Extraction and residual antinutritional components in protein fractions of *Sinapis alba* and *Brassica napus* oil-free meals

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ABSTRACT

Proteins were sequentially extracted from Australian canola (*Brassica napus*) and mustard (*Sinapis alba*) meals and two industrial canola meal samples, according to Osborne method (OSB), based on their solubilities in water, 5% NaCl, 0.1 M NaOH and 70% ethanol. These extracts were then compared to the glutelin fraction obtained by direct alkaline extraction method (DIR) in terms of protein yield, recovery, and residual antinutritional components. The OSB method was found to be more effective for protein extraction which resulted in higher cumulative protein yield and recovery of water-soluble protein fractions, although the DIR method produced a glutelin fraction of higher protein content. Phenolic compounds were observed in all the extracted protein fractions although sinapine, the major phenolic compound in canola, was present only in the albumin fractions. The glucosinolate content of all extracts was below the detection limit of the method employed (<3 µmol/g). Residual antinutritional components in protein fractions extracted from *S. alba* meal were, in general, higher than those of *B. napus* meal.

Key words: *Brassica* – *Sinapis* – Osborne fractions – glucosinolates – sinapine – total phenolics

INTRODUCTION

Canola meal has always been recognised as having the potential to be an alternative protein source for human consumption (Uppstrom, 1995). The most recent reviews on the subject have concluded that the presence of antinutritional factors such as glucosinolates, phytic acid, and phenolics, including sinapine and tannins remains a major drawback to their utilisation for food manufacture (Aider and Barbana, 2011; Tan et al., 2011). Proteins are the major constituent in oilseed meal and the growing demand for canola oil worldwide implies that more meal will be produced. Therefore, this alternative protein source needs further investigation.

Oilseed protein isolates are normally prepared by direct alkaline extraction (DIR) in an environment of high pH followed by acid precipitation, presumably due to the high nitrogen yield obtained in the isolate (Pedroche et al., 2004). Apart from poor solubility issues, there is little information about the level of antinutritional factors in the isolates (Yoshie-Stark et al., 2008). It is likely, however, that the Osborne (OSB) method which involves the sequential extraction of proteins using water, salt solution, alkaline and alcoholic solutions (Osborne and Mendel, 1914), could produce canola proteins with better characteristics and functional properties.

In comparison to canola standard *Brassica* oilseeds, *S. alba*, which is from the same *Brassicaceae* family, has been grown mostly as a condiment crop. *S. alba* has many agronomic advantages such as excellent heat and drought tolerance, large seeds, highly shatter-resistant seed pods, high resistance to blackleg disease and useful levels of flea beetle resistance without insecticidal protection. It is, however, not grown as an oilseed due to the low oil content, relatively high erucic acid and high glucosinolate content (Raney and Rakov, 2007). While the oil-free meal of *S. alba* possesses a relatively high protein content (45-48%) and the amino acid composition of the meal is fairly well balanced, its protein extracts also show useful technological functionalities (Aluko et al., 2005).

In this study, therefore, we extracted the protein fractions from *S. alba* and *B. napus* oilseed meals by using both DIR and OSB methods to study the protein extractability and the contents of major antinutritional components of each fraction namely total phenolics, sinapine, and glucosinolate contents. The influence of industrial processing of the canola meal on protein preparation was also reported.

MATERIALS AND METHODS

Sources of materials and chemicals
B. napus oilseeds, and *S. alba* were supplied by, NSW DPI, Australia. Industrial pre-toast and toasted *B. napus* meals were supplied by Cargill Oilseeds (Footscray, VIC, Australia). The industrial toasted meal is the by-product of an oil (solvent) extraction process that involves heating the meal at very high temperatures of up to 110 °C for the recovery of solvent as an economic requirement for viability of the process. Reagents and chemicals were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated.

Preparation of canola meals
Whole oilseed samples were ground into powder using a coffee grinder. Petroleum spirit at a meal to solvent ratio of 1:4 was then used to defat the ground seeds. The mixture was filtered through Whatman No. 1 paper, and the wet meal was allowed to dry in a fume hood at room temperature overnight. Industrial meals were used as supplied.

Protein extraction using the Osborne (OSB) method
Protein fractions were prepared using the classical procedure of Osborne and Mendel (1914) with modifications. Meal samples were extracted with 10 volumes of de-ionised water for 1 hr on an end-over-end shaker. The extracts were centrifuged at 3,000 g for 10 min to obtain the water soluble albumin fractions (supernatant). This process was repeated twice on the subnatant after which the albumin fractions were pooled and filtered under vacuum through Whatman No.1 paper. The residues were then similarly extracted with 5% NaCl, 0.1 M NaOH and 70% ethanol sequentially, to obtain globulin, glutelin and prolamin fractions respectively. The meal residues were rinsed with five volumes of de-ionised water and collected for the determination of total protein recovery. Salt in the globulin extract was removed by dialysis at 4 °C using cellulose membrane (molecular weight cut-off of 12.4 kDa) against 20 volumes of de-ionised water, for 72 hr with water changes every 24 hr. For glutelin fractions, pH of the extracts was adjusted to 4.0 with 1 M HCl and centrifuged at 3,000 g for 10 min to obtain the precipitates which were then re-suspended in five volumes of de-ionised water and adjusted to pH 7.0 with 1 M NaOH. The supernatant (glutelin residues) was dialysed as above to remove the salt generated as a result of the initial pH adjustment; this was kept and analysed for the determination of mass balance of the entire process. Ethanol in the prolamin fractions was evaporated from the extracts using Rotavapor R-210 (Buchi Labortechnik, Flawil, Switzerland) at 40 °C. All extracted protein fractions and residues were freeze-dried and stored in the freezer (−20 °C) until further analysis.

Protein extraction using the direct alkaline (DIR) method
Defatted meal was extracted three times, directly by 0.1 M NaOH solution at a meal to solvent ratio of 1:10 as described for preparation of the glutelin fractions by the OSB method above.

Chemical analyses
Proximate analysis of the meals was determined by standard Association of Official Analytical Chemists methods (AOAC, 1996). Total phenolic content was determined by Folin–Ciocalteu's reagent method according to Asami et al. (2003) with the absorbance readings measured using FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg, Germany) at 750 nm. The total phenolic content was standardised against sinapic acid and expressed as sinapic acid equivalents (SAE) per gram sample. Sinapine content was determined by high performance liquid chromatography (HPLC) according to the procedure described by Mailer et al. (2008). Glucosinolate content was determined as glucose from the hydrolysis of the glucosinolates according to the official Australian Oilseeds Federation method 4-1.22 and reported as µmol/g oil-free meal at 10% moisture (Mailer et al. 2008).

Statistical analysis

All extractions and analyses were carried out in triplicate and the means with standard deviation were reported. Data collected were subjected to analysis of variance (ANOVA), and Fisher's least significant difference (LSD) test was used to determine the means that were significantly different ($P \leq 0.05$) using SPSS™ statistical software version 17 (SPSS Inc, Chicago, IL, USA).

RESULTS

Extraction yield and protein recovery

Protein yield of albumin fractions obtained from all meal samples (4.96-13.61%) was significantly higher than that of all the other respective Osborne fractions (Fig. 1). Albumin yield of industrial pre-toast meal (12.15%) while similar to *B. napus* meal (12.16%), was significantly higher than that of industrial toasted meal (4.96%) and *S. alba* meal (9.32%). Protein yield of globulin extracted from industrial pre-toast meal (6.50%) and *S. alba* meal (7.74%) was significantly higher than that extracted from *B. napus* and industrial toasted sample. Protein yield of glutelin and prolamin fractions extracted from industrial pre-toast meal was significantly higher than that extracted from non-industrial samples. Prolamins were the minor fraction extracted from all samples and were ≤2% of the total extracted proteins. Toasting of industrial meal resulted in a significantly decreased yield in albumin, globulin and glutelin fractions, but increased the yield of the prolamin fractions. Protein yield of the DIR fraction (glutelins) obtained from industrial pre-toast sample (18.89%) was significantly higher in comparison to the non-industrial *B. napus* meal samples (15.50%). The desolventising process (toasting) significantly reduced the yield of DIR glutelins to the lowest at 9.61%.

As shown in Fig. 1, proteins remaining in the total residues (meal and glutelin residues) obtained from the DIR method were significantly higher than those produced from the OSB method. Irrespective of the extraction method used, industrial toasted meal had the highest amount of proteins in total residues (17.78%, OSB; 17.66%, DIR), left behind after protein extraction in comparison to other meal samples (4.14–5.14%, OSB; 9.16–14.22%, DIR). Furthermore, as shown in Table 1, the recovery of desired protein fractions by OSB method for all the meal samples (39.18–67.81%) was significantly and consistently higher than the recovery by DIR method (26.70–47.23%) while industrial toasted meal had the lowest recovery of desired protein fractions irrespective of extraction method.
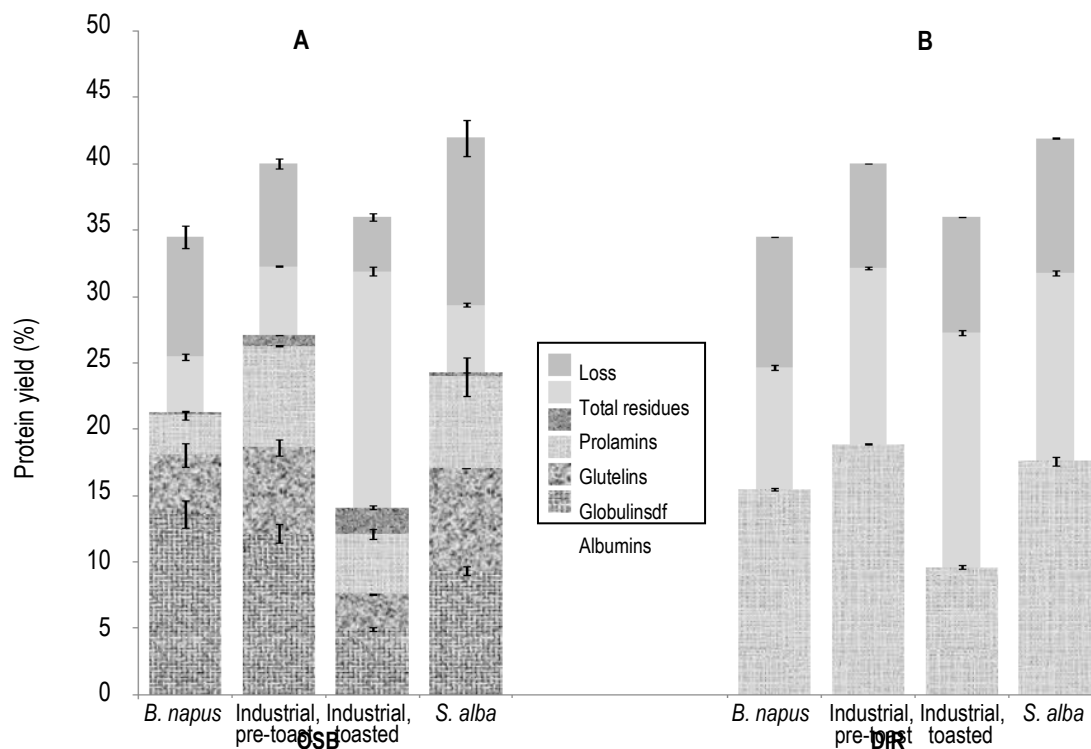
Fig 1. Protein yield (%) of various samples prepared using (A) Osborne (OSB) and (B) direct alkaline (DIR) extraction methods.

Table 1. Protein recovery (%) from meal samples using Osborne (OSB) and direct alkaline extraction (DIR) methods.

Extraction method	Sample	Desired fractions[1]	Total[2]
OSB method	*B. napus*	61.79 ± 2.68[a]	73.77 ± 2.42[a]
	Ind. pre-toast	67.81 ± 0.93[b]	80.65 ± 0.90[b]
	Ind. Toasted	39.18 ± 1.37[c]	88.56 ± 0.76[c]
	S. alba	57.91 ± 3.22[a]	70.06 ± 3.22[a]
DIR method	*B. napus*	44.94 ± 0.19[w]	71.49 ± 0.11[x]
	Ind. pre-toast	47.23 ± 0.16[x]	80.43 ± 0.02[y]
	Ind. Toasted	26.70 ± 0.26[y]	75.76 ± 0.10[z]
	S. alba	41.94 ± 0.53[z]	75.85 ± 0.19[z]

Data are means of triplicates ± standard deviation. Within each column, means with different superscripts are significantly different ($p \leq 0.05$).
[1] Desired fractions refer to the sum of % protein in the albumin, globulin, glutelin and prolamin fractions for the OSB method or the glutelin fractions only for the DIR method.
[2] Total refers to the sum of % protein in the desired fractions and all residues.

Composition of protein fractions
Protein content
As shown in Table 2, protein content of albumin fractions extracted from non-industrial *B. napus* (39.88%), industrial pre-toast (38.00%) and *S. alba* (33.88%) meals was significantly higher than the protein content of albumins extracted from industrial toasted meal (20.00%). Significantly higher protein content was observed in the globulin fractions extracted from industrial pre-toast meal (68.00%) and *S. alba* meal (62.44%) in comparison to that extracted from other meal samples (37.94 and 46.88%). Furthermore, protein content of glutelin fractions extracted by the OSB method from both industrial pre-toast and toasted meals (73.69%, 77.31%) was significantly higher than that extracted from non-industrial *B. napus* (46.25%) and *S. alba* meals (39.63%). The same trend was observed for prolamin fractions. Protein content of DIR fraction (glutelin) obtained from industrial pre-toast meal (83.50%) was not significantly different to that of toasted (75.94%) or *S. alba* meal (72.31%), but significantly higher than those extracted from the non-industrial *B. napus* (57.94%).

Antinutritional content
The composition of several antinutritional components of the various extracted protein fractions is also shown in Table 2. Compared to the other fractions, albumin fractions obtained using the OSB method contained the highest amount of total phenolics, with the fraction obtained from *S. alba* having the highest phenolics content (240.35 SAE/g), about twice the phenolics content in other samples (120.19-130.58 SAE/g). Relative to the albumin fractions, all extracted globulin fractions had significantly different and lower total phenolics content, ranging from 21.01 to 42.20 SAE/g. Similar trends were observed for the OSB glutelin (12.38–39.47 SAE/g) and prolamin (34.27–70.12 SAE/g) fractions. Phenolics content of DIR fractions extracted from *S. alba* meal (62.44 SAE/g) was significantly higher than that of other meal samples (39.72-51.40 SAE/g).

Sinapine was detected only in the albumin fractions and was significantly different depending on meal samples, with albumin fractions of *S. alba* having the highest (34.36 g/kg), while those of *B. napus* had the lowest sinapine content (9.78 g/kg). Glucosinolate content of all protein fractions extracted by both OSB and DIR methods was below the detection limit (<3 µmol/g).

DISCUSSION
Extraction yield and protein recovery
Protein recovery of desired OSB fractions and DIR fractions in this study was comparable to the protein recovery values by various extraction methods reported previously (Gillberg and Tornell, 1976; Xu and Diosady, 1994). The significantly lower protein yield of DIR fractions in comparison to OSB fractions could possibly be due to the acid precipitation step. According to Gillberg and Tornell (1976), rapeseed (canola) has a very complex protein composition, with widely different isoelectric points. In this study, however, only the fractions that were insoluble at pH 4.0 were collected. This has apparently caused a considerably large amount of proteins which solubilised at pH 4.0 to be left behind. The acid precipitation step of the DIR method, although resulting in low protein yield, is an effective technique in preparing extracts of higher protein content or purity. The overall lower protein content of individual OSB fractions, especially the albumins and the globulins in comparison to DIR fractions could also be due to the solubilisation of other non-protein components such as carbohydrates and antinutritional components along with the extracted protein fractions in the different solvents used accordingly (Pedroche et al., 2004).

The desolventising process, which decreased the meal protein content by only 10%, translated into a significant decrease in recovery of desired protein fractions by a margin of at least 40% for both OSB and DIR method. Results indicated that the industrial toasted meal had a significantly higher amount of (non-extractable) proteins in the residues in comparison to the other meal samples irrespective of protein extraction method, suggesting that the desolventising process carried out at high temperature affected the overall meal solubility in all extraction solvents, resulting in reduced protein extractability.

Table 2. Protein content and antinutritional factors of protein fractions from canola meal prepared using Osborne (OSB) and direct (DIR) extraction method.

Sample	Meal	Protein fractions					
		OSB				DIR
		Albumins	Globulins	Glutelins	Prolamins	Glutelins
Protein content (Nx6.25), %						
B. napus	34.50 ± 1.95[a]	39.88 ± 2.26[a]	37.94 ± 2.15[a]	46.25 ± 2.62[a]	9.63 ± 0.54[a]	57.94 ± 3.28[a]
Ind. pre-toast	40.00 ± 2.26[ab]	38.00 ± 2.15[ac]	68.00 ± 3.85[b]	73.69 ± 4.17[b]	23.75 ± 1.34[b]	83.50 ± 4.72[b]
Ind. toasted	36.00 ± 2.04[ab]	20.00 ± 1.13[b]	46.88 ± 2.65[c]	77.31 ± 4.37[b]	36.88 ± 2.09[c]	75.94 ± 4.30[b]
S. alba	41.94 ± 2.37[a]	33.88 ± 1.92[c]	62.44 ± 3.53[b]	39.63 ± 2.24[a]	11.13 ± 0.63[a]	72.31 ± 4.09[b]
Total phenolics content, SAE*/g						
B. napus	52.80 ± 1.05[a]	125.57 ± 0.11[a]	21.01 ± 0.27[a]	17.64 ± 0.24[a]	34.27 ± 1.55[a]	39.73 ± 0.96[a]
Ind. pre-toast	50.77 ± 1.85[a]	120.19 ± 1.16[b]	42.20 ± 0.19[b]	25.38 ± 0.45[b]	45.63 ± 0.55[b]	51.28 ± 0.50[b]
Ind. toasted	27.93 ± 0.22[b]	130.58 ± 1.63[c]	32.88 ± 0.69[c]	39.47 ± 0.22[c]	62.31 ± 1.14[c]	51.40 ± 0.58[b]
S. alba	74.50 ± 1.62[c]	240.35 ± 1.95[d]	36.68 ± 1.01[d]	12.38 ± 0.16[d]	70.12 ± 0.87[d]	62.44 ± 0.86[c]
Sinapine content, g/kg						
B. napus	11.24 ± 0.06[a]	9.78 ± 0.11[a]				
Ind. pre-toast	10.60 ± 1.07[a]	19.61 ± 0.01[b]		- not detected		
Ind. toasted	7.51 ± 0.13[b]	18.21 ± 0.29[b]				
S. alba	12.49 ± 0.98[a]	34.36 ± 1.07[c]				
Glucosinolate content, µmol/g						
B. napus	9.09 ± 0.99[a]					
Ind. pre-toast	10.47 ± 1.14[a]	- Glucosinolate content of all the extracted protein fractions were below the detection limit (<3 µmol/g).				
Ind. toasted	<3					
S. alba	143.50 ± 15.63[b]					

* Sinapic acid equivalent
 a,b,c Within each column, means with different superscripts are significantly different ($p \le 0.05$).

Antinutritional content

The major phenolic compounds in rapeseed or canola are sinapate esters with sinapine being the most prominent one (Shahidi and Naczk, 1992). The detection of sinapine only in the albumin fractions could possibly be due to the solubilisation of sinapine in water at the first step of the OSB method, which also explains the absence of sinapine in the subsequent protein fractions. Higher sinapine content in extracted albumins in relation to the meals may be due to the removal of other water insoluble components as precipitates during the extraction of albumin fractions, thus concentrating the sinapine in the water extracts. It is notable that sinapine content of albumin fractions was higher than its respective meals except for the non-industrial *B. napus* meal sample. Furthermore, sinapine content of *S. alba* albumin fractions was significantly higher than that of non-industrial *B. napus* and industrial pre-toast meal samples although there was no significant difference in sinapine content in the starting meals. All of these results suggest a species-specific reason and requires further investigation. Sinapine was not detected in the DIR fraction as a result of the alkaline extraction conditions whereby sinapine was hydrolysed to sinapic acid and choline (Austin and Wolff, 1968).

The significantly higher content of glucosinolates in *S. alba* meal in comparison to other industrial and non-industrial canola grade *Brassica* meals was consistent with an earlier study by Katepa-Mipondwa et al. (1999), confirming that *S. alba* meal is neither suitable as animal feed nor human food applications. Nevertheless, glucosinolate content of all protein fractions prepared by either OSB or DIR methods were below the detection limit. The absence of glucosinolates could be due to the decomposition of glucosinolates in the presence of water as demonstrated by Tripathi and Mistra (2007). This does not necessarily imply, however, that these fractions are safe for human consumption. For example, in the OSB method, presence of water and myrosinase enzyme from the meals would result in hydrolysis of glucosinolates and the formation of toxic degradation products such as thiocyanates and nitriles (Gillberg and Tornell, 1976) which are also biologically active (Tripathi and Mishra, 2007).

It is noteworthy that, all protein fractions extracted from industrial toasted meal by OSB method had higher content of total phenolics in comparison to the original meal on a gram protein per gram meal basis. Comparatively, for other meal samples, all protein fractions had a reduced content of total phenolics except for albumin fractions. Consequently, the albumin fractions that show significant sinapine content and possible accumulation of toxic degradation products of glucosinolates need to be further investigated, if this fraction of canola meal protein is to be viable as a human food ingredient. This is especially important if technological functionality of the albumin fractions was proved to be significant. Our results conclude that the utility of *S. alba* meal as a potential food protein source is less attractive owing to the significantly higher contents of sinapine and glucosinolates in comparison to protein isolates from *B. napus* meals.

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