Determination of Condensed Tannins Utilizing Microwave Technology

Christina Johnson (cjohnson@eplbas.com)

EPL BIO ANALYTICAL SERVICES

Abstract

Red anthocyanidins, which cause the various red shades found in many foods and plants, are produced by an oxidation reaction of proanthocyanidins, or condensed tannins (1). The red color caused by the oxidation reaction is commonly used to measure the amount of condensed tannins with a spectrophotometer at 550 nanometers. Currently, the procedure for extracting condensed tannins from canola seed and canola meal is lengthy and potentially dangerous. A new procedure utilizing microwave technology to defat and hydrolyze offers a wide variety of benefits to the determination of tannins. Samples are defatted with petroleum ether and acetone. After drying, soluble condensed tannins (SCT) are extracted. Solutions are added to the SCT extracts in a microwavable tube to allow for hydrolysis of the samples. These microwavable tubes eliminate the use of pressurized glass tubes which can be a safety concern.

Abstract (continued)

A similar process is used for the extraction and hydrolysis for insoluble condensed tannins (ICT). Converting these steps to utilize the microwave will eliminate overnight defatting and cut hydrolysis time to a third of what is currently required. Microwave technology results in better temperature control and even heating for more consistent and repeatable results. Results for 7 canola seed SCT samples show a mean of 0.0883% dry basis (DB) with a coefficient of variation (CV) of 8.04%. The 11 canola seed ICT samples had a mean of 0.201% DB with a CV of 8.78%. The 9 canola meal SCT samples averaged 0.106% DB with a CV of 2.96%. The 11 canola meal ICT samples produced a mean of 0.334% DB with a CV of 12.0%. The new procedure utilizing microwave technology can create faster, safer, and more uniform conditions for determining the presence of condensed tannins.

Objective

The objective was to validate a safer and more reliable analytical method for the determination of tannins in canola seed and canola meal utilizing microwave technology.

Chemicals and Reagents

Procyanidin B2 analytical standard was obtained from Sigma-Aldrich. A 2,000 µg/mL stock standard was prepared in 5.26 mM sodium meta-bisulfite ($Na_2S_2O_5$) in 70:30 (v/v) acetone:deionized (DI) water. A 200 µg/mL working standard was prepared using the $Na_2S_2O_5$ solution as well. All standards were stored in a freezer (approximately -20°C). DI water was obtained from a Barnstead NANOPure water system. Additional reagents used included:

- 1-Butanol, Sigma-Aldrich, St. Louis, MO
- Petroleum ether, Omnisolv®, EMD, Billerica, MA
- Acetone, Omnisolv®, EMD, Billerica, MA
- Concentrated Hydrochloric Acid, Macron Fine Chemicals, Avantor, Center Valley, PA
- Sodium meta-bisulfite, Sigma-Aldrich, ACS, Steinheim, Germany
- Ammonium iron (III) sulfate dodecahydrate, Sigma-Aldrich, ACS, Steinheim, Germany
- 6N HCl, J.T. Baker®, Avantor, Center Valley, PA

Chemicals and Reagents

- 5.26 mM sodium meta-bisulfite in 70:30 (v/v) acetone:DI water was prepared by weighing approximately 0.5 g of sodium meta-bisulfite and combining this with 350 mL of acetone and 150 mL of DI water.
- 95:5 (v/v) butanol:HCl solution was made by mixing 950 mL of 1-butanol with 50 mL of concentrated HCl.
- 2% ferric ammonium sulfate solution was prepared by mixing approximately 1 g of ammonium iron (III) sulfate dodecahydrate with 50 mL of 2N HCl.
- 2N HCl was prepared using 100 mL of 6N hydrochloric acid and 200 mL of DI water.

Equipment

- Centrifugal mill grinder
- Balances, 0.1, 1.0, and/or 10.0 mg accuracy
- Class A volumetric glassware
- Cellulose thimble
- Spectrophotometer, Spectronic Genesys 5, or equivalent
- Mars Microwave or Equivalent
- GreenChem Microwave Vessels and Turntable
- Torque Wrench (set at 5 ft/lbs)
- Fiber optical sensor
- Pipetters (fixed, adjustable, and repeating)

Equipment

- Plastic centrifuge tube, 15 mL
- Centrifuge
- Gyratory Shaker
- Spatula
- Weigh boats or weigh paper
- Beakers
- Scintillation vials
- Cuvettes

Test Matrices

This method was validated using canola meal and canola seed (*Brassica napus*). Samples were ground and homogenized using a centrifugal mill grinder to pass through a 0.75 mm sieve. Samples were stored in a freezer at approximately -20°C when not needed in the laboratory.

Procedures

Canola Seed

- Weighed 0.8 g (±0.02 g) of ground canola seed onto weigh paper then transferred to microwave vessels.
- Defatting was achieved using 40 mL of 2:1 petroleum ether:acetone solution with microwave heating (Table 1).
- The fat extract was decanted and discarded in flammable waste. Samples were dried and transferred to a 15 mL centrifuge tube.

Canola Meal

- 0.5 g (±0.02 g) of ground canola meal was weighed directly into 15mL plastic centrifuge tubes. Defatting was not necessary.

Procedures

Canola Seed and Canola Meal

- A spike sample was fortified using 0.25 mL of the 2,000 µg/mL stock standard.
- Soluble condensed tannins (SCT) were extracted using 5 mL of the $Na_2S_2O_5$ solution.
- Samples were placed on a shaker for 15 minutes then centrifuged for 10 minutes at approximately 3,000 rpm.
- The extract was decanted into a separate 15 mL graduated centrifuge tube.
- The previous three steps were repeated two more times.
- Following the final cycle, the extracts were brought to a volume of 15 mL using the $Na_2S_2O_5$ solution.

Procedures

Canola Seed and Canola Meal

- 1 mL of the sample extract, 8 mL of 95:5 (v/v) butanol:HCl and 0.1 mL of the ferric ammonium sulfate solution were added to a microwave vessel.
- A blank was prepared using the same solutions except substituting 1 mL of the $Na_2S_2O_5$ solution, rather than sample extract.
- Microwave vessels were assembled and the samples were microwaved (Table 1).
- Samples were allowed to cool then brought to volume in a 10 mL volumetric flask using 1-butanol.
- Samples were poured into cuvettes and absorbance at 550 nanometers was determined.
- After extraction of the SCT samples, a matrix pellet remained in the bottom of each centrifuge tube. Acetone was used to transfer the pellet from the 15 mL centrifuge tube into a cellulose thimble.

Procedures

Canola Seed and Canola Meal

- Samples were left in a cellulose thimble to air dry for a minimum of two hours.
- After complete drying, a total ICT weight was recorded. A sub-sample (0.0300-0.0330 g) was weighed on weigh paper, then transferred to a microwave vessel.
- 0.1 mL ferric ammonium sulfate, 7 mL 95:5 (v/v) butanol: HCl, and 1 mL of the 5.26 mM $Na_2S_2O_5$ in 70:30 (v:v) acetone:DI water were added to the microwave vessels.
- Vessels were assembled and samples were microwaved (Table 1).
- Samples were poured into individual 15 mL centrifuge tube and centrifuged at approximately 3,000 rpm for 10 minutes.

Procedures

Canola Seed and Canola Meal

- Supernatant liquid was decanted into a 25 mL volumetric flask. The blank was decanted into a 10 mL flask.
- The previous 4 steps were repeated two more times for all samples except the blank. The butanol solution aided in removing all sample from the centrifuge tube.
- Following the final cycle, the 25 mL volumetric flasks containing samples and the 10 mL volumetric flask containing the blank were brought to volume using 1-butanol.
- Samples and blank were poured into cuvettes and absorbance at 550 nanometers was determined.

Procedures

Calibration Standards

- 1 mL of the 200 µg/mL working standard, 8 mL of 95:5 (v/v) butanol:HCl and 0.1 mL of the ferric ammonium sulfate solution were added to a microwave vessel.
- Calibration standards were microwaved concurrently with the SCT set.
- After cooling, the calibration standard was poured into a 10 mL volumetric flask and brought to volume with 1-butanol.
- Aliquots of 3 mL, 2 mL, and 1 mL were taken from the standard and brought to volume in 5mL flasks. 0.5 mL and 0.2 mL aliquots were taken from the 3 mL (12 µg/mL) standard. Absorbance at 550 nm was determined.

Procedures

Table 1. Microwave Parameters

Step	Power	Pressure (psi)	Temperature Ramp	Hold Time (min)
Defatting (canola seed only)	1600 watts, 100%	100	①RT to 100°C in 10 minutes	35
SCT Hydrolysis	1600 watts, 100%	100	①RT to 95 °C in 2 minutes	20
ICT Hydrolysis	1600 watts, 100%	100	①RT to 95 °C in 2 minutes	18

① RT= Room Temperature

Results and Discussion

A minimum of seven replicate sub-samples of each matrix were analyzed to determine the precision of the developed method via calculation of the relative standard deviation (RSD). Tannins content was calculated in percent dry basis (Table 2).

Results and Discussion

Table 2. Unfortified Sample Results

Matrix and Step	Replicates	Mean (%DB)	Standard Deviation	RSD (%)
Canola Seed SCT	7	0.0883	0.00710	8.04
Canola Seed ICT	11	0.201	0.0176	8.78
Canola Meal SCT	9	0.106	0.00313	2.96
Canola Meal ICT	11	0.334	0.0401	12.0

Results and Discussion

To verify accuracy, three samples in each SCT set were fortified with procyanidin B2 and percent recovery was calculated. The overall mean (n=6) recovery was 90.7%. The mean (n=3) recovery for each matrix can be found in table 3.

Table 3. SCT fortification recoveries

Matrix	Fortification Level (%DB)	Mean (n=3) Recovery (%)
Canola Seed	0.0670	102
Canola Meal	0.107	79.4

Results and Discussion

To verify accuracy, three samples in each SCT set were fortified with procyanidin B2 and percent recovery was calculated. The overall mean (n=6) recovery was 90.7%. The mean (n=3) recovery for each matrix can be found in table 3.

Table 3. SCT fortification recoveries

Matrix	Fortification Level (%DB)	Mean (n=3) Recovery (%)
Canola Seed	0.0670	102
Canola Meal	0.107	79.4

Conclusions

The validation of an analytical method using microwave technology was successful. The updated method produced acceptable accuracy and precision.

Figure 1. SCT Standard Curve

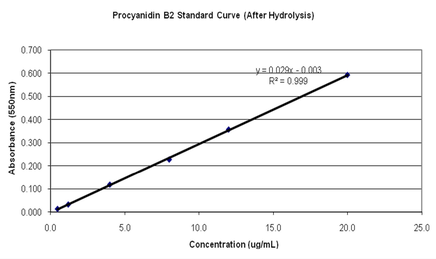
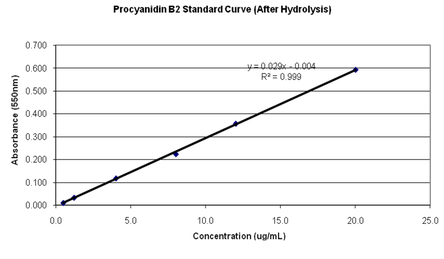


Figure 2. ICT Standard Curve



References

(1) "Animal Science-Plants Poisonous to Livestock." *Cornell University Department of Animal Science*. Cornell University, n.d. Web. 09 Sep 2013.