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Rapeseed napin and cruciferin are readily digested by poultry

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Title: Rapeseed napin and cruciferin are readily digested by poultry

Running title: Rapeseed proteins are readily digested by poultry

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Summary

Rapeseed proteins have been considered as being poorly digestible in the gut of non-ruminants. The aim of the study was to assess the digestibility of napin and cruciferin in ileal digesta of broiler chickens, testing sixteen samples of rapeseed co-products with protein levels ranging from 293 g/kg to 560 g/kg dry matter. Each sample was included into a semi-synthetic diet at a rate of 500 g/kg and evaluated with broiler chickens in a randomised design. Dietary and ileal digesta proteins were extracted and identified by gel-based liquid chromatography tandem mass spectrometry (LC-MS/MS). Three isomers of napin (a 2S albumin) and nine cruciferins (an 11S globulin) were identified in the rapeseed co-products, whereas six endogenous enzymes such as trypsin (I-P1, II-P29), chymotrypsin (elastase and precursor), carboxypeptidase B, and α-amylase were found in the ileal digesta. It is concluded that as none of the rapeseed proteins were detected in the ileal digesta, rapeseed proteins can be readily digested by broiler chickens, irrespective of the protein content in the diet.

Keywords: napin; cruciferin; protein; rapeseed meal; rapeseed cake; chickens.

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Introduction

The seed storage proteins of rapeseed (*Brassica napus*) consist of approximately 60% cruciferin (known as 11S globulin, rich in lysine and methionine), 20% napin (2S albumin, rich in glutamine, proline, and cysteine), and minor proteins such as thionins, trypsin inhibitor and lipid transfer protein (Berot et al., 2005; Bos et al., 2007). Cruciferin (molecular weight, MW 300-360 kDa) consists of six subunits that are arranged as two trimers, held together by hydrogen bonds and salt bridges (Wanasundara and McIntosh, 2013). The cruciferin subunit of this hexameric assembly (~50 kDa) contains an acidic or α-chain (29-33 kDa) and a basic or β-chain (20-23 kDa), that are linked by single disulphide bond (Schatzki et al., 2014). Napin (MW ~13-18 kDa), is a dimer of a large or heavy polypeptide (10-12 kDa) and a small or light (3-6 kDa) polypeptide that are connected by four disulphide bonds (Rask et al., 1998; Wanasundara and McIntosh, 2013; Schatzki et al., 2014).

Rapeseed co-products are of considerable interest as a protein source in animal feeds due to a high content of protein with a greater content of sulphur-rich amino acids (cysteine, methionine) compared to a standard soybean meal (Wickramasuriya et al., 2015). During rapeseed oil production, whole seeds are de-fatted by hexane extraction producing a rapeseed meal (RSM), or by cold-pressing producing a rapeseed cake (RSC) (Untersmayr and Jensen-Jarolim, 2008). The crude protein content of the co-products may range from 329 to 437 g/kg dry matter (DM) (Seneviratne et al., 2011a, b; Maison et al., 2014). However, protein content and individual amino acid levels will vary depending on rapeseed variety and oil extraction method used (Kasprzak et al., 2016). Several studies have shown that rapeseed protein is less digestible (by an absolute decrease of 14-16 %) than soybean protein or casein protein in standard diets (Savoie et al., 1988; Adedokun et al., 2008). This difference in nutritional value of protein is not only attributed to variation in chemical composition between the co-products, but also to the compact structure and relatively high content of disulphide bonds in rapeseed protein. When *in vitro* models were used, napin was reported to be extremely resistant to pepsin digestion and denaturation caused by heat and low pH (Murtagh et al., 2003; Abeysekara and Wanasundara, 2009; Wanasundara, 2011).

To the best of our knowledge, there is no *in vivo* study focusing on digestibility of napin and cruciferin in the gastro-intestinal tract of non-ruminants when examining rapeseed proteins.

The aim of the current study was to identify proteins in de-fatted rapeseed co-products, and the corresponding ileal digesta from broilers fed rapeseed diets.

Materials and methods

Rapeseed co-products and diets

Thirteen rapeseed varieties were grown and harvested in four different counties in Great Britain in 2013. Four rapeseed varieties were cold-pressed producing RSC, and eleven rapeseed varieties were softly processed and hexane-extracted producing soft rapeseed meal (SRSM). The soft processing was used in order to minimise the possibility of overriding the variety variation across the SRSM.

The conditioning, seed crushing and hexane extraction was conducted in a pilot plant (Pessac, Bordeaux, France), while cold-pressing was performed at a local plant in Norfolk (United Kingdom) according to previously described methods (Kasprzak et al., 2016). The resulting four RSC and twelve SRSM samples were ground (4 mm sieve) and included in a semi-synthetic diet at 500 g/kg as previously published by Kasprzak et al. (2016). The rapeseed co-products were the only source of protein in the diets. Each of the diets also contained, in addition to the rapeseed co-products, wheat starch (200 g/kg), glucose (195 g/kg), vitamins and minerals (50 g/kg), rapeseed oil (50 g/kg) and an inert digestibility marker - titanium dioxide (5 g/kg).

Bird study

Day old male Ross Broilers 308 (n = 192) were obtained from a British designated breeder (PD Hook Hatcheries Ltd., Thirsk, UK) and housed in the Animal Facility at the School of Bioscience, University of Nottingham, UK. The chickens were housed in pairs, in cages of 42 cm tall, 30 cm deep and 37 cm wide. All bird protocols were approved by the relevant Ethical Review Committee and all experimental conditions followed official guidelines for the care and management of birds.

Birds were weighed to ensure that individuals in a pair are as close as possible to each other in terms of weight to avoid any dominance. The chickens were located in pairs of a similar body weight to the cages. Weighing and allocation of birds to cages were prior to feeding the starter

diet and the experimental diets. All chickens were fed a standard commercial broiler starter diet based on wheat and de-hulled SBM with content of protein 190 g/kg as-fed (Chick Starter Crumb, Dodson and Horrell Ltd., Northamptonshire, UK) for 14 days. Afterwards, chickens weighing 445± 56.0 g were allocated to each of sixteen experimental diets (n=6) in a randomized complete block design and fed for eight days. On day 22, birds were culled by asphyxiation with carbon dioxide followed by cervical dislocation to confirm death and the ileal region of the gut was dissected out from the Meckel's diverticulum to the ileal-caecal junction. Ileal digesta were collected from both birds per cage and pooled providing six replicates for each experimental diet. The samples were stored at -20 ^{o}C until further analysis.

Analytical methods

RSC and SRSM were analysed for dry matter (DM) in duplicate samples weighing 60-65 g that were dried at 100 ^{o}C in a forced air convection oven. DM of ileal digesta was measured by freeze-drying the ileal content. Total nitrogen was determined using the Dumas method 968.06 (AOAC). Crude protein (CP) was calculated as 6.25 × total nitrogen. Amino acid were oxidized with performic acid and further neutralised with sodium metabisulphite (Llames and Fontaine, 1994). Then, the content of amino acids was determined by an ion-exchange chromatography for post-column derivatisation with ninhydrin. The content of oil was determined using continuous-wave low-resolution nuclear magnetic resonance spectrometry (EN ISO).

Solubilisation of proteins from rapeseed co-products and freeze-dried ileal digesta

Proteins were extracted from rapeseed co-products and ileal digesta according to a method by Wanasundara and McIntosh (2013) with a minor modification. Twenty mg of rapeseed co-products or ileal digesta was mixed with 1000 µl of acidulated water (1µS conductance water, 2% NaCl, adjusted with HCl to pH=3) for 2 hours at 20 ^{o}C by rolling (Roller mixer SRT1, Stuart Scientific, UK). Subsequently, the slurry was centrifuged (23.500 g, 20 min) and the supernatant was collected.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

22.5 µl of sample supernatant and 7.5 µl of 4X Laemmli buffer with (0.35 M) and without reducing agent (dithiothreitol, +DTT, -DTT) were heated (100 ^{o}C for 5 min) and then centrifuged (16.000 g, 10 min). DTT was used to cleave disulphide linkages between cysteine groups in proteins. 15 µl of supernatant sample as well as low and high molecular weight standards (10 µl, 1.4-26.6 kDa; 15 µl, 10-250 kDa, Bio-Rad Laboratories, Hercules CA, US) were loaded onto a 10-20% Tris/Tricine polyacrylamide gradient gel (Bio-Rad, UK). The electrophoresis was run at 80 V for 20 min and 120 V for 1 h 40 min using Tris/Tricine running buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS (Bio-Rad, UK). Afterwards, gels were fixed (methanol 40%, acetic acid 10%) for 30 min, stained in coomassie Blue (acetic acid 10%, coomassie blue G 0.25 g/l) for 1 h and destained in 10% acetic acid solution for at least 3 x 15 minutes washes. The images of the gels were recorded (GS-800 calibrated densitometer, Bio-Rad, UK).

Processing and *in vitro* tryptic digestion

Protein bands were excised from gels using a sterile scalpel into ~1 mm^3 cubes, and processed in gel pieces using the robotic liquid handling station (Proteome Works Mass PREP, Waters, UK). The samples were incubated three times in 100 µl of de-stain solution (50 mM ammonium bicarbonate, 50% acetonitrile), and dehydrated in 50 µl of acetonitrile for 5 minutes. After the evaporation of acetonitrile, the sample was treated with reducing solution (10 mM DTT, 100 mM ammonium bicarbonate) and alkylation solution. Following washing with ammonium bicarbonate and acetonitrile, the microtitre plate containing the gel plugs was cooled to 6 °C and 25 µl of trypsin gold (Promega) was added per well. Sample was diluted to 10 ng/µl in trypsin digestion buffer (50 mM ammonium bicarbonate), subsequently incubated at 6°C for a further 20 minutes in order to permit trypsin entry into the gel plugs, followed by incubation at 40 °C for 5 hours.

Mass spectrometry and protein identification

Samples were analysed by liquid chromatography-tandem mass spectrometry on a Q-TOFII fitted with a nanoflow ESI (electrospray ionization) source (Waters Ltd). Peptides were trapped, desalted and separated on a short pre-column (PepMap C18 reverse phase, 5-mm [Thermo]) and delivered on-line to the MS via a CapLC HPLC system. Tandem MS data were acquired using an automated data-dependent switching between MS and MS/MS scanning based upon

ion intensity, mass and charge state (data directed analysis (DDA™)). In this automated acquisition type of experiment, a method was created in the MassLynx 4.0 software in which charge state recognition was used to select doubly, triply and quadruply charged precursor peptide ions for fragmentation. The collision energy was automatically selected based on charge and mass of each precursor and varied from 15 to 55 eV. Protein Lynx Global Server version 2.0 (Waters, Ltd) was used to process the uninterpreted MS data into peak list (pkl) files which were searched against all entries in Swissprot 2014_11, 2015_02 and/or NCBInr 20141208, 20150208, 20150213 databases using the web version of the MASCOT MS/MS ions search tool (http://www.matrixscience.com/). Carbamidomethylation of cysteine and oxidation of methionine were set as variable modifications. One missed cleavage by trypsin was accepted. Other than file type (Micromass pkl) and instrument type (ESI-QUAD-TOF), all remaining search values were the present defaults. Positive identification was based on the Mascot score, significant peptide coverage of the protein sequence.

Results

Content of protein in diets and ileal digesta

The chemical characterisation of rapeseed co-product and ileal digesta is shown in Table 1 (all data on DM basis). The content of CP varied between 293 g/kg and 339 g/kg in RSC, and ranged from 419 g/kg to 560 g/kg DM in SRSM. Similarly, total amino acid (TAA) content ranged from 256 g/kg DM in RSC, to 457 g/kg DM in SRSM. Thus, the RSC batch was relative low in CP, whereas SRSM was richer in CP. CP level ranged from 109 g/kg DM in ileal digesta of Compass RSC to 164 g/kg DM in ileal digesta of Incentive SRSM, respectively. The sum of methionine and cysteine varied from 16 to 34 g/kg DM in rapeseed co-products, while the methionine and cysteine content ranged from 7 to 11 g/kg DM in ileal digesta.

Identification of proteins in rapeseed co-products

Across all sixteen rapeseed co-products, the polypeptide profiles of proteins showed the same pattern of protein bands under non-reducing conditions, irrespective of the rapeseed variety and processing method. Similarly, the profiles were almost identical under reducing conditions

across all of the samples. Figure 1 shows a polypeptide profile of proteins in two rapeseed varieties (DK Cabernet, Compass) that were processed by both methods (hexane extraction and cold pressing). Under non-reducing condition, the predominant rapeseed proteins mainly migrated at ~50 kD and ~14 kD. Also, two peptides in bands of ~26 kD and one in a band of ~18 kD were migrated. After the incubation under reducing conditions of 0.35 M DTT, the intensity of the two bands at ~26 kD and one band at ~18 kD substantially increased, and two new bands have appeared above 26 kD. Simultaneously, the intensity of band in ~50 kD band diminished considerably. The change from non-reducing to reducing condition was a consequence of intensity shift in a band at ~14 kD towards two intensive bands appeared at ~10 kD and 4 kD. Tandem MS analysis and database searching identified nine isomers of cruciferin, and three isomers of napin from Brassica napus (Table 2). The peptides derived from intact napin were not significantly mapped to napin 2SS3 (data not shown) but were significantly fitted to cruciferin CRU4.

Identification of proteins in ileal digesta

Ninety six polypeptide profiles of ileal digesta showed the same pattern of the protein migration across the gels, regardless of rapeseed variety and processing. All protein bands of ileal digesta appeared to be similar to that of the rapeseed proteins obtained under non-reducing conditions. However, mass spectrometric identification of the ileal digesta proteins showed that all the protein bands examined were endogenous chicken enzymes (Figure 2, Table 3). The proteins were identified as trypsin (I-P1, II-P29), chymotrypsin (elastase and precursor) (all ~20 kDa), carboxypeptidase B (~30 kDa), and α-amylase (~50 kDa). Under non-reducting condition, although the polypeptide profiles showed a similar pattern of these enzymes across all samples of ileal digesta, the ileal digesta of four cold pressed varieties (DK Cabernet, Compass, Sesame, NK Grandia) resulted in slightly lower relative abundance at 10 kDa and 50 kDa compared to the ileal digesta of all hexane extracted varieties.

Discussion

The high concentration of methionine and cysteine in the rapeseed co-products might reflect the abundance of sulphide bonds in napin as well as cruciferin (Table 1). However, the content of sulphur-rich amino acids in ileal digesta might potentially derive from indigestible dietary proteins or endogenous enzymes.

Both napin and cruciferin are reported as allergenic proteins in rapeseeds and mustards in European Union or Canada (Menendezarias et al., 1990; Palomares et al., 2005; Puumalainen et al., 2015). The allergenicity of the protein has been linked often with its resistance to digestion by hydrolysis enzymes (Untersmayr and Jensen-Jarolim, 2008). Thus, the poor digestibility or allergenicity of rapeseed protein, is considered as a negative factor in the nutritional value of rapeseed co-products either in animal feeds or human diets (Wanasundara, 2011). However, in contrast to many investigations reporting a low digestibility value of CP and amino acids in RSM (Adedokun et al., 2008; Zhou et al., 2013; Kozlowski and Jeroch, 2014; Le et al., 2014; Li et al., 2015) a recent growth performance trial testing RSM resulted in a very similar rates of body weight gain to the control non-rapeseed diet when evaluated in non-ruminants (Parr et al., 2015). This suggests that protein rich co-products might have a good nutritional quality.

The digestibility of dietary protein and thus the overall estimation of the nutritional value of protein varies, depending on the protein type, solubility, protein interaction with other components (concentrate vs. food matrix) and type of digestion models (*in vitro* vs. *in vivo*) (Ren et al., 2012; Zhang and Vardhanabhuti, 2014; Overduin et al., 2015). Pantoja-Uceda et al. (2004) investigated the structure of the precursor form of the recombinant napin BnIb (rproBnIb, 2S albumin) from the seeds of *Brassica napus*, using an *in vitro* proteolytic digestion by the standard simulated gastric fluid, and circular dichroism analysis by heat treatment up to 80 ^{o}C and cooling to 20 ^{o}C. The highly compact and thermal structure of rproBnIb appeared to be a very resistant to digestion, and showed very limited unfolding pattern, recovering after cooling to 20 ^{o}C. In contrast, the rapeseed cruciferin exhibited a surface hydrophobicity with a low thermal stability (Salleh et al., 2002). Withana-Gamage et al. (2014) tested the Arabidopsis hetero- and homo-hexameric cruciferin forms composed only of CRUA, CRUB or CRUC subunits using simulated gastric fluid degradation kinetics; they showed that all cruciferins were easily cleaved by proteolytic enzyme during the 2 hours, but CRUC was digested at a slower rate than CRUA

and CRUB. A study of Bos et al. (2007) investigating the nutritional value of rapeseed protein isolates using an *in vivo* digestion model of humans, has reported that both napin and cruciferin were not completely digested in the ileal stage, based only on SDS-PAGE assay.

In the current study, the ileal digesta were collected from broiler chickens that were fed 2 hours prior to sampling. We did not observe any cruciferin or napin in digesta, all the ileal digesta proteins were assigned to endogenous digestive enzymes.

Application of SDS-PAGE is often used to illustrate the napin and cruciferin abundance and di-sulphate bond cleavages at different stages of protein degradation using either *in vitro* or *in vivo* digestion models (Bos et al., 2007). As the molecular weights of cruciferin and napin, as well as their degradation products, exhibit very similar apparent MWs to that of the digestive enzymes observed (such as α–amylase, chymotrypsin, carboxypeptidase, trypsin, trypsinogen) in SDS-PAGE, the migrated protein bands from ileal digesta might be mismatched and incorrectly assigned to the rapeseed proteins when MS-based identification is not undertaken on ileal samples (Bos et al., 2007; Abeysekara and Wanasundara, 2009; Rommi et al., 2014).

The secretion of endogenous enzymes in the gut depends on diet, the animal species and its physiological state (Brzek et al., 2013). The “adaptive modulation hypothesis” describes the course of digestion as a process, in which the activity of digestive enzymes is adjusted to the content of the substrates in the diet, such that animals fully utilize available resources but at the same time do not waste energy on synthesising the excess enzymes (Karasov and Diamond, 1988; Diamond and Hammond, 1992). In the current study, all diets consisted of the same amount of wheat starch, glucose, vitamins and minerals. Although added rapeseed oil was the same between test diets (50 g/kg), total rapeseed oil content varied as RSC had greater levels of residual oil than SRSM. Thus, the difference in relative abundance of endogenous enzyme in SDS-PAGE profiles between RSC and SRSM ileal digesta might be mainly due to the different content of CP and oil in diets.

An understanding of fate of rapeseed protein and functionality of the digestive system, in terms of secretion of endogenous enzymes, is far from being completely understood across bird species. However, to our knowledge this is the first study showing the lack of presence of rapeseed protein following the changes in abundance of endogenous enzymes in ileal digesta. The evidence of abundance of trypsin/chymotrypsin after feeding low or high protein diets might

299 explain the reason of a low and varied nutritional value of rapeseed protein often reported
300 (Maison, 2013). The evaluation of nutritional value in dietary protein rich feed, is based on the
301 content of protein in diets, ileal digesta and endogenous protein. Endogenous losses are
302 calculated based on the endogenous proteins that are excreted in the human or animal gastro-
303 intestinal tract after consumption of protein-free diets (Stein et al., 2007). However, when
304 various protein-concentrated diets are tested, the estimation of endogenous and dietary protein
305 is challenging due to almost identical molecular weights between rapeseed protein and
306 endogenous enzyme proteins, and variation in endogenous protein secretion depending on
307 individual components in the diets.
308 To conclude, despite published evidence of *in vitro* based-experiments describing the low
309 digestibility of rapeseed protein, in the current study napin and cruciferin were not detected in
310 the ileal digesta of broiler chickens regardless of dietary protein content, rapeseed variety and
311 type of oil-extraction process. The absence of rapeseed proteins in the terminal ileum suggests
312 that they could be readily digested. A molecularly based approach, such as using the proteomic
313 tools in the current study, is applicable to investigate the true fate of dietary rapeseed proteins
314 and their dynamics within the entire tract. This will help to further our understanding of in order
315 to measure the nutritional value of rapeseed co-products.
316
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319
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Table 1. Concentration of crude protein and sulphur- rich amino acids in rapeseed co-products and ileal digesta (g/kg dry matter)

Rapeseed variety	DM	Rapeseed co-products					Ileal digesta			
		Met	Cys	TAA	CP	Oil	Met	Cys	TAA	CP
Rapeseed cake										
Compass	899	5.7	10.5	255.8	293.2	259.6	1.0	5.7	84.8	109.1
Sesame	890	6.5	14.1	292.8	331.8	293.0	1.1	6.1	86.6	110.6
NK Grandia	892	6.8	14.3	302.9	335.0	268.9	1.0	6.0	83.3	111.7
DK Cabernet	881	6.5	16.8	305.0	339.7	292.5	1.2	6.2	90.7	114.5
Mean	890	6.4	13.9	289.1	324.9	278.5	1.1	6.0	86.4	111.5
SE	3.6	0.24	1.28	11.41	10.71	8.46	0.03	0.10	1.60	1.13
Soft rapeseed meals										
DK Cabernet1*	866	8.8	19.0	395.5	418.6	30.8	1.4	7.9	113.8	137.7
DK Cabernet2*	864	9.1	19.2	411.3	456.9	31.2	1.5	8.9	120.8	155.5
Quartz	866	9.1	18.8	400.4	430.4	31.9	1.7	8.9	128.9	162.3
Trinity	868	8.8	19.9	399.1	442.8	33.7	1.3	7.3	105.7	133.9
Compass	848	7.8	16.7	385.8	467.5	30.4	1.4	6.6	105.0	130.8
Incentive	853	9.4	18.6	439.8	469.1	34.7	1.6	8.6	128.0	163.7
Excalibur	833	9.4	21.2	429.6	494.8	30.3	1.5	9.4	125.5	158.6
Avatar	856	9.0	19.2	409.8	495.1	38.4	1.8	8.4	127.3	146.5
PR46W21	822	9.9	23.7	452.9	507.3	35.6	1.5	7.8	112.4	139.0
Palmedor	859	9.9	20.9	450.5	516.7	28.2	1.5	7.3	114.2	145.2
L2750L	838	9.6	20.9	444.4	521.2	44.8	1.6	8.4	119.7	148.8
Ability	821	8.9	21.7	456.5	560.2	48.1	1.7	8.0	121.7	149.9
Mean	849	9.1	20.0	423.0	481.7	34.8	1.5	8.1	118.6	147.6
SE	5.0	0.16	0.53	7.33	12.01	1.76	0.04	0.23	2.41	3.15

DM, dry matter; Cys, cysteine; Met, methionine; TAA, total amino acids; CP, crude protein; SE, standard error.

* A variety of DK Cabernet was grown on two different farms and further processed by hexane extraction.

21 Table 2. Identified proteins in rapeseed co-products

Band ID	Identification	Taxonomy	Accession number	Mascot score	Matched peptides	Matched sequences	Sequence coverage (%)
CRU_n	Cruciferin BnC1	B.napus	CRU1_BRANA	576	14	6	25
	Cruciferin CRU4	B.napus	CRU4_BRANA	529	13	6	22
	Cruciferin CRU1	B.napus	CRU3_BRANA	452	12	5	17
CRU_r	Cruciferin CRU4	B.napus	CRU4_BRANA	418	11	5	18
	Cruciferin BnC1	B.napus	CRU1_BRANA	417	10	4	22
	Cruciferin CRU1	B.napus	CRU3_BRANA	283	6	4	13
α CRU_n1	Cruciferin BnC1	B.napus	CRU1_BRANA	546	8	6	26
	Cruciferin CRU1	B.napus	CRU3_BRANA	336	6	3	12
	Cruciferin CRU4	B.napus	CRU4_BRANA	330	4	3	20
α CRU_n2	Cruciferin CRU4	B.napus	CRU4_BRANA	534	18	7	23
	Cruciferin CRU1	B.napus	CRU3_BRANA	311	3	3	16
α CRU_r1	Cruciferin CRU1	B.napus	CRU3_BRANA	733	20	9	32
	Cruciferin/Cruciferin BnC1	B.napus	CRUA_BRANA/CRU1_BRANA	142/142	3/3	2/2	5/5
α CRU_r2	Cruciferin CRU1	B.napus	461840	*481*	3	3	25
	Cruciferin subunit/BnaC01g09900D	B.napus	12751302/674894422	*468/468*	3/3	3/3	26/26
	BnaA09g04300D	B.napus	674913375	*364*	3	3	21
	BnaA08g13680D	B.napus	674918950	*256*	2	2	9

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23 Table 2. Identified proteins in rapeseed co-products (continued)

Band ID	Identification	Taxonomy	Accession number	Mascot score	Matched peptides	Matched sequences	Sequence coverage (%)
α CRU_r3	Cruciferin CRU4	B.napus	CRU4_BRANA	331	4	2	18
	Cruciferin BnC1	B.napus	CRU1_BRANA	278	6	4	12
α CRU_r4	Cruciferin CRU4	B.napus	CRU4_BRANA	355	8	6	20
β CRU_n	Cruciferin CRU4	B.napus	CRU4_BRANA	598	21	4	23
	Cruciferin/Cruciferin BnC1	B.napus	CRUA_BRANA/CRU1_BRANA	424/424	10/10	4/4	13/13
	Cruciferin CRU1	B.napus	CRU3_BRANA	237	4	3	7
β CRU_r	Cruciferin CRU1	B.napus	CRU3_BRANA	402	11	5	18
	Cruciferin CRU4	B.napus	CRU4_BRANA	387	7	3	16
Nap	Cruciferin CRU4	B.napus	CRU4_BRANA	375	6	4	20
Nap L	napin large chain L2A	B.napus	1699238	*243*	2	1	69
	napin large chain L2C	B.napus	1699240	*174*	1	1	60
	napin-3/large peptide	B.napus	2SS3_BRANA	335	7	2	58
Nap S	napin 3	B.napus	2SS3_BRANA	170	1	1	27

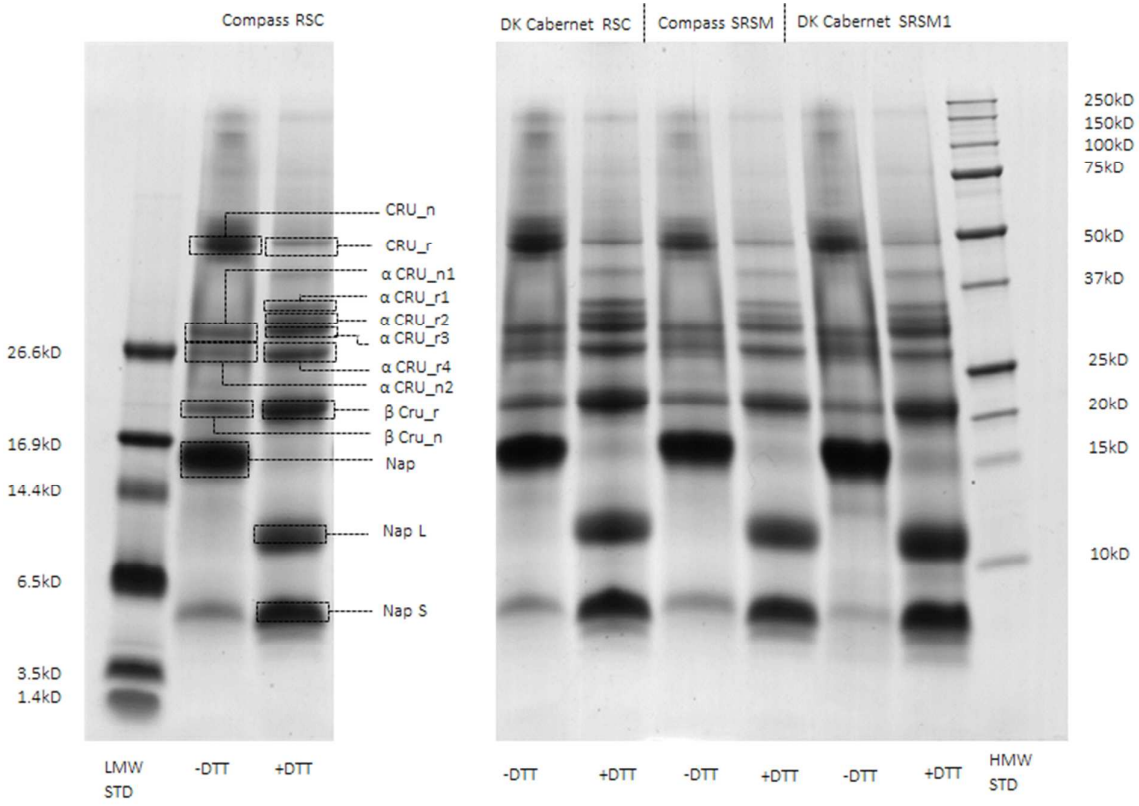
Mascot score, is derived from the ions scores for all the matched peptides. Number of matched peptides and matched sequences, is a number of significantly peptides/sequences associated with protein identified by Mascot. Percentage coverage, is percentage of the database sequence entry that is covered by the peptides matched to the Mascot data. Mascot scores in italics and non-italics indicate NCBInr and SwissProt scores, respectively.

Table 3. Identified proteins in ileal digesta

ID Band	Identification	Taxonomy	Accession number	Mascot Score	Matched peptides	Matched sequences	Sequence coverage (%)
Amy	α-amylase 2A, pancreatic precursor	Gallus gallus	377520154	*936*	7	7	53
Carb	Carboxypeptidase B preproprotein	Gallus gallus	476007880	*681*	7	6	38
ChymTryp	Chymotrypsin-like elastase family member 2A precursor	Gallus gallus	157817197	*461*	5	4	40
	Chymotrypsin-C precursor	Gallus gallus	483968280	*278*	6	4	31
Tryp	Trypsin II-P29	Gallus gallus	TRY3_CHICK	381	10	4	36
	Trypsin I-P1	Gallus gallus	TRY1_CHICK	267	3	3	31

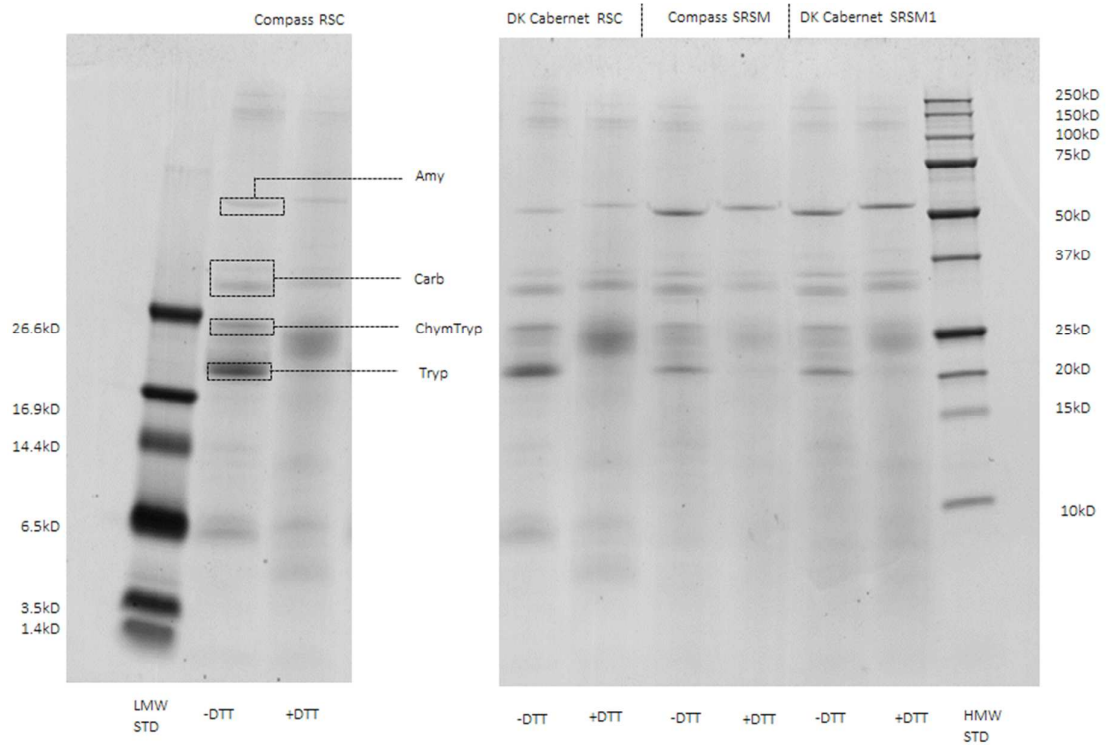
Mascot score, is derived from the ions scores for all the matched peptides. Number of matched peptides and matched sequences, is a number of significantly peptides/sequences associated with protein identified by Mascot. Percentage coverage, is percentage of the database sequence entry that is covered by the peptides matched to the Mascot data. Mascot scores in italics and non-italics indicate NCBInr and SwissProt scores, respectively.

Figure 1. SDS-PAGE profiles of rapeseed proteins extracted from rapeseed cake and meal.



RSC, rapeseed cake; SRSM, soft rapeseed meal; LMW STD, low molecular weight standard (1.4, 3.5, 6.5, 14.4, 16.9 and 26.6 kDa); HMW STD, high molecular weight standard (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa); DTT, dithiothreitol, indicating that sample was analysed with (+DTT) or without (-DTT).

Figure 2. SDS-PAGE profile of proteins extracted from ileal digesta after feeding with two rapeseed cake and soft rapeseed meal (Compass and DK Cabernet1).



RSC, rapeseed cake; SRSM, soft rapeseed meal; LMW STD, low molecular weight standard (1.4, 3.5, 6.5, 14.4, 16.9 and 26.6 kDa); HMW STD, high molecular weight standard (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa); DTT, dithiothreitol, indicating that sample was analysed with (+DTT) or without (-DTT).