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# Interference of phenolic compounds in *Brassica napus*, *Brassica rapa* and *Sinapis alba* seed extracts with the Lowry protein assay

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**Abstract**

The protein content of aqueous extracts of *Brassica napus*, *Brassica rapa* and *Sinapis alba* meal was determined by the Lowry and Kjeldahl nitrogen assays. Phenolic compounds interfered with the Lowry method to different extents based on the lines studied as well as the extraction procedure used. Three ways to correct for this interference were studied; acid precipitation of the protein before analysis, analyzing in the presence and absence of copper and the binding of free phenolics using non-ionic, porous polystyrene (Amberlite XAD-4). Analysis in presence and absence of copper, and using the difference in absorption at 660 nm between these analyses, proved to be the best way to correct for phenolic interference in the Lowry assay. Extractability of *Cruciferae* seed phenolics may be pH dependant thus the contribution of phenolics to the Lowry protein assay varies with the pH used for extraction.
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*Keywords:* Lowry assay; Protein; Phenolics; *Cruciferae*

## 1. Introduction

Most plant materials contain phenolic compounds that could interfere with the existing protein assays. Commonly used protein quantification methods are based on colour complex development (e.g. Lowry, Biuret assays), binding of proteins to certain dyes or fluorophores (e.g. Bradford assay, *o*-phthalaldehyde method), measuring intrinsic properties of proteins (e.g. fluorescence, UV absorption, functional groups in infra red region) or total nitrogen content (e.g. Kjeldahl and Dumas methods). For plant materials the official analytical methods that are accepted (American of Association Cereal Chemists; AACC, Association of Official Analytical Chemists; AOAC, American Oil Chemists' Society; AOCS) are based on the Kjeldahl N analysis. The Kjeldahl method measures total nitrogen in the sample, not just protein nitrogen, it is time consuming, uses corrosive reagents and needs a relatively large amount of protein to be accurate.

The method introduced by Lowry, Rosebrough, Farr, and Randall (1951) (Lowry method) has been the most popular colorimetric method to determine soluble protein content because it is simple, sensitive, precise and fast. This method is derived from the assay by Wu (1922) that used the Folin–phenol reagent (originally described by Folin & Denis, 1912) to quantify proteins based on their tyrosine (amino acid with phenolic group) residues. The Folin–Ciocalteu reagent that is employed in the Lowry method was first used for determining the tyrosine and tryptophan content in proteins by Folin and Ciocalteu (1927). The same reagent is utilized for measuring phenolic compounds such as monohydric phenols, polyphenols, flavanoids and tannins (Singleton & Rossi, 1965; Swain & Hillis, 1959). The modification to the Wu (1922) method by Lowry et al. (1951) was the addition of a step that proteins partake with cupric ions under alkaline conditions to increase the sensitivity of the assay by 3–15 fold.

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The active ingredients of the yellow coloured Folin–Ciocalteu reagent are polymeric ions from phosphomolybdic and phosphotungstic heteropoly acids ($3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5MoO_2{\cdot}10H_2O$ and $3H_2O{\cdot}P_2O_5{\cdot}14WO_3{\cdot}MoO_2{\cdot}10H_2O$) (Folin & Ciocalteu, 1927; Singleton & Rossi, 1965). Loss of 1, 2, or 3 oxygen atoms from tungstate and/or molybdate under alkaline conditions and in the presence of proteins results in one or more of several complexes that have a characteristic blue colour with a $\lambda_{max}$ 745–750 nm and $\lambda_{min}$ 405 nm (Peterson, 1979). Tyrosine and tryptophan and to a lesser extent histidine, cystine and cysteine can reduce the Folin–Ciocalteu reagent. The exact role of copper is not very clear. According to Winters and Minchin (2005) the cupric ions ($Cu^{2+}$) bind with protein resulting in a reduction to the cuprous ($Cu^{+}$) form that then reacts with the Folin–Ciocalteu reagent. Instead of the direct reduction of the reagent by $Cu^{+}$, Peterson (1979) explained that the proteins reduce the reagent, whereby copper chelates in the peptide structure and facilitates electron transfer from the protein to the reagent.

The main shortcomings of the Lowry method are that color development depends on the composition of the protein and that many substances may interfere. Hartree (1972) measured the color complex at 660 nm instead of 750 nm to reduce the effect of protein composition. Potty (1969) and later Winters and Minchin (2005) used the difference between analysis with and without copper ions to reduce the interference of phenolic compounds and other oxidants that are present in plant extracts. However, these two studies used model situations with well defined and purified phenolics, phenolic concentration, protein and protein concentrations.

Flour and meal of canola (*B. napus*) contain 6.4–12.8 g/kg and 13.2–18.1 g/kg phenolic compounds, compared to 0.23 g/kg and 4.6 g/kg in soybean flour and soybean meal in the soluble (free, esterified and glycosided) and insoluble bound form. Several *Cruciferae* species, like canola and mustard seed (*S. alba*) have been studied as sources for food and non-food protein (Aluko, Reaney, McIntosh, Ouellet, & Katepa-Mupondwa, 2004; Bérot, Compoint, Larré, Malabat, & Guéguen, 2005; Ghodsvali, Vosoughi Khodaparast, & Diosady, 2005; Kozlowska, Zadernowski, Nowak, & Piskula, 1991; Naczk, Diosady, & Rubin, 1986; Naczk, Amarowicz, Sullivan, & Shahidi, 1998; Xu, Lui, Luo, & Diosady, 2003). The free and bound phenolics present in the seed co-extract with proteins at alkaline pH, which is often used for protein extraction and could interfere with the Lowry protein assay (Naczk et al., 1998; Xu & Diosady, 2000). The objectives of this research were to quantify and reduce this interference. Acid precipitation of the protein, analyzing in presence and absence of copper and binding of free phenolics to a non-ionic resin (amberlite XAD-4) were used. The contribution of phenolics to the Lowry protein assay of protein extracts prepared at different pH was also studied. The Kjeldahl N-based protein quantification was used as the reference analysis method.

## 2. Materials and methods

### 2.1. Seed and meal preparation

Protein extracts were prepared from seed meals of five genotypes each of *Brassica napus* (Argentine, AC Excel, Westar, 46A65 and Q2), *Brassica rapa* (ACS-C7, Torch, AC Sunbeam, TR29 and TR8) and, *Sinapis alba* (HS3, HS4, HS5, AC Base and AC Pennant). The seeds were produced in 2004 at the Saskatoon Research Farm as part of the Brassica Oilseed Breeding Program of Agriculture and Agri-Food Canada.

The seeds were cracked in a stone mill (Morehouse Cowles, Chino, CA) and air classified to separate the hull and the cotelydons. The cotelydons were defatted using hexane (8:1 v/w) in Swedish tubes that were shaken at high speed for 20 min followed by filtration using a Whatman No. 1 filter paper (Whatman International Ltd, Maidstone, UK). The defatted meals were air dried and ground with a coffee grinder to pass through a #40 (425 µm, Tyler, Mentor, OH) mesh screen. The material so obtained has <3% (weight basis) residual oil content.

### 2.2. Preparation of protein extracts and precipitates

Proteins were extracted according to the method of Bérot et al. (2005). One gram of meal was slurried in 10 mL of 50 mM Tris–HCl buffer, pH 8.5, containing 750 mM NaCl, 5 mM EDTA and 28 mM sodium bisulphite, mixed for 1 h and then centrifuged for 10 min at 15,000*g*. The pellet was re-extracted under the same conditions. The supernatants of the two extraction steps were combined, filtered through a Whatman no. 1 filter paper and referred to as protein extract or whole extract.

The proteins of the extracts were precipitated according to the method of Winters and Minchin (2005). An aliquot of 0.75 mL of a solution containing 20% (w/v) trichloroacetic acid (TCA) and 0.4% (w/v) phosphotungstic acid (PTA) was added to 0.75 mL protein extract. After 30 min at 4 °C the acidified protein extract was centrifuged at 15,000*g* for 10 min. The protein pellets were recovered, dissolved in 7.5 mL 0.1 M NaOH and, referred to as pellets or neutralized pellets. The supernatant (480 µL) obtained from centrifugation was neutralized by adding 256 µL of 1 M NaOH and referred to as supernatants.

### 2.3. Protein content

The Lowry protein assay was performed including the modifications suggested by Markwell, Haas, Bieber, and Tolbert (1978). The scale of the assay was reduced to a total volume of 150 µL to be compatible with microplate well capacity. The protein extracts, redissolved pellets and neutralized supernatants, which were made by protein precipitation from the extracts, were analyzed after 200, 200 and 20 times dilution with distilled water, respectively. An aliquot of 105 µL of Lowry reagent C [A: 2.0% (w/v) $Na_2CO_3$,

0.4% (w/v) NaOH, 0.16% (w/v) Sodium tartrate, 1% (w/v) SDS; B: 4% (w/v) $CuSO_4 \cdot 5H_2O$; C: 100:1, A:B] was mixed with 35 μL of diluted sample. In a second well on the same plate reagent A that did not contain copper was mixed with 34.9 μL of diluted sample. The plate was incubated for 15 min at 22 °C and 10.5 μL of 1 N Folin–Ciocalteu reagent (Sigma, St Louis, MO) was added to each well. After incubating for 45 min the microplate was flushed with air to remove air bubbles and the absorbance was read at 660 nm on a SPECTRAmax® microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Bovine serum albumin (BSA 10–100 μg/mL Sigma) was used as the standard protein. The protein content of the extract was given as mg/mL BSA equivalents and that of the pellet and supernatant as percentage of the total protein present in extract. The absorbance without copper was given as a percentage of the absorbance in the presence of copper ($\%A_{660} \pm$ Cu).

The protein content of the extract and pellet was determined by Kjeldahl N analysis according to the American Association of Cereal Chemists' method 46-12 (AACC, 2000) using a nitrogen conversion factor of 5.5 (Tkachuk, 1969). The protein content of the extract was calculated as percentage and converted to mg/mL. For the pellets the protein content was calculated as percentage of protein precipitable from the extract and the protein content of supernatant was determined by difference.

### 2.4. Phenolic content

The phenolic content of the extract was determined by the method of Spies (1957). The protein extract was diluted 200 times with distilled water. In a microplate well 50 μL of diluted sample was mixed with 50 μL of 0.45 M $Na_2CO_3$ in 0.14 M NaOH and 50 μL 1 N Folin–Ciocalteu reagent. After incubating for 30 min at room temperature, the absorbance was read at 660 nm. Sinapic acid (7–112 μg/mL Sigma) was used as the standard. Free phenolics were expressed as mg sinapic acid equivalent (SAE) per mL sample.

### 2.5. Adsorbent treatment

The protein extracts of *S. alba* lines HS3 and AC Base as well as *B. rapa* line TR8 were treated with the non-ionic, porous polystyrene resin, Amberlite XAD-4 (Rohm and Haas, Philadelphia, PA) as described by Loomis, Lile, Sanstrom, and Burbott (1979). To 1 mL of extract 0.2 g of Amberlite XAD-4 beads was added, mixed by vortexing for 3 min and filtered through a 0.45 μL filter (Whatman Inc., Clifton, NJ). The proteins of the treated extract was precipitated as described in Section 2.2 and the protein contents of the treated extract, protein pellets and supernatants were measured by the Lowry method.

### 2.6. Release of phenolic compounds – pH dependence

Protein was extracted from 0.5 g meal (HS3 or AC Base) by stirring with 10 mL distilled water for 1 h, whereby the slurry had a pH between 3.5 and 10 adjusted by addition of 1 M NaOH or 1 M HCl, followed by centrifugation for 10 min at 15,000*g*. During the extraction the pH was checked every 15 min and adjusted if needed. The supernatants were analyzed for protein content using the Lowry method.

### 2.7. Calculating corrected protein content

Corrected protein content was calculated in three ways.

Calculation I:

Protein content corrected using precipitable protein: Corrected protein (mg/mL) = protein content of the extract by Lowry * (% precipitable protein/100).

Calculation II:

Protein content, corrected by analysis in the presence and absence of copper of the protein extract was calculated in four steps:

i. $\%A_{660} \pm \text{Cu} = (A_{660\text{nm}}$ by Lowry assay without $CuSO_4/A_{660\text{nm}}$ by Lowry assay with $CuSO_4) * 100$
ii. $A_{660\text{Tyr\&Trp}} = (18/82) * (100 - \%A_{660} \pm \text{Cu})$ assuming that in *Cruciferae* protein the ratio between Tyr and Trp and peptide bonds is similar to BSA (18:82)
iii. $A_{660\text{Phenolics}} = \%A_{660} \pm \text{Cu} - \%A_{660\text{Tyr\&Trp}}$
iv. Corrected protein (mg/mL) = protein content of the extract by Lowry * $[(100 - A_{660\text{Phenolics}})/100]$

Calculation III:

Correction using protein precipitation followed by analysis in the presence and absence of copper of the pellet was calculated by a combination of Calculation I and II.

### 2.8. Statistical analysis

All measurements were done a minimum of three times and mean values are reported. Analysis of variance (ANOVA) was used to determine whether the protein and phenolic contents of the extracts differed among lines. Least significant differences were calculated using the General Linear Model (GLM) procedure. SAS software (SAS Institute Inc., 1990) was used.

## 3. Results and discussion

### 3.1. Protein content: Lowry vs. Kjeldahl method

The analysis of the 15 seed extracts by the Lowry method gave a range of 22.8–32.6 mg/mL protein as BSE equivalents (Table 1). By the Kjeldahl method a range of 22.4–26.3 mg/mL protein (Table 1) was observed for the same seed extracts. Kjeldahl protein values depend on the nitrogen content of the extract and a conversion factor for calculation. Based on amino acid composition, Tka-

Table 1
Protein content of *B. napus*, *S. alba* and *B. rapa* protein extracts, their protein pellets and supernatants obtained from protein precipitation with TCA-PTA as determined by the Kjeldahl and Lowry assays

| Seed line | Protein content (mg/mL)[1] | | % Precipitable protein in the extract[1,2] | | % Apparent protein in the supernatant[1,3] | |
|---|---|---|---|---|---|---|
| | Kjeldahl[4] | Lowry[5] | Kjeldahl[4] | Lowry[5] | Kjeldahl N[6] | Lowry[5] |
| *B. napus* | | | | | | |
| Argentine | 25.2 abc | 27.4 def | 87.2 bcd | 71.0 ab | 12.8 | 23.5 fg |
| AC Excel | 23.0 de | 26.6 f | 88.9 abc | 68.4 abcd | 11.1 | 27.2 fg |
| Westar | 24.3 bcd | 26.3 f | 82.5 defg | 64.2 bcde | 17.5 | 35.0 cd |
| 46A65 | 23.8 cde | 29.9 b | 87.4 bcd | 61.9 bcde | 12.6 | 23.4 g |
| Q2 | 26.3 a | 29.0 bc | 89.2 ab | 66.7 abcde | 10.8 | 28.7 efg |
| *B. rapa* | | | | | | |
| ACS-S7 | 24.0 cde | 27.7 cdef | 84.9 bcdef | 69.0 abc | 15.1 | 41.7 b |
| Torch | 24.3 bcd | 30.0 b | 85.7 bcde | 61.0 bcde | 14.3 | 30.8 def |
| AC Sunbeam | 23.6 cde | 27.1 ef | 85.3 bcdef | 67.1 abcde | 14.7 | 35.9 bcd |
| TR29 | 22.8 de | 26.5 f | 84.6 bcdef | 61.5 bcde | 15.4 | 39.4 bc |
| TR8 | 23.1 de | 22.8 g | 88.3 bcd | 75.9 a | 11.7 | 38.7 bc |
| *S. alba* | | | | | | |
| HS3 | 24.9 abc | 28.4 cde | 78.7 g | 57.3 e | 21.3 | 34.8 cde |
| HS4 | 25.8 ab | 29.1 bc | 80.6 efg | 59.2 cde | 19.4 | 30.8 def |
| HS5 | 25.2 abc | 28.6 bcd | 79.5 fg | 66.3 abcde | 20.5 | 36.8 bcd |
| AC Base | 22.4 e | 32.6 a | 85.9 bcde | 58.7 cde | 14.1 | 62.0 a |
| AC Pennant | 23.7 cde | 32.3 a | 83.1 cdefg | 57.9 de | 16.9 | 64.5 a |

[1] Values in the same column followed by the same letter are not significantly different ($P < 0.05$, $n = 4$).
[2] Protein precipitate obtained by TCA-PTA addition was redissolved and neutralized. Remaining supernatant after protein precipitation was also neutralized.
[3] Almost all proteins were precipitated and the chromophore development was due to interfering substances like non-protein nitrogen in Kjeldahl and phenolics in Lowry assay, i.e. referred as "apparent protein".
[4] % $N \times 5.5$.
[5] Measured as BSA equivalents.
[6] Calculated using the difference between the amount of Kjeldahl N-based protein in the whole extract and the pellet.

chuk (1969) reported that the *N* conversion factor for rapeseed meal is 5.53 and yellow mustard meal is 5.40. In this study a conversion factor of 5.5 for all three species was used. The Kjeldahl data showed little variation among species. The protein contents of the *B. rapa* extracts were in the lower end, while *S. alba* and B. *napus* extracts were spread throughout the range (Table 1).

The response on the Lowry assay is based on the peptide bonds and tyrosine residues of proteins and most likely some phenolic compounds present in the extract. The extracts of *S. alba* lines AC Base and AC Pennant showed a relatively high protein value (32.3 and 32.6 mg/mL) whereas *B. rapa* line TR8 had a relatively low protein value (22.8 mg/mL) according to the Lowry assay (Table 1). The high protein content of AC Base and AC Pennant extracts could be due to a high content of phenolics instead of high actual protein content, since Kjeldahl N-based protein values were similar to the other lines. All the extracts with the exception of TR8 exhibited a much higher protein content by the Lowry assay compared to Kjeldahl, indicating probable interference from the phenolics present in the extract. The low protein value of the TR8 extract by the Lowry assay may be due to a relatively low amount of phenolics present in the extract.

The seeds used in this study were dehulled before defatting to avoid the interference of polymeric phenolics and seed coat polysaccharides (especially in *S. alba*) with the protein extraction. The phenolic components present in the seed cotyledons were the only contributors that could co-extract with proteins. Potty (1969) demonstrated that accurate protein measurement using the Lowry assay could be made in protein solutions containing up to 40 μg/mL of phenolic compounds. The phenolic content in the extracts from *B. napus*, *S. alba* and *B. rapa* ranged from 2.44 to 2.72, 2.66 to 3.56 and 2.40 to 2.72 mg/mL as sinapic acid equivalents, respectively (Table 2). The extracts of the lines AC Pennant and AC Base had significantly higher ($P < 0.05$) phenolic content than all other extracts. The TR8 extract gave a comparatively low content of phenolics though this was not significant. It should be noted here that the extracts of the present study may not represent the total extractable phenolics of these seeds; one reason was the use of dehulled seed and the other was the use of aqueous extraction medium.

To correct for the phenolic interference in the Lowry assay the following methods were used: protein precipitation, analysis in the presence and absence of copper, a combination of both and the treatment of the extracts with Amberlite XAD-4.

### 3.2. Protein precipitation

The combination of trichloroacetic acid and phosphotungstic acid (TCA-PTA) was expected to precipitate all

Table 2
Phenolic content of the protein extracts of *B. napus*, *S. alba* and *B. rapa*

| Seed line | Phenolics in the protein extract (mg/mL SAE)[1,2] |
|---|---|
| *B. napus* | |
| Argentine | 2.72 ± 0.03 |
| AC Excel | 2.46 ± 0.04 |
| Westar | 2.44 ± 0.00 |
| 46A65 | 2.64 ± 0.04 |
| Q2 | 2.78 ± 0.28 |
| *B. rapa* | |
| ACS-S7 | 2.52 ± 0.10 |
| Torch | 2.72 ± 0.01 |
| AC Sunbeam | 2.52 ± 0.09 |
| TR29 | 2.58 ± 0.06 |
| TR8 | 2.40 ± 0.14 |
| *S. alba* | |
| HS3 | 2.66 ± 0.13 |
| HS4 | 2.72 ± 0.03 |
| HS5 | 2.68 ± 0.03 |
| AC Base | 3.52 ± 0.11 |
| AC Pennant | 3.56 ± 0.04 |

[1] SAE, sinapic acid equivalent.
[2] Mean value ± standard deviation.

the proteins (Yeang, Yusof, & Abdullah, 1995). For the supernatant the term "apparent protein" was used, since all proteins were most likely acid precipitated and the chromophore development was due to interfering substances like non-protein nitrogen components (e.g. alkaloids, free amino acids, polyamines, glucosinolates) for Kjeldahl and phenolics and other strong oxidants that reduce the Folin–Ciocalteu reagent for Lowry.

Using the Kjeldahl method 10.8–21.3% of the protein in the extract was apparent and remained in the supernatant (Table 1). By the Lowry assay 23.4–64.5% of the protein was apparent, whereby AC Base and AC Pennant had nearly double the apparent protein content of all the other lines. These lines may have a high amount of free phenolics. According to the values obtained for phenolics, AC Base and AC Pennant had 3.52 and 3.56 mg/ml SAE, respectively. These are exceptionally high values compared to the other lines used in this study (Table 2).

Protein precipitation followed by Kjeldahl analysis of the pellet showed that 82.5–89.2%, 78.8–85.9% and 84.9–88.3% of the protein can be precipitated from *B. napus*, *S. alba* and *B. rapa*, respectively (Table 1). This would be the actual amount of protein in the extract. The protein values of the whole extracts by Kjeldahl when corrected for the amount of non-protein nitrogen that remained in the supernatant varied from 19.2 mg/mL for *S. alba* line AC Base to 23.5 mg/mL for *B. napus* line Q2 (Table 3). This assumed that all the precipitated material was protein and that no protein remained in the supernatant. According to the Lowry assay 61.9–71.0%, 61.0–75.9% and 57.3–66.3% of the measured protein was precipitated from the extracts of the *B. napus*, *B. rapa* and *S. alba* lines by TCA-PTA (Table 1). In the supernatant mainly the free phenolics would react in the Lowry assay as though they were proteins.

Protein values as determined by Lowry and corrected for free phenolics by means of protein precipitation were between 16.3 mg/mL and 19.5 mg/mL (Table 3) for these extracts. The extracts from AC Base and AC Pennant that showed high protein values and the extract of TR8 that exhibited very low protein content originally gave values similar to the other extracts of the study after this correction. The corrected Lowry values were slightly lower than the corrected Kjeldahl values. The reason for this is not clear. The main assumption for this correction calculation was that all the precipitated material was protein and that no protein remained in the supernatant. However, some of the protein-bound phenolics may reduce the Folin–Ciocalteu (phenol) reagent too and contribute to chromophore formation by the pellet. Presence of protein-bound phenolics in Brassica seed protein preparations has been reported (Amarowicz, Ciska, & Kmita-Glazewska, 1993; Amarowicz, Panasiuk, & Pari, 2003). The work by Smyk (2003) showed that sinapic acid in particular has high affinity to proteins depending on the pH of the medium.

### 3.3. Protein analysis in presence and absence of copper

Potty (1969) suggested that assaying the samples with and without copper may be a suitable way to correct for the interference of phenolic compounds in the Lowry assay. The values without copper reflect the color complex generated by the bound phenolics and amino acids (mainly Tyr and Trp) that give a phenol-type response. The additional response in the presence of copper is due to protein–copper interaction. Table 4 gives the $\%A_{660} \pm$ Cu of all extracts, which is their absorption when analyzed by the Lowry assay without the copper reduction as percentage of the absorption when analyzed with the copper reduction step.

For the protein extracts of *B. napus*, *B. rapa* and three of the *S. alba* lines the $\%A_{660} \pm$ Cu ranged from 33.0% to 39.7%. The $\%A_{660} \pm$ Cu of AC Base and AC Pennant were 58.8% and 54.6%, which was exceptionally high. This indicated that a high content of phenolics in the extracts of these two lines, since a large difference in amino acid profile, especially in the contents of Tyr and Trp compared to the other lines is most unlikely.

Fig. 1 presents the BSA standard curve as analyzed in the presence and absence of copper. The BSA used is a purified protein and virtually free of phenolics. The absorbance without copper was due to the ionisable Tyr and Trp residues of this protein. From Fig. 1 it can be calculated that 18% of the total absorbance was due to Tyr and Trp and 82% was due to the peptide bonds that interacted with copper. To calculate a correction factor for phenolic interference using the data with and without copper reduction, it was assumed that the proteins of *S. alba*, *B. rapa* and *B. napus* contain the same amount of Tyr and Trp relative to the amount of total peptide bonds as BSA. BSA is a widely used protein as the standard for protein quantification methods.

Table 3
Correction of Lowry assay protein data for the interference of phenolics using protein precipitation and analysis in the presence and absence of copper

| Seed line | Kjeldahl[1,2] (mg/mL) | Lowry[1, 2] (mg/mL) | Corrected protein content (mg/mL)[1] | | | |
|---|---|---|---|---|---|---|
| | | | Kjeldahl | Lowry | | |
| | | | Precipitation[3] | Precipitation[4] | With and without Cu[5] | Precipitation[4] & With and without Cu[5,6] |
| *B. napus* | | | | | | |
| Argentine | 25.2 ± 2.4 | 27.4 ± 0.9 | 22.0 | 19.5 | 20.8 | 18.0 |
| AC Excel | 23.0 ± 0.5 | 26.6 ± 0.3 | 20.4 | 18.2 | 20.1 | 16.8 |
| Westar | 24.3 ± 0.5 | 26.3 ± 1.6 | 20.0 | 16.9 | 20.3 | 16.0 |
| 46A65 | 23.8 ± 0.8 | 29.9 ± 0.5 | 20.8 | 18.5 | 22.1 | 17.3 |
| Q2 | 26.3 ± 2.8 | 29.0 ± 0.2 | 23.5 | 19.3 | 23.7 | 18.5 |
| *B. rapa* | | | | | | |
| ACS-S7 | 24.0 ± 0.7 | 27.7 ± 0.2 | 20.4 | 19.1 | 21.2 | 18.4 |
| Torch | 24.3 ± 0..3 | 30.0 ± 0.1 | 20.8 | 18.3 | 22.2 | 17.4 |
| AC Sunbeam | 23.6 ± 0.3 | 27.1 ± 0.4 | 20.1 | 18.2 | 21.1 | 17.2 |
| TR29 | 22.8 ± 0.2 | 26.5 ± 0.7 | 19.3 | 16.3 | 20.4 | 14.7 |
| TR8 | 23.1 ± 0.6 | 22.8 ± 0.2 | 20.4 | 17.3 | 17.1 | 15.6 |
| *S. alba* | | | | | | |
| HS3 | 24.9 ± 0.7 | 28.4 ± 0.3 | 19.6 | 16.3 | 20.9 | 14.7 |
| HS4 | 25.8 ± 0.3 | 29.1 ± 0.9 | 20.8 | 17.2 | 22.4 | 15.4 |
| HS5 | 25.2 ± 1.2 | 28.6 ± 0.5 | 20.0 | 19.0 | 21.5 | 17.7 |
| AC Base | 22.4 ± 0.9 | 32.6 ± 0.1 | 19.2 | 19.1 | 16.4 | 16.7 |
| AC Pennant | 23.7 ± 0.6 | 32.3 ± 1.2 | 19.7 | 18.7 | 17.9 | 16.1 |

[1] Mean value ± standard deviation.
[2] Same values as columns 2 and 3 of Table 1, respectively.
[3] Values are calculated based on the protein content obtained from Kjeldahl N analysis of the precipitate.
[4] Obtained using Calculation I in Section 2.
[5] Obtained using Calculation II in Section 2.
[6] Obtained using Calculation III in Section 2.

Table 4
Comparison of Lowry assay values with and without copper reduction step for the protein extracts, protein pellets and supernatants of *B. napus*, *S. alba* and *B. rapa*

| Seed line | $\%A_{660} \pm$ Cu[1] | | |
|---|---|---|---|
| | Whole extract[2] | Pellet[2,3] | Supernatant[2,3] |
| *B. napus* | | | |
| Argentine | 37.8 b | 24.2 cdef | 75.5 a |
| AC Excel | 38.1 b | 24.5 cde | 68.7 a |
| Westar | 36.6 bc | 22.2 efg | 54.3 bcd |
| 46A65 | 39.3 b | 23.4 defg | 66.0 ab |
| Q2 | 33.0 c | 21.5 fg | 65.7 ab |
| *B. rapa* | | | |
| ACS-S7 | 37.3 bc | 21.1 g | 62.8 abc |
| Torch | 39.2 b | 22.0 efg | 68.5 a |
| AC Sunbeam | 36.1 bc | 22.5 efg | 51.2 cd |
| TR29 | 37.0 bc | 25.8 bcd | 46.9 d |
| TR8 | 38.6 b | 26.2 bcd | 47.1 d |
| *S. alba* | | | |
| HS3 | 39.7 b | 26.0 bcd | 63.9 abc |
| HS4 | 36.9 bc | 26.7 abc | 70.7 a |
| HS5 | 38.4 b | 23.4 defg | 66.6 ab |
| AC Base | 58.8 a | 28.4 ab | 74.0 a |
| AC Pennant | 54.6 a | 29.4 a | 66.7 ab |

[1] Protein extracts, pellets and supernatant were analyzed with the Lowry method in the presence and absence of copper. Refers to Calculation II.i of Section 2.
[2] Values in the same column followed by the same letter are not significantly different ($P < 0.05$, $n = 4$).
[3] Protein precipitate obtained by TCA-PTA addition was redissolved and neutralized. Remaining supernatant was also neutralized.

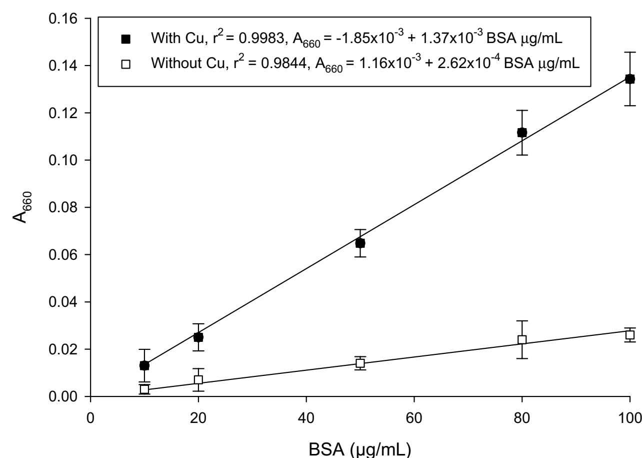


Fig. 1. Standard curve used for Lowry method with BSA with copper reduction step and without.

The corrected protein content of most of the extracts using $\%A_{660} \pm$ Cu ranged from 16.5% (AC Excel) to 19.4% (Q2), whereas the protein contents of AC Base and AC Pennant would only be 13.4% and 14.6% (Table 3). This low corrected protein content for the samples that were originally high in phenolics might be due to the reduced response of the phenolics in the presence of copper. According to Winters and Minchin, 2005 the type of phenolic compound play a role in the response observed in the Lowry assay, especially among *ortho*-diphenols. Since the seed extract contains a mixture of phenolic acids the response may vary depending on the source. Winters and Minchin (2005) have suggested a mathematical calculation to correct this reduced response. However, such mathematical correction may not be applicable to this study since the amount and type of phenolics present in the extracts are not known and might vary considerably within and between species used in the study.

### 3.4. Protein precipitation followed by analysis in presence and absence of copper

A major shortcoming of the precipitation method is that a correction for bound phenolics that co-precipitate with proteins are not included in the calculation. By determining $\%A_{660} \pm$ Cu for the redissolved and neutralized pellets, the effect of bound phenolics on the protein assay could be reduced. The assay of the pellet without copper reduction is based on the Tyr and Trp content as well as the bound phenolics. Assaying the pellets with copper resulted in an increase in blue colour due to the interaction between the peptide bonds with copper. Most pellets had a $\%A_{660} \pm$ Cu of 21.1–26.7% (Table 4). AC Base and AC Pennant had slightly higher $\%A_{660} \pm$ Cu (28.4% and 29.4%) values than other lines. Because of the absence of free phenolics in the precipitated protein these values were low compared to the supernatant and whole protein extract.

The $\%A_{660} \pm$ Cu values of the supernatants were, except in two cases, well above 50% and even to 75.5%. The *B. rapa* lines TR29 and TR8 gave values of 46.9% and 47.1%, respectively. In the supernatant it is mainly the free phenolics that reduce the Folin–Ciocalteu reagent.

### 3.5. Adsorbent treatment

The adsorbent chosen, Amberlite® XAD-4, is a nonionic styrene-divinylbenzene polymer that was successfully used for binding of phenolics in plant tissue enzyme extracts by Loomis et al. (1979). Binding of the phenolics to the resin is primarily by hydrophobic interaction. Amberlite XAD-4 binds little or no protein. The protein extracts of three selected seed lines HS3, AC Base and TR8 were treated with the Amberlite. These samples were chosen based on their phenolic content (Table 2). HS3 had the lowest phenolic content of the *S. alba* lines whereas AC Base had the high phenolic content similar to AC Pennant. The *B. rapa* line TR8 had the lowest phenolic content of all lines studied. The hypothesis was that the Amberlite treatment would result in a reduced protein content when measured with the Lowry method and this reduction would be much more for AC Base than for the two other lines. All samples showed reduction in measured protein content when treated with Amberlite; 3.6% (TR8), 7.3% (HS3) and 7.7% (AC Base) reduction compare to the protein content of untreated extract was observed. But this reduction

was not significantly different among samples. Amberlite treatment seemed not a good way to correct for phenolics in *Cruciferae* seed extracts or the interference of phenolics with the Lowry protein assay.

### 3.6. Protein extraction at different pHs

The interference of phenolics in the Lowry assay appeared to be line and species dependent. When the efficiencies of different protein extraction methods using just one line are compared, this interference would be irrelevant if the ratio between proteins and phenolics extracted are identical. However, apart from the starting material used for the protein extraction, the extraction conditions, such as pH, salt concentration etc. may also affect the amount of phenolics and its ratio to the amount of actual protein in the final extract. To study this experimental effect, proteins were extracted from *S. alba* lines HS3 and AC Base with water over a pH range of 3.5 to 10. The protein contents of the extracts assayed by the Lowry method and are shown in Fig. 2. The absorbance of the Lowry assay without copper as percentage of with copper over this pH range is shown in Fig. 3. The extraction pH showed an effect on the protein content, but more important information was that $\%A_{660} \pm \mathrm{Cu}$ was pH dependent. According to this observation, the pH of the medium in which the protein extract was prepared, affected the amount of phenolics that was extracted and present in the solution. This interference was maximal around pH 7.5 (Fig. 3) for AC Base, which is also the pH where seemingly a lot of protein was extracted (Fig. 2). The extract of AC Base was one of the two extracts that exhibited a relatively high amount of phenolics (Table 2). Around neutral pH AC Base phenolics were more extractable, however this extractability diminished with increase in pH of the medium. Within the same pH range the HS3 line indicated low phenolic content when extracted at pH 8.5. However the same material exhibited

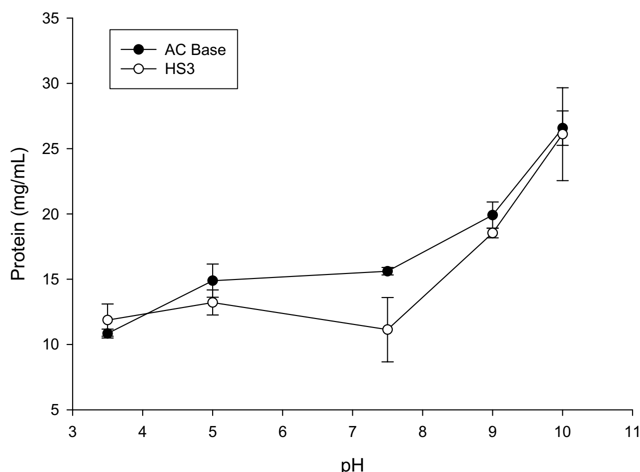


Fig. 2. Protein content as determined by the Lowry method of the protein extracts of *S. alba* lines HS3 and AC Base obtained at different pHs.

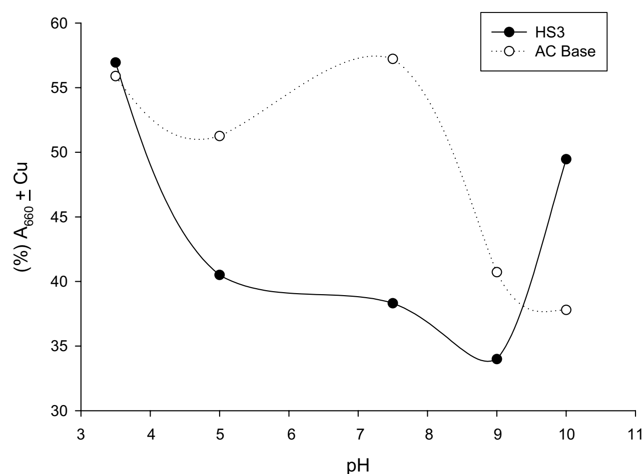


Fig. 3. The absorbance at 660 nm without copper as percentage of with copper for the extracts of *S. alba* lines HS3 and AC Base at different pHs using the Lowry assay.

a very high extractability for phenolics at pH above 9 and below 5. Phenolic choline esters are abundant in crucifer seeds such as canola and mustard (Naczk et al., 1998). Sinapine (sinapic acid choline ester) which is the predominant phenolic compound in the seed material used in this study may be solubilized in both acidic and basic pH due to the bifunctional nature of the molecule. This difference in extractability of phenolics may also depend on whether the molecules are free, esterified or bound form. It is clear that the extractability of seed phenolics is pH dependant and varies with the type of genetic material used. Therefore, if Lowry assay is employed to estimate the soluble protein content of *Cruciferae* seed extracts careful interpretation of the results is essential and a possible correction for phenolic interference is required.

## 4. Conclusions

Phenolic compounds of *Cruciferae* seed extracts interfere in the Lowry protein assay. The extent of this interference depends on the genotype studied and was especially clear among the *S. alba* lines. These *S. alba* lines vary in their soluble phenolic content, which lead to an overestimation of the protein content by means of the Lowry method. Also the extraction procedure used, in particular the extraction pH, affected the amount of phenolic interference. This interference need to be accounted for if data among different genotypes and extraction procedures are compared. The best way to do this would be to analyze the extracts in presence and in absence of copper ($\%A_{660} \pm \mathrm{Cu}$). Compared to the other methods of correction, e.g. protein precipitation with TCA-PTA and treatment with Amberlite, the determination of $\%A_{660} \pm \mathrm{Cu}$ is fast, especially when the Lowry method is carried out on a microscale. Additionally, it resulted in very similar protein values as with the non-protein nitrogen corrected protein values obtained from Kjeldahl analysis.

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