available RSM (Table 2). These were collected over a two-year period, scanned by two NIRS instruments and transferred to AFBI for wet chemistry analysis and for formulation into pig and broiler diets. NIRS 1 was based at Aunir and was a NIRSystems 6500 spectrophotometer (Foss, Hillerød, Denmark). The RSM samples were scanned in duplicate in a coarse transport quarter cell over the wavelength range 400-2498nm with readings taken at 2nm gaps. The scans were analysed using the Foss Chemometrics software Win ISI4. The mathematical treatment of standard normal variate and detrend (SNVD), first derivative, gap of 4 and smooth of 4 was applied. Modified partial least squares regression was performed on the data set on the range 400 nm – 2500nm and NIRS calibration and validation statistics generated to predict CP, NDF amino acid content and digestibility co-efficients and compared with analysed values obtained through wet chemistry and pig and broiler trials.

NIRS 2 was based at QUB and was an Antaris II FT-NIR instrument (Thermo Fisher Scientific, Dublin, Ireland). Each sample was added to a sample cup with a spinning capability in the Integrating Sphere module of the instrument. The samples were analysed in triplicate following a background scan for each, and 32 scans were acquired at each analysis at a resolution of 16cm^{-1} in the NIR range, 4000-9000cm^{-1}. The spectra were then further analysed using a quantitative calibration database from Aunir (Towcester, England). These calibrations included Group 20 High Protein High Oil, and Group 30 High Protein Low Oil. Quantitative nutritional results were then obtained from these calibrations for each sample to result in two predictions from NIRS 2. NIRS 2A was Group 20 High Protein High Oil and NIRS 2B was Group 30 High Protein Low Oil.

Sub-samples were taken of the 90 RSM samples and analysed for glucosinolate profile at the James Hutten Institute according to the method developed by Bennett *et al.* (1994).

The selected 90 samples were analysed for DM, CP and NDF according to AOAC methods (AOAC 1990) and for amino acid content (Biochrom 30+ ion-exchange chromatography system).

3.2. Determination of total tract and ileal digestibility of RSM in pigs

3.2.1. Experimental design and diets

All procedures described were approved by the AFBI Animal Welfare Ethical Review Body and were conducted under the Animal Scientific Act (1986). RSM was included at 500g/kg, mixed with maize starch and dextrose to produce the test diets. An N-free maize starch/dextrose diet was formulated to determine basal endogenous losses (Stein *et al.,* 2006). Titanium dioxide was included at 4g/kg in all diets as an indigestible marker (Table 3). RSM was milled through a 5mm screen (hammer mill), diet ingredients were mixed together in a Hobert mixer for at least 30 minutes and offered as meal.

The diets were offered to eight batches of 12 post valve T caecum cannulated (PVTC) pigs (Landrace X Large white; average initial weight 33kg) in a partially balanced, eight period change over design. Each RSM diet was offered to eight pigs and therefore there were eight replicates per RSM. The N-free diet was offered four times to eight pigs and therefore, there were 32 replicates. PVTC was carried out according to the procedure of Van Leeuwen *et al.* (1991), and TiO_2 was included as an indigestible marker. Pigs were fed daily at 08:30 and 16:00 h for the duration of the experiment and given a fixed allowance for the eight day period. Feed allowance for the period was calculated at 4% of bodyweight at the beginning of the period (Pedersen and Lindberg, 2010). Each period consisted of a 4-day pre-feed, followed by a 2-day faeces collection and 2-day x 7.5-h collections of ileal digesta. Samples of faeces and ileal digesta were oven dried at 80^{o}C, milled through a 1mm screen and analysed.

Diet samples were analysed for DM, gross energy, crude protein, NDF and TiO2 (AOAC 1990 and Peddie *et al.,* 1982). Amino acid content of diets were calculated by using the amino acid content of the RSM sample. Faeces samples were analysed for DM, CP, NDF and gross energy and ileal samples were analysed for DM, TiO2 and amino acid content (as per diets and RSM).

3.3. Determination of total tract and ileal digestibility of RSM in broilers

3.3.1. Experimental design and diets

All procedures described were approved by the AFBI Animal Welfare Ethical Review Body and were conducted under the Animal Scientific Act (1986). RSM was included at 500g/kg, mixed with maize starch and dextrose to produce the test diets. An N-free maize starch/dextrose diet was formulated to determine basal endogenous losses (Stein *et al.,* 2006). Titanium dioxide was included at 4g/kg in all diets as an indigestible marker (Table 4). RSM was milled through a 5mm screen (hammer mill), diet ingredients were mixed together in a Hobert mixer for at least 30 minutes and offered as meal.

The diets were offered to 15 batches of 64 male broilers (Ross 308) over 15 experimental trials. Each batch contained two controls (the N-free diet and RSM3) in order to compare results between batches. Therefore, there were eight diets offered to eight birds in each batch. This resulted in eight replicates per RSM sample (and 120 replicates for N-free and RSM3 diets). For each experimental trial, 100 male broiler chicks were obtained at hatching from Moy Park Ltd (Dungannon, Northern Ireland). They were placed in a commercial brooder for 7 d with *ad libitum* access to water and a crumbled starter diet (Hi-Grain Chick Crumbs, John Thompson and Sons, Belfast). At 7 d, all birds were weighed and the heaviest and lightest discarded, leaving 64 to be allocated to treatment diet and metabolisable cage according to a pre-determined randomisation based on liveweight. Birds were placed in the individual wire metabolism cages at an initial room temperature of 33^{o}C, reduced by 1^{o}C every 2d down to 24^{o}C. The light:dark cycle was 18:6 h and relative humidity was set at 50%. All birds were offered water and feed (crumbled starter diet *ad libitum*) from 7-18d. At 18d, birds which were allocated to RSM diets were offered standard crumbled starter diet and the appropriate RSM treatment diet on a 50:50 mix until 21d. From 21-25d, birds were offered the appropriate RSM treatment diet as a pre-feed period and a total excreta collection was made from 25-28d for determination of apparent metabolisable energy (AME) and DM, NDF and energy digestibility. The individual bird excreta were collected daily and stored at 4^{o}C. At the end of the balance collection period, the excreta were weighed and then oven-dried at 80^{o}C. The sample weights were allowed to equilibrate and the sample was then milled through a hammer mill fitted with a 0.75mm screen and stored for subsequent analysis. Birds allocated to the N-free diet were offered the diet from 25-28d. At 28d, the birds were humanely killed by dislocation of the spinal cord and the contents of the ileum collected to determine ileal digestibility of DM and amino acids. Ileal digesta was freeze dried and milled as for excreta samples.

Diet samples were analysed for DM, energy, crude protein, NDF and TiO_2 (AOAC 1990 and Peddie *et al.,* 1982). Amino acid contents of diets were calculated by using the amino acid content of the RSM sample. Excreta samples were analysed for DM, CP, NDF and gross energy and ileal samples were analysed for DM, TiO_2 and amino acid content (as per diets and RSM).

3.4. Calculations

Total tract and AID co-efficients of the diet were determined using TiO2 as an indigestible marker according to the equations of Stein *et al.* (2006). SID co-efficients of amino acids were calculated from AID and measured endogenous losses (Table 7), also according to Stein *et al.* (2006). As RSM was the only source of amino acids and NDF in the test diets, these values also represent the digestibility for each RSM sample. The total tract energy digestibility of each RSM sample can be calculated from the total tract energy digestibility of the diet by the difference method which assigns known energy to the other energy contributing dietary ingredients (dextrose, maize starch and

sugar). These ingredients have been calculated to have a total tract digestibility co-efficient of 0.85 and 0.82 for pigs and broilers, respectively (Hilton *et al.*, 1987).

Table 1. Proximate analysis (%) of 517 RSM samples processed by Cargill UK (as predicted by NIRS)

	Moisture	Oil B	Protein	Fibre	Starch	Sugar	NDF
Average	11.1	3.2	35.9	10.9	6.2	8.3	26.8
Minimum	8.2	1.9	34.2	9.9	4.8	6.9	22.7
Maximum	15.2	6.9	37.6	12.4	7.8	9.6	30.0
SD	0.54	0.35	0.50	0.45	0.30	0.37	0.86

Table 2. Selection matrix to target 90 RSM samples for pig and broiler trials (n=92 selected)

		Crude Protein (%)					
NDF content (%)		34.2-35.4	35.4-35.6	35.6-35.8	35.8-36.0	36.0-36.4	36.4-37.6
	22.7-26.2	5 (0)	5 (2)	6 (1)	4 (0)	4 (0)	0 (1)
	26.2-26.5	2 (0)	2 (1)	2 (0)	2 (2)	2 (0)	1 (0)
	26.5-26.8	2 (2)	2 (1)	2 (2)	3 (0)	2 (1)	1 (0)
	26.8-27.1	2 (0)	2 (0)	1 (1)	3 (2)	2 (4)	0 (2)
	27.1-27.5	2 (2)	1 (0)	2 (1)	2 (2)	4 (1)	3 (1)
	27.5-28.0	1 (2)	1 (0)	1 (0)	2 (0)	3 (2)	4 (3)
	28.0-30.0	0 (43)	0 (2)	1 (0)	1 (1)	1 (1)	3 (9)

Numbers in brackets indicate actual samples received

Table 3. Ingredient and formulated nutrient composition (g/kg) of experimental pig diets

	RSM sample	N-Free

Ingredient inclusion (g/kg)		
Rapeseed meal	500	0
Dextrose	160	500
Limestone flour	2	3.2
Di-calcium phosphate	12	22
Salt	4	4
Soya Oil	60	20
Maize starch	256	402
Titanium dioxide (TiO_2)	4	4
Calcined Magnesite (MgO)	0	1
Potassium Carbonate (K_2CO_3)	0	4
Vitacel R200 (99.5% cellulose)[a]	0	40
Vitamin and mineral premix[b]	2.5	2.5
Formulated nutrient content (g/kg)		
Crude protein	168.5	28.7
Crude fibre	64.3	0.72
Oil B	81.5	43.4
Digestible energy (MJ/kg)	14.58	14.87

[a] J. Rettenmaier and Sohne GmbH, Rosenberg, Germany.

[b] Supplied per kg of diet: 160000IU of vitamin A; 40000IU of vitamin D3; 32mg iodine as calcium iodate; 5333mg of iron as ferrous sulphate; 133.33mg of selenium as sodium selenite; 960mg of copper as cupric sulphate; 967.74mg of manganese as manganese oxide; 109500mg of calcium as calcium carbonate, 28000mg of sodium, 35000mg phosphorus, 7900mg of lysine, 6500mg of methionine.

Table 4. Ingredient and formulated nutrient composition (g/kg) of experimental broiler diets

	RSM sample	N-Free
Ingredient inclusion (g/kg)		
Rapeseed meal	500	0
Dextrose	100	420
Limestone flour	8	12
Di-calcium phosphate	18	22
Salt	1	3
Soya Oil	60	30
Maize starch	300	450
Titanium dioxide (TiO_2)	3	3
Sodium bicarbonate (Na_2CO_3)	5	5
Vitacel R200 (99.5% cellulose)[a]	0	50
Vitamin and mineral premix[b]	5	5
Formulated nutrient content (g/kg)		
Crude protein	176.5	3.1
Crude fibre	59.3	50.0
Oil B	66.0	28.6
Metabolisible energy (MJ/kg)	11.9	14.1

[a] J. Rettenmaier and Sohne GmbH, Rosenberg, Germany.

[b] Supplied per kg of diet: 2000000IU of vitamin A; 600000IU of vitamin D3; 8000mg of vitamin E; 400mg/kg of vitamin K; 400mg of vitamin B1, 1500mg of vitamin B2, 2400mg of pantothenic acid; 600mg of pyridoxine; 9000mcg of vitamin B12; 6000mg of niacin; 300mcg of folic acid; 30000mcg of biotin; 30000mg of choline chloride; 2400mg of copper as cupric sulphate; 18000mg of manganese as manganese oxide; 300mg iodine as calcium iodate; 40mg of selenium as sodium selenite; 6000mg of iron as ferrous sulphate; 100mg of molybdenum as sodium molybdate; 800mg of copper (cupric chelate of amino acid); 700mg of manganese (manganese chelate of amino acid); 1000mg of zinc (zinc chelate of amino acid); 731000mg of calcium as calcium carbonate; 35000mg of magnesium.

4. Results

4.1 RSM chemical composition

The minimum, mean and maximum values for CP, NDF and amino acid content for the 92 RSM samples as analysed and as predicted by NIRS are presented in Table 5. Analysed CP content ranged from 351.1 to 425.2g/kg DM and predicted values were slightly higher and ranged from 372.0 to 439.2g/kg DM. The three NIRS prediction equations resulted in similar values for predicted CP with similar SDs. Analysed NDF content ranged widely from 233.6 to 595.9g/kg DM, but predicted ranges for NDF were lower as were predicted mean values (Table 5). Table 6 presents the relationship between the analysed and predicted chemical composition of RSM using the three NIRS scans. The relationship between analysed CP and NIRS-predicted CP are highly significant ($P<0.001$) although the R^2 are not particularly strong ($R^2=0.58$, 0.47 and 0.38) for NIRS 1, NIRS 2A and NIRS 2B, respectively. The relationship between analysed NDF and NIRS predicted NDF are also highly significant ($P<0.001$) for NIRS 1 and NIRS 2B ($R^2=0.31$ and 0.27) but not for NIRS 2A ($P=0.243$, $R^2=0.40$).

Table 5. Minimum, mean, maximum and standard deviation values of analysed and predicted CP, NDF and lysine content (g/kg DM) of RSM samples (n=92)

	Minimum	Mean	Maximum	Standard deviation
Analysed content (g/kg)				

Crude protein	351.1	389.0	425.2	14.1
NDF	233.6	411.2	595.9	73.5
Indispensable amino acids				
Arginine	15.0	21.7	31.0	2.56
Histidine	8.56	9.76	11.89	0.702
Isoleucine	12.78	14.8	17.9	0.927
Leucine	22.67	25.7	31.1	1.59
Lysine	13.9	18.5	24.1	1.95
Methionine	6.7	7.4	9.1	0.417
Phenylalanine	13.0	15.2	19.9	1.32
Threonine	15.6	16.8	20.1	0.736
Valine	16.4	19.0	24.1	1.18
Dispensable amino acids				
Alanine	15.0	16.4	20.1	0.896
Asparate	25.2	27.5	33.8	1.72
Cysteine	7.8	9.1	11.4	0.630
Glutamate	56.3	63.2	76.0	4.10
Glycine	17.2	19.2	23.3	1.04
Proline	16.2	22.0	33.6	2.47
Serine	15.0	16.4	19.9	0.883
Tyrosine	8.22	9.76	11.9	0.784
Predicted content (g/kg)				
Crude protein (NIRS 1)	379.7	401.1	439.4	11.8
Crude protein (NIRS 2A)	375.9	406.8	435.0	13.0
Crude protein (NIRS 2B)	372.0	408.1	437.0	12.7
NDF (NIRS 1)	265.4	318.5	350.0	15.1
NDF (NIRS 2A)	328.9	357.7	426.6	24.1
NDF (NIRS 2B)	292.9	360.3	458.9	55.1
Lysine (NIRS 1)	15.2	18.7	21.8	1.3

Table 6. Relationship (R^2) between analysed CP, NDF and lysine of RSM samples and NIRS predicted content (n=92)

	Analysed vs. NIRS 1	Analysed vs. NIRS 2A	Analysed vs. NIRS 2B

	R^2	P	R2	P	R2	P
Crude protein	0.580	<0.001	0.473	<0.001	0.378	<0.001
NDF	0.310	<0.001	0.400	0.246	0.266	<0.001
Lysine	0.144	<0.001	Not predicted			

4.2 Determination of endogenous losses in pigs and broilers

Using the N-free diet, basal endogenous losses of amino acids were determined for pigs and broilers (Table 7). The highest endogenous losses for indispensable amino acids were for threonine in both pigs and broilers (1.330 and 2.457g/kg DMI respectively) and the lowest losses for indispensible amino acids were for methionine in pigs (0.226g/kg DMI) and histidine in broilers (0.075g/kg DMI). In terms of dispensable amino acids, the highest losses were for proline in pigs (7.657g/kg DMI) and glutamate in broilers (4.50g/kg DMI). The lowest losses for dispensable amino acids in pigs were for cysteine in pigs (0.556g/kg DMI) and tyrosine in broilers (1.010g/kg DM).

Table 7. Endogenous losses (g/kg DMI) as determined for pigs and poultry using N-free diets

	Pigs	Broilers
Indispensable amino acids		
Arginine	0.819	1.578

Histidine	0.272	0.075
Isoleucine	0.848	1.635
Leucine	1.356	2.375
Lysine	1.064	2.354
Methionine	0.226	0.673
Phenylalanine	0.855	1.388
Threonine	1.330	2.457
Valine	1.291	2.278
Dispensable amino acids		
Alanine	1.390	1.645
Asparate	1.944	3.510
Cysteine	0.556	1.275
Glutamate	2.642	4.500
Glycine	2.632	2.317
Proline	7.657	2.572
Serine	1.021	2.253
Tyrosine	0.650	1.010

4.3 Determination of total tract and ileal digestibility of RSM in pigs

A wide range in AID was observed across the 92 samples (Table 8). Minimum values for some parameters were below 0.3 and maximum values were all above 0.8. The SD values were high and were reflective of the variability of individual RSM digestibility. Similar variability was observed for SID, with wide ranges for all parameter (Table 9). Again, the high SD values were reflective of the variability of individual RSM digestibility at the ileal level. The range in total tract DM and energy digestibility was lower (0.461 to 0.898 and 0.606 to 0.807 for DM and energy, respectively) than for NDF digestibility (0.201 to 0.758). The SD values indicate that total tract DM and energy digestibility were the least variable parameters, whereas NDF digestibility was the most variable parameter (Table 10).

Table 8. Apparent ileal digestibility (AID) of DM and amino acids in pigs (n=92)

	Minimum	Mean	Maximum	Standard Deviation
Ileal DM digestibility	0.210	0.631	0.829	0.076

Indispensable amino acids				
Arginine	0.422	0.818	0.913	0.069
Histidine	0.194	0.798	0.899	0.099
Isoleucine	0.156	0.633	0.829	0.105
Leucine	0.357	0.703	0.861	0.088
Lysine	0.258	0.658	0.899	0.105
Methionine	0.160	0.782	0.899	0.105
Phenylalanine	0.378	0.725	0.898	0.088
Threonine	0.274	0.594	0.826	0.097
Valine	0.257	0.619	0.848	0.095
Dispensable amino acids				
Alanine	0.213	0.604	0.822	0.106
Asparate	0.279	0.607	0.846	0.109
Cysteine	0.182	0.629	0.821	0.101
Glutamate	0.493	0.740	0.886	0.077
Glycine	0.167	0.550	0.818	0.134
Serine	0.305	0.634	0.848	0.096
Tyrosine	0.276	0.664	0.885	0.092

Table 9. Standardised ileal digestibility (SID) of amino acids in pigs

	Minimum	Mean	Maximum	Standard Deviation
Indispensable amino acids				

Arginine	0.499	0.896	0.991	0.064
Histidine	0.247	0.855	0.955	0.098
Isoleucine	0.279	0.750	0.945	0.103
Leucine	0.467	0.811	0.970	0.086
Lysine	0.354	0.774	1.021	0.097
Methionine	0.225	0.844	0.959	0.104
Phenylalanine	0.503	0.839	1.018	0.085
Threonine	0.432	0.756	0.995	0.095
Valine	0.391	0.757	0.994	0.093
Dispensable amino acids				
Alanine	0.338	0.777	0.994	0.104
Asparate	0.431	0.751	0.996	0.105
Cysteine	0.320	0.754	0.952	0.099
Glutamate	0.579	0.826	0.975	0.075
Glycine	0.448	0.828	1.102	0.130
Serine	0.435	0.761	0.968	0.094
Tyrosine	0.418	0.800	1.028	0.09

Table 10. Total tract digestibility in pigs

	Minimum	Mean	Maximum	Standard Deviation
DM	0.461	0.758	0.898	0.051
Energy	0.606	0.741	0.807	0.052
NDF	0.201	0.480	0.758	0.099

*Energy digestibility of RSM calculated assuming other ingredients have an energy digestibility of 0.85

4.4 Determination of total tract and ileal digestibility of RSM in broilers

Table 11 presents the AID of RSM in broilers. The range in values were closer than for pigs, with all minimum values being higher than 0.4. However, there were still wide variations in the AID of individual RSM for DM and indispensable and dispensable amino acids. Similar variability is observed for SID of amino acids (Table 12). Total tract digestibility was least variable for DM and energy (Table 13). DM total tract digestibility ranged from 0.466 to 0.937 with mean co-efficient being 0.850. Energy digestibility ranged from 0.463 to 0.844, with the mean being 0.809. NDF digestibility was highly variable and ranged from 0.174 to 0.831, with the mean being 0.613. The SD values reflect the variability in total tract digestibility of individual RSM samples.

Table 11. Apparent ileal digestibility (AID) of DM and amino acids in broilers

	Minimum	Mean	Maximum	Standard Deviation
Ileal DM digestibility	0.387	0.654	0.873	0.076
Indispensable amino acids				
Arginine	0.395	0.794	0.946	0.076
Histidine	0.524	0.739	0.749	0.041
Isoleucine	0.474	0.699	0.847	0.068
Leucine	0.436	0.734	0.893	0.085
Lysine	0.486	0.677	0.905	0.072
Methionine	0.458	0.769	0.956	0.112
Phenylalanine	0.422	0.734	0.838	0.083
Threonine	0.415	0.624	0.792	0.075
Valine	0.447	0.678	0.823	0.070
Dispensable amino acids				
Alanine	0.498	0.731	0.928	0.073
Asparate	0.471	0.669	0.823	0.070
Cysteine	0.401	0.619	0.900	0.087
Glutamate	0.475	0.783	0.870	0.075
Glycine	0.481	0.682	0.831	0.068
Proline	0.411	0.641	0.862	0.085
Serine	0.443	0.654	0.889	0.078
Tyrosine	0.501	0.696	0.826	0.076

Table 12. Standardised ileal digestibility (SID) of amino acids in broilers

	Minimum	Mean	Maximum	Standard Deviation
Indispensable amino acids				

Arginine	0.604	0.942	1.098	0.070
Histidine	0.539	0.755	0.766	0.042
Isoleucine	0.700	0.919	1.049	0.067
Leucine	0.629	0.922	1.085	0.084
Lysine	0.733	0.930	1.104	0.062
Methionine	0.547	0.948	1.121	0.127
Phenylalanine	0.631	0.921	1.051	0.085
Threonine	0.685	0.922	1.086	0.074
Valine	0.684	0.923	1.063	0.072
Dispensable amino acids				
Alanine	0.698	0.934	1.099	0.073
Asparate	0.720	0.929	1.075	0.069
Cysteine	0.681	0.906	1.131	0.083
Glutamate	0.631	0.929	1.027	0.075
Glycine	0.710	0.926	1.082	0.068
Proline	0.637	0.883	1.108	0.077
Serine	0.701	0.934	1.114	0.075
Tyrosine	0.714	0.906	1.044	0.071

Table 13. Total tract digestibility in broilers

	Minimum	Mean	Maximum	Standard Deviation
DM	0.466	0.850	0.937	0.056
Energy*	0.463	0.809	0.844	0.061
NDF	0.174	0.613	0.831	0.125

*Energy digestibility of RSM calculated assuming other ingredients have an energy digestibility of 0.82

4.5 NIRS prediction of amino acid content in RSM

A glossary of terms used to assist in the interpretation of NIRS statistics are presented in Table 14.

The calibration and validation statistics for the prediction of amino acid content in RSM are presented in Table 15. The relationship between analysed and predicted parameters were reasonably robust with R^2>0.6, except for threonine, alanine, proline, serine and tyrosine (R^2=0.365, 0.574, 0.043, 0.483 and 0.500, respectively). The SEC and SECV were low for some parameters. It is generally accepted that SECV as % of mean below 5% is indicative of a good relationship between analysed and predicted. SECV as % of mean was above 5% for arginine, proline and tyrosine but below 5% for the prediction of the other amino acid contents. Several of the RPD calibration values were above

1.5, which suggests calibrations are able to distinguish between high and low values in the dataset. RPD value (2.1) for arginine content was above 2, which indicates that the prediction equation is quantitative. The highest RPD calibration value was observed for lysine content (2.5), and this suggests that the NIRS calibration prediction for lysine is good. The independent cross validation statistics showing the strength of relationship between analysed and predicted amino acid content in RSM are presented in Table 16. Relationships (R^2) were weakened when compared with the calibration statistics, with arginine and lysine having the strongest relationship between analysed and predicted (R^2 = 0.788 and 0.715, respectively). RPD validation values were also the best for these amino acids (1.83 and 1.79, respectively). The majority of SEP as % of the mean values were below 5%.

4.6 NIRS prediction of ileal and total tract digestibility in pigs

The calibration and validation statistics for the prediction of AID of DM and amino acids in pigs are presented in Table 17. The relationship between analysed and predicted parameters were reasonably robust with R^2>0.6, except for cystine and tyrosine AID (R^2 = 0.515 and 0.453, respectively). SEC and SECV were low for some parameters. SECV as % of mean was below 5% for arginine, methionine, phenylalanine and glutamate AID, but above 5% for DM AID and the AID of other amino acids. The majority of RPD calibration values were below 1.5, which suggests calibrations are unsatisfactory. Only RPD values for isoleucine, lysine, methionine, valine, alanine and glycine AID were greater than 1.5, indicating that while these calibrations are poor, they can distinguish between high and low values in the dataset. The independent cross validation statistics showing the strength of relationship between analysed and predicted AID of DM and amino acids are presented in Table 18. Relationships (R^2) were weakened when compared with the calibration statistics, with serine and DM AID having the strongest relationship between analysed and predicted (R^2 = 0.757 and 0.714, respectively). Only DM, arginine, leucine and glutamate AID predictions resulted in SEP as % of mean below 5% and the majority of RPD prediction values were low, suggesting poor NIRS prediction of AID in pigs.

Table 19 presents the calibration and validation statistics for the prediction of SID of amino acids in pigs. In general, the relationship between analysed and predicted SID assessed in terms of R^2 was quite robust with the majority of R^2 values being greater than 0.6. Also, SEC and SECV as % of mean were low for SID of arginine, histidine, methionine, phenylalanine, theronine, glutamic, serine and tyrosine. However, the RPD calibration values were low (<1.5), indicative of an unsatisfactory calibration prediction equation. Only the calibration predictions for SID of isoleucine, lysine, threonine, alanine, asparate, glycine and serine (RPD calibration >1.5) were able to distinguish between high and low values in the dataset. When independent cross validation statistics were applied to SID in pigs (Table 20) relationship between analysed and predicted weakened (R^2>0.6 for

only six parameters and SEP as % of mean <5% for only five parameters). All RPD prediction values were below 1.5, which suggests that the NIRS prediction is unsatisfactory.

The relationship between analysed and predicted total tract digestibility in pigs (Table 21) was reasonably robust in terms of R^2 (>0.6) and low SECV as % of mean for DM and energy digestibility (2.9 and 3.3%, respectively). However, the RPD calibration values were low. The independent cross validation statistics showing the strength of relationship between analysed and predicted total tract digestibility of DM, energy and NDF in pigs are presented in Table 22. As for ileal digestibility, relationships weakened upon cross validation (R^2<0.5). While SEP as % of the mean remained low (<5%) for DM and energy digestibility, the RPD values (all <1.5) indicated an unsatisfactory NIRS prediction.

4.7 NIRS prediction of ileal and total tract digestibility in broilers

The calibration and validation statistics for the prediction of AID of DM and amino acids in pigs are presented in Table 23. The majority of relationships between analysed and predicted AID (as assessed by R^2) were reasonably strong, apart from the relationship between analysed and predicted histidine AID (R^2=0.26). Contradictorily, SECV as % of the mean was only below 5% for histidine AID (3.1%). The highest RPD calibration values were observed for the prediction of AID of glumatate, phenylalanine, tyrosine, arginine, and alanine (1.91, 1.86, 1.55, 1.54 and 1.52, respectively). When independent cross validation statistics were applied to AID in broilers (Table 24), the relationship between analysed and predicted weakened (R^2>0.5 for AID of DM and all indispensable amino acids). SEP as % of mean was >5% for all AID parameters and the majority of RPD validation values were below 1.5, indicating an unsatisfactory NIRS prediction. The highest RPD validation value was for AID of alanine (2.05), which suggests that the NIRS is quantitative.

The relationships between analysed and predicted SID of amino acids in broilers (Table 25) were reasonably robust in terms of R^2 values. The lowest R^2 were observed for histidine and methionine SID (R^2=0.30 and 0.35, respectively). Very few SID predictions resulted in SECV as % of mean of less than 5% (arginine, 5.0% and histidine, 3.2%) and only five RPD calibration values were >1.5 (SID of leucine, phenylanine, alanine, glutamate and serine). When independent cross validation statistics were applied (Table 26), relationships between analysed and predicted SID weakened (R^2<0.6 for 13 of the amino acids). All SEP as % of the mean values were above 5% and only three RPD validation values were >1.5 (SID of threonine (1.53), alanine (1.92), glutamate (1.55) and glycine (1.63).

Table 27 presents the calibration and validation statistics for the prediction of total tract digestibility in broilers. R^2 values for the prediction of DM and energy digestibility were low (<0.1) but high for NDF digestibility (R^2=0.91). SECV as % of mean values were below 5% for DM and energy

digestibility but RPD calibrations values were below 1.5. For NDF digestibility, the SECV as % of mean was high (9.5%) but RPD calibration value was 1.86, which indicated that the NIRS prediction could distinguish between high and low values in the dataset. The independent cross validation statistics showing the strength of relationship between analysed and predicted total tract digestibility in broilers are presented in table 28. Relationships were weakened in terms of R^2 values and all SEP as % of the mean values were greater than 5%. Only the RPD validation for NDF digestibility was above 1.5.

Table 14. Definitions of terms used in NIRS

Term	Definition
N	Number of observations used in final calibration
Mean	Mean of experiential observations
SD	Standard deviation
R^2(RSQ)	Fraction of variance accounted for by the NIR calibration when all accepted observations are included in the relationship (i.e. relationship between actual and predicted)
SEC	Standard error of calibration when all accepted observations are included in the relationship
1-VR	1-Variance ratio – Fraction of variance accounted for NIR prediction when some observations are used for cross validation of the calibration
SECV	Standard error of cross validation when some observations are used for cross validation of the calibration
SECV as % of the mean	Indication of accuracy of calibration, with values of less than 5% being acceptable
RPD calibration (Black 2008)	Ratio of prediction to deviation = SD/SECV RPD<1.5 = calibration unsatisfactory RPD 1.5-2.0 = calibration can distinguish between high and low values RPD 2.0-2.5 = calibration quantitative RPD 2.5-3.0 = calibration predictions good RPD >3.0 = calibration predictions excellent
SEP	Standard error of prediction when some values are used for independent validation
SEP as % of the mean	Indication of accuracy of prediction, with values of less than 5% being acceptable
RPD prediction	Ratio of prediction to deviation = SD/SEP RPD<1.5 = calibration unsatisfactory RPD 1.5-2.0 = calibration can distinguish between high and low values RPD 2.0-2.5 = calibration quantitative RPD 2.5-3.0 = calibration predictions good RPD >3.0 = calibration predictions excellent

Table 15. The calibration and validation statistics for the prediction of amino acid content in RSM

Constituent	N	Mean	SD	Est. Min	Est. Max	SEC	R^2	SECV	SECV as % of the mean	RPD calibration
Indispensable amino acids										
Arginine	73	1.944	0.217	1.295	2.594	0.077	0.874	0.103	5.283	2.108
Histidine	75	0.877	0.055	0.712	1.041	0.029	0.722	0.034	3.913	1.598
Isoleucine	75	1.324	0.071	1.112	1.535	0.038	0.710	0.044	3.308	1.610
Leucine	75	2.307	0.113	1.969	2.645	0.064	0.677	0.076	3.294	1.484
Lysine	75	1.694	0.176	1.167	2.222	0.056	0.899	0.069	4.096	2.535
Methionine	75	0.660	0.030	0.569	0.750	0.023	0.421	0.026	3.941	1.158
Phenylalanine	75	1.367	0.103	1.058	1.675	0.047	0.788	0.054	3.915	1.921
Threonine	76	1.505	0.051	1.351	1.658	0.041	0.365	0.045	2.971	1.145
Valine	74	1.697	0.083	1.449	1.945	0.048	0.666	0.054	3.165	1.540
Dispensable amino acids										
Alanine	73	1.471	0.061	1.288	1.654	0.040	0.574	0.044	2.971	1.396
Asparate	76	2.468	0.128	2.084	2.852	0.070	0.703	0.082	3.335	1.554
Cystine	74	0.813	0.046	0.675	0.951	0.026	0.676	0.030	3.630	1.556
Glutamate	74	5.673	0.323	4.704	6.641	0.153	0.776	0.181	3.185	1.786
Glycine	75	1.723	0.075	1.499	1.948	0.045	0.637	0.051	2.971	1.459
Proline	74	1.976	0.154	1.514	2.438	0.151	0.043	0.154	7.789	1.001
Serine	75	1.470	0.064	1.279	1.661	0.046	0.483	0.051	3.484	1.242
Tyrosine	77	0.881	0.064	0.690	1.072	0.045	0.500	0.047	5.334	1.353

Table 16. Statistics of cross validation for the prediction of amino acid content in RSM

		Means		R^2		SD		RPD
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
Indispensable amino acids								
Arginine	0.111	2.174	1.968	0.788	5.640	0.256	0.203	1.829
Histidine	0.042	0.976	0.885	0.549	4.746	0.070	0.048	1.143
Isoleucine	0.058	1.480	1.336	0.576	4.341	0.093	0.060	1.034
Leucine	0.104	2.566	2.323	0.494	4.477	0.159	0.100	0.962
Lysine	0.099	1.882	1.697	0.715	5.834	0.195	0.177	1.788
Methionine	0.034	0.740	0.665	0.203	5.113	0.042	0.021	0.618
Phenylalanine	0.071	1.524	1.377	0.662	5.156	0.132	0.093	1.310
Threonine	0.063	1.677	1.515	0.159	4.158	0.074	0.031	0.492
Valine	0.080	1.899	1.714	0.513	4.667	0.118	0.070	0.875
Dispensable amino acids								
Alanine	0.067	1.641	1.485	0.359	4.512	0.090	0.046	0.687
Asparate	0.111	2.752	2.489	0.514	4.460	0.172	0.106	0.955
Cystine	0.039	0.905	0.817	0.534	4.774	0.063	0.041	1.051
Glutamate	0.257	6.321	5.722	0.515	4.491	0.410	0.292	1.136
Glycine	0.072	1.924	1.739	0.465	4.140	0.104	0.062	0.861
Proline	0.223	2.204	2.001	0.057	11.14	0.247	0.045	0.202
Serine	0.068	1.637	1.482	0.282	4.588	0.088	0.044	0.647
Tyrosine	0.049	0.976	0.885	0.460	5.537	0.078	0.047	0.959

Table 17. The calibration and validation statistics for the prediction of AID of DM and amino acids in pigs

Constituent	N	Mean	SD	Min	Max	SEC	R^2	SECV	SECV as % of mean	RPD calibration
Ileal DM digestibility	87	0.627	0.056	0.458	0.795	0.024	0.824	0.042	6.656	1.345
Indispensable amino acids										
Arginine	86	0.823	0.042	0.700	0.949	0.022	0.728	0.031	3.760	1.339
Histidine	82	0.816	0.045	0.682	0.949	0.027	0.632	0.034	4.169	1.309
Isoleucine	85	0.643	0.065	0.447	0.839	0.029	0.801	0.043	6.640	1.532
Leucine	86	0.710	0.052	0.554	0.865	0.031	0.644	0.038	5.285	1.384
Lysine	85	0.667	0.066	0.468	0.867	0.036	0.715	0.041	6.174	1.612
Methionine	82	0.798	0.043	0.669	0.927	0.020	0.776	0.026	3.246	1.664
Phenylalanine	87	0.844	0.054	0.682	1.005	0.032	0.639	0.041	4.896	1.305
Threonine	85	0.599	0.062	0.413	0.785	0.030	0.774	0.043	7.228	1.432
Valine	86	0.617	0.064	0.424	0.810	0.033	0.731	0.042	6.875	1.517
Dispensable amino acids										
Alanine	84	0.614	0.063	0.424	0.804	0.035	0.688	0.042	6.775	1.522
Asparate	90	0.610	0.081	0.367	0.853	0.048	0.647	0.057	9.283	1.431
Cystine	83	0.639	0.056	0.471	0.808	0.040	0.515	0.048	7.477	1.174
Glutamate	87	0.743	0.051	0.591	0.896	0.026	0.741	0.035	4.679	1.460
Glycine	85	0.560	0.086	0.302	0.818	0.038	0.800	0.050	8.857	1.732
Serine	88	0.640	0.062	0.456	0.825	0.023	0.858	0.042	6.621	1.453
Tyrosine	84	0.666	0.047	0.526	0.807	0.037	0.453	0.040	5.927	1.185

Table 18. Statistics of cross validation for AID of DM and amino acids in pigs

		Means		R^2		SD		RPD
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
Ileal DM digestibility	0.031	0.631	0.630	0.714	4.921	0.076	0.052	1.677
Indispensable amino acids								
Arginine	0.034	0.818	0.820	0.503	4.146	0.069	0.037	1.088
Histidine	0.085	0.798	0.795	0.113	10.692	0.099	0.037	0.435
Isoleucine	0.045	0.633	0.635	0.612	7.087	0.105	0.059	1.311
Leucine	0.035	0.703	0.704	0.628	4.972	0.088	0.043	1.229
Lysine	0.046	0.658	0.660	0.589	6.970	0.105	0.056	1.217
Methionine	0.092	0.782	0.776	0.059	11.856	0.105	0.038	0.413
Phenylalanine	0.046	0.725	0.724	0.479	6.354	0.088	0.045	0.978
Threonine	0.038	0.594	0.598	0.693	6.355	0.097	0.056	1.474
Valine	0.038	0.619	0.619	0.692	6.139	0.095	0.057	1.500
Dispensable amino acids								
Alanine	0.045	0.604	0.605	0.585	7.438	0.106	0.054	1.200
Asparate	0.047	0.607	0.610	0.659	7.705	0.109	0.066	1.404
Cystine	0.067	0.629	0.625	0.234	10.720	0.101	0.042	0.627
Glutamate	0.030	0.740	0.741	0.680	4.049	0.077	0.044	1.467
Glycine	0.050	0.550	0.553	0.698	9.042	0.134	0.079	1.580
Serine	0.033	0.634	0.637	0.757	5.181	0.096	0.057	1.727
Tyrosine	0.054	0.664	0.662	0.208	8.157	0.092	0.032	0.593

Table 19. The calibration and validation statistics for the prediction of SID of amino acids in pigs

	N	Mean	SD	Est. Min	Est. Max	SEC	R^2	SECV	SECV as % of mean	RPD calibration
Indispensable amino acids										
Arginine	86	0.902	0.038	0.789	1.015	0.020	0.725	0.030	3.316	1.264
Histidine	80	0.874	0.042	0.748	1.001	0.024	0.676	0.041	4.701	1.024
IsoLeucine	85	0.760	0.064	0.569	0.950	0.034	0.706	0.041	5.357	1.560
Leucine	86	0.817	0.051	0.665	0.969	0.031	0.635	0.037	4.553	1.360
Lysine	85	0.783	0.061	0.599	0.967	0.034	0.687	0.040	5.160	1.520
Methionine	83	0.860	0.044	0.729	0.990	0.021	0.766	0.029	3.374	1.500
Phenylalanine	87	0.844	0.054	0.682	1.005	0.032	0.639	0.041	4.896	1.305
Threonine	84	0.761	0.061	0.577	0.945	0.034	0.684	0.040	5.216	1.544
Valine	86	0.755	0.063	0.565	0.945	0.033	0.728	0.042	5.603	1.494
Dispensable amino acids										
Alanine	86	0.783	0.064	0.591	0.975	0.035	0.706	0.042	5.415	1.509
Asparate	87	0.759	0.075	0.535	0.983	0.044	0.650	0.048	6.381	1.543
Cystine	83	0.765	0.056	0.597	0.933	0.040	0.493	0.046	6.003	1.220
Glutamate	87	0.829	0.050	0.679	0.979	0.026	0.737	0.035	4.174	1.442
Glycine	84	0.840	0.081	0.596	1.083	0.043	0.716	0.051	6.015	1.610
Serine	85	0.769	0.060	0.588	0.950	0.027	0.800	0.035	4.563	1.721
Tyrosine	84	0.802	0.045	0.666	0.938	0.031	0.532	0.036	4.489	1.258

Table 20. Statistics of cross validation for SID of amino acids in pigs

		Means		R^2		SD		RPD
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
Indispensable amino acids								
Arginine	0.031	0.896	0.897	0.522	3.456	0.064	0.033	1.065
Histidine	0.083	0.855	0.852	0.154	9.742	0.098	0.036	0.434
IsoLeucine	0.052	0.750	0.752	0.471	6.915	0.103	0.053	1.019
Leucine	0.035	0.811	0.812	0.613	4.310	0.086	0.041	1.171
Lysine	0.044	0.774	0.776	0.565	5.670	0.097	0.051	1.159
Methionine	0.085	0.844	0.839	0.175	10.131	0.104	0.039	0.459
Phenylalanine	0.040	0.839	0.839	0.552	4.768	0.085	0.043	1.075
Threonine	0.045	0.756	0.759	0.582	5.929	0.095	0.051	1.133
Valine	0.037	0.757	0.757	0.691	4.888	0.093	0.055	1.486
Dispensable amino acids								
Alanine	0.043	0.777	0.778	0.608	5.527	0.104	0.055	1.279
Asparate	0.048	0.751	0.753	0.620	6.375	0.105	0.062	1.292
Cystine	0.069	0.754	0.751	0.177	9.188	0.099	0.041	0.594
Glutamate	0.030	0.826	0.827	0.671	3.628	0.075	0.043	1.433
Glycine	0.056	0.828	0.831	0.587	6.739	0.130	0.071	1.268
Serine	0.039	0.761	0.763	0.645	5.111	0.094	0.056	1.436
Tyrosine	0.053	0.800	0.799	0.236	6.633	0.095	0.033	0.623

Table 21. The calibration and validation statistics for the prediction total tract digestibility in pigs (NB. Energy digestibility of diet)

	N	Mean	SD	Est. Min	Est. Max	SEC	R^2	SECV	SECV as % of mean	RPD calibration
DM	85	0.757	0.028	0.674	0.840	0.017	0.635	0.022	2.946	1.247
Energy	87	0.759	0.033	0.662	0.858	0.020	0.628	0.025	3.290	1.304
NDF	85	0.473	0.080	0.233	0.712	0.041	0.734	0.052	10.900	1.548

Table 22. Statistics of cross validation for total tract digestibility in pigs (NB. Energy digestibility of diet)

		Means		R^2		SD		RPD validation
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
DM	0.025	0.758	0.756	0.409	3.307	0.051	0.022	0.880
Energy	0.025	0.759	0.757	0.491	3.303	0.052	0.026	1.040
NDF	0.064	0.480	0.472	0.470	13.559	0.099	0.070	1.094

Table 23. The calibration and validation statistics for the prediction of AID of DM and amino acids in broilers

	N	Mean	SD	Est. Min	Est. Max	SEC	R^2	SECV	SECV as % of mean	RPD calibration
Ileal DM digestibility	83	0.635	0.050	0.485	0.786	0.039	0.388	0.044	6.862	1.149
Indispensable amino acids										
Arginine	80	0.788	0.076	0.561	1.015	0.033	0.815	0.049	6.246	1.539
Histidine	79	0.741	0.026	0.664	0.818	0.022	0.255	0.023	3.145	1.099
Isoleucine	79	0.690	0.075	0.467	0.914	0.035	0.775	0.055	8.010	1.347
Leucine	80	0.727	0.087	0.464	0.989	0.038	0.807	0.059	8.107	1.484
Lysine	75	0.668	0.070	0.459	0.876	0.041	0.649	0.049	7.385	1.412
Methionine	76	0.742	0.109	0.413	1.070	0.077	0.506	0.102	13.766	1.071
Phenylalanine	76	0.723	0.087	0.461	0.985	0.035	0.844	0.047	6.497	1.857
Threonine	79	0.613	0.082	0.368	0.859	0.045	0.703	0.057	9.310	1.433
Valine	77	0.672	0.074	0.450	0.894	0.034	0.795	0.055	8.197	1.345
Dispensable amino acids										
Alanine	81	0.718	0.081	0.475	0.962	0.031	0.859	0.054	7.449	1.518
Asparate	78	0.660	0.074	0.439	0.881	0.030	0.839	0.057	8.606	1.299
Cystine	81	0.610	0.089	0.343	0.877	0.042	0.781	0.070	11.514	1.268
Glutamate	78	0.776	0.080	0.538	1.015	0.036	0.797	0.042	5.371	1.906
Glycine	80	0.672	0.075	0.447	0.896	0.035	0.786	0.060	8.861	1.257
Proline	79	0.633	0.089	0.365	0.901	0.039	0.812	0.063	9.926	1.424
Serine	79	0.643	0.081	0.400	0.886	0.045	0.687	0.057	8.896	1.414
Tyrosine	77	0.686	0.076	0.458	0.913	0.045	0.650	0.049	7.118	1.551

Table 24. Statistics of cross validation for AID of DM and amino acids in broilers

		Means		R^2		SD		RPD val
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
DM	0.048	0.654	0.640	0.280	7.500	0.076	0.033	0.688
Indispensable amino acids								
Arginine	0.057	0.794	0.783	0.523	7.280	0.076	0.070	1.228
Histidine	0.039	0.739	0.733	0.196	5.321	0.041	0.014	0.359
Isoleucine	0.058	0.699	0.687	0.493	8.443	0.068	0.068	1.172
Leucine	0.063	0.734	0.722	0.556	8.726	0.085	0.080	1.270
Lysine	0.049	0.677	0.670	0.556	7.313	0.072	0.055	1.122
Methionine	0.080	0.769	0.739	0.490	10.825	0.112	0.080	1.000
Phenylalanine	0.063	0.734	0.721	0.542	8.738	0.083	0.081	1.286
Threonine	0.056	0.624	0.614	0.551	9.121	0.075	0.071	1.268
Valine	0.056	0.678	0.670	0.497	8.358	0.070	0.069	1.232
Dispensable amino acids								
Alanine	0.037	0.731	0.717	0.799	5.160	0.073	0.076	2.054
Asparate	0.035	0.669	0.658	0.777	5.319	0.070	0.068	1.943
Cystine	0.058	0.619	0.608	0.606	9.539	0.087	0.082	1.414
Glutamate	0.054	0.783	0.773	0.586	6.986	0.075	0.072	1.333
Glycine	0.041	0.682	0.670	0.708	6.119	0.068	0.067	1.634
Proline	0.062	0.641	0.630	0.562	9.841	0.085	0.084	1.355
Serine	0.055	0.654	0.642	0.553	8.567	0.078	0.070	1.273
Tyrosine	0.060	0.696	0.686	0.430	8.746	0.076	0.064	1.067

Table 25. The calibration and validation statistics for the prediction of SID of amino acids in broilers

	N	Mean	SD	Est. Min	Est. Max	SEC	R^2	SECV	SECV as % of mean	RPD calibration
Indispensable amino acids										
Arginine	81	0.937	0.068	0.734	1.141	0.028	0.826	0.047	4.993	1.449
Histidine	76	0.756	0.026	0.678	0.834	0.022	0.300	0.024	3.187	1.079
Isoleucine	79	0.907	0.069	0.700	1.114	0.042	0.634	0.049	5.435	1.398
Leucine	80	0.915	0.088	0.652	1.177	0.037	0.820	0.057	6.277	1.524
Lysine	76	0.922	0.055	0.759	1.086	0.038	0.520	0.048	5.160	1.145
Methionine	77	0.917	0.125	0.542	1.293	0.101	0.350	0.126	13.680	0.997
Phenylalanine	75	0.912	0.089	0.645	1.178	0.032	0.870	0.045	4.980	1.958
Threonine	78	0.914	0.077	0.682	1.146	0.034	0.806	0.053	5.810	1.454
Valine	76	0.919	0.073	0.702	1.137	0.034	0.783	0.054	5.852	1.349
Dispensable amino acids										
Alanine	81	0.922	0.081	0.678	1.165	0.034	0.829	0.054	5.815	1.515
Asparate	77	0.918	0.072	0.703	1.133	0.046	0.596	0.055	5.979	1.306
Cystine	79	0.899	0.084	0.646	1.151	0.037	0.804	0.059	6.544	1.432
Glutamate	79	0.922	0.078	0.687	1.156	0.028	0.874	0.045	4.871	1.742
Glycine	79	0.918	0.072	0.701	1.136	0.033	0.790	0.054	5.848	1.348
Proline	79	0.874	0.082	0.628	1.120	0.037	0.794	0.061	6.970	1.348
Serine	77	0.926	0.075	0.702	1.150	0.038	0.739	0.048	5.128	1.573
Tyrosine	77	0.898	0.069	0.692	1.104	0.041	0.642	0.046	5.100	1.498

Table 26. Statistics of cross validation for SID of amino acids in broilers

		Means		R^2		SD		RPD
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
Indispensable amino acids								
Arginine	0.050	0.942	0.932	0.565	5.365	0.070	0.062	1.240
Histidine	0.040	0.755	0.748	0.170	5.348	0.042	0.015	0.375
Isoleucine	0.052	0.919	0.903	0.504	5.759	0.067	0.057	1.096
Leucine	0.062	0.922	0.910	0.567	6.813	0.084	0.080	1.290
Lysine	0.049	0.930	0.925	0.374	5.297	0.062	0.040	0.816
Methionine	0.106	0.948	0.914	0.312	11.597	0.127	0.077	0.726
Phenylalanine	0.067	0.921	0.907	0.514	7.387	0.085	0.084	1.254
Threonine	0.047	0.922	0.912	0.637	5.154	0.074	0.072	1.532
Valine	0.058	0.923	0.915	0.478	6.339	0.072	0.068	1.172
Dispensable amino acids								
Alanine	0.039	0.934	0.920	0.771	4.239	0.073	0.075	1.923
Asparate	0.052	0.929	0.918	0.481	5.664	0.069	0.057	1.096
Cystine	0.066	0.906	0.896	0.474	7.366	0.083	0.081	1.227
Glutamate	0.047	0.929	0.918	0.676	5.120	0.075	0.073	1.553
Glycine	0.040	0.926	0.914	0.722	4.376	0.068	0.065	1.625
Proline	0.060	0.883	0.871	0.531	6.889	0.077	0.077	1.283
Serine	0.057	0.934	0.921	0.503	6.189	0.075	0.067	1.175
Tyrosine	0.057	0.906	0.897	0.385	6.355	0.071	0.058	1.018

Table 27. The calibration and validation statistics for the prediction total tract digestibility in broilers (NB. Energy digestibility of the diet)

	N	Mean	SD	Est. Min	Est. Max	SEC	R^2	SECV	SECV as % of mean	RPD calibration
DM digestibility	76	0.860	0.021	0.798	0.922	0.020	0.073	0.021	2.384	1.010
Energy digestibility	77	0.872	0.025	0.797	0.948	0.024	0.067	0.025	2.855	1.012
NDF digestibility	81	0.611	0.108	0.288	0.933	0.033	0.909	0.058	9.468	1.860

Table 28. Statistics of cross validation for total tract digestibility in broilers (NB. Energy digestibility of the diet)

		Means		R^2		SD		RPD
	SEP	Analysed	Predicted		SEP as % of mean	Analysed	Predicted	
DM	0.043	0.850	0.849	0.017	5.065	0.056	0.007	0.163
Energy	0.047	0.863	0.861	0.059	5.459	0.061	0.007	0.149
NDF	0.061	0.613	0.601	0.747	10.150	0.125	0.109	1.787

5. Discussion

5.1 RSM chemical composition

The actual CP content of the RSM samples in this study were within reported ranges in the literature (351-425g/kg DM, average 389g/kg DM). Maison *et al.* (2015) reported an average CP content of 416g/kg DM and a range between 369 and 437g/kg DM (based on a sample size of 17). The sample size in this study was large (n=92) and presumably reflected the range in chemical composition of RSM available for the feed industry. NDF content for the RSM samples in Maison *et al.* (2015) ranged from 274-386 g/kg DM, average 340g/kg DM, which was a narrower range than in the current study (234-596g/kg DM, average 411g/kg DM). This can be explained by the large sample set in the current study and also by the planned selection of samples containing higher levels of NDF. Overall, the range in content of amino acids of RSM samples in this study was similar to reported values but average values were somewhat lower. For this study average lysine content was 18.5g/kg DM whereas McDonald *et al.,* 1995, Khajali and Slominski 2012 and Grageola *et al.,* 2013 reported average lysine content to be 23.9, 22.2 and 19.4g/kg DM, respectively.

There are few studies in the literature to compare NIRS predictions of CP and NDF content in RSM as the majority of work has been conducted on intact seeds (Chen *et al.,* 2011). However, Fontaine *et al.* (2001) reported that the CP and amino acid content of RSM could be accurately predicted by NIRS. In addition, Daszykowski *et al.* (2008) have developed prediction models to determine the CP, fibre and oil content of RSM in Polish crushing plants, with prediction errors of less than 5%. Similarly, Aunir have developed in-house prediction models to determine a range of chemical constituents including CP, NDF and lysine (NIRS1). These models were then used on the QUB NIRS instrument as described in the materials and methods to predict CP and NDF (NIRS2A and NIRS2B). When simple regression analysis was conducted to determine the relationship between analysed and predicted values, significant ($P<0.001$) correlation was observed for NIRS1 and NIRS2B. This indicated that NIRS has the ability to accurately predict chemical composition. Splitting the dataset into two groups (high protein, high oil and high protein, low oil) resulted in significant relationships between actual and predicted CP for both groups but only for the high protein, low oil group for NDF content. The relationships achieved by NIRS2A and NIRS2B showed that the prediction equations developed using a FOSS instrument (Aunir) can be successful transferred to an Antaris instrument (QUB), which strengthens the commercial application of these prediction equations. However, splitting the dataset into two groups based on CP and oil content did not result in stronger correlation between actual and predicted and suggested that the original prediction equations were adequate to predict a wide range of CP and NDF contents in RSM.

The prediction of lysine content using the Aunir in-house equations was positively related ($P<0.001$) to analysed lysine content but the relationship as described by R^2 was weak (0.144). Given the

importance of lysine in pig and broiler nutrition, accurate prediction of both lysine content and lysine digestibility in raw ingredients is crucial to optimal ration formulation and ultimately optimal animal performance. While this weak (although significant) relationship between analysed and predicted lysine content demonstrates the potential for NIRS to predict lysine content in RSM, it was recognised that more work was required to develop the prediction equation for lysine content. NIRS prediction of lysine content was not a primary objective of this current study but as all RSM were analysed for lysine and other amino acids, it was possible to develop prediction equations from the current dataset. While none of the prediction equations for any of the amino acids could be described as particularly "good" in that the ratio of prediction to deviation (RPD) values were not high, the high R^2 and low errors (both SECV and SEP) indicate that there is potential for NIRS to accurately predict amino acid content in RSM. The strongest prediction was for lysine content and, as already stated, this is an important amino acid in pig and broiler diets, therefore prediction of content in RSM by NIRS would be a significant step forward in accurate ration formulation. Chen *et al.* (2011) conducted a study which investigated the use of NIRS in predicating the analysed and relative amino acid content in intact rapeseed. These workers reported "excellent" calibration predictions based on RPD values (>3.0) for the majority of actual amino acid content (including lysine). However, a criticism of their work is that there was no independent validation conducted, which would further test the prediction equations for each amino acid. In the current study, independent validation was conducted and while this lowered the R^2 and RPD values, this exercise fully tested the calibration equations. The prediction of lysine content remained the strongest and, with an RPD value of greater than 1.5, it can be concluded that the current prediction has limited value and could be of use in ration formulation. Perhaps a greater number of samples to add to the calibration and validation dataset would yield stronger prediction equations. The dataset tested by Chen *et al.* (2011) contained more than 200 rapeseed samples, whereas only a maximum of 92 RSM samples were used in this current study.

5.2 Determination of endogenous losses in pigs and broilers

Endogenous losses have been measured in several studies using a N-free diet formulated from varying quantities of sugar, maize starch and a fibre source. Values for endogenous losses in both pigs and broilers are highly variable across studies (e.g. Moter and Stein 2004, Stein *et al.,* 2006, Grageola *et al.,* 2013, Woyengo *et al.,* 2015 and Liu *et al.,* 2016 for pigs; Golian *et al.,* 2008, Valencia *et al.,* 2009, Kong and Adeola, 2013 and Adedokum *et al.,* 2014 for broilers). The values obtained in this current study fall within the ranges reported by others. Endogenous losses can be affected by feed intake, the ratio of sugar to starch in the diet and the age of the animal (Moter and Stein, 2004, Adedokun *et al.,* 2014) and it is important that these factors are consistent when determining endogenous losses to calculate SID from AID. It was therefore, necessary to determine endogenous losses in this study rather than simply using a literature value, as this ensured that the endogenous losses were applicable to the level of feed intake applied and the animal used in the study. For pigs

and broilers the highest loss for indispensible amino acids was for threonine and this is in keeping with what is reported in the studies listed above. It can therefore be concluded that endogenous losses were accurately determined for pigs and broilers in this study. These values can be used in other studies on different feed ingredients, thus the measurement of endogenous losses in this study has resulted in the establishment of standard values which will reduce the numbers of animals required for *in vivo* studies in future which follows the same scientific protocol.

5.3 The digestibility of RSM by pigs and broilers

While there are several reports in the literature on the digestibility of RSM in pigs and broilers, there are no studies which have evaluated a large number of RSM samples to produce a dataset of digestibility co-efficient and to examine the variability of RSM digestibility for pigs and broilers as has been achieved in this current study. Several studies in the literature have compared the digestibility of RSM with other sources of protein (e.g. soyabean meal, fish meal or rapeseed cake) and report average values for RSM digestibility. The average values quoted in these studies correlate well with the average values determined in this study. For example, Liu *et al.* (2016) list pig AID and SID values for amino acids in conventional and high protein canola meal (double"00" rapeseed meal) in comparison with soyabean meal. These researchers observed no difference in AID and SID between the two rapeseed meals but found that AID and SID of rapeseed meal was lower than for soyabean meal. There was no assessment of the variability in RSM digestibility. Similarly, Grageola *et al.* (2013) compared AID and SID of rapeseed meal and rapeseed cake and, in general, average AID and SID values for amino acids correlated well with those observed in this study. For broilers, average AID values observed in this study were slightly lower than values reported by other workers (e.g. Toghyani *et al.,* 2015 and Kasprzak *et al.,* 2016) and average SID values slightly higher (e.g. Kim *et al.,* 2012). However, again, variability in digestibility co-efficients has not been well established in broilers. The development of the large dataset of AID and SID co-efficients in this current study highlights the variability in nutritive value of RSM for pigs and broilers and the need for an accurate means to predict this variability.

Maison *et al.* (2015) identified the limited knowledge available on total tract RSM digestibility of energy and fibre and conducted a study to determine digestibility co-efficients in six samples of canola meal, 11 samples of 00-RSM and five samples of 00-RSM expellers. On average, values for energy and NDF digestibility corresponded with average values in this study (0.729 vs. 0.741 and 0.556 vs. 0.480 for energy and NDF digestibility, respectively). The average value for energy digestibility in RSM for pigs also was in keeping with that reported by Woyengo *et al.,* 2015. The wide range in digestibility for both energy and NDF observed in this study has not been reported previously, although some variability in energy digestibility has been observed (Maison *et al.,* 2015). This variability has been attributed to differences in processing methods but, as all RSM samples in this study were crushed at the same plant (Cargill UK), this cannot explain the wide variability

observed. The variability is most likely due to genetic differences in RSM meal and variations in growing and harvesting conditions. Indeed, inherent variability within wheat and other raw materials used in diet production is well documented (Ball *et al.,* 2013) and again highlights the need for a means to predict digestibility of RSM rather than the use of "book" values in diet formulation.

5.4 NIRS prediction of RSM digestibility in pigs and broilers

NIRS has been shown to be accurate in predicting the nutritive value of forages for ruminants and of wheat for broilers (Park *et al.*, 1998 and Ball *et al.,* 2016) but little work has been published on the use of NIRS to predict the digestibility of RSM in pigs and broilers. In this current study, the relationships between analysed and predicted digestibility co-efficients were reasonably strong and in some cases the errors associated with prediction both for cross-validation (SECV) and independent validation (SEP) were low and as a % of the mean, less than 5%. However, a low error value does not necessarily indicate a strong prediction equation, and ratio of prediction to deviation (RPD) is regarded as the criterion for judging the strength of prediction (Black *et al.,* 2008). Unfortunately, none of the RPD values for with cross-validation or independent validation were above 2.0 which would suggest that the prediction equation was of quantitative value. However, RPD values for several digestibility parameters were above 1.5 which indicates that the prediction equation can distinguish between high and low values in a dataset and may be of some value. Chen *et al.* (2011) suggested that while values of above 1.5 and below 2.0 have limited prediction strength, the results may still be included in breeding programmes for oil seed rape and therefore the digestibility co-efficients with RPD values of above 1.5 in the independent validation in this study should be considered for further development. For pigs, this would include AID of DM, glycine and serine but none for SID. For broilers, this would include AID of alanine, glycine and asparate and SID of threonine, alanline, glutamate and glycine (with SID of threonine being of the most interest as it is an indispensable amino acid).

5.5 Conclusions

- There is wide variation in CP and NDF content of RSM available for use in animal feed in the UK.
- NIRS can accurately predict CP, NDF and lysine content of RSM and has the potential to predict other amino acid content.
- Endogenous losses in pigs and broilers were successfully determined, providing a useful basis for future research on other feed ingredients.
- The variability in ileal and total tract digestibility of RSM in both pigs and broilers is huge and supports the requirement for an alternative to "book" values in feed formulations.
- NIRS has the potential to predict AID and SID of some amino acids (particularly threonine in broilers) but prediction calibrations are not robust.

- The NIRS prediction equations could be further developed by increasing the dataset.

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APPENDIX

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Glucosinolate profiling of oil seed rape meal

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This report presents the findings of the glucosinolate analysis component of WP2.

CONTENTS

Executive Summary

Glucosinolates are glucosides, consisting of a central carbon bound to a thioglucose group via a sulphur atom and to a sulphate group via a nitrogen atom (i.e. *b*-thioglucoside-*N*-hydroxysulphates (*cis*-*N*-hydroximinosulphate esters)). The central carbon is also bound to a side (R) group which varies between the different glucosinolates and is responsible for their structural and functional variation. The huge diversity of R groups has resulted in the characterisation of approaching 200 structurally distinct glucosinolates.

Classically glucosinolate content has been determined by destructive techniques, whereby degradative levels are measured by colourimetric approaches. Methods have also been developed with HPLC-UV approaches, however, due to the huge diversity of glucosinolates and the challenge of chromatographically resolving them, as well as the fact that UV alone cannot differentiate the glucosinolates structurally, the HPLC-UV approach results in over estimation of glucosinolate concentration where different species co-elute by HPLC.

Therefore, HPLC-MS methods were developed since MS analysers are capable of differentiating the co-eluting glucosinolates based upon differences in their molecular weight and their MS-MS fragmentation patterns.

As a first step, a non-targeted accurate mass based HPLC-MS method was developed employing a Thermo Orbitrap XL MS system. The method was applied to the oil seed rape meal samples where 14 confirmed glucosinolates (Gluconapin, Progoitrin, Glucobrassicanapin Sinalbin, Sinigrin, Glucoraphanin, Gluconasturtiin, Glucotropaeolin, Glucoerucin, Glucobrassicin, Neoglucobrassicin, Methoxyglucobrassicin, Hydroxyglucobrassicin and Gluconapoleiferin) were identified. By employing quality assurance (reference) samples, the method was validated and showed typically lower than 10% RSD values for the majority of the measured glucosinolates over an analytical run of 150 analyses.

Since the non-targeted approach only provides a relative comparison of glucosinolate levels on an arbitrary scale, a more targeted quantitative approach was developed based upon an HPLC-triple quadrupole (QqQ)-MS instrument. HPLC-QqQ-MS is a more sensitive MS analyser, however, its application is limited by the availability of glucosinolate reference standards which are required to develop the HPLC-QqQ-MS methods as well as being applied to peak quantification. The targeted HPLC-QqQ-MS was first developed and validated across a range of concentrations for each available glucosinolate standard with typical limits of detection (LOD) between 3-9 nM and limits of quantification (LOQ) between 5-30 nM. The HPLC-QqQ-MS method was next applied to the full

series of oil seed rape meal samples and calibration curves were generated with serial dilutions of a glucosinolate reference standard cocktail.

In summary, Gluconapin, Progoitrin and Glucobrassicanapin, were found to be the major glucosinolates, with levels approaching tenfold or greater of the minor glucosinolate components. Gluconapin (2.8-106 µM) and Progoitrin (2.5-92 µM) both ranged from approximately 2-100µM and revealed similar trends across the sample set. Samples 13-15, 41-43, 58-61, 73, 97 and 98, classified as being high erucic acid content rape meal samples, showed extremely high levels of Progoitrin (70-92 µM) and Gluconapin (60-107 µM). Samples 1-40 (excluding 13-15) and samples 74-96 showed a concentration range of 15-22 µM Progoitrin and 10-15 µM Gluconapin. Samples 44-64 showed slightly elevated concentration levels of Progoitrin 25-30 µM and Gluconapin 15-25 µM, whereas samples 65-72 showed the lowest concentrations of Progoitrin and Gluconapin ranging between 3-5 µM. Unfortunately, an analytical reference standard was not available for Glucobrassicanapin and thus, fully quantitative values cannot be reported, however, by comparison to the relative abundance of Glucobrassicanapin to the relative abundances of Progoitrin and Gluconapin obtained with the non-targeted profiling method, the concentration range would be estimated to be similar (2-100 µM). Glucobrassicanapin showed identical trends throughout the samples as described above for Progoitrin and Gluconapin.

The minor glucosinolate components, Sinalbin, Sinigrin, Glucoraphanin, Gluconasturtiin, Glucotropaeolin, Glucoerucin, Glucobrassicin, Neoglucobrassicin, Methoxyglucobrassicin, Hydroxyglucobrassicin and Gluconapoleiferin, all ranged in concentration between 0.01-2 µM, with the exception of Sinalbin (0.18-35 µM). The sample trends for the minor glucosinolates, with the exception of Sinalbin, revealed very similar patterns as for the major glucosinolates, with the typical sample concentration range of 0.01-0.8 µM, increasing by two-three folds in the case of the high erucic acid content rape meal samples.

Given the massive elevation in the levels of both major and minor glucosinolates within high erucic acid containing rape meal and the aim of producing low glucosinolate content animal feeds, it perhaps indicates that high erucic acid rape meal should not be processed for animal feed stocks.

If AFBI take the glucosinolate data provided to them and perform comparative analysis with their digestibility and nutrition studies performed in broilers and pigs (or make the full dietary datasets available), then a greater depth of nutritional understanding will be gained in relation to glucosinolate content. Likewise, by comparison of the HPLC-MS glucosinolate results to the NIR spectroscopy data, it may be found that NIR spectroscopy is capable of at least indicating a total glucosinolate level, in which case the technique could be applied as a rapid screening approach to indicate high glucosinolate containing rape meal prior to production of animal feeds.

List of Tables and Figures

Table S3: Glucosinolate cocktail quantification curve R^2, Limits of Detection (LOD) and Limits of Quantification (LOQ) values for the eleven glucosinolates within the targeted method

Figure S4. Calibration curves of the eleven glucosinolates within the reference compound cocktail applied in the targeted quantification method

7. Introduction

Glucosinolates are a natural class of sulphur and nitrogen containing anionic secondary metabolites found almost exclusively within the plant family Brassicaceae (e.g. Cruciferae, Capparidaceae, and Caricaceae). Within the Brassica, the glucosinolates impart a pungent taste due to their breakdown to isothiocyanates (also known as mustard oils) via the action of the enzyme myrosinase. Glucosinolates are generally regarded as the storage form of their biologically active aglycones (isothiocyanates). The glucosinolate contents of plants are highly variable, although in vegetables of the Brassica glucosinolates approximately account for 1% dry weight (Rosa *et al.,* 1997), with their levels rising as high as 10-25% total dry weight in the case of seeds of certain species (O'Hare *et al.*, 2005).

Glucosinolates are derived from the metabolism of glucose and a range of different amino acids. Glucosinolates are glucosides, consisting of a central carbon bound to a thioglucose group via a sulphur atom and to a sulphate group via a nitrogen atom (i.e. *b*-thioglucoside-*N*-hydroxysulphates (*cis*-*N*-hydroximinosulphate esters)). The central carbon is also bound to a side (R) group which varies between the different glucosinolates and is responsible for their structural and functional variation. The R groups are generally classed as alkyl, aromatic, benzoate, indole, multiple glycosylated and sulphur containing side chains, which then may contain double bonds, oxo, hydroxyl, methoxy, carbonyl or di-sulphide linkages (Clarke, 2010). The huge diversity of R groups have resulted in the characterisation of 120 structurally distinct glucosinolates as defined in a 2001 survey (Fahey *et al.,* 2001), with the true number of characterised glucosinolates having since risen to approaching 200 (Clarke, 2010). The synthesis of glucosinolates is under enzymatic control and the structures are derived from both protein and non-protein L-amino acids. There are two major groups: firstly the so called aliphatic glucosinolates which are derived largely from methionine, or its chain-elongated homologues, as well as valine, alanine, leucine and isoleucine, with one example being glucoraphanin which is derived from dihomomethionine; secondly, there are the so-called aromatic glucosinolates, which include the indolic glucosinolates, such as glucobrassicin which is derived from tryptophan, and sinalbin derived from tyrosine, as well as a plethora of other examples derived from phenylalanine and chain elongated homologues such as homophenylalanine (Clarke, 2010).

In-planta, the enzyme myrosinase in the presence of water, cleaves off the glucose group from glucosinolates, with the remaining molecule being rapidly converted to form an isothiocyanate. The isothiocyanates that are metabolised from the glucosinolates represent their active form and are known to serve as defence compounds for the plant and are especially associated with responses against insect herbivory (Hopkins *et al.,* 2009). Certain feeding insects have adapted mechanisms to cope with high glucosinolate containing food sources. The large white butterfly for example

selectively oviposit on glucosinolate containing plants where the compounds have been shown to enhance larval survival, which is believed to be due to their possession of a nitrile specifier protein which diverts glucosinolate metabolism towards nitriles rather than reactive isothiocyanate forms. Other insects have been shown to de-sulphate glucosinolates rendering them unfit for degradation by myrosinase to isothiocyanates. Insects have even been shown to actively sequester glucosinolates and detoxify them within the body tissues or convert them to forms that are toxic against insects competing for the same food sources, illustrating complex evolutionary adaption between multiple insect species and their Brassica food sources (Jansen *et al.,* 2008; Hopkins *et al.,* 2009). Glucosinolates are not just toxic to insects, but have been shown to have negative effects in animals and humans feeding on plant materials that contain them in high concentrations. Certain glucosinolates have been shown to have toxic effects, largely acting as goitrogens, in both humans and animals, although tolerance towards the compounds varies even within species (Anilakumar *et al.,* 2006). Therefore, it is of great importance to study glucosinolate content in both foods destined for human consumption, as well as in glucosinolate rich animal feeds such as oil seed rape meal, and to correlate glucosinolate content with negative and positive dietary and health effects.

Classically glucosinolate content has been determined by destructive techniques, whereby degradative levels are measured by colourimetric approaches. Such methods have been based upon glucose determination, primarily as hexokinase coupled to NADH production and glucose oxidase and peroxidase coupled to various coloured dyes (Tholen *et al.,* 1993), as well as determination of isothiocyanate and benzenedithiol content following cyclocondensation, with either colourimetric assays or HPLC-UV detection (Zhang *et al.,* 1996; Clarke *et al.,* 2010). Near Infra-Red Spectroscopy has also been validated as a non-destructive technique where bond vibrations associated with O–H, C–H and N–H groups are applied as an indication of total glucosinolate content, with the added advantage that total protein and lipid content can also be estimated within a single assay (Clarke, 2010). Determination of total glucosinolate content has also been made via TMS derivatisation of glucosinolates coupled with GC-MS quantification, as well as direct GC-MS quantification of the more volatile and GC amenable isothiocyanates following myrosinase digestion from intact glucosinolates. The disadvantage of such approaches is that they typically provide an indirect measurement of total glucosinolate content and that the individual glucosinolates cannot be differentiated and measured directly and therefore, cannot be differentiated with respect to their positive or negative health or dietary outcomes in the case of human food-stuffs and animal feeds. HPLC-UV approaches have been commonly applied to quantify de-sulphated glucosinolates, but are regarded as not being appropriate for the measurement of individual intact glucosinolates (Francisco *et al.,* 2009). The use of methods that measure the degradative products of glucosinolates by which to infer the quantification of the parent glucosinolates are commonly still in use, however, the specificity and accuracy of such approaches is clearly limited (Clarke, 2010).

HPLC-UV approaches have in the past been applied to the quantification of a small number of glucosinolate targets, and in the case of the current study, could be applied to the oil seed rape meal samples, however the lack of specificity and accuracy of such an approach is a major limitation (Francisco *et al.,* 2009; Clarke, 2010). Such methods based upon UV quantification, assume that the HPLC system can completely resolve each targeted glucosinolate from all other glucosinolates and for that matter from all other compounds that would produce a UV response. Otherwise, if the target glucosinolate is not cleanly resolved through HPLC, then the UV absorbance peak area that it is quantified by could in fact be based upon the UV response to potentially tens of compounds that are co-eluting with the target glucosinolates, reducing specificity and accuracy of the assay. The only way to overcome such lack of specificity is by employing a HPLC detector that is capable of differentiating the various forms of intact glucosinolates, therefore, providing the assay with the desired level of specificity to quantify the many diverse individual glucosinolates. Given the high complexity and very high numbers of potential glucosinolates that have been determined in previous studies (>200; Clarke, 2010), it would in fact, be extremely analytically naïve to have confidence that a HPLC-UV method alone, where UV cannot differentiate the co-eluting glucosinolate and alternative metabolite species, is accurately quantifying each individual target glucosinolate.

The current state-of-the-art in the determination and quantification of glucosinolates is based upon HPLC separation combined with mass spectrometry (MS) based detection (Clarke, 2010). Whilst attempts have been made to apply super-critical fluid LC (Buskov *et al.,* 2000), capillary electrophoresis (Bringmann *et al.,* 2005) and variants of hydrophobic interaction liquid chromatography (HILIC; Troyer *et al.,* 2001; Wade *et al.,* 2007), to glucosinolate analysis, their applications have been limited to date and it is generally considered that applying C_{18} based reversed phase column chemistries, such as the Phenomenex Luna C18 RP column, with formic acid buffered water-acetonitrile mobile phases is the chromatographic method of choice for both the analysis of intact and de-sulphated glucosinolates (Cataldi *et al.,* 2007; de Vos *et al.,* 2007; Rochfort *et al.,* 2008; Clarke, 2010). A large range of MS detectors, from nominal mass accuracy single and triple quadrupoles (Bennett *et al.* . 2004; Song *et al.,* 2005) and ion traps (Cataldi *et al.* . 2007), to time-of-flight (TOF) and quadrupole-TOF MS (Fabre *et al.,* 2007; Rochfort *et al.,* 2008; Jansen *et al.,* 2008), have been applied to the detection of glucosinolates. Accurate mass TOF instruments have been shown to be particularly appropriate for intact glucosinolate analysis, but without MS^2 abilities cannot differentiate isomeric forms. Q-TOF since it has MS^2 capabilities has been further considered to be the most appropriate platform, however, with the recent advances in accurate mass ion trap instruments with MS^n capabilities (i.e. detectors such as the Fourier Transform Ion Cyclotron Resonance MS or Thermo Orbitrap MS), they along with Q-TOF are now regarded as the current state of art in the MS analysis of intact glucosinolates.

Since within the LC-MS analytical facility at the James Hutton Institute, both triple quadrupole MS and Thermo Orbitrap MS detectors are available in conjunction with HPLC, two strategies were developed for the measurement of glucosinolates. The first strategy focused on the application of a non-targeted approach employing HPLC-Orbitrap MS whereby as many different metabolites are measured in conjunction as what can be detected within a typical non-targeted or enriched methanolic extract of oil seed rape meal, inclusive of glucosinolates, amino acids and potential tannins. This non-targeted approach is particularly appropriate in this case since it makes no pre-conceptions as to which glucosinolates will be the major compounds that are detected. As reported, up to 200 glucosinolates structures have been previously characterised across the Brassica (Clarke, 2010), of which many are isomeric and which due to the common core structure of the glucosinolate, produce identical fragmentation patterns in MS^2 analysis, therefore, accurate mass based metabolite profiling was considered as being the optimal approach with which to survey and relatively quantify the large potential diversity of these compounds within oil seed rape meal. Secondly, an attempt was made to develop a more targeted method applying HPLC- triple quadrupole (QqQ) MS where based upon pre-characterised MS^2 fragmentation patterns, specific glucosinolates previously reported in oil seed rape meal were targeted. However, the limitation of this second approach is that certified analytical standards for each targeted glucosinolate must be sourced and applied in method development, thus the approach is restricted to the compounds where these difficult to resource glucosinolate reference standards are available.

8. Materials and methods

Untargeted metabolite profiling analysis

All oil seed rape meal samples were first homogenised to fine powders and a reference (henceforth called Quality Assurance – QA) sample was prepared with an equal mix of all of the individual samples. The samples were extracted in 75% methanol: 24.9% water: 0.1% formic acid. The HPLC-MS analysis were performed with a Thermo Accela 600 HPLC system coupled with a PDA and Orbitrap XL MS system effectively according to the methods of de Vos *et al. .* (2007), only optimised for faster LC separations permitted by applying a Phenomenex C18 core shell column (00F-4462-E0 Kinetex C18 2.6 µm 150 x 4.6 mm 100Ä) (Figure 1a) as opposed to the conventional Phenomenex C18 HPLC column (00F-4251-B0 Luna 3µm 150 x 2 mm 100Ä) (Figure 1b). Data were preliminarilly collected in the full-scan accurate mass MS mode for glucosinolate quantification, MS^2 data were also collected for the QA samples and glucosinolate analytical reference standards to aid unambigiuous identification. HPLC-MS data were chromatographicly aligned and deconvolved, following which glucosinolates were identified against two libraries, the Wageningen University glucosinolate and flavanoid library and a glucosinolate library based upon the 200 characterised Brassica glucosinolates (Clarke, 2010). Based upon HPLC retention time, accurate mass MS measurements and matching of MS^2 spectra (Figure S1), the following glucosinolates and amino

acids were unambiguously identified, Phenylalanine, Tryptophan, Glucotropaeolin, Sinigrin, Progoitrin, Glucoerucin, Sinalbin, Gluconasturtiin, Glucobrassicin and Gluconapin. Compounds were otherwise identified at a putative level on the basis of accurate mass based matching to the molecular formula and compound databases.

Full descriptions of the non-targeted LC-MS analytical methods are provided in supplementary section S1.

Targeted profiling of glucosinolates

With respect to the targeted analytical approach for glucosinolate measurement, the sample preparation was identical to those applied to the non-targeted analysis, only the extract concentration was doubled. HPLC-MS was performed with an Agilent 1260 Infinity HPLC system coupled to an Infinity PDA detector and an Agilent 6460 triple quadrupole mass spectrometry (QQQ-MS) system operated under Agilent MassHunter (Agilent Ltd. Stockport U.K). The HPLC gradient method was identical to that applied to the non-targeted analysis, only once all glucosinolates had eluted from the column, the gradient efficiency was increased to maximise sample throughput (Figure 1c). Mass spectra were primarilly collected in Multiple Reaction Monitoring (MRM) scan mode where a quantification fragment ion (based upon the core glucosinolate fragment ions of *m/z* 259 or 275) and a qualification fragment ion (which where possible was unique to each glucosinolate, or minimally unique to each glucosinolate within their expected retention time range) were monitored (Table S2). The HPLC-QQQ-MS method was applied to both the sample extracts as well as a cocktail of eleven available glucosinolate standards ranging in concentration (0 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, 100 µM), with the standards being applied to produce calibration curves, against which the sample concentration of each monitored glucosinolate was relatively compared for estimates of true concentration (uM).

Full descriptions of the targeted glucosinolate analysis methods are provided in supplementary section S2.

9. Results and Discussion

Non-targeted metabolite profiling results

The oil seed rape meal sample extracts proved to be incredibly rich in metabolic compounds with a total of 822 identified (putatively and unambiguously) metabolites being detected. 111 compounds were annotated as potential glucosinolates, 18 were annotated as amino acids, 18 were annotated as organic acids, 54 were annotated as potential tannins, 13 were annotated as sugars and sugar-derivatives (sugar alcohols, sugar phosphates, polyols), 60 were annotated as potential flavonoids, 335 were annotated as potential lipids (fatty acids, MAGs, DAGs, TAGs, Phospholipids, Ceramides, lipophilic vitamins, sterols, etc.), and a further 213 compounds were placed into a group of varying compound chemistries. Of the 822 metabolites, those that showed a lower than 30% RSD across

the QA samples were taken forward to have their significance considered in relation to the analysed individual oil seed rape meal samples.

As a means to indicate which of the samples of oil seed rape meal were closely related and which showed distinct groupings away from the more typical samples, principal components analysis (PCA) was applied to the normalised peak areas for the 822 metabolites (Figure 2), with the PCA scores presented as a conventional PCA bi-plot (Figure 2a) and as a dendrogram (Figure 2b). PCA indicated a sample cluster formed of samples 41-to-44, 58-to-61, 73, 97 and 98, which were discriminated on the basis of highly elevated (5-20 fold) glucosinolate levels (Figure 2c), and were interestingly later found to be the OSRM samples categorised as being of a high erucic acid content. Given the massive elevation in the levels of glucosinolates within high erucic acid containing OSRM and the aim of producing low glucosinolate content animal feeds, it perhaps indicates that high erucic acid OSRM should not be processed for animal feed stocks.

As a next step, bar charts were generated based upon the normalised peak area response for each individual sample, as well as blank sample controls and the average normalised peak area across all 18 QA sample analyses (with the standard error being indicated by the error bar). In Figure 3 bar charts for the major identified glucosinolates of oil seed rape meal are presented. On the basis of the eleven major glucosinolates detected in the oil seed rape meal sample extracts, Progoitrin, Gluconapin and Glucobrassicanapin, proved to be by far the most dominant species (Table 1 presents the relative concentrations of glucosinolates for all individual samples and Table 2 presents a summary of the relative glucosinolate concentrations). In Figure 4, bar charts are presented for the major amino acids. In Figure 5, total amino acid, total potential tannins, total organic acids, total lipids, total vitamins, total flavanoid, and total sugar and sugar derivative, contents are provided.

Targeted glucosinolate profiling

As a first step, the targeted glucosinolate method was assessed against the cocktail of the eleven defined glucosinolate reference standards. For concentrations of 500 nM and greater, all eleven glucosinolates revealed RSD's that fell below 8% (most typically 0-2%). The calibration curves were next plotted for each of the eleven glucosinolates, R^2 values calculated (all eleven compounds ranged from 0.995-1 R^2) (Table S3), and finally, limits of detection (LOD) and limits of quantification (LOQ) calculated (all eleven glucosinolates showed LOD between 3-9 nM and LOQ between 5-30nM, with the exceptions of glucoiberin, with an LOD of 17 nM and LOQ of 59 nM, and progoitrin, with an LOD of 25 nM and LOQ of 85 nM) (Table S3). On the basis of the application of the targeted glucosinolate quantification method to the cocktails of glucosinolate standards, the developed method is impressive in terms of repeatability and with respect to the calculated R^2, LOD and LOQ values.

Applying the targeted glucosinolate method to the oil seed rape meal extracts, it was possible to cleanly resolve and extract peak areas for seven of the target compounds only. The glucosinolates that could be accurately resolved, deconvolved and peak areas extracted, included gluconapin, gluconasturtiin, glucoraphanin, progoitrin, sinalbin and sinigrin. Unfortunately, due to near co-elution (HPLC peak 'shouldering'; Figure 6) of multiple compounds (which are highly likely to be other closely structurally related glucosinolates), it was not possible to accurately extract (deconvolve) peak areas for the following target glucosinolates; glucoiberin, glucocheirolin, glucotropaeolin, glucoerucin and glucobrassicin. This observation, whilst disappointing with respect to the applicability of the targeted approach, especially given the extremely labour intensive procedure to producing and validating such methods, really does highlight the huge complexity and diversity of glucosinolate compounds within the oil seed rape meal sample matrix, and further validates the application of the non-targeted metabolite profiling method.

The glucosinolates where peak areas could be extracted accurately for the quantification ion (gluconapin, gluconasturtiin, glucoraphanin, progoitrin, sinalbin and sinigrin) were next quantified against the calibration curves produced with the cocktail of defined glucosinolate reference standards and finally bar plots (Figure 7) and a quantification results table (Table 3 presents the relative concentrations of glucosinolates for all individual samples and Table 4 presents a summary of the relative glucosinolate concentrations) were produced. Comparisons between the targeted concentration measurements (Figure 7) and the non-targeted relative peak abundance data (Figure 3) indicated that the two independently generated datasets produced highly complementary results, other than for glucoraphanin which was only detected with the targeted HPLC-MS method most likely due to the greater sensitivity afforded by the QQQ-MS detector.

In summary, Gluconapin, Progoitrin and Glucobrassicanapin, were found to be the major glucosinolates, with levels approaching tenfold or greater of the minor glucosinolate components identified within the rape meal matrix. Gluconapin (2.8-106 µM) and Progoitrin (2.5-92 µM) both ranged from approximately 2-100µM and revealed similar trends across the sample set. Samples 13-15, 41-43, 58-61, 73, 97 and 98, classified as being high erucic acid content rape meal samples, showed extremely high levels of Progoitrin (70-92 µM) and Gluconapin (60-107 µM). Samples 1-40 (excluding 13-15) and samples 74-96 showed a concentration range of 15-22 µM Progoitrin and 10-15 µM Gluconapin. Samples 44-64 showed slightly elevated concentration levels of Progoitrin 25-30 µM and Gluconapin 15-25 µM, whereas samples 65-72 showed the lowest concentrations of Progoitrin and Gluconapin ranging between 3-5 µM. Unfortunately, an analytical reference standard was not available for Glucobrassicanapin and thus, fully quantitative values cannot be reported. However, by comparison to the relative abundance of Glucobrassicanapin to the relative abundances of Progoitrin and Gluconapin obtained with the non-targeted profiling method, the

concentration range would be estimated to be similar (2-100 µM). Glucobrassicanapin showed identical trends throughout the samples as described above for Progoitrin and Gluconapin.

The minor glucosinolate components, Sinalbin, Sinigrin, Glucoraphanin, Gluconasturtiin, Glucotropaeolin, Glucoerucin, Glucobrassicin, Neoglucobrassicin, Methoxyglucobrassicin, Hydroxyglucobrassicin and Gluconapoleiferin, all ranged in concentration between 0.01-2 µM, with the exception of Sinalbin (0.18-35 µM). The sample trends for the minor glucosinolates, with the exception of Sinalbin, revealed very similar patterns as for the major glucosinolates, with the typical sample concentration range of 0.01-0.8 µM, increasing by two-three folds in the case of the high erucic acid contend OSRM samples. Sinalbin showed more variable levels across the sample population typically ranging between 1-15 µM, with the exception of the high erucic acid samples 61 and 13 that showed higher concentrations of 20 and 35 µM, respectively. Given the massive elevation in the levels of both major and minor glucosinolates within high erucic acid containing OSRM and the aim of producing low glucosinolate content animal feeds, it perhaps indicates that high erucic acid OSRM should not be processed for animal feed stocks.

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Table 1: Glucosinolate, amino acid, total flavanoid, total tannin, total lipid, total vitamin and total sugar and sugar derivative, normalised relative abundances as determined through the non-targeted metabolite profiling approach

COMPOUND	IDENTIFICATION LE	RT (MINUTES)	Blank_Sta	Blank_en	QC AVERAGE	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Glucotropaeolin	Unambiguous	9.35	0	0	0.000101293	4.26626E-05	4.20477E-05	3.36605E-05	3.64346E-05	3.49E-05	4.02E-05
Sinigrin	Unambiguous	4.39	0	0	8.6037E-05	0.000117212	7.79564E-05	6.40476E-05	9.5252E-05	6.44E-05	7.68E-05
Progoitrin	Unambiguous	3.63	0	0	0.00930485	0.007996023	0.00771908	0.005268898	0.006053413	0.00587	0.007644
Glucoerucin	Unambiguous	9.59	0	0	0.000364615	0.000308258	0.000321806	0.000243685	0.000262587	0.000252	0.000294
Sinalbin	Unambiguous	5.34	0	0	0.002186724	0.004418272	0.004778518	0.004531285	0.004605116	0.004601	0.005917
Gluconasturtiin	Unambiguous	12.84	0	0	0.001207456	0.001221842	0.001087747	0.000892117	0.000917987	0.00095	0.001092
Glucobrassicin	Unambiguous	10.99	0	0	0.000405905	0.000265847	0.000287226	0.000239178	0.000227821	0.000207	0.000269
Gluconapin	Unambiguous	6.16	0	0	0.011059108	0.009015484	0.008757813	0.007643586	0.008704394	0.007837	0.009009
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	Putative	16.23	0	0	0.000400791	0.000362554	0.000406485	0.000303651	0.000328713	0.000314	0.000355
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	Putative	12.53	0	0	0.000498961	0.000432988	0.000433008	0.000360738	0.000372722	0.000364	0.00043
Glucobrassicanapin	Putative	8.94	0	0	0.007580917	0.004056549	0.003931974	0.003144116	0.003342309	0.003327	0.003781
Gluconapoleiferin	Putative	5.44	0	0	0.002481947	0.001166356	0.001091046	0.000863027	0.001039339	0.000899	0.001073
Hydroxy-glucobrassicin	Putative	7.74	0	0	0.002814123	0.001866928	0.001978569	0.001372831	0.001532499	0.001468	0.001818
TOTAL GLUCOSINOLATES	NA	NA	0	0	0.038492727	0.031270977	0.030913277	0.02496082	0.027518587	0.026188	0.031797
Phenylalanine	Unambiguous	6.77	0	0	0.000132598	0.000131004	0.000146908	0.000164464	0.0001498	0.000145	0.000134
Tryptophan	Unambiguous	9.13	0	0	9.33205E-05	9.7022E-05	0.000104257	0.000116511	0.000110492	9.87E-05	0.000104
Threonine OR homoserine OR O-methylserine;;	Putative	3.15	0	0	1.23762E-05	1.36277E-05	1.58085E-05	1.57077E-05	1.55391E-05	1.52E-05	1.47E-05
Histidine	Putative	2.99	0	0	1.36544E-05	1.68124E-05	1.72373E-05	1.60511E-05	1.52141E-05	1.65E-05	1.78E-05
Valine	Putative	10.29	0	0	9.63089E-06	1.27248E-05	1.20883E-05	9.6598E-06	8.99215E-06	1.05E-05	1.36E-05
Asparagine	Putative	3.12	0	0	0.000210529	0.000187688	0.000203029	0.000223953	0.000201593	0.00019	0.000209
Alanine	Putative	3.16	0	0	5.21344E-06	5.13061E-06	5.44194E-06	6.10352E-06	5.32849E-06	4.92E-06	6.17E-06
Arginine	Putative	2.99	0	0	4.65621E-05	5.52729E-05	5.90324E-05	5.90212E-05	5.61259E-05	5.94E-05	6.1E-05
acetyl-L-Lysine	Putative	4.92	0	0	0.000187466	0.000140317	0.000153857	0.000149088	0.000153468	0.000142	0.000144
TOTAL AMINO ACIDS	NA	NA	0	0	0.000711351	0.000659599	0.00071766	0.000760559	0.000716553	0.000682	0.000704
TOTAL POTENTIAL TANNINS	NA	NA	0	0	0.000841903	0.000829152	0.000746534	0.000866208	0.000796229	0.000978	0.000995
TOTAL LIPIDS	NA	NA	0	0	0.068899202	0.06978161	0.068454223	0.072757251	0.071944862	0.073789	0.06871
TOTAL FLAVANOIDS	NA	NA	0	0	0.017964822	0.017538713	0.017204719	0.016752137	0.017199762	0.016818	0.017915
TOTAL ORGANIC ACIDS	NA	NA	0	0	0.011013946	0.010971147	0.011451853	0.011652388	0.011690566	0.011068	0.011013
TOTAL VITAMINS	NA	NA	0	0	0.002378711	0.001948664	0.001905608	0.001873275	0.00193459	0.001878	0.001959

Table 1 cont.

COMPOUND	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17
Glucotropaeolin	3.93E-05	3.65096E-05	3.267E-05	3.68781E-05	4.48633E-05	3.74281E-05	0.000181422	0.000164119	0.000152435	2.53007E-05	3.46994E-05
Sinigrin	8.81E-05	8.97157E-05	6.683E-05	0.000124759	0.000145076	6.49266E-05	0.000265786	0.000310762	0.000106134	8.17785E-05	7.33726E-05
Progoitrin	0.007322	0.007173984	0.0048347	0.006286737	0.008536056	0.005509278	0.019971964	0.024605025	0.025832651	0.005517438	0.007890942
Glucoerucin	0.000287	0.000268816	0.0002452	0.000268624	0.000327264	0.00026863	0.000858072	0.000867518	0.000841911	0.000241981	0.000258079
Sinalbin	0.005067	0.004795564	0.004293	0.004042624	0.00434407	0.004725129	0.00722902	0.005005936	0.004852398	0.002693376	0.003539922
Gluconasturtiin	0.00107	0.000990078	0.000885	0.001054658	0.001309965	0.00095598	0.002614835	0.002346435	0.0026758	0.001022015	0.001138535
Glucobrassicin	0.000124	0.000263011	0.0002128	0.000281374	0.000192037	0.000230767	0.000754411	0.000237	0.00036557	0.000215487	0.000237835
Gluconapin	0.008977	0.008538031	0.007464	0.008605491	0.009584559	0.007894859	0.02064871	0.023610224	0.023073939	0.008120002	0.009047129
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000356	0.000308044	0.0003005	0.000325683	0.000417762	0.000309503	0.000958986	0.000828391	0.001033261	0.000248248	0.000245315
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000412	0.000405046	0.0003585	0.000382205	0.000500645	0.000387862	0.000930795	0.000981825	0.001068815	0.000335588	0.000352784
Glucobrassicanapin	0.003836	0.003407232	0.0032075	0.00354095	0.004459936	0.003349645	0.01248467	0.011207915	0.010570446	0.003370906	0.003491294
Gluconapoleiferin	0.001162	0.001037381	0.0008608	0.001055122	0.001255378	0.000974307	0.004001434	0.004304345	0.003905627	0.000908116	0.001066331
Hydroxy-glucobrassicin	0.001592	0.001425574	0.0013156	0.001466939	0.001972928	0.001634589	0.00843947	0.009130579	0.008172557	0.001068352	0.001243432
TOTAL GLUCOSINOLATES	0.030333	0.028738988	0.024077	0.027472044	0.033090538	0.026342903	0.079339576	0.083600074	0.082651545	0.023848589	0.02861967
Phenylalanine	0.000138	0.000110925	0.0001439	0.000143417	0.000123941	0.000148331	0.00023938	0.000235918	0.0001939	0.000147416	0.000114378
Tryptophan	0.000106	7.99765E-05	0.0001296	0.000121263	8.75868E-05	0.00011232	0.000148546	0.000134564	0.000117816	0.000114653	9.01712E-05
Threonine OR homoserine OR O-methylserine;;	1.41E-05	1.31862E-05	1.388E-05	1.55943E-05	1.39112E-05	1.5109E-05	1.08027E-05	1.00838E-05	9.27715E-06	1.71442E-05	1.71597E-05
Histidine	1.69E-05	1.93708E-05	1.682E-05	1.42697E-05	1.64426E-05	1.3967E-05	2.60456E-05	1.82584E-05	2.85266E-05	1.37825E-05	1.59847E-05
Valine	1.14E-05	1.29316E-05	8.673E-06	8.52236E-06	1.00314E-05	9.31013E-06	7.04975E-06	6.34048E-06	8.82875E-06	8.7714E-06	1.45786E-05
Asparagine	0.000184	0.000199494	0.0002147	0.000217988	0.000173498	0.000214366	0.000251763	0.000204713	0.00022091	0.000226582	0.000205786
Alanine	4.74E-06	5.99357E-06	5.435E-06	5.98609E-06	5.87118E-06	5.04819E-06	6.13178E-06	3.59051E-06	5.09302E-06	6.04263E-06	7.25768E-06
Arginine	5.7E-05	6.02675E-05	5.51E-05	5.15134E-05	5.24481E-05	5.56442E-05	6.73418E-05	6.42746E-05	6.79665E-05	5.25367E-05	5.49707E-05
acetyl-L-Lysine	0.000137	0.000148951	0.0001616	0.000160142	0.000138852	0.000162255	0.00016293	0.000272889	0.000183727	0.000110862	8.2427E-05
TOTAL AMINO ACIDS	0.000668	0.000651096	0.0007497	0.000738697	0.000622582	0.000736351	0.000919991	0.000950632	0.000836045	0.00069779	0.000602714
TOTAL POTENTIAL TANNINS	0.000943	0.000764196	0.0007965	0.000807647	0.000727506	0.000772329	0.000810331	0.000723515	0.000720296	0.000758241	0.000797016
TOTAL LIPIDS	0.071566	0.069144042	0.068683	0.070281786	0.067721868	0.070958015	0.0444142	0.04827967	0.048163309	0.074499875	0.068688655
TOTAL FLAVANOIDS	0.017242	0.017181586	0.0164449	0.016385016	0.01731784	0.016616951	0.02655686	0.029019942	0.028285186	0.017541631	0.018298022
TOTAL ORGANIC ACIDS	0.010723	0.010323446	0.0121886	0.011845791	0.011085742	0.011392939	0.008094361	0.008325898	0.007552923	0.01371535	0.012145838
TOTAL VITAMINS	0.001914	0.001822162	0.001853	0.001845011	0.001924294	0.001814088	0.001605997	0.001640795	0.001640623	0.001914943	0.001841896

Table 1 cont.

COMPOUND	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28
Glucotropaeolin	2.81032E-05	2.95466E-05	2.36304E-05	2.4354E-05	2.96762E-05	2.37175E-05	3.2039E-05	2.6243E-05	2.68635E-05	2.54677E-05	5.06075E-05
Sinigrin	6.06108E-05	6.04135E-05	6.0946E-05	4.5395E-05	4.4868E-05	9.83473E-05	5.2128E-05	7.96545E-05	6.47972E-05	7.10234E-05	4.09807E-05
Progoitrin	0.00647232	0.006286474	0.007607495	0.00537152	0.005536819	0.006396931	0.00637015	0.005105801	0.006962442	0.006958611	0.007948068
Glucoerucin	0.000261792	0.00024311	0.000239342	0.00021837	0.000252673	0.000233609	0.00026599	0.000251747	0.00023467	0.000247456	0.000247817
Sinalbin	0.002897056	0.002620933	0.003065357	0.00272186	0.001979137	0.002924118	0.00257049	0.002633326	0.002691728	0.00291775	0.001749319
Gluconasturtiin	0.001028171	0.000996637	0.001031648	0.00091627	0.001009361	0.001016514	0.00103436	0.001023191	0.00101272	0.000959679	0.001061868
Glucobrassicin	0.000236966	0.000230644	0.000219847	0.0002019	0.000243227	0.000225898	0.00024106	0.000244475	0.000240105	0.000222622	0.00022519
Gluconapin	0.008852557	0.008389301	0.008608023	0.00828656	0.007194185	0.008329567	0.00943835	0.008590392	0.008776514	0.008683991	0.009435014
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000268238	0.000280043	0.000223758	0.00023969	0.000296229	0.000232197	0.00030509	0.000274517	0.000254176	0.000250895	0.000388703
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000366757	0.000376312	0.000354499	0.00032408	0.000363375	0.000341251	0.00040431	0.000359157	0.000368355	0.000354354	0.000471839
Glucobrassicanapin	0.003485265	0.003501638	0.00328971	0.0031279	0.003570546	0.003296312	0.0040359	0.003343519	0.003379503	0.003446603	0.004392594
Gluconapoleiferin	0.001029196	0.001024691	0.001006666	0.00088232	0.000914179	0.000912887	0.0011663	0.00092605	0.000988657	0.001032821	0.001650516
Hydroxy-glucobrassicin	0.001166478	0.001122881	0.000918349	0.00081821	0.001034733	0.000869871	0.00113718	0.000920492	0.000995886	0.00098777	0.00117492
TOTAL GLUCOSINOLATES	0.02615351	0.025162624	0.026649269	0.02317844	0.022469007	0.02490122	0.02705335	0.023778567	0.025996418	0.026159044	0.028837436
Phenylalanine	0.000143582	0.000143989	0.000107333	0.00014425	0.000139961	0.000138094	0.00013692	0.000154486	0.000140277	0.000140846	0.000132716
Tryptophan	0.000110824	0.000116019	8.88938E-05	0.00012158	0.000121584	0.000114133	0.00011214	0.00012505	0.000116677	0.000106148	9.87505E-05
Threonine OR homoserine OR O-methylserine;;	1.81362E-05	1.69052E-05	1.77843E-05	1.6247E-05	1.81046E-05	1.87676E-05	1.7665E-05	1.77302E-05	1.93278E-05	1.67396E-05	1.20281E-05
Histidine	1.41157E-05	1.33019E-05	1.74992E-05	1.5289E-05	1.76108E-05	1.62982E-05	1.444E-05	1.49987E-05	1.58902E-05	1.55606E-05	1.0512E-05
Valine	9.14108E-06	9.21393E-06	1.28681E-05	8.919E-06	9.59671E-06	1.02341E-05	8.4339E-06	9.64833E-06	9.5439E-06	9.53315E-06	8.81869E-06
Asparagine	0.000236226	0.00021868	0.000223691	0.00024109	0.00026619	0	0.0002264	0.000285948	0.000230453	0.000238684	0.000193975
Alanine	5.5503E-06	5.99124E-06	6.98767E-06	6.6189E-06	5.73403E-06	6.1171E-06	6.3099E-06	7.22265E-06	5.44978E-06	6.20463E-06	4.89941E-06
Arginine	5.42177E-05	4.75577E-05	5.56605E-05	5.0775E-05	5.39903E-05	5.39009E-05	5.0987E-05	5.392E-05	5.13617E-05	5.09363E-05	3.97263E-05
acetyl-L-Lysine	0.000120904	0.000119339	8.48917E-05	0.00011174	0.000121372	0.000104269	0.00012848	0.000115981	0.000116936	0.000111196	0.000200565
TOTAL AMINO ACIDS	0.000712697	0.000690997	0.000615609	0.00071651	0.000754143	0.000461815	0.00070178	0.000784985	0.000705916	0.000695848	0.000701991
TOTAL POTENTIAL TANNINS	0.000747892	0.000784402	0.00075105	0.00080422	0.000731089	0.000807314	0.00083261	0.000766778	0.000773149	0.000797191	0.000730138
TOTAL LIPIDS	0.069411276	0.070378985	0.068165211	0.07108691	0.074033583	0.070586959	0.06933729	0.070319022	0.070216982	0.070971372	0.07173742
TOTAL FLAVANOIDS	0.017621047	0.017253729	0.018744077	0.01679913	0.016636688	0.017925366	0.01797458	0.017115412	0.01777369	0.01693244	0.017382777
TOTAL ORGANIC ACIDS	0.013898007	0.013867585	0.012193421	0.01406317	0.014451328	0.014153664	0.01356551	0.014322017	0.014234425	0.013221868	0.01296069
TOTAL VITAMINS	0.001868122	0.001847045	0.001889008	0.00183175	0.001962266	0.001918237	0.00195473	0.001879418	0.001906089	0.001822797	0.002058155

Table 1 cont.

COMPOUND	Sample 29	Sample 30	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40
Glucotropaeolin	5.13592E-05	5.42533E-05	4.84965E-05	4.23841E-05	5.27916E-05	5.75377E-05	5.7E-05	4.82E-05	4.8E-05	4.79119E-05	4.73298E-05
Sinigrin	4.00474E-05	3.80747E-05	4.10511E-05	0.000178709	4.04444E-05	5.82553E-05	6.79E-05	7.36E-05	3.68E-05	4.46756E-05	5.4421E-05
Progoitrin	0.008679858	0.008228134	0.006390302	0.006924196	0.007353851	0.008222095	0.008898	0.007323	0.006958	0.009771014	0.00711234
Glucoerucin	0.000215592	0.000287947	0.000180839	0.000151673	0.000215301	0.000247658	0.000246	0.000191	0.000214	0.000228916	0.000252031
Sinalbin	0.002198539	0.0013892	0.002329113	0.003360145	0.002463444	0.002225508	0.002405	0.002359	0.001972	0.001837323	0.001519826
Gluconasturtiin	0.000980639	0.001217788	0.00086293	0.00089513	0.000986475	0.001111885	0.000999	0.000873	0.000938	0.001034873	0.001066268
Glucobrassicin	0.000264335	0.000215097	0.000247726	0.000235233	0.000272971	0.000275428	0.000255	0.000281	0.000226	0.000221461	0.000195781
Gluconapin	0.008496103	0.009380948	0.008035597	0.007336422	0.009133674	0.009302375	0.009333	0.008644	0.008362	0.009194656	0.008374329
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000382425	0.000462851	0.000339843	0.000298345	0.000370443	0.000409713	0.000373	0.000318	0.000358	0.000354847	0.000385477
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000469966	0.000500449	0.000443707	0.000382846	0.000473244	0.000503868	0.00046	0.000401	0.000414	0.000439576	0.000416373
Glucobrassicanapin	0.00418739	0.004779712	0.003937707	0.003535091	0.004305547	0.004815243	0.004742	0.004185	0.004086	0.004136673	0.004328625
Gluconapoleiferin	0.001557529	0.001704529	0.001356003	0.001222148	0.001608271	0.001738307	0.001784	0.001557	0.001528	0.001706372	0.001459428
Hydroxy-glucobrassicin	0.001102367	0.001285413	0.00091354	0.000945357	0.001193862	0.001228128	0.001208	0.00095	0.001051	0.00111819	0.001001457
TOTAL GLUCOSINOLATES	0.028626149	0.029544396	0.025126855	0.025507678	0.028470319	0.030196	0.030827	0.027202	0.02619	0.030136488	0.026213686
Phenylalanine	9.91195E-05	0.000139064	0.000123925	0.0001314	0.000141849	0.000132925	0.000145	0.000133	0.000141	9.73044E-05	0.00014034
Tryptophan	7.51125E-05	9.44933E-05	0.000101576	9.16179E-05	0.00010483	9.2507E-05	0.000117	0.000105	0.000101	5.93627E-05	9.84393E-05
Threonine OR homoserine OR O-methylserine;;	1.22782E-05	1.1847E-05	1.20038E-05	1.17041E-05	1.32104E-05	1.23535E-05	1.3E-05	1.47E-05	1.28E-05	1.08368E-05	1.11816E-05
Histidine	1.19665E-05	1.12387E-05	1.11882E-05	1.24852E-05	1.04473E-05	1.16236E-05	1.12E-05	1.15E-05	1.09E-05	1.16734E-05	1.22188E-05
Valine	1.11914E-05	8.19788E-06	9.05653E-06	1.19095E-05	9.08755E-06	9.00674E-06	1.18E-05	9.29E-06	8.93E-06	1.2779E-05	8.29798E-06
Asparagine	0.000177848	0.000209882	0.000186533	0.000180371	0.00018581	0.000184395	0.000184	0.000224	0.000199	0.000178473	0.000212595
Alanine	4.93436E-06	5.59473E-06	4.68153E-06	4.66794E-06	5.52064E-06	4.93569E-06	4.85E-06	5.71E-06	5.73E-06	5.62535E-06	5.86347E-06
Arginine	4.17351E-05	3.87602E-05	4.05052E-05	3.95141E-05	4.20225E-05	4.05119E-05	4.09E-05	4.15E-05	4.08E-05	4.03641E-05	4.1066E-05
acetyl-L-Lysine	0.000160419	0.000212051	0.000186	0.000123701	0.000187949	0.000187854	0.00018	0.000195	0.000199	0.00016895	0.000203507
TOTAL AMINO ACIDS	0.000594605	0.000731129	0.000675469	0.000607371	0.000700726	0.000676114	0.000707	0.00074	0.000718	0.000585369	0.000733509
TOTAL POTENTIAL TANNINS	0.000826666	0.000752341	0.000793929	0.000781697	0.000775062	0.000804609	0.000992	0.001032	0.001005	0.000835418	0.000855706
TOTAL LIPIDS	0.066851085	0.073467594	0.074070946	0.075135714	0.07450995	0.064755453	0.067609	0.070595	0.073892	0.066440196	0.070246149
TOTAL FLAVANOIDS	0.016484264	0.017429625	0.016637179	0.016840755	0.016967605	0.016402454	0.017965	0.016659	0.016709	0.017731554	0.016756242
TOTAL ORGANIC ACIDS	0.010690349	0.013242591	0.012729903	0.012090497	0.012918348	0.011713745	0.011858	0.013037	0.01232	0.010763946	0.012196199
TOTAL VITAMINS	0.00189988	0.002133062	0.001956546	0.001896554	0.001994844	0.001937188	0.00206	0.002	0.001964	0.001945287	0.002046585

Table 1 cont.

COMPOUND	Sample 41	Sample 42	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53
Glucotropaeolin	0.0003518	0.000396143	0.00033136	0.000114521	0.000121079	0.00012153	0.000128026	0.0001204	0.000112	0.000128	8.617E-05	0.0001081
Sinigrin	7.034E-05	0.00014534	7.9325E-05	5.00043E-05	4.489E-05	5.4086E-05	4.18844E-05	5.545E-05	4.42E-05	4.62E-05	5.945E-05	0.0002027
Progoitrin	0.0260063	0.02406387	0.02269094	0.008650297	0.010755396	0.01160841	0.009087801	0.0087967	0.008089	0.009651	0.0073041	0.0096577
Glucoerucin	0.0008593	0.000774213	0.00078827	0.000307839	0.000325784	0.00032623	0.000331167	0.0003102	0.000301	0.000338	0.0002916	0.0003379
Sinalbin	0.0012193	0.001061066	0.002629	0.00109699	0.001142805	0.00141261	0.001271164	0.0011762	0.001017	0.001012	0.0023183	0.0015421
Gluconasturtiin	0.0023076	0.002445108	0.00220433	0.001135429	0.001305477	0.00134619	0.001172527	0.0011091	0.001127	0.001178	0.0010408	0.0011986
Glucobrassicin	0.0003139	0.000299234	0.00034922	0.00033759	0.000285514	0.00019206	0.000311019	0.0003159	0.000281	0.000342	0.0002671	0.0003137
Gluconapin	0.0247966	0.022280541	0.02135575	0.010817872	0.010981116	0.01169293	0.011850081	0.011339	0.010368	0.01173	0.0094394	0.0114397
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.0006881	0.000645806	0.00082425	0.000301395	0.000332229	0.00031161	0.00034809	0.0003085	0.000296	0.000335	0.0003624	0.0004068
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.0009104	0.000924115	0.00095399	0.000483357	0.000556911	0.00058728	0.000521197	0.0004984	0.000489	0.000503	0.0004692	0.0005423
Glucobrassicanapin	0.0184183	0.019623565	0.01648319	0.008318288	0.008512076	0.00824455	0.008856055	0.0080722	0.007651	0.008665	0.0073541	0.0077913
Gluconapoleiferin	0.0063271	0.006079219	0.00577942	0.002929702	0.003039749	0.00399494	0.003181581	0.0029489	0.002808	0.003313	0.0024536	0.0029119
Hydroxy-glucobrassicin	0.0085758	0.008168503	0.00816117	0.001504872	0.001675485	0.00181878	0.001738379	0.001504	0.001392	0.001596	0.0015588	0.0020225
TOTAL GLUCOSINOLATES	0.0908447	0.086906722	0.0826302	0.036048156	0.039078511	0.04171123	0.03883897	0.0365549	0.033974	0.038836	0.0330051	0.0384753
Phenylalanine	0.0002159	0.000185863	0.00022921	0.000123146	0.000103014	6.6176E-05	0.000115499	0.0001222	0.000117	0.000116	0.0001283	0.0001214
Tryptophan	0.000115	9.68502E-05	0.00012267	9.57915E-05	7.00421E-05	3.8356E-05	8.01443E-05	8.557E-05	8.59E-05	8.99E-05	9.32E-05	8.137E-05
Threonine OR homoserine OR O-methylserine;;	1.189E-05	9.88097E-06	1.3065E-05	1.25785E-05	1.39871E-05	1.9143E-05	1.32138E-05	1.324E-05	1.36E-05	1.55E-05	1.351E-05	1.343E-05
Histidine	1.927E-05	1.91823E-05	2.1708E-05	1.11464E-05	1.11804E-05	1.6982E-05	9.83841E-06	1.003E-05	1.09E-05	9.82E-06	1.098E-05	1.052E-05
Valine	6.465E-06	9.20761E-06	6.1315E-06	7.35169E-06	1.13079E-05	1.4981E-05	7.81153E-06	7.732E-06	7.53E-06	7.96E-06	7.854E-06	7.773E-06
Asparagine	0.0002545	0.000231253	0.00027766	0.000222835	0.000192802	0.00031457	0.000209601	0.0002117	0.000204	0.000229	0.0002038	0.0002067
Alanine	4.071E-06	4.66653E-06	3.9961E-06	4.96172E-06	3.97023E-06	7.2326E-06	4.38056E-06	5.018E-06	4.24E-06	5.16E-06	4.904E-06	5.008E-06
Arginine	7.028E-05	6.72633E-05	6.4514E-05	4.02161E-05	4.02092E-05	5.4949E-05	4.1529E-05	4.071E-05	3.97E-05	4.03E-05	3.83E-05	3.743E-05
acetyl-L-Lysine	0.0003649	0.000311943	0.00027622	0.000235692	0.000200011	0.00017689	0.000231538	0.0002315	0.000224	0.000239	0.00019	0.0001562
TOTAL AMINO ACIDS	0.0010622	0.000936109	0.00101518	0.00075372	0.000646525	0.00070928	0.000713555	0.0007277	0.000708	0.000752	0.0006908	0.0006398
TOTAL POTENTIAL TANNINS	0.0008818	0.000848665	0.00083708	0.000813763	0.000852185	0.00084226	0.000840046	0.0008534	0.000844	0.000858	0.000806	0.0010058
TOTAL LIPIDS	0.0446363	0.050138354	0.04648412	0.067840545	0.067495462	0.06557114	0.064272534	0.0639135	0.067078	0.070658	0.0687807	0.0670232
TOTAL FLAVANOIDS	0.0303479	0.03223289	0.03060167	0.01818881	0.020141453	0.02056475	0.018588475	0.0188454	0.018766	0.018757	0.0187207	0.0190462
TOTAL ORGANIC ACIDS	0.0069411	0.007007059	0.00727026	0.011533618	0.010380219	0.01020248	0.011068818	0.0112276	0.011231	0.011014	0.0116073	0.0111539
TOTAL VITAMINS	0.0024893	0.002680022	0.0024532	0.002807084	0.002915593	0.00251974	0.00274913	0.0028836	0.002865	0.002883	0.0027622	0.0028453

Table 1 cont.

COMPOUND	Sample 54	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60	Sample 61	Sample 62	Sample 63	Sample 64	Sample 65	Sample 66	Sample 68
Glucotropaeolin	0.0001216	9.992E-05	8.85E-05	0.0002606	0.000239	0.000236	0.0002909	0.0001811	0.000111	6.113E-05	2.137E-05	2.989E-05	4.439E-05
Sinigrin	3.858E-05	4.339E-05	5.395E-05	6.557E-05	0.0006237	0.0003855	0.0008054	2.331E-05	1.215E-05	2.598E-05	1.035E-05	8.344E-06	1.677E-05
Progoitrin	0.0071389	0.0075564	0.0089867	0.0221959	0.0290293	0.0247136	0.0261134	0.0133104	0.0062885	0.0106024	0.0029477	0.0020012	0.0040892
Glucoerucin	0.0003412	0.0002961	0.0002882	0.0008875	0.0008486	0.000813	0.0007503	0.0009069	0.0004919	0.0003732	0.000117	7.205E-05	0.0001073
Sinalbin	0.0009041	0.0023988	0.002641	0.000337	0.0029658	0.0041908	0.0028691	0.0002283	0.0002805	5.691E-06	0	0.0001911	0.0003014
Gluconasturtiin	0.0012463	0.001044	0.0010335	0.0019949	0.0024764	0.002446	0.0023295	0.0008829	0.0004898	0.00094	0.0003042	0.0003788	0.0005213
Glucobrassicin	0.0003162	0.0002777	0.0003102	0.0006408	0.0003414	0.0007966	0.0005244	0.0001139	7.193E-05	0.0005488	0.0001532	0.0001172	0.0001843
Gluconapin	0.0106539	0.0095865	0.0095649	0.0195136	0.0269575	0.0211807	0.022595	0.0140244	0.009306	0.0103257	0.0045754	0.0033362	0.0047288
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.0003776	0.0003556	0.0003369	0.0007786	0.0009723	0.0009423	0.0009371	0.0003588	0.0001966	0.0012367	0.0004196	8.884E-05	0.000128
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.0005049	0.0004773	0.0004749	0.000904	0.0010976	0.0009467	0.0009273	0.0006415	0.0003478	0.0013027	0.0004852	0.0001608	0.0002295
Glucobrassicanapin	0.0089427	0.0072309	0.0067021	0.012248	0.0204762	0.0195103	0.0206124	0.0073981	0.0045908	0.0135243	0.0053907	0.0025929	0.0036044
Gluconapoleiferin	0.0028868	0.0022809	0.0023418	0.0038583	0.0067435	0.0059296	0.0067199	0.0037146	0.0020942	0.0048971	0.0018306	0.0007592	0.0010613
Hydroxy-glucobrassicin	0.0014167	0.0016541	0.0013893	0.0087676	0.0082123	0.007017	0.0073897	0.0034926	0.0013821	0.003655	0.0007058	0.0003478	0.0005997
TOTAL GLUCOSINOLATES	0.0348896	0.0333017	0.0342119	0.0724523	0.1009837	0.0891081	0.0928644	0.0452768	0.0256633	0.0474987	0.0169612	0.0100843	0.0156164
Phenylalanine	0.0001195	0.0001301	0.0001122	0.0002624	0.0002376	0.0002403	0.0002529	9.635E-05	0.0001018	0.0001474	9.74E-05	5.674E-05	5.753E-05
Tryptophan	9.259E-05	9.832E-05	8.415E-05	0.0001592	9.646E-05	0.0001353	0.0001136	4.114E-05	5.082E-05	0.0001066	8.817E-05	5.398E-05	4.707E-05
Threonine OR homoserine OR O-methylserine;;	1.255E-05	1.222E-05	1.388E-05	1.001E-05	1.191E-05	1.066E-05	1.337E-05	8.492E-06	9.313E-06	1.321E-05	1.255E-05	1.387E-05	1.561E-05
Histidine	1.153E-05	1.068E-05	1.359E-05	2.21E-05	2.389E-05	2.187E-05	1.986E-05	1.501E-05	1.081E-05	2.322E-05	1.285E-05	3.259E-06	5.652E-06
Valine	7.585E-06	7.402E-06	8.664E-06	7.311E-06	5.74E-06	6.503E-06	7.146E-06	4.504E-06	4.556E-06	1.367E-05	1.24E-05	8.243E-06	1.206E-05
Asparagine	0.0002411	0.0002101	0.0002051	0.000279	0.0002183	0.0002553	0.0002286	0.0002166	0.0001969	0.0001869	0.0001801	0.000147	0.0001453
Alanine	5.088E-06	4.01E-06	4.714E-06	4.644E-06	4.027E-06	4.798E-06	3.368E-06	5.56E-06	4.629E-06	5.486E-06	6.733E-06	4.945E-06	4.675E-06
Arginine	4.271E-05	3.714E-05	4.145E-05	8.132E-05	7.399E-05	6.766E-05	6.405E-05	4.417E-05	4.18E-05	3.566E-05	3.086E-05	1.716E-05	2.29E-05
acetyl-L-Lysine	0.0002324	0.000188	0.0001614	0.0002657	0.0003921	0.0002346	0.0002399	0.0003988	0.0003198	0.0004775	0.0004113	0.0001371	0.000116
TOTAL AMINO ACIDS	0.000765	0.000698	0.0006451	0.0010918	0.001064	0.000977	0.0009427	0.0008306	0.0007405	0.0010097	0.0008523	0.0004423	0.0004268
TOTAL POTENTIAL TANNINS	0.0009312	0.0008532	0.0009327	0.0010039	0.0009742	0.0010303	0.0006655	0.0006429	0.000661	0.000704	0.0007165	0.0007878	0.0006983
TOTAL LIPIDS	0.0638735	0.0699352	0.0699304	0.0571167	0.050336	0.0588561	0.0559314	0.0517485	0.0661064	0.0571671	0.0639087	0.0766671	0.0711834
TOTAL FLAVANOIDS	0.0180799	0.0185022	0.0190069	0.0313214	0.0282761	0.0281763	0.0299329	0.0248907	0.0241328	0.0189211	0.0181549	0.0188375	0.0197542
TOTAL ORGANIC ACIDS	0.0107726	0.011172	0.0108245	0.0077495	0.0073154	0.0084764	0.0087783	0.008167	0.0095736	0.0104242	0.0111204	0.0150633	0.0122613
TOTAL VITAMINS	0.0028614	0.0028468	0.0027225	0.0025153	0.0022358	0.0023724	0.0022448	0.002945	0.0030813	0.0018903	0.0019638	0.0030345	0.0029334

Table 1 cont.

COMPOUND	Sample 69	Sample 70	Sample 71	Sample 72	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 80	Sample 81
Glucotropaeolin	3.8238E-05	3.67E-05	5.19E-05	3.55888E-05	0.00029727	7.82541E-05	8.4837E-05	8.61318E-05	8.63125E-05	9.21512E-05	7.88E-05	7.98975E-05
Sinigrin	1.2448E-05	1.48E-05	2.39E-05	1.1701E-05	0.000122292	5.0442E-05	3.30027E-05	4.74864E-05	2.73203E-05	4.15598E-05	2.76E-05	3.75642E-05
Progoitrin	0.00277282	0.00247	0.004256	0.002587962	0.024858508	0.00493892	0.005635273	0.006695762	0.004375361	0.005222155	0.005345	0.004492817
Glucoerucin	9.1645E-05	9.28E-05	0.000113	8.97084E-05	0.000840815	0.000116441	0.000154395	0.000148641	0.000146328	0.000151686	0.000121	0.000152211
Sinalbin	0.0002776	0.000285	0.000468	0.000236748	0.001851722	0.001005794	0.000911471	0.000936953	0.00075964	0.000903169	0.000856	0.000794781
Gluconasturtiin	0.00042324	0.000435	0.000583	0.000396133	0.002528017	0.000698782	0.00074962	0.00081426	0.000722348	0.000753735	0.000687	0.000725141
Glucobrassicin	0.00015238	0.000158	0.000201	0.00014305	0.000693566	0.000192682	0.000171902	0.000205504	0.000183034	0.000229579	0.000195	0.000211151
Gluconapin	0.00402904	0.004058	0.005095	0.003821197	0.023529481	0.007369019	0.00805343	0.007732471	0.006969205	0.007561917	0.00725	0.007208366
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.00011947	0.000114	0.00014	0.00011452	0.000958679	0.000187417	0.000206519	0.000229801	0.000222296	0.000241738	0.000197	0.000219856
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.00018939	0.000194	0.000254	0.000173914	0.001019922	0.000365804	0.000386344	0.000408118	0.000380682	0.000404851	0.000366	0.000384526
Glucobrassicanapin	0.0030264	0.003096	0.003949	0.002904575	0.018293044	0.004704646	0.005369743	0.005617503	0.005539442	0.00568085	0.005195	0.005364469
Gluconapoleiferin	0.00096615	0.000905	0.001182	0.000907777	0.005742003	0.00149109	0.001664076	0.001720409	0.001421365	0.001752577	0.001445	0.001487576
Hydroxy-glucobrassicin	0.000482	0.000452	0.000711	0.000419825	0.007158602	0.000974284	0.00120941	0.001036324	0.001125057	0.001141093	0.00096	0.000950759
TOTAL GLUCOSINOLATES	0.0125808	0.01231	0.017028	0.011842699	0.087893918	0.022173577	0.024630022	0.025679364	0.021958389	0.02417706	0.022724	0.022109116
Phenylalanine	7.2962E-05	6.96E-05	5.67E-05	7.53126E-05	0.000246168	0.000125795	0.000132376	8.76026E-05	0.000132638	0.000131591	0.00011	0.000118386
Tryptophan	6.3808E-05	6.17E-05	4.18E-05	6.16354E-05	0.000133691	7.55684E-05	7.8507E-05	5.30733E-05	8.21972E-05	8.01334E-05	6.88E-05	8.84314E-05
Threonine OR homoserine OR O-methylserine;;	1.3754E-05	1.44E-05	1.39E-05	1.57444E-05	1.16074E-05	1.13462E-05	1.16683E-05	9.19774E-06	9.28645E-06	9.94158E-06	1.02E-05	1.09906E-05
Histidine	5.027E-06	6.36E-06	6.36E-06	4.80233E-06	2.18614E-05	7.89088E-06	7.70407E-06	9.6077E-06	8.41499E-06	7.84007E-06	7.32E-06	9.12939E-06
Valine	8.7846E-06	8.05E-06	1.28E-05	8.71578E-06	6.81063E-06	9.0728E-06	9.15101E-06	1.28185E-05	8.69193E-06	8.66885E-06	9.7E-06	8.71446E-06
Asparagine	0.00016004	0.000184	0.000145	0	0.000286255	0.000184777	0.000167294	0	0.000196647	0.000202579	0.000167	0.000222052
Alanine	4.5464E-06	5.81E-06	4.63E-06	4.76391E-06	4.80449E-06	5.11735E-06	4.63357E-06	4.59757E-06	3.83852E-06	5.34529E-06	4.65E-06	4.92488E-06
Arginine	2.3736E-05	2.3E-05	2.46E-05	2.29021E-05	7.16627E-05	3.8637E-05	3.70712E-05	4.06795E-05	3.84713E-05	3.87648E-05	3.8E-05	3.89394E-05
acetyl-L-Lysine	0.0001552	0.00015	0.00012	0.000162219	0.000418665	0.000220687	0.000225629	0.000185391	0.000223492	0.000229563	0.000186	0.000230331
TOTAL AMINO ACIDS	0.00050785	0.000523	0.000426	0.000356095	0.001201526	0.000678892	0.000674035	0.000402968	0.000703677	0.000714427	0.000602	0.000731899
TOTAL POTENTIAL TANNINS	0.00069781	0.00074	0.000985	0.00079895	0.000856296	0.000818443	0.000850306	0.0008342	0.000781342	0.000814428	0.000809	0.000765605
TOTAL LIPIDS	0.07517004	0.083917	0.077831	0.073795093	0.055337707	0.079110572	0.079827316	0.083952475	0.082053003	0.082296228	0.082715	0.088039953
TOTAL FLAVANOIDS	0.0189261	0.018543	0.02056	0.018748225	0.029270445	0.018726716	0.019351615	0.019519644	0.018144077	0.018485263	0.01894	0.01860149
TOTAL ORGANIC ACIDS	0.01456912	0.015273	0.011743	0.014568426	0.00762784	0.010495701	0.010897704	0.009526943	0.011523031	0.011299609	0.01028	0.010939223
TOTAL VITAMINS	0.00294602	0.002937	0.002995	0.002937654	0.002401942	0.002887098	0.002859166	0.002891878	0.002981181	0.002983398	0.002879	0.003021698

Table 1 cont.

COMPOUND	Sample 82	Sample 83	Sample 84	Sample 85	Sample 86	Sample 87	Sample 88	Sample 89	Sample 90	Sample 92	Sample 93	Sample 94
Glucotropaeolin	7.99996E-05	8.56896E-05	8.80711E-05	7.6112E-05	8.31583E-05	4.79672E-05	6.71392E-05	4.5022E-05	6.9461E-05	5.73552E-05	5.88743E-05	6.29901E-05
Sinigrin	3.59734E-05	3.30038E-05	3.2933E-05	4.321E-05	2.91561E-05	3.09604E-05	2.07359E-05	2.2462E-05	2.13476E-05	3.16082E-05	2.81411E-05	2.59221E-05
Progoitrin	0.006414896	0.006377043	0.006969649	0.00432497	0.004848612	0.005269452	0.007535747	0.00436861	0.005714317	0.005325973	0.006214527	0.006705613
Glucoerucin	0.000146374	0.000156344	0.000161205	0.00013939	0.000154029	0.000192639	0.000243455	0.00017197	0.000255131	0.00020858	0.000213607	0.00022902
Sinalbin	0.000895023	0.000975129	0.001094757	0.00078037	0.000853889	0.001458932	0.000270955	0.00103693	0.000258683	0.000457491	0.000486356	0.000339448
Gluconasturtiin	0.000703144	0.000732405	0.000771838	0.00070935	0.000749431	0.000646755	0.000763554	0.00059566	0.000767378	0.000708997	0.00069598	0.000748678
Glucobrassicin	0.000219248	0.000250513	0.000215481	0.00017148	0.000201983	0.000138485	0.00015146	0.00015349	0.000207288	0.000176384	0.00016374	0.000176848
Gluconapin	0.00782511	0.008028145	0.007968415	0.00712758	0.007437752	0.006603328	0.007821511	0.00587342	0.007876311	0.006918266	0.007175323	0.007481185
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000188991	0.000206759	0.000219033	0.00020267	0.000210639	0.000262193	0.000264032	0.00023959	0.000290185	0.000238269	0.000260459	0.000257039
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000370613	0.000376065	0.000379981	0.00035257	0.000389748	0.000340187	0.000402511	0.00030638	0.00042958	0.000374915	0.000378033	0.00040502
Glucobrassicanapin	0.005226655	0.005553188	0.005573609	0.00527636	0.005626043	0.004378396	0.006362744	0.0041229	0.006654602	0.005326765	0.005704204	0.005815017
Gluconapoleiferin	0.001739594	0.001776496	0.001868946	0.00158979	0.001700023	0.001691214	0.002324941	0.00152935	0.002264207	0.001931797	0.002060435	0.002191409
Hydroxy-glucobrassicin	0.000912984	0.000944022	0.001133693	0.0008553	0.001038649	0.000959379	0.00131003	0.00089924	0.001247354	0.001145305	0.001218952	0.001339971
TOTAL GLUCOSINOLATES	0.024758605	0.025494801	0.026477611	0.02164916	0.023323113	0.022019889	0.027538816	0.01936502	0.026055846	0.022901705	0.024658632	0.025778159
Phenylalanine	0.000112286	0.000126874	9.61484E-05	0.00012764	0.000122282	0.000117388	0.000114463	0.00010909	0.000117116	0.000114102	0.000114037	0.000113696
Tryptophan	7.81572E-05	8.88917E-05	6.48018E-05	8.3773E-05	8.41316E-05	9.39947E-05	7.79019E-05	8.3522E-05	8.79997E-05	8.36441E-05	8.08978E-05	7.86329E-05
Threonine OR homoserine OR O-methylserine;;	1.10502E-05	1.25805E-05	1.09732E-05	1.1389E-05	1.02282E-05	1.54376E-05	1.57183E-05	1.4087E-05	1.42728E-05	1.33974E-05	1.28847E-05	1.30238E-05
Histidine	9.28232E-06	9.88727E-06	9.10797E-06	9.2217E-06	7.86445E-06	1.23652E-05	1.20722E-05	1.137E-05	1.326E-05	9.90026E-06	1.10717E-05	1.14522E-05
Valine	9.92442E-06	9.83594E-06	1.0237E-05	7.8816E-06	8.0964E-06	7.50842E-06	8.44925E-06	6.7371E-06	7.55643E-06	7.03861E-06	7.13843E-06	7.08456E-06
Asparagine	0.000205984	0.000206559	0.000179403	0.00019306	0.000191233	0.000228939	0.000204404	0.00022547	0.000235846	0.000208509	0.000191273	0.000191593
Alanine	4.38981E-06	4.43241E-06	5.35619E-06	4.1416E-06	5.43616E-06	6.37698E-06	4.84506E-06	5.1684E-06	5.29696E-06	5.33264E-06	4.13413E-06	3.76317E-06
Arginine	4.07915E-05	3.91275E-05	3.89184E-05	3.878E-05	3.62085E-05	3.87909E-05	3.82065E-05	3.7382E-05	4.00899E-05	3.5729E-05	3.91577E-05	3.61222E-05
acetyl-L-Lysine	0.000221614	0.000230042	0.000205069	0.00023505	0.000225497	0.000132409	0.000130239	0.00011464	0.000142722	0.0001311	0.000108292	0.00012952
TOTAL AMINO ACIDS	0.00069348	0.00072823	0.000620015	0.00071093	0.000690978	0.000653209	0.000606298	0.00060746	0.00066416	0.000608753	0.000568886	0.000584888
TOTAL POTENTIAL TANNINS	0.000807688	0.000797509	0.000813769	0.00083445	0.000923189	0.000871389	0.000894698	0.0007875	0.000857825	0.000843975	0.00088258	0.000829776
TOTAL LIPIDS	0.080072584	0.078381344	0.074483474	0.08348718	0.077813818	0.057537132	0.057615361	0.05591917	0.064173032	0.061690524	0.060235201	0.061487325
TOTAL FLAVANOIDS	0.018851584	0.019426118	0.019283974	0.01827898	0.018405826	0.018766156	0.018944283	0.01774599	0.018527078	0.017539675	0.018265207	0.018242324
TOTAL ORGANIC ACIDS	0.010280875	0.011168861	0.010339698	0.01119567	0.011222845	0.012079637	0.010874014	0.01132018	0.011205906	0.011089351	0.011220964	0.010327574
TOTAL VITAMINS	0.002829823	0.00289256	0.00275331	0.00296262	0.002900765	0.00248891	0.002583345	0.0024132	0.002473513	0.002392708	0.002390899	0.002369782

Table 1 cont.

COMPOUND	Sample 95	Sample 96	Sample 97	Sample 98	Minimum	Maximum	Mean	RSD all samples	SE all samples	QC RSD	QC SE	Detected in total
Glucotropaeolin	7.02582E-05	6.13309E-05	0.000344	0.000324	2.13745E-05	0.000396	9.21E-05	91.93140051	8.77914E-06	4.306646726	1.02822E-06	93/93 samples
Sinigrin	2.49415E-05	6.20379E-05	9E-05	5.98E-05	8.34357E-06	0.000805	7.96E-05	141.1884728	1.16519E-05	13.72548903	2.78341E-06	93/93 samples
Progoitrin	0.00769481	0.006081704	0.023098	0.019105	0.002001205	0.029029	0.009153	69.86990368	0.000663165	15.81178375	0.00034678	93/93 samples
Glucoerucin	0.000248068	0.000171112	0.001032	0.001022	7.20538E-05	0.001032	0.000327	73.32009977	2.48987E-05	3.09098106	2.65641E-06	93/93 samples
Sinalbin	0.000405467	0.000931582	0.001317	0.001372	0	0.007229	0.002053	76.76085664	0.000163379	8.821603208	4.54679E-05	92/93 samples
Gluconasturtiin	0.000805513	0.00073771	0.002555	0.002318	0.000304195	0.002676	0.001115	51.02709046	5.89841E-05	4.956102781	1.41051E-05	93/93 samples
Glucobrassicin	0.000193619	0.000197851	0.000234	0.000376	7.19321E-05	0.000797	0.00026	47.7814919	1.28965E-05	6.969381864	6.6678E-06	93/93 samples
Gluconapin	0.008201494	0.006923548	0.018017	0.017055	0.00333618	0.026958	0.01025	50.06603419	0.000532121	6.498228613	0.000169387	93/93 samples
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000262463	0.00024617	0.000788	0.00078	8.88431E-05	0.001237	0.000377	62.26133936	2.43137E-05	3.602394207	3.40308E-06	93/93 samples
Neoglucobrassicin or methoxy glucobrassicin (putative, no standard for RT ma	0.000394195	0.000349666	0.00092	0.000821	0.000160799	0.001303	0.000489	46.4148513	2.3524E-05	4.552475346	5.35399E-06	93/93 samples
Glucobrassicanapin	0.006286242	0.004790574	0.017977	0.017274	0.002592873	0.020612	0.006661	68.7882925	0.000475151	3.484679044	6.22656E-05	93/93 samples
Gluconapoleiferin	0.002530709	0.001636866	0.007492	0.007119	0.000759195	0.007492	0.002268	72.62348533	0.000170791	9.22767789	5.3982E-05	93/93 samples
Hydroxy-glucobrassicin	0.001342585	0.000912398	0.008435	0.006993	0.000347833	0.009131	0.002221	109.3451389	0.000251779	7.848373596	5.20579E-05	93/93 samples
TOTAL GLUCOSINOLATES	0.028460366	0.02310255	0.082298	0.07462	0.01008431	0.100984	0.035345	60.42554507	0.002214644	7.145832086	5.89183E-05	93/93 samples
Phenylalanine	0.000120217	0.000111018	0.000187	0.000225	5.67328E-05	0.000262	0.000136	32.25239145	4.54312E-06	13.3832492	4.18274E-06	93/93 samples
Tryptophan	9.51062E-05	8.33981E-05	7.44E-05	0.000125	3.83564E-05	0.000159	9.37E-05	25.21733658	2.45056E-06	17.97793656	3.9544E-06	93/93 samples
Threonine OR homoserine OR O-methylserine;;	1.47322E-05	1.39165E-05	1.1E-05	1.18E-05	8.49153E-06	1.93E-05	1.34E-05	18.48714004	2.57171E-07	6.821394203	1.98987E-07	93/93 samples
Histidine	1.23296E-05	1.17398E-05	2.27E-05	2.01E-05	3.25912E-06	2.85E-05	1.35E-05	36.38282344	5.10155E-07	8.845694946	2.84687E-07	93/93 samples
Valine	8.79761E-06	9.82722E-06	1.04E-05	7.36E-06	4.50432E-06	1.5E-05	9.17E-06	23.02047033	2.18908E-07	19.23376068	4.36611E-07	93/93 samples
Asparagine	0.000211282	0.000212084	0.000227	0.000271	0	0.000315	0.000204	23.69573693	5.01765E-06	8.742314771	4.33813E-06	90/93 samples
Alanine	4.96418E-06	5.64127E-06	4.11E-06	5.11E-06	3.36806E-06	7.26E-06	5.17E-06	15.82417269	8.48946E-08	10.15050375	1.24731E-07	93/93 samples
Arginine	3.79536E-05	4.08111E-05	7.19E-05	6.5E-05	1.71644E-05	8.13E-05	4.66E-05	27.44181432	1.32611E-06	3.916407633	4.29818E-07	93/93 samples
acetyl-L-Lysine	0.000139036	0.000129653	0.00027	0.000315	8.2427E-05	0.000478	0.000193	40.50679106	8.0895E-06	8.070974674	3.56626E-06	93/93 samples
TOTAL AMINO ACIDS	0.000644418	0.000618089	0.000879	0.001045	0.000356095	0.001202	0.000714	21.164393	2.194645319	10.79358183	2.413518268	93/93 samples
TOTAL POTENTIAL TANNINS	0.000880739	0.000883896	0.001116	0.001068	0.000642927	0.001116	0.000832	11.13858585	9.61083E-06	10.0533523	3.24459E-07	93/93 samples
TOTAL LIPIDS	0.06044855	0.068707508	0.051315	0.051148	0.0444142	0.08804	0.067696	13.98364217	0.000981623	11.42075706	4.7013E-06	93/93 samples
TOTAL FLAVANOIDS	0.018705558	0.019526697	0.035214	0.019158	0.016385016	0.035214	0.019747	21.43879121	0.000439005	8.852144038	4.58796E-06	93/93 samples
TOTAL ORGANIC ACIDS	0.010501857	0.012178954	0.007537	0.008272	0.006941061	0.015273	0.011211	17.11839525	0.000199009	13.89575734	1.78646E-05	93/93 samples
TOTAL VITAMINS	0.002496047	0.002782984	0.002233	0.002166	0.001605997	0.003081	0.002353	19.19087042	4.68221E-05	7.61040885	1.34941E-06	93/93 samples

Table 2: Relative glucosinolate concentration ranges across the entire sample population based upon the non-targeted LC-MS profiling approach

COMPOUND	IDENTIFICATION LEVEL	RT (MINUTES)	Minimum	Maximum	Mean	SD (all samples)	RSD (all samples)	RSD (QA samples)	Detected in total
Glucotropaeolin	Unambiguous	9.35	0.0000213745	0.0003961431	0.0000920937	0.0000851214	91.93	4.31	93/93
Sinigrin	Unambiguous	4.39	0.0000083436	0.0008053734	0.0000795867	0.0001129810	141.19	13.73	93/93
Progoitrin	Unambiguous	3.63	0.0020012055	0.0290293438	0.0091531993	0.0064303548	69.87	15.81	93/93
Glucoerucin	Unambiguous	9.59	0.0000720538	0.0010323290	0.0003274876	0.0002413982	73.32	3.09	93/93
Sinalbin	Unambiguous	5.34	0.0000000000	0.0072290203	0.0020525656	0.0015841371	76.76	8.82	93/93
Gluconasturtiin	Unambiguous	12.84	0.0003041951	0.0026758003	0.0011147459	0.0005718558	51.03	4.96	93/93
Glucobrassicin	Unambiguous	10.99	0.0000719321	0.0007965702	0.0002602869	0.0001241050	47.78	6.97	93/93
Gluconapin	Unambiguous	6.16	0.0033361801	0.0269575346	0.0102496384	0.0051590006	50.07	6.50	93/93
Neoglucobrassicin or methoxy glucobrassicin	Putative	16.23	0.0000888431	0.0012367302	0.0003765942	0.0002357436	62.26	3.60	93/93
Neoglucobrassicin or methoxy glucobrassicin	Putative	12.53	0.0001607986	0.0013026983	0.0004887594	0.0002280975	46.41	4.55	93/93
Glucobrassicanapin	Putative	8.94	0.0025928725	0.0206124150	0.0066613006	0.0046062834	68.79	3.48	93/93
Gluconapoleiferin	Putative	5.44	0.0007591947	0.0074916498	0.0022679246	0.0016559172	72.62	9.23	93/93
Hydroxy-glucobrassicin	Putative	7.74	0.0003478333	0.0091305790	0.0022205590	0.0024405762	109.35	7.85	93/93
TOTAL GLUCOSINOLATES	NA	NA	0.0100843098	0.1009837162	0.0353447417	0.0214717165	60.43	7.15	93/93

Table 3: Glucosinolate levels determined by the targeted profiling approach for all individual samples

	Blank Start	Blank End	QC Average	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17
Gluconapin	0	0	21.74	12.1	10.5	8.9	10.7	9.9	11.1	12.0	10.7	9.6	12.2	14.5	11.2	89.5	82.0	90.6	10.3	11.5
Gluconasturtiin	0	0	0.86	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.7	0.7	0.7	0.7	2.5	1.7	1.7	0.7	0.6
Glucoraphanin	0	0	0.09	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1
Progoitin	0	0	29.66	18.9	17.5	15.2	17.4	15.6	18.1	20.3	17.8	15.6	19.2	23.2	17.8	79.2	76.2	77.4	15.3	16.7
Sinalbin	0	0	5.70	12.9	12.5	12.5	12.6	12.7	15.2	13.5	12.2	13.1	11.8	12.3	14.5	33.5	15.8	17.0	7.9	8.6
Sinigrin	0	0	0.22	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.4	0.3	0.2	0.2

	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39
Gluconapin	10.6	11.5	10.2	9.7	10.7	10.5	12.4	11.0	10.6	10.9	13.2	14.1	16.3	11.1	9.6	12.9	16.2	13.5	12.6	12.5	13.6
Gluconasturtiin	0.7	0.7	0.6	0.7	0.8	0.6	0.7	0.8	0.6	0.6	0.8	0.6	1.0	0.7	0.6	0.7	0.7	0.6	0.7	0.7	0.6
Glucoraphanin	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Progoitin	16.5	17.4	15.7	14.3	16.4	16.0	18.7	16.9	16.3	16.4	23.0	22.8	27.5	17.9	15.4	20.5	25.9	22.2	20.7	20.9	23.3
Sinalbin	7.2	6.7	7.5	7.3	6.2	7.3	6.5	8.1	7.1	7.9	4.5	5.6	3.8	6.7	10.3	6.3	5.2	5.4	6.8	4.6	4.4
Sinigrin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

	Sample 40	Sample 41	Sample 42	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53	Sample 54	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60	Sample 61	Sample 62
Gluconapin	15.8	86.2	82.4	80.0	20.0	21.6	23.1	23.9	21.0	19.2	22.7	15.2	19.1	24.4	16.2	15.2	78.0	106.7	93.1	87.3	40.8
Gluconasturtiin	1.0	1.8	1.6	1.8	0.9	0.8	0.8	0.9	0.9	0.9	0.8	0.8	0.8	1.1	0.9	0.7	2.0	1.8	2.4	1.8	0.7
Glucoraphanin	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2
Progoitin	26.4	84.3	77.9	82.4	30.8	31.3	33.0	33.1	31.5	29.3	33.0	26.1	30.1	33.5	26.2	26.1	74.1	92.2	85.4	80.5	50.1
Sinalbin	4.6	3.3	3.2	8.1	2.5	2.4	2.3	3.0	2.8	2.3	2.6	6.0	3.8	2.9	6.6	6.8	1.3	10.1	20.8	9.5	1.0
Sinigrin	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.6	0.5	0.6	0.2

	Sample 63	Sample 64	Sample 65	Sample 66	Sample 68	Sample 69	Sample 70	Sample 71	Sample 72	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 80	Sample 81	Sample 82	Sample 83	Sample 84	Sample 85
Gluconapin	13.3	20.7	4.7	2.8	4.1	3.6	3.6	4.6	3.3	92.5	9.4	9.7	10.2	11.1	11.1	9.4	10.6	10.5	10.4	11.0	9.7
Gluconasturtiin	0.4	0.6	0.3	0.4	0.3	0.4	0.4	0.4	0.4	2.3	0.6	0.6	0.6	0.7	0.7	0.6	0.8	0.5	0.6	0.5	0.7
Glucoraphanin	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Progoitin	23.3	25.1	5.6	2.5	3.9	3.7	3.5	4.5	3.6	88.1	14.7	16.0	16.5	17.2	17.3	14.5	17.2	16.8	17.6	17.5	15.5
Sinalbin	0.5	0.2	0.2	0.5	0.7	0.6	0.7	1.0	0.7	7.0	2.4	2.0	2.2	2.0	2.4	1.7	2.0	2.1	2.3	2.1	2.3
Sinigrin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

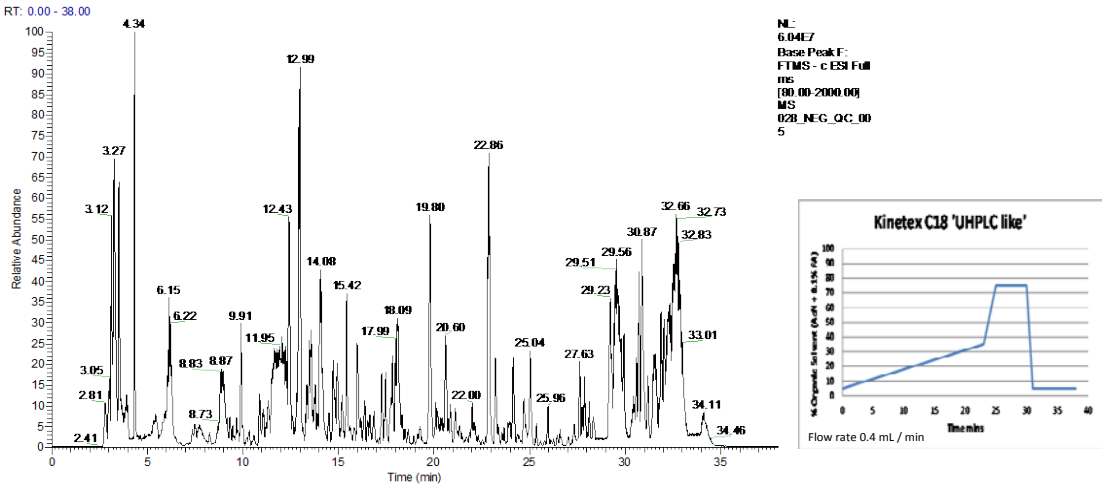
	Sample 86	Sample 87	Sample 88	Sample 89	Sample 90	Sample 92	Sample 93	Sample 94	Sample 95	Sample 96	Sample 97	Sample 98	Minimum	Maximum	Mean	RSD all samples	SE all samples	Detected in total
Gluconapin	10.0	6.6	9.8	6.4	11.4	8.4	8.5	9.6	10.1	7.4	61.6	58.0	2.84	106.70	22.35	115.639926	11.86440607	93/93 SAMPLES
Gluconasturtiin	0.7	0.6	0.6	0.6	0.8	0.7	0.6	0.6	0.6	0.5	1.4	1.8	0.32	2.53	0.83	56.5101006	5.797813989	93/93 SAMPLES
Glucoraphanin	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.1	0.01	0.25	0.08	40.22676687	4.127179198	93/93 SAMPLES
Progoitin	16.8	15.7	24.6	14.9	25.3	19.4	20.5	23.1	25.2	15.0	71.4	68.8	2.49	92.18	28.16	79.32294532	8.138362472	93/93 SAMPLES
Sinalbin	2.0	2.7	0.8	2.6	0.8	1.3	1.2	0.8	1.0	2.1	3.5	4.4	0.18	33.53	5.85	92.40516461	9.480569851	93/93 SAMPLES
Sinigrin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.20	0.64	0.23	29.65043162	3.042070097	93/93 SAMPLES

Table 4: Absolute glucosinolate concentration ranges across the entire sample population based upon the targeted LC-MS profiling approach

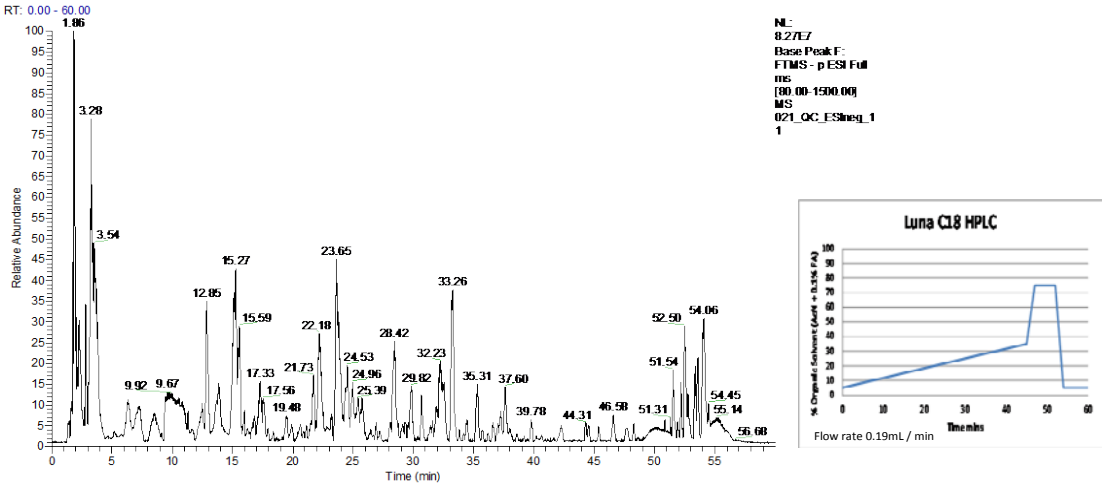
	Minimum (uM)	Maximum (uM)	Mean (uM)	SD (all samples)	RSD (all samples)	RSD (QC samples)	Detected in total
Gluconapin	2.84	106.70	22.35	25.99	115.64	2.64	93/93
Gluconasturtiin	0.32	2.53	0.83	0.47	56.51	12.1	93/93
Glucoraphanin	0.01	0.25	0.08	0.03	40.23	6.81	93/93
Progoitin	2.49	92.18	28.16	22.46	79.32	2.42	93/93
Sinalbin	0.18	33.53	5.85	5.44	92.41	2.26	93/93
Sinigrin	0.20	0.64	0.23	0.07	29.65	1.91	93/93

Figure 1. Chromatographic development throughout the non-targeted and targeted approaches

(a) Non-targeted analysis –C18 'UHPLC like' (Phenomenex Kinetex C18 2.6µm 150 x 4.6 mm 100Ä)



(b) Non-targeted analysis – standard C18 HPLC (Phenomenex Luna 3um 150 x 2 mm 100Ä)



(b) Targeted analysis –C18 'UHPLC like' (Phenomenex Kinetex C18 2.6µm 150 x 4.6 mm 100Ä)

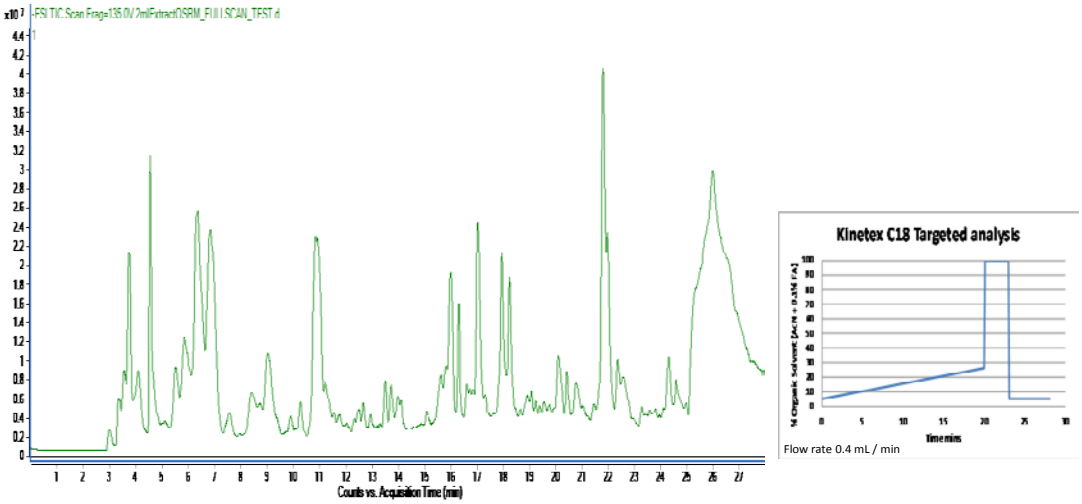


Figure 2. Principal Component Analysis of all metabolite classes. (a) PCA scores plot

(a)

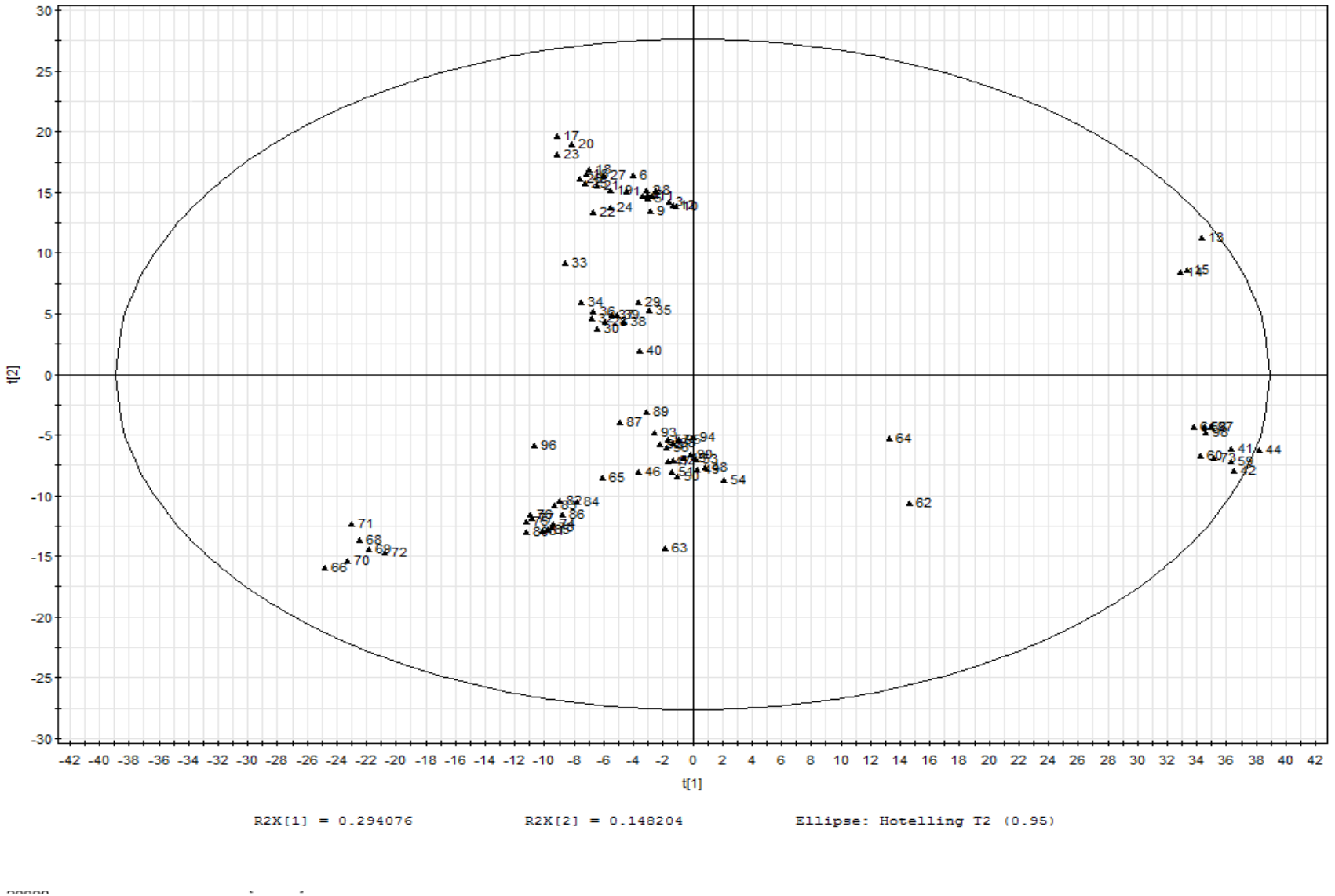


Figure 2. Principal Component Analysis of all metabolite classes. (b) Dendrogram of PC scores

(b)

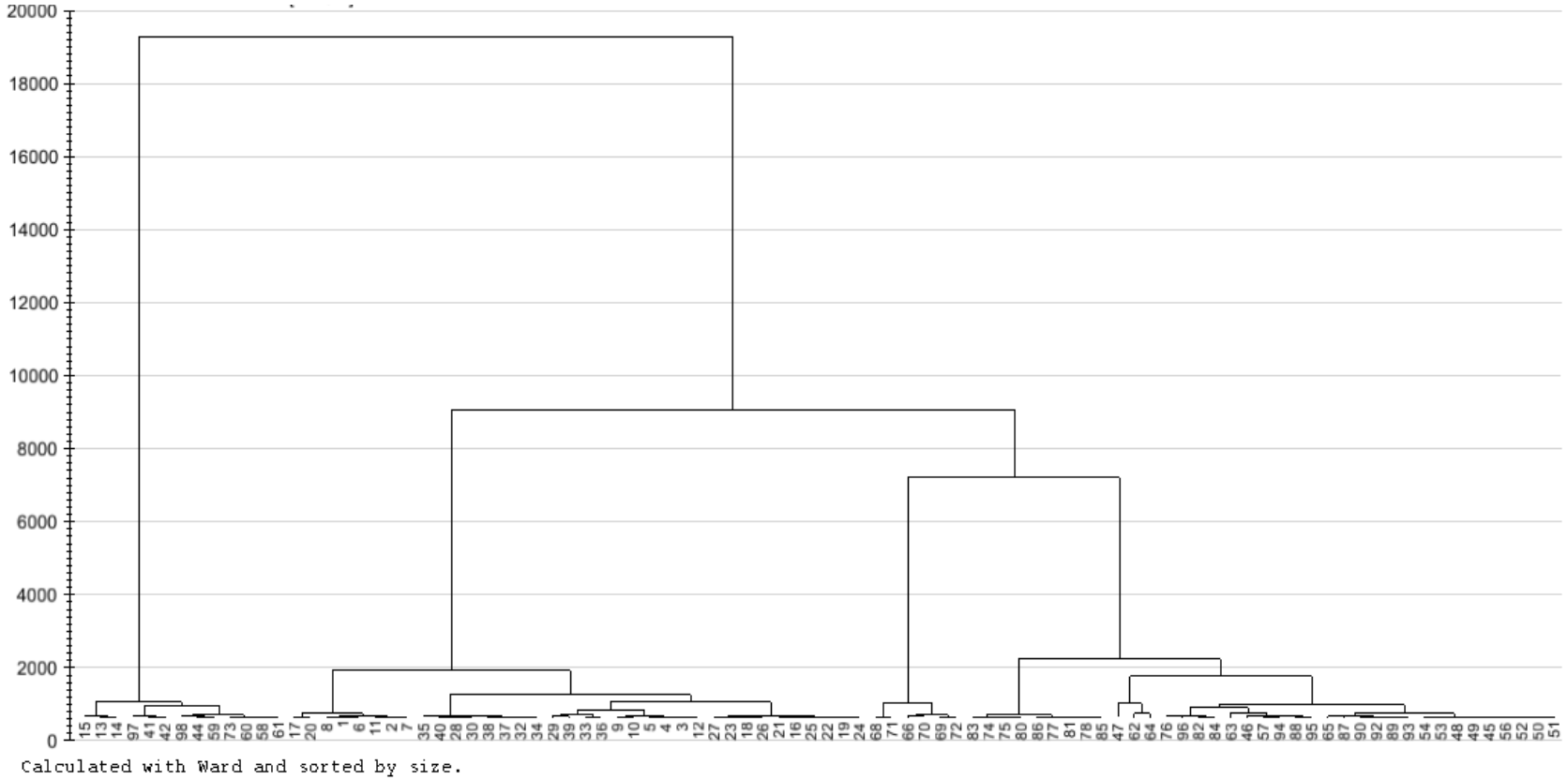


Figure 2. Principal Component Analysis of all metabolite classes. (c) PCA loading plot

(C)

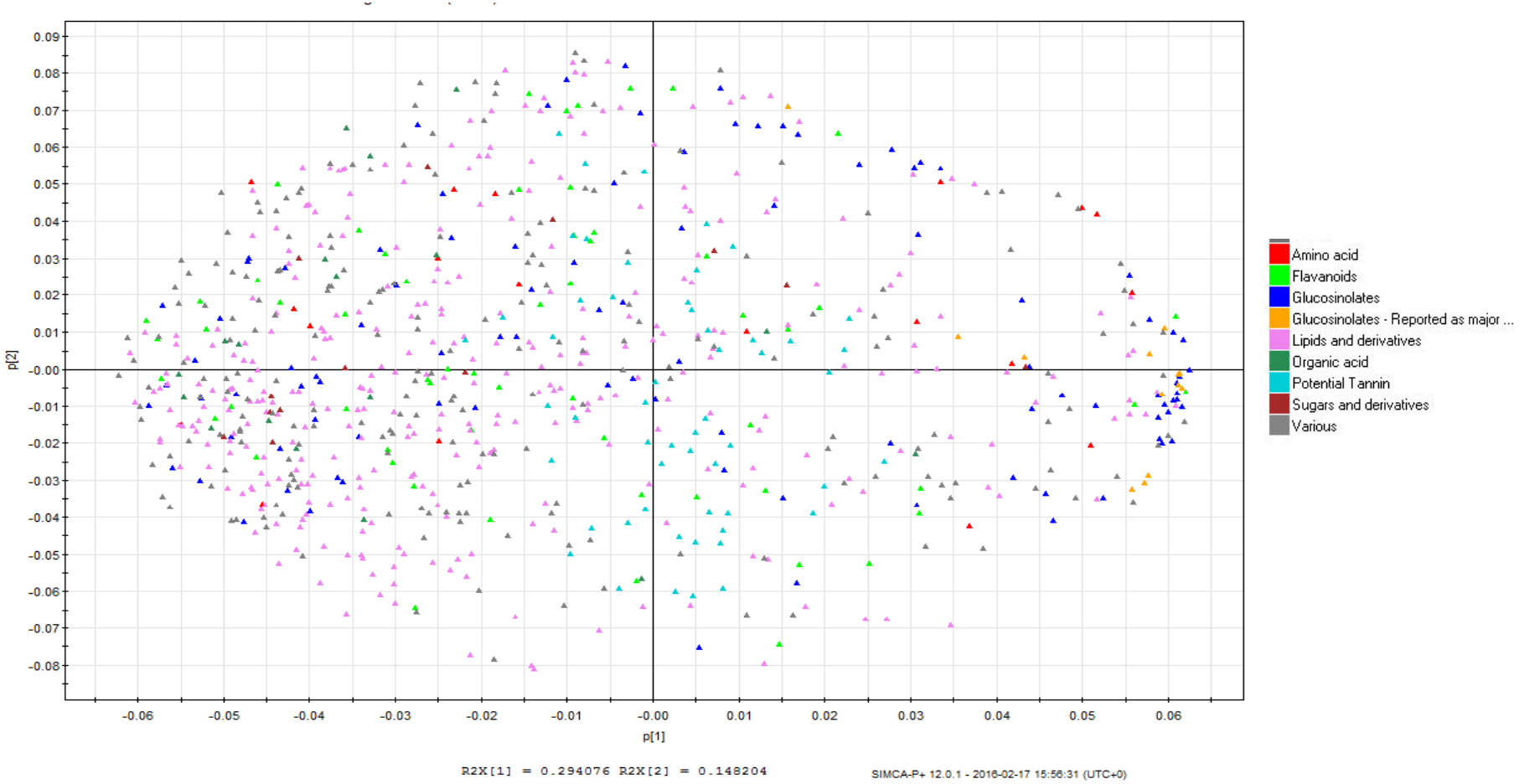


Figure 3. Bar graphs of the major glucosinolates of oil seed rape meal

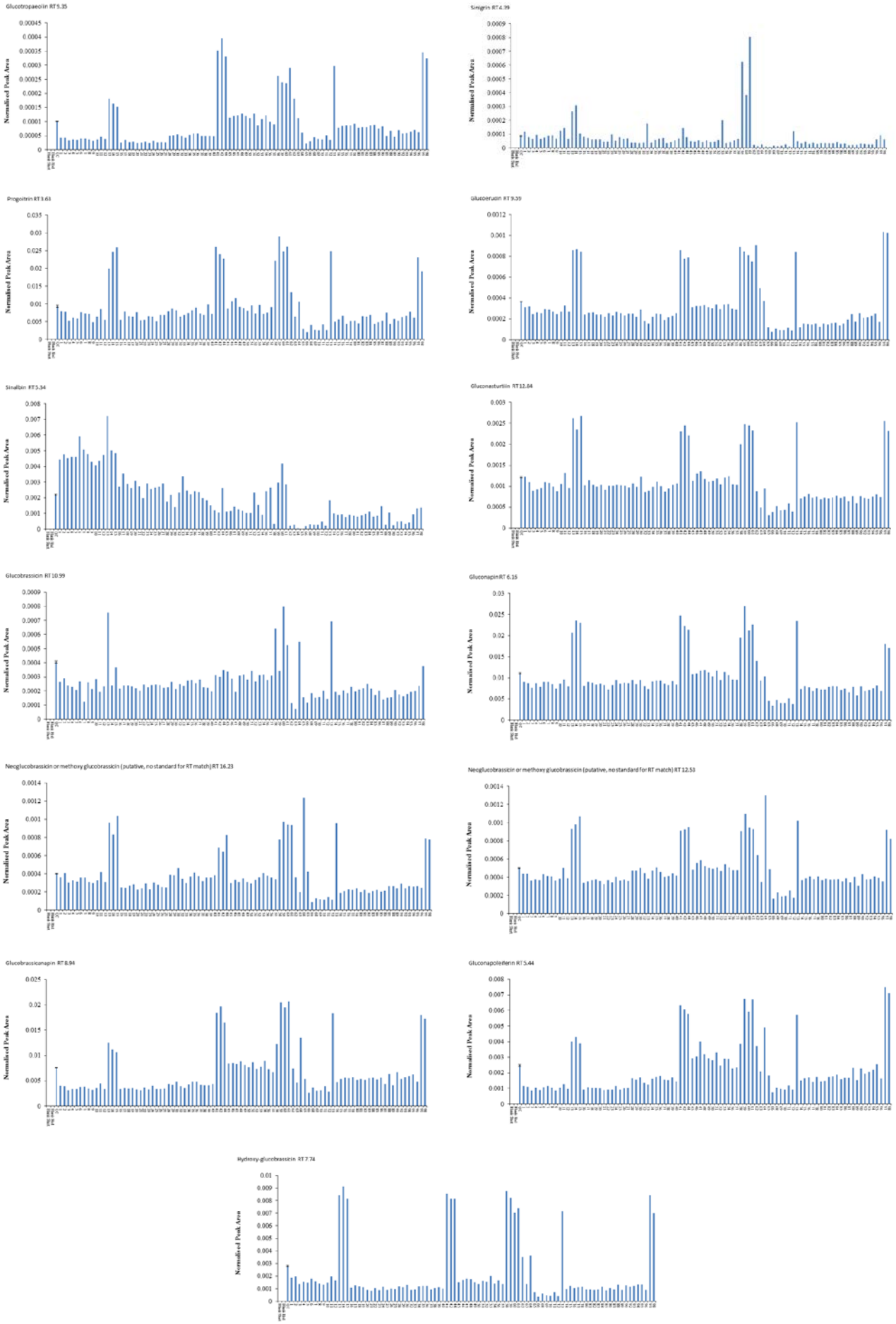


Figure 4. Bar graphs of the amino acids of oil seed rape meal

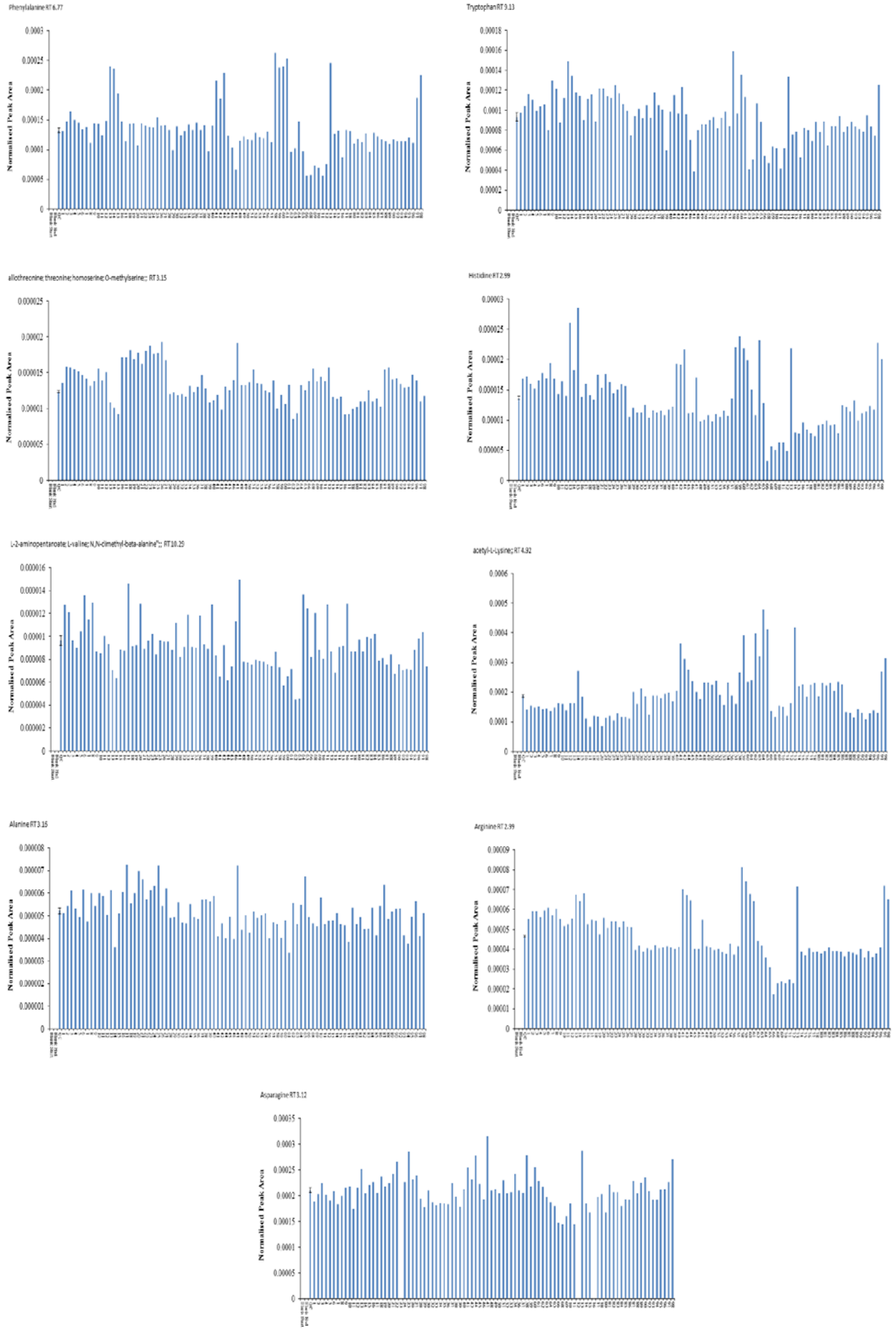
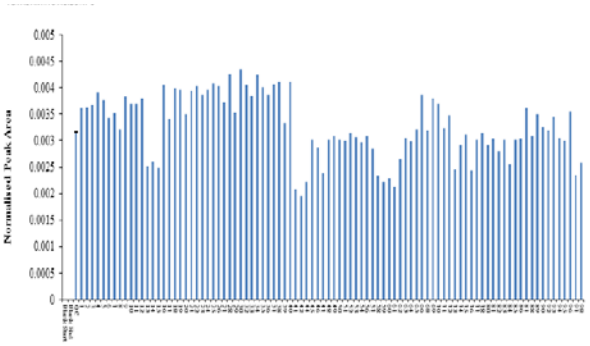
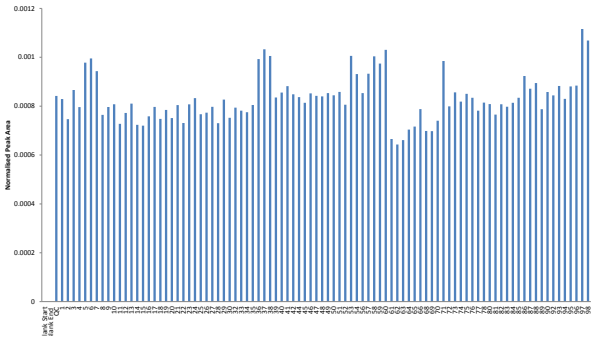


Figure 5. Bar graphs for the total potential tannin levels, organic acid levels, total lipid levels, total vitamin levels, total flavanoid levels and total sugar and sugar derivative levels

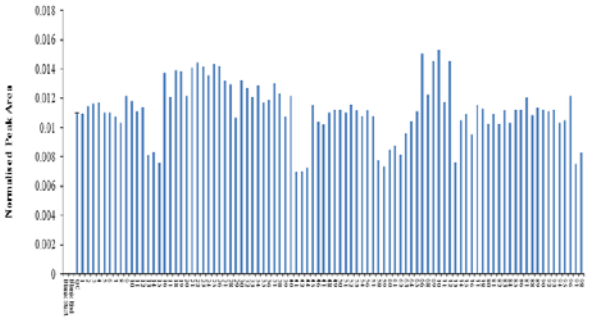
TOTAL AMINO ACIDS



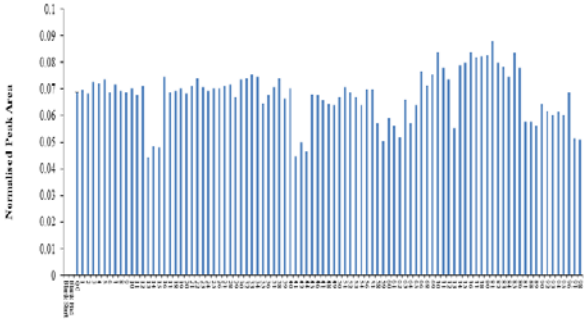
TOTAL POTENTIAL TANNINS



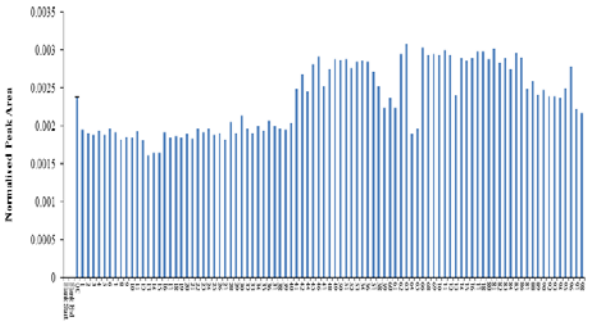
TOTAL ORGANIC ACIDS



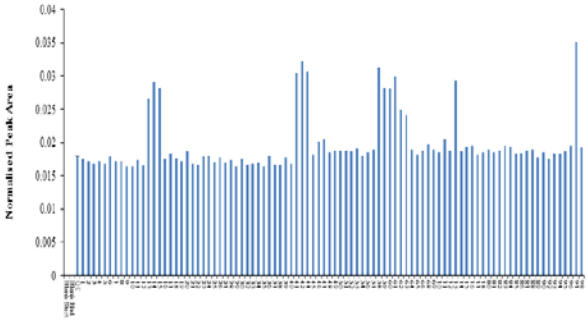
TOTAL LIPIDS



TOTAL VITAMINS



TOTAL FLAVANOIDS



TOTAL SUGARS, SUGAR ALCOHOLS, SUGAR PHOSPHATES, POLYOLS

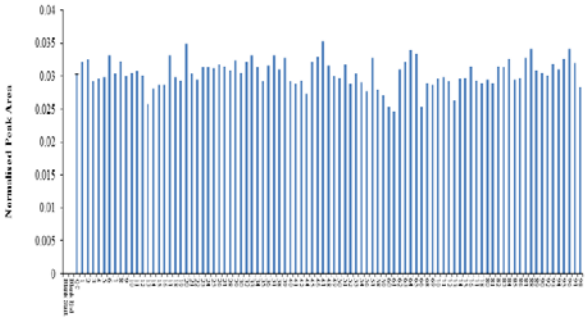
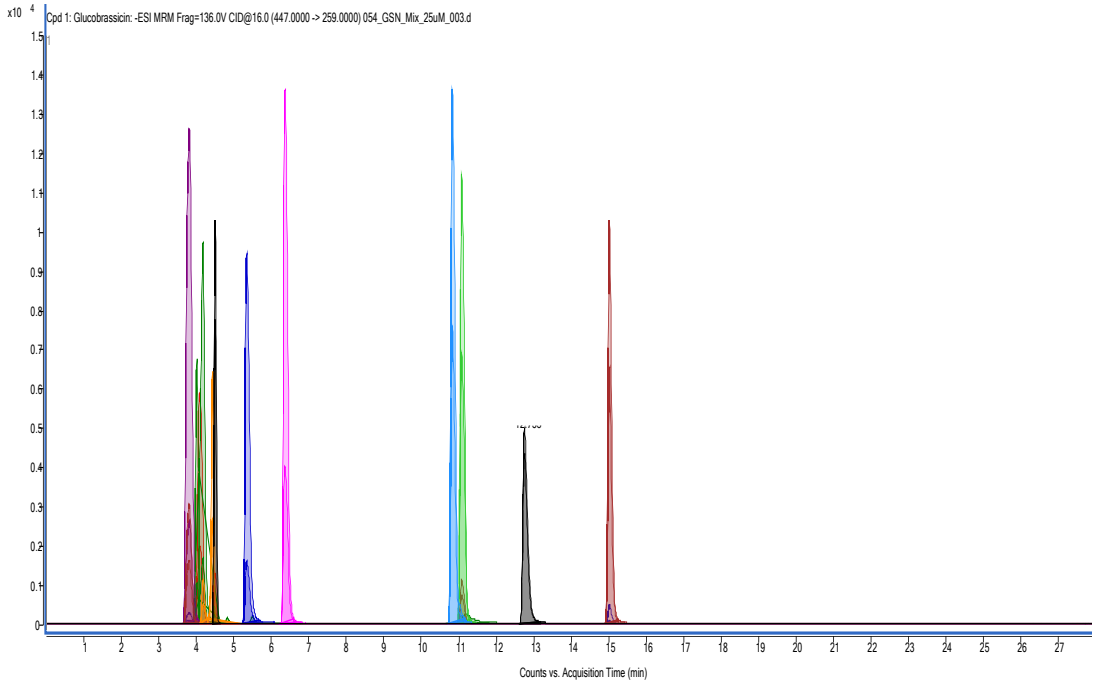


Figure 6. Targeted glucosinolate quantification, example MRM transition chromatograms for the glucosinolate reference standard cocktail and oil seed rape meal extract

(a) Targeted glucosinolate analysis – MRM transitions based upon the cocktail of glucosinolate standards



(b) Targeted glucosinolate analysis – MRM transitions based upon the oil seed rape meal extract

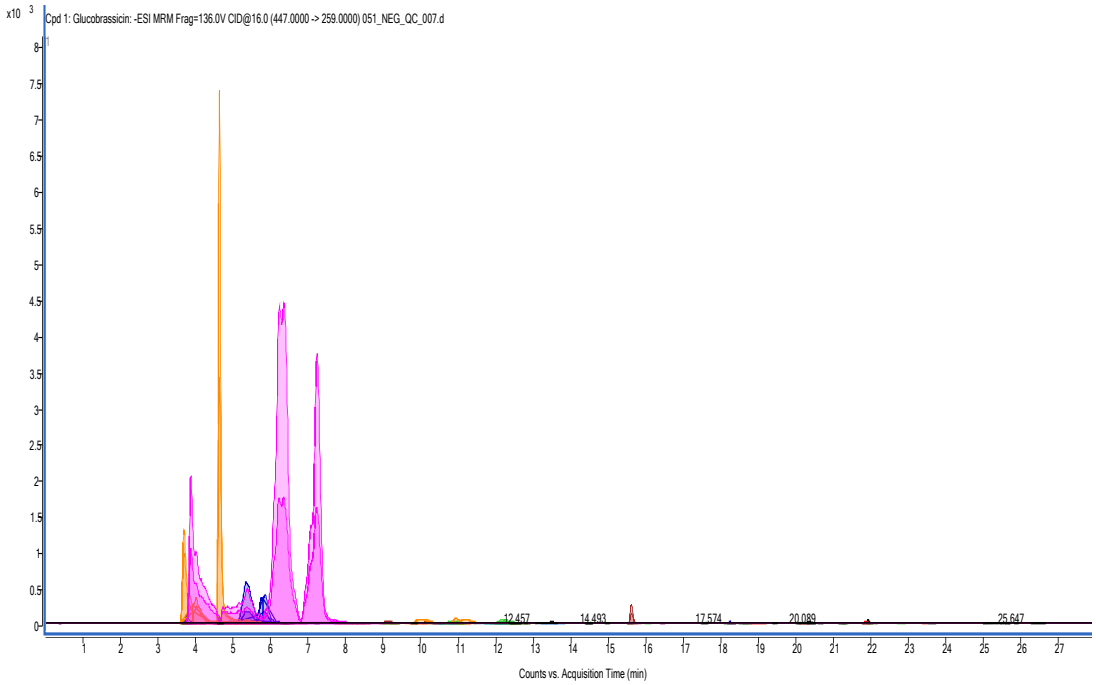
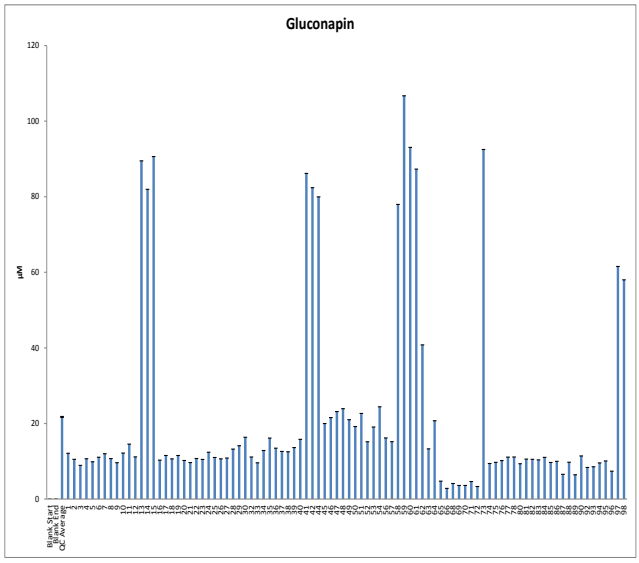
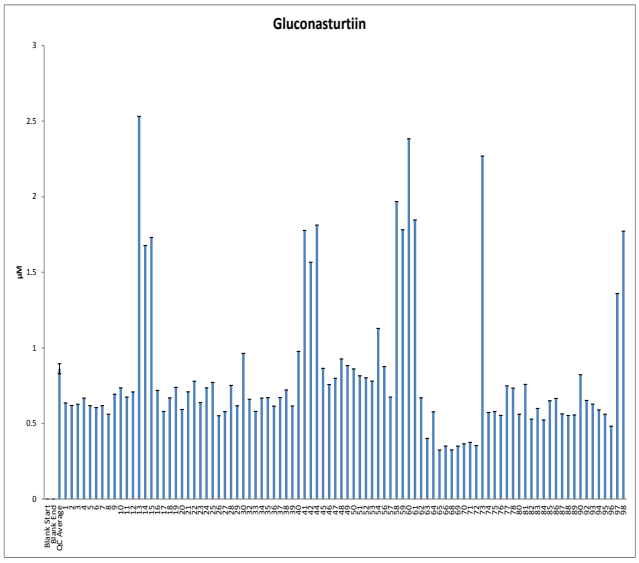
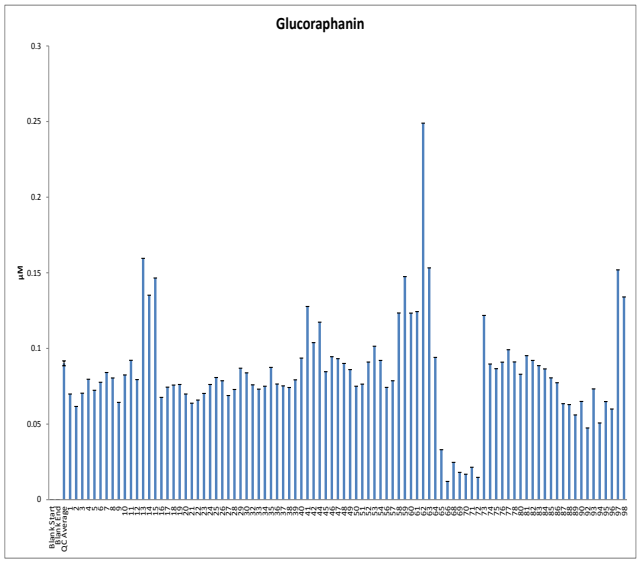
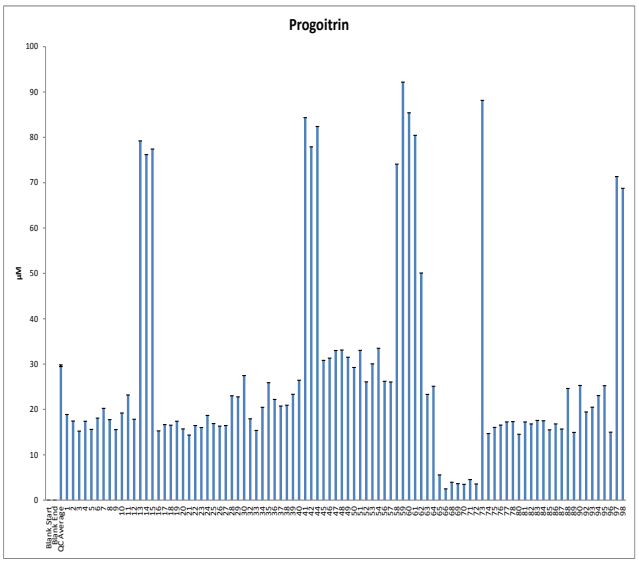


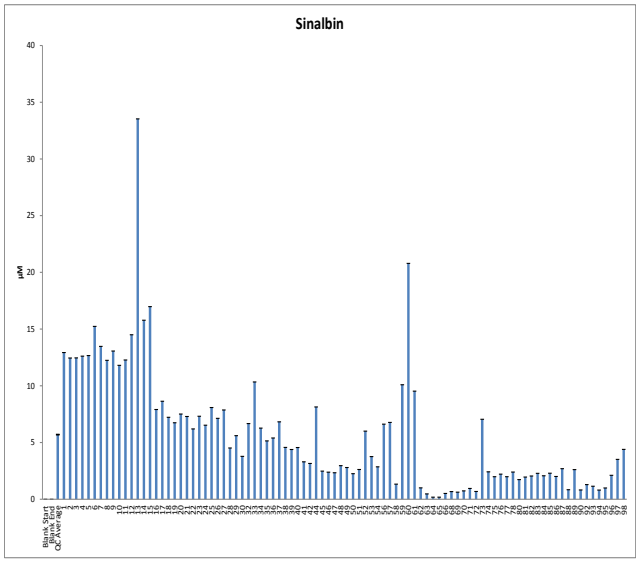
Figure 7. Bar graphs of the major glucosinolates of oil seed rape meal detected by the targeted approach











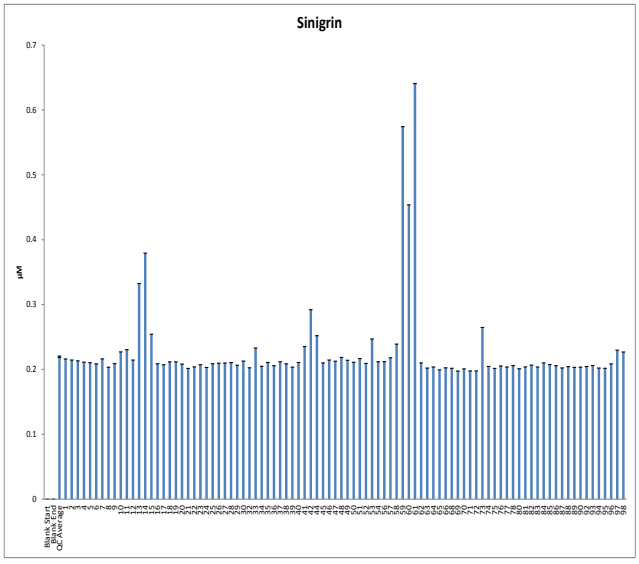


Figure S1. RT, accurate mass MS, and MS^2 spectral match of sinalbin standard and sinalbin within the oil seed rape meal QA extract as an example of an unambiguously identified glucosinolate

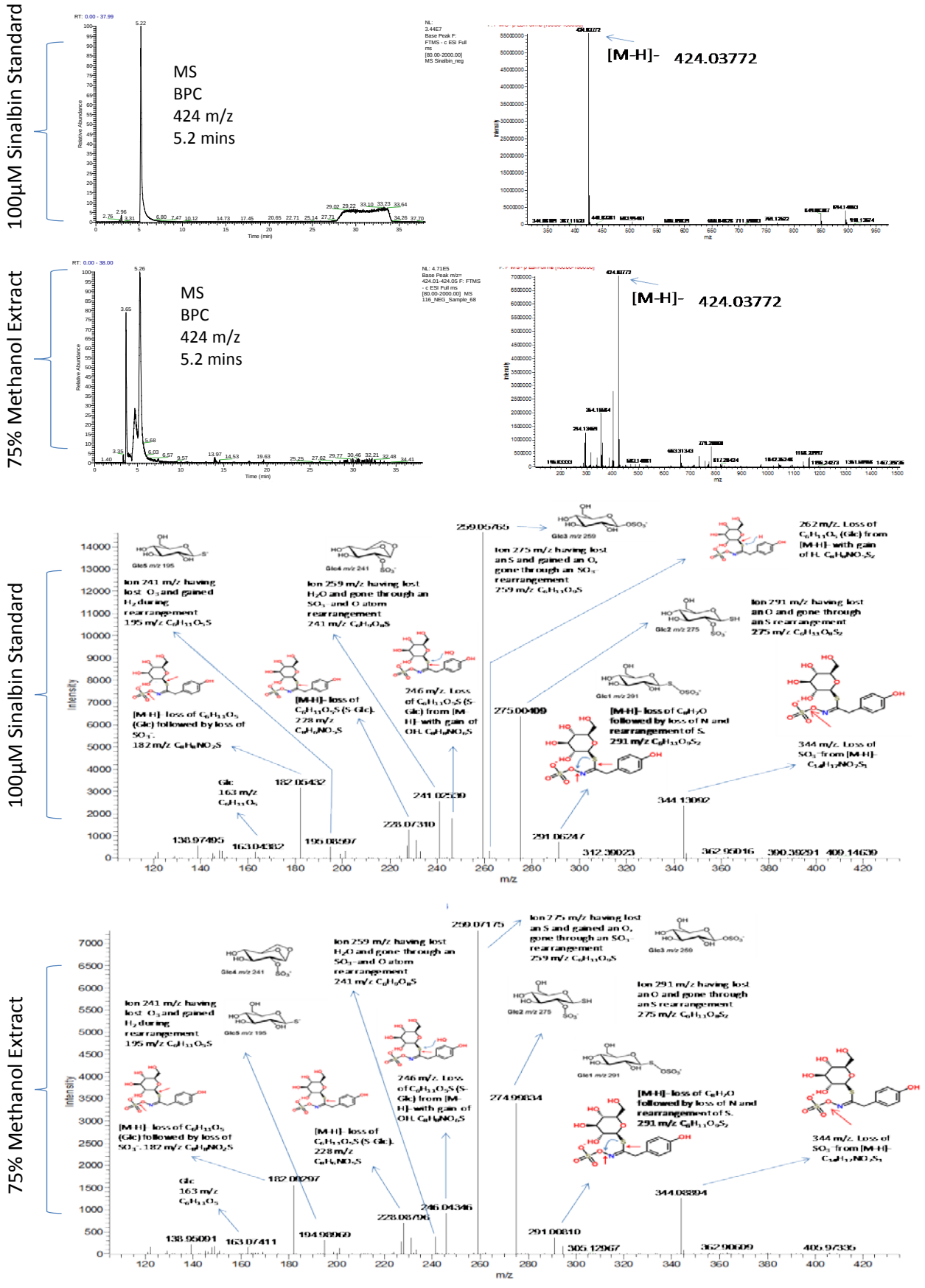


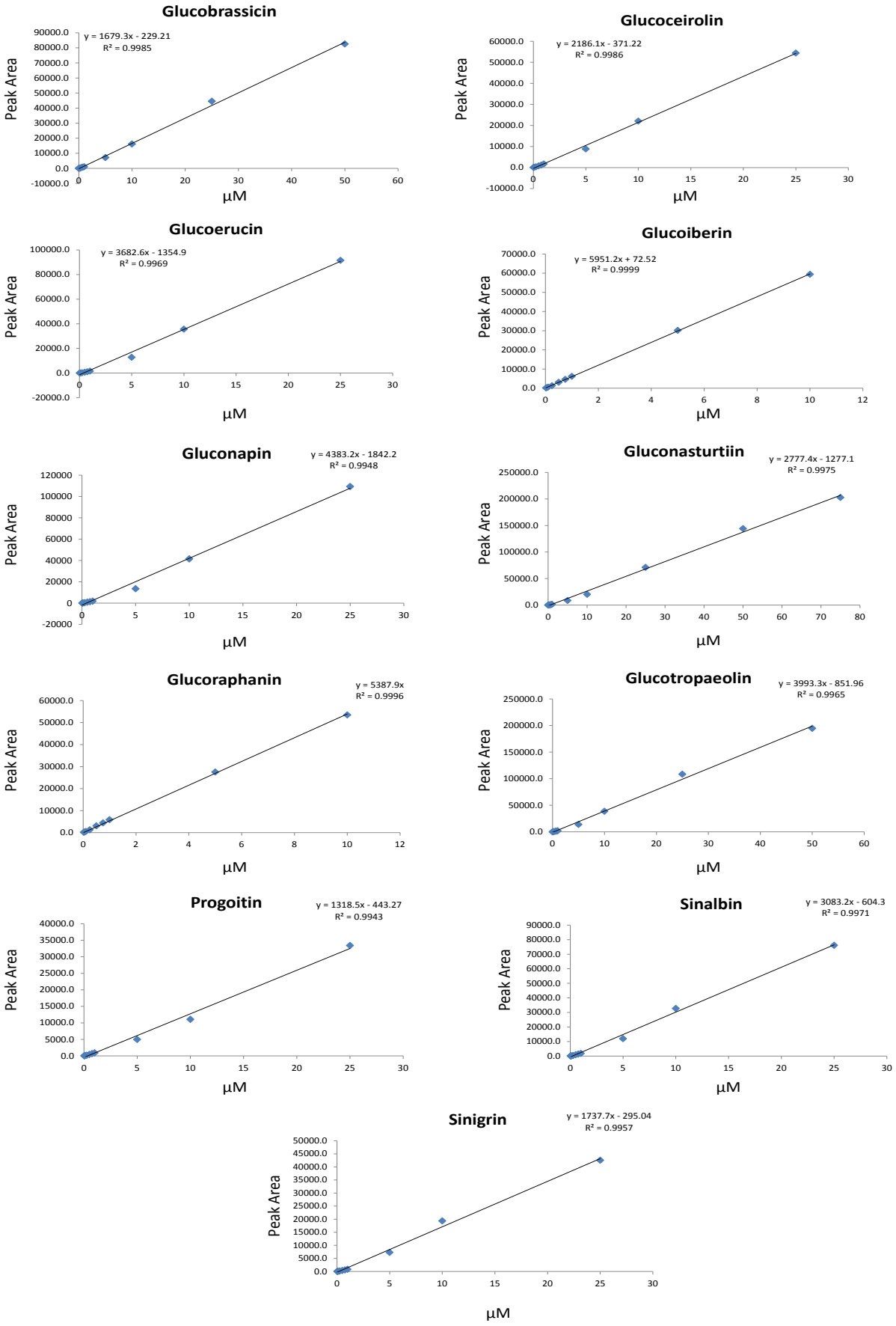
Table S2: Optimised MRM method parameters for eleven target glucosinolates developed for the targeted analysis

Compound	Formula	Mode	m/z	Fragment ion	Fragmentor V	CE	Intensity	RT
Glucoiberin	C11H21NO10S3	Negative	422	358	131	12	19740	3.9
Glucoiberin	C11H21NO10S3	Negative	422	196	131	20	5131	
Glucocheirolin	C11H21NO11S3	Negative	438	259	143	20	6109	4.4
Glucocheirolin	C11H21NO11S3	Negative	438	275	143	20	3091	
Sinigrin	C10H17NO9S2	Negative	358	259	131	12	10303	4.5
Sinigrin	C10H17NO9S2	Negative	358	241	131	12	6272	
Glucoraphanin	C12H23NO10S3	Negative	436	372	136	14	8245	4.5
Glucoraphanin	C12H23NO10S3	Negative	436	259.1	136	18	1545	
Progoitrin	C11H19NO10S2	Negative	388	259	131	12	1899	4.5
Progoitrin	C11H19NO10S2	Negative	388	195	131	16	1759	
Sinalbin	C14H19NO10S2	Negative	424	258.9	131	16	5693	5.3
Sinalbin	C14H19NO10S2	Negative	424	195.1	131	16	2476	
Gluconapin	C11H19NO9S2	Negative	372	259	136	12	9565	6.4
Gluconapin	C11H19NO9S2	Negative	372	275	136	12	3766	
Glucotropaeolin	C14H19NO9S2	Negative	408	259	146	16	7850	10.6
Glucotropaeolin	C14H19NO9S2	Negative	408	166	146	20	5171	
Glucoerucin	C12H23NO9S3	Negative	420	259	141	16	11229	10.9
Glucoerucin	C12H23NO9S3	Negative	420	178.1	141	16	7034	
Glucobrassicin	C16H20N2O9S2	Negative	447	259	136	16	6506	12.5
Glucobrassicin	C16H20N2O9S2	Negative	447	205	136	16	6010	
Gluconasturtiin	C15H21NO9S2	Negative	422.1	259	146	16	18733	15.1
Gluconasturtiin	C15H21NO9S2	Negative	422.1	180.1	146	16	12357	

Table S3: Glucosinolate cocktail quantification curve R^2, Limits of Detection (LOD) and Limits of Quantification (LOQ) values for the eleven glucosinolates within the targeted method

	Glucobrassicin	Glucocheirolin	Glucoerucin	Glucoiberin	Gluconapin	Gluconasturtiin	Glucoraphanin	Glucotropaeolin	Progoitrin	Sinalbin	Sinigrin
Slope	1679.340662	2186.062647	3481.94903	4212.11748	4383.18901	2012.336939	3649.924251	3993.260186	1096.392	3083.2143	1737.6873
Intercept	-229.2066819	-371.2174967	-1049.98219	4275.78643	-1842.2132	-350.7985366	1397.762601	-851.962878	-105.88981	-604.2969	-295.04373
R^2	0.998489059	0.998639624	0.99688848	0.9999178	0.99480426	0.99475126	0.994546805	0.996489286	0.9979394	0.9970952	0.9957208
Range (uM)	0.025 - 20	0.025 - 10	0.025 - 10	0.025 - 10	0.025 - 25	0.025 - 10	0.025 - 25	0.025 - 50	0.025 - 10	0.025 - 25	0.025 - 25
Mass g/mol	486.56	439.03	421.5	461.6	411.5	462.16	475.58	447.5	427.5	463.5	397.5
LOD uM	0.004589026	0.003342147	0.00323603	0.01772619	0.00425143	0.007418048	0.007733466	0.005891608	0.0255875	0.0088886	0.0080422
LOQ uM	0.015296752	0.011140489	0.01078677	0.0590873	0.01417145	0.024726826	0.025778221	0.019638695	0.0852915	0.0296286	0.0268072
LOD nM	4.589025694	3.342146771	3.23603191	17.7261914	4.2514338	7.418047847	7.733466156	5.891608414	25.587456	8.8885945	8.042171
LOQ nM	15.29675231	11.14048924	10.786773	59.0873047	14.171446	24.72682616	25.77822052	19.63869471	85.291522	29.628648	26.807237
LOD ug L	2.232836342	1.467302697	1.36398745	8.18240996	1.74946501	3.428324993	3.677881835	2.636494765	10.938638	4.1198636	3.196763
LOQ ug L	7.442787805	4.891008989	4.54662483	27.2746999	5.83155002	11.42774998	12.25960612	8.788315884	36.462125	13.732879	10.655877

Figure S4. Calibration curves of the eleven glucosinolates within the reference compound cocktail applied in the targeted quantification method



Supplementary Information

S1 Non Targeted metabolite profiling experimental methodology

S1.1 Sample Extraction

So that as many different metabolites as possible were detected, inclusive of glucosinolates, amino acids, and many other metabolic classes, a non-biased and non-targeted extraction was performed in 75% methanol: 24.9% water: 0.1% formic acid. Each individual sample was first homogenised to a very fine powder using a coffee mill, 500 mg aliquots were weighed out to be extracted in 15 ml centrifuge tubes, in addition a mixture of equal quantities of all of the samples was employed as a quality assurance (QA) and reference sample (that is both extracted and analysed multiple times throughout the entire analytical sequence and therefore define which detected compounds show high relative standard deviations that indicate compounds that are unreliably extracted and/or detected). Briefly, the extraction involves addition of eight volumes of extraction buffer to the homogenised oil seed rape cake mix (4 ml of buffer to 500 mg of material). The sample is vortex mixed for 30 seconds, prior to ultra-sonication for 15 minutes, followed by a further 15 minutes of agitation, after which the sample is centrifuged to pellet any solid matter and the supernatant taken forward to LC-MS analysis.

S1.2 High Performance Liquid Chromatography - Mass Spectrometry

The extract supernatants were transferred to Single Step 0.45 µm PTFE filter analytical vials fitted with pre-slit silicon septa caps (Thomson Ltd. Oceanside, California U.S., P/N 35540-500). The samples were stored in the auto sampler at 9 °C and analysed within 120 h of extraction in negative electrospray ionisation (ESI) mode. HPLC separations were performed with a Thermo Accela 600 HPLC system coupled with an Accela PDA detector (Thermo-Fisher Ltd. Hemel Hempstead U.K) essentially according to the methods of de Vos *et al.* (2007), only optimised for faster LC separations permitted by applying a Phenomenex C18 core shell column (00F-4462-E0 Kinetex C18 2.6 µm 150 x 4.6 mm 100Ä) (Figure 1a) as opposed to the conventional Phenomenex C18 HPLC column (00F-4251-B0 Luna 3µm 150 x 2 mm 100Ä) (Figure 1b).

The HPLC was operated at a flow rate of 400 µL/min, the column was maintained at a temperature of 40 ^{o}C. The solvent A, 18.2 MΩ.cm deionised water (ELGA-PureLab option-Q, Elga Ltd., High Wycombe U.K.), and solvent B, HPLC grade acetonitrile (Fisher Scientific Ltd. Loughborough U.K.) were acidified with 0.1% [v/v] mass spectrometry grade formic acid (Fisher Scientific Ltd. U.K., P/N A117-50). Prior to sample analysis a new HPLC column was conditioned with solvents A and B for a minumum of 40 min at a flow rate of 400µL/min. A sample injection volume of 5 µL was employed in partial-loop mode. The gradient programme was as follows: 5-35% B 0-23 min, 35-75% B 23-25 min, hold 75% B 25-30 min, 75-5% B 30-31 min, hold 5% B 31-38 min. Autosampler syringe and line washes were performed with 80% HPLC grade acetonitrile. The HPLC column eluent was first monitored by the Accela PDA detector where spectra were collected in wavelength/absorbance

mode from 200-600 nm with a filter bandwidth and wavelength step of 1 nm, the filter rise time was 1 sec, the sample rate was 10 Hz. Additionally three chanal set points were employed, Channel A 280 nm, Channel B 365 nm, Channel C 520 nm, with a bandwidth of 9 nm and a sample rate of 10 Hz.

The PDA detector eluent was next transfered to the Thermo LTQ-Orbitrap XL mass spectrometry system operated under Xcalibur software (Thermo-Fisher Ltd. U.K.). Mass spectra were primarilly collected in full scan mode (*m/z* 80-2000, at a mass resolution of 30,000 (FWHM defined at *m/z* 400) within the FT for high mass precision. In addition, a second method was devised where as well as full scan MS data being colllected within the FT, a data-dependent secondary scan event was applied to collect MS^2 CID fragmentation spectra within the LTQ based upon the top three most intense ions as defined within the preliminary full MS scan. For both methods, full scan MS and MS^2, data were collected in the centroid mode. A scan speed of 0.1 seconds and 0.4 seconds were apllied in the LTQ and FT respectively. The Automatic Gain Control was set to $1x10^5$ and $1x10^6$ for the LTQ and FT respectively. Prior to the analytical run the LTQ and FT-MS were tuned to optimise conditions for the detection of ions in the mid detection range of *m/z* 80-2000, as well as being calibrated with the manufacturers recomended calibration mixture and procedure. The ESI conditions were optimised to allow efficient ionisation and ion transmission without causing insource fragmentation. The following settings were applied to ESI: Spray voltage -3.5kV; Sheath gas 35; Aux gas 15; Capilary voltage 35V; Tube lens voltage -100V; Capilary temperture 380^o. A control extraction blank sample was analysed at the start and end of the analytical block, thus providing a measure of the sample back-ground and also a measure of compound carry over resulting from dirtying of the ESI source throughout the sample run. The LC-MS system was initially conditioned with eight analyses of the QA sample mixture which provides system stability, a further two analyses of QA sample were performed, this was followed by analysis of seven individual samples and was followed by the analysis of another QA sample. This procedure was followed until all samples were analysed and the run concluded with three further analyses of QA sample and the final end run blank. The individual oil seed rape meal samples were completely randomised prior to analysis. The QA samples provide a mechanism by which the data is assessed to define peaks that are of a high quality. Metabolite peaks that show greater than a 30% relative standard deviation within the QA samples spread throughout the analytical run were flagged as peaks that should not be considered as significant due to not being reproducibly detected.

S1.3 HPLC-MS profile deconvolution and data pre-treatment

The LC-MS raw data profiles were first converted into an MZML centroid format within the freely available Proteowizard MSConvert software package (http://proteowizard.sourceforge.net/). Each MZML based three-dimensional data matrix (intensity × *m*/*z* × time – one per sample) was converted (or deconvolved) into a vector of *peak responses* (extracted peak areas), where a *peak response* is defined as the sum of intensities over a window of specified mass and time range (e.g.

m/*z* = 102.1 ± 0.01 and time = 130 ± 30 s). In this experiment the deconvolution was performed using the freely available XCMS software (http://masspec.scripps.edu/xcms/xcms.php). A full description of the data deconvolution method performed within XC-MS is available in Dunn *et al.,* (2008), in this study the band width (bw) setting was adjusted from 10 to 20 to accommodate the wider peak widths that result in HPLC in comparison to Ultra High Performance Liquid Chromatography (UHPLC). In development of the deconvolution method several band width settings were assessed including bw 10, 20, 30 and 40, with bw 20 producing the most satisfactory results. The XC-MS deconvolution results in the production of an MS Excel based XY matrix. Data normalisation was based upon the correction of each individual peak area to the total ion chromatogram (TIC) signal (i.e. all individual metabolite signals are scaled to the samples total signal). The normalisation effectively corrects for instrument inaccuracies that largely result from the sample injection apparatus. The data were next filtered based upon the relative standard deviation (RSD) of each detected feature across the QA samples that are interspersed across the entire analytical batch run, compound features that show a greater than 30% RSD across the QA samples were filtered out and deemed not to be suitable to monitor the feed quality of the oil seed rape meal samples due to not being robustly measured.

S1.4 Construction and application of glucosinolate and flavanoid targeted as well as plant-kingdom and lipid compound specific databases for annotation of HPLC-MS profiles

The first step in compound identification was the production of the three databases applied for metabolite annotation. The first database was produced from two libraries, the Wageningen University glucosinolate and flavanoid library and the glucosinolate library gifted by Dr. Don Clark (FERA). The libraries were sorted so that for each entry we had a compound name(s), an associated molecular formula, and the correct accurate (monoisotopic) mass for each molecular formula. Two text files are then created, one which takes *m/z* accurate mass information from the LC-MS data, converts it to a neutral compound mass which is then possible to match to a molecular formula(s), and a second that associates the given molecular formula(s) to actual compound identities. A second library based upon the Plant metabolic Network PlantCyc database (http://www.plantcyc.org) was produced in a synonymous fashion. The final metabolite library was based upon the LipidMaps database and was applied for the annotation of a large range of lipid species including free fatty acids, MAGs, DAGs and TAGs, phospholipids, ceramides, sterols and related lipophilic vitamins.

The Excel data matrix produced by the XCMS workflow was finally annotated applying the PutMedID set of workflows within the Taverna Workbench 1.7.2 software package (Brown *et al..* 2009). PutMedID first calculates *m/z*-to-*m/z* based correlations, where for example if two or more *m/z* within a given retention time tolerance (+/- 30 secs) show high levels of Pearson correlation coefficient (greater than 0.9), they are deemed to be *m/z* features that result from a common metabolite. In a second step the mass differences between correlated *m/z* features allows us to define the type of ion that is formed in ESI, for example, H+, Na+, K+, etc. (ESI positive mode), H-, Cl-, CHOOH-, etc.

(ESI negative mode). This permits the calculation of the compounds neutral mass from the accurate mass MS information for the charged ion (i.e. *m/z*). Once the neutral mass is known, it can then be matched to the possible molecular formula against the compound libraries that have been developed. As a third step, the molecular formulas annotated for each *m/z* feature are then matched to the second database file where the matched molecular formula(s) are associated with the compound name(s).

S1.5 Unambiguous identification of glucosinolates

To confirm the putative identifications that are made through the accurate mass data annotation procedure, it is necessary to obtain analytical standard(s) for those putatively annotated compounds, with the aim of matching an LC based retention time (RT), an MS based accurate mass and an MS^2 based fragmentation spectra, between the analytical standard and the compound within the biological extract when analysed on the same LC-MS instrument and under identical conditions. Only by matching of two orthogonal properties, RT and MS^2 based fragmentation spectra, are we able to define an unambiguous identification for a given metabolite feature (Figure S5). In total, eleven glucosinolate analytical standards were obtained based upon global availability. The standards were first dissolved in either water or 80% methanol depending upon compound solubility to produce a stock solution of 1 mg/mL. The stock solutions were further diluted to provide a solution of 100 µM in concentration, that was then transferred to an HPLC analytical vial and analysed under identical conditions and applying the same HPLC-MS^2 method as to the oil seed rape meal extracts. Based upon HPLC retention time, accurate mass MS measurements and matching of MS^2 spectra, the following glucosinolates and amino acids were unambiguously identified, Phenylalanine, Tryptophan, Glucotropaeolin, Sinigrin, Progoitrin, Glucoerucin, Sinalbin, Gluconasturtiin, Glucobrassicin and Gluconapin. Compounds were otherwise identified at a putative level on the basis of accurate mass based matching to the molecular formula and compound databases as described in section.

S2 Targeted glucosinolate profiling experimental methodology

S*2.1 Sample Extraction*

For targeted analysis the same extraction was performed in 75% methanol: 24.9% water: 0.1% formic acid. Each individual sample was first homogenised to a very fine powder using a coffee mill, 500 mg aliquots were weighed out to be extracted in 15 ml centrifuge tubes, in addition a mixture of equal quantities of all of the samples was employed as a quality assurance (QA) and reference sample (that is both extracted and analysed multiple times throughout the entire analytical sequence and therefore define which detected compounds show high relative standard deviations that indicate compounds that are unreliably extracted and/or detected). Briefly, the extraction involves addition of four volumes of extraction buffer to the homogenised oil seed rape cake mix (2 ml of buffer to 500 mg of material, as opposed to 4 ml of buffer to 500mg of material as applied in the non-targeted

analysis). The sample is vortex mixed for 30 seconds, prior to ultra-sonication for 15 minutes, followed by a further 15 minutes of agitation, after which the sample is centrifuged to pellet any solid matter and the supernatant taken forward to LC-MS analysis.

S2.2 High Performance Liquid Chromatography - Mass Spectrometry

The extract supernatants were transferred to Single Step 0.45 µm PTFE filter analytical vials fitted with pre-slit silicon septa caps (Thomson Ltd. Oceanside, California U.S., P/N 35540-500). The samples were stored in the auto sampler at 9 °C and analysed within 60 h of extraction in negative electrospray ionisation (ESI) mode. HPLC separations were performed with an Agilent 1260 Infinity HPLC system coupled to an Agilent 1260 Infinity PDA detector (Agilent Ltd. Stockport U.K) essentially according to the methods of de Vos *et al.* (2007), only optimised for faster LC separations permitted by applying a Phenomenex C18 core shell column (00F-4462-E0 Kinetex C18 2.6 µm 150 x 4.6 mm 100Ä) as opposed to the conventional Phenomenex C18 HPLC column (00F-4251-B0 Luna 3µm 150 x 2 mm 100Ä).

The HPLC was operated at a flow rate of 400 µL/min, the column was maintained at a temperature of 40 ^{o}C. The solvent A, 18.2 MΩ.cm deionised water (ELGA-PureLab option-Q, Elga Ltd., High Wycombe U.K.), and solvent B, HPLC grade acetonitrile (Fisher Scientific Ltd. Loughborough U.K.) were acidified with 0.1% [v/v] mass spectrometry grade formic acid (Fisher Scientific Ltd. U.K., P/N A117-50). Prior to sample analysis a new HPLC column was conditioned with solvents A and B for a minumum of 40 min at a flow rate of 400µL/min. A sample injection volume of 10 µL was employed. The gradient programme was identical to that employed for the non-targeted analysis up to 20 minutes into the gradient when all expected glucosinolates have eluted, the gradient was then changed to rapidly elute off any remaining compounds with 100% acetonitrile prior to column re-equilibriation, which shortened the total gradient time to 28.1 minutes (Figure 1c). The final gradient method was as follows: 5-26% B 0-20 min, 26-100% B 20-20.1 min, hold 100% B 20.1-23 min, 100-5% B 23-23.1 min, hold 5% B 23.1-28.1 min. Autosampler syringe and line washes were performed with 80% HPLC grade acetonitrile. The HPLC column eluent was first monitored by the PDA detector where spectra were collected in wavelength/absorbance mode from 200-600 nm with a filter bandwidth and wavelength step of 1 nm, the filter rise time was 1 sec, the sample rate was 10 Hz. Additionally three chanal set points were employed, Channel A 280 nm, Channel B 365 nm, Channel C 520 nm, with a bandwidth of 9 nm and a sample rate of 10 Hz.

The PDA detector eluent was next transfered to the Agilent 6460 triple quadrupole mass spectrometry (QQQ-MS) system operated under Agilent MassHunter workstation software (Agilent Ltd. Stockport U.K.). Prior to analysis the QQQ-MS was tuned and calibrated with the manufacturers recomended calibration mixture and procedures. The ESI conditions were optimised to allow efficient ionisation and ion transmission without causing insource fragmentation. The following settings were applied to ESI: Spray voltage -3.5kV; Gas flow 9 L/min and temperature 350 ^{o}C; Nebuliser pressure 50 psi; Sheath gas flow 11 L/min and temperature 250 ^{o}C; Nozzle voltage 500 V; the MS1 and MS2

heater temperatures were both set to 100 ^{o}C. Mass spectra were primarilly collected in Multiple Reaction Monitoring (MRM) scan mode. For each glucosinolate where standards were available, the pure compound was first directly infused (without HPLC separation) and a range of fragmentor voltages (50-200 V) and collision energies (10-100 normalised colision energy (NCE)) were applied with a 20 ms dwell time to optimise the fragmentations of each compound. For each glucosinolate two optimised fragment ions were selected, the first fragment ion (the quantification ion) was for most glucosinolates based upon the core glucosinolate fragment ions of *m/z* 259 or 275, whereas the second fragment ion was selected as a qualification ion which where possible was unique to each glucosinolate, or minimally unique to each glucosinolate within their expected retention time range. Each pair of optimised product ions applied to monitor each individual glucosinolate along with their optimised fragmentor voltages and collision energies are presented in Table 2. On the basis of the eleven optimised glucosinolates each having two product ions, the developed MRM method had an MS duty cycle time of 517 ms or 1.93 cycles per second (20 ms dwell time x (22 scan events + 3.5 ms inter-scan delay) = 517 ms), which was considered appropriate for the expected peak widths of the targeted glucosinolates.

Once the optimised MRM method had been finalised, the HPLC, PDA and MS methods were combined and applied to both the sample extracts as well as a cocktail of the eleven glucosinolate standards ranging in concentration (0 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, 100 µM). Each concentration of the glucosinolate cocktail was first analysed four times starting with the lowest and running through to the highest concentration, thus avoiding compound carryover that may potentially result from the analysis of the higher concentrations. Once the glucosinolate cocktails had been analysed in order to produce calibration curves to perform relative quanitification against, the ESI source of the QQQ-MS was fully cleaned and needle wash vials replaced, again to prevent any carryover of residual glucosinolates from the calibration cocktails to the oil seed rape meal sample extract profiles. A control extraction blank sample was analysed at the start and end of the sample extracts analytical block, thus providing a measure of the sample back-ground and also a measure of compound carry over resulting from dirtying of the ESI source throughout the sample run. The LC-MS system was initially conditioned with eight analyses of the QA sample mixture which provides system stability, a further two analyses of QA sample were performed, this was followed by analysis of seven individual samples and was followed by the analysis of another QA sample. This procedure was followed until all samples were analysed and the run concluded with two further analyses of QA sample and the final end run blank. The individual oil seed rape meal samples were completely randomised prior to analysis. The QA samples provide a mechanism by which the data is assessed to define target compounds that are detected with a high quality.