Effect of Supercritical CO_2 on Myrosinase Activity and Glucosinolate Degradation in Canola

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The effect of supercritical carbon dioxide (SC-CO_2) extraction conditions (temperature, pressure, extraction time, and moisture content of the samples) on the myrosinase activity and glucosinolate hydrolysis in flaked and whole canola seeds was studied. Combined effects of high temperature (75 °C), pressure (62.1 MPa), and moisture (~20% w/w) were necessary to achieve 90% enzyme inactivation in canola flakes in 3 h. Similar extraction conditions resulted in only 44% enzyme inactivation in whole canola seeds. Significant glucosinolate degradation was observed after 5 h of extraction of low moisture content (8.5% w/w) whole canola seeds. Glucosinolate degradation in canola flakes was minimal at all moisture levels studied. Therefore, myrosinase inactivation prior to SC-CO_2 extraction of canola flakes may not be necessary. However, the presence of enzyme activity in canola extracted at low moisture or temperature should be taken into account for further utilization of meal.

Keywords: *Supercritical CO_2; myrosinase; moisture; canola; glucosinolate*

INTRODUCTION

Glucosinolates are found mainly in the Cruciferae crops, e.g. cabbage, turnips, radishes, mustard, rapeseed, and canola, and contribute to the characteristic pungent odor and bitter taste of these plants. Intact glucosinolates are not toxic; however, their hydrolytic products were associated with goitrogenicity, toxicity, and antinutritional effects in animals (Vermorel et al., 1988). Recently, hydrolysis products of indole glucosinolates have received special attention due to their anticarcinogenic functions (McDanell et al., 1988).

The presence of glucosinolates in rapeseed has been a major limitation for its use as animal feed. Several methods have been developed for removing the undesirable glucosinolates and their hydrolysis products from rapeseed meal. Hydrothermal treatment of intact seeds in sodium sulfate (Mothadi-Nia et al., 1986), extraction of ground seeds with a two-phase solvent system (Diosady et al., 1985), and soaking of intact seeds with citric acid or ammonium carbamate (Schwenke et al., 1990) were some of the methods investigated. Procedures designed to extract glucosinolates from canola flour were not efficient (Sosulski and Dabrowski, 1984). Plant breeding seems to be the primary technique used to overcome the glucosinolate problem during the development of canola varieties from rapeseed, by which the glucosinolate content was reduced from 100−205 μmol/g (traditional rapeseed) to <30 μmol/g (canola).

Although glucosinolates are mainly hydrolyzed by enzyme reactions, thermal degradation of various glucosinolates was also reported (MacLeod et al., 1981; Sosulski and Dabrowski, 1984; Goronowitz et al., 1978). However, nonenzymatic thermal degradation of glucosinolates requires higher temperatures (>100 °C) than enzymatic degradation. Canola is heat treated to condition the seeds for improved oil extraction, to inactivate myrosinase enzyme, to remove solvent, and to dry the meal throughout the conventional process. Seeds are flaked, preheated at 20−50 °C, and cooked at 80−95 °C for 15−30 min. Generally, water or steam is not added to flakes during the cooking process to avoid acceleration of enzymatic glucosinolate hydrolysis (Campbell, 1984). However, crushing brings myrosinase and glucosinolates in close contact, and some hydrolysis is inevitable until the enzyme is inactivated. Hydrolytic products, which are oil soluble, enter the oil during extraction. Sulfur compounds in oil cause catalyst poisoning during subsequent hydrogenation and unpleasant odors in heated oil (Daun and Hougen, 1976). Campbell and Slominski (1990) showed that major glucosinolate decomposition occurred during desolventization of meal. The effect of meal drying on the glucosinolate degradation was minimal.

Myrosinase (thioglucoside glucohydrolase, EC 3.2.1) is present in all plants containing glucosinolates (Fenwick et al., 1983) and catalyzes the hydrolysis of glucosinolates. Myrosinase is located outside the myrosin cells (protein accumulating idioblasts) and has the tendency to adhere to membrane surfaces (Höglund et al., 1991), whereas glucosinolates are found in the myrosin cells. Therefore, myrosinase and glucosinolates do not come in contact unless the seed tissues are damaged. Myrosinase specifically cleaves the thioglucosidic bond, and the resulting aglucone spontaneously undergoes a Lossen rearrangement to produce isothiocyanate (MacLeod and Rossiter, 1986).

Kozlowska et al. (1983) studied inactivation of rapeseed myrosinase and showed that enzyme activity was lost rapidly above 65 °C in the crude extract, while inactivation in intact and flaked seeds started after 5 min of incubation at 90−100 °C and 90−100% relative humidity. Myrosinase inactivation was easier in flaked seeds than in intact seeds. No glucosinolate degradation was observed in intact rapeseed. However, glucosinolates were substantially hydrolyzed in flaked seeds during myrosinase inactivation since the enzyme was already in contact with the substrate. Therefore, Kozlowska et al. (1983) proposed that the rapeseed-processing industry should consider inactivation of

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myrosinase in intact seed prior to rather than after the flaking process. In another study (Appelqvist and Josefsson, 1967), a small loss of myrosinase activity was observed after heat treatment of rapeseed at 90 °C and 4% moisture content for 15 min, while the same heat treatment at 6−8% moisture content effectively inactivated the enzyme. Similarly, Eapen et al. (1968) showed that dry heat treatment (104 °C for 30 min) did not decrease myrosinase activity, while steam blanching (5−30 min) was effective in completely inactivating the enzyme. Soaking of *Brassica napus* and *Brassica campestris* in boiling water for 1.5 and 1 min, respectively, resulted in complete myrosinase inactivation (Eapen et al., 1968). After studying microwave inactivation of myrosinase in canola seeds, Owusu-Ansah and Marianchuk (1991) concluded that the exposure time needed for enzyme inactivation at a specific power was moisture dependent. More recently, McCurdy (1992) reported that infrared processing of canola fines at 78 °C had little effect on myrosinase activity, while processing at 105 °C resulted in a high level of enzyme inactivation. This treatment was more effective in canola fine screenings than in canola seeds due to their higher moisture content (9.9% for screenings vs 5.3% for seeds) (McCurdy, 1992).

The effect of supercritical carbon dioxide (SC-CO_2) on enzyme activity varies with processing conditions and type of protein. Studies with soy protein showed that lipoxygenase was inactivated during SC-CO_2 extraction of oil at 73−85.4 MPa and 80−100 °C (Eldridge et al., 1986). Christianson et al. (1984) reported that peroxidase activity was reduced 10-fold during SC-CO_2 extraction of corn germ at 34.5−55.2 MPa and 50 °C. Pectinesterase in single-strength orange juice could be inactivated with SC-CO_2 at 40 °C and 31 MPa (Balaban et al., 1991). Several commercial enzyme preparations studied by Taniguchi et al. (1987a) retained over 90% of their initial activity following SC-CO_2 treatment at 20.3 MPa and 35 °C for 1 h. The presence of ethanol in SC-CO_2 and increasing moisture content of samples were factors improving the effectiveness of enzyme inactivation. Taniguchi et al. (1987b) extracted oil from mustard seeds with SC-CO_2 without lowering the myrosinase activity and sinigrin content of the samples.

To date, the effect of SC-CO_2 on the endogenous myrosinase enzyme, its activity, and glucosinolate degradation reactions in canola has not been reported. Glucosinolate hydrolysis reactions under SC-CO_2 conditions should be investigated for proper optimization of SC-CO_2 extraction conditions and to determine the extent of seed heat pretreatment necessary prior to oil extraction congruent with evaluation of the quality of extracted oil and residual meal. Therefore, the objective of this study was to examine the effect of SC-CO_2 extraction conditions (temperature, pressure, extraction time, and moisture content of the samples) on the myrosinase activity and glucosinolate hydrolysis in flaked and whole canola seeds.

MATERIALS AND METHODS

Flaked canola (*B. napus* and *B. campestris*) and whole seeds were obtained from CanAmera Foods, Fort Saskatchewan, AB, and kept below −30 °C until used. Canola seeds were flaked commercially using a series of roller mills. Canola flake samples were obtained prior to the conventional cooking process and were referred to as "preheated", since they were heated to 40 °C in less than 5 min following flaking. Moisture content of the canola flakes and whole seeds was modified by adding 7.0 mL of deionized water to 45 g of sample followed by overnight tempering in the refrigerator (4 °C) prior to each extraction. Unmodified samples were referred to as "original" preheated flakes or seeds. The moisture content of the samples just before the SC-CO_2 extraction was determined according to AACC Method 44-31 (AACC, 1969) and reported on a w/w % basis.

SC-CO_2 Extraction. A laboratory scale supercritical fluid extraction (SCFE) unit (Newport Scientific Inc., Jessup, MD) as described by Temelli (1992) was used for this study. The extractor cell was loaded with 35 g of canola flakes for each experiment. SC-CO_2 extractions were carried out at 35−75 °C and 21.4−62.1 MPa for 3 h. CO_2 flow rate was maintained at 2.5 ± 0.5 g/min. The experimental procedure for whole seeds was similar to that of the flaked seeds except extraction conditions were kept constant at 75 °C and 62.1 MPa while the extraction time was varied at 1, 3, and 5 h. Residual samples after SC-CO_2 extraction were kept at −80 °C until they were analyzed for their enzyme activity and glucosinolate content.

Enzyme Activity. Myrosinase activity was measured according to the method of Owusu-Ansah and Marianchuk (1991) after modifications as follows: A 10 g canola sample was homogenized in 50 mL of chilled acetone (reagent grade Omni solvent, BDH Inc., Toronto, ON) for 2 × 30 s using a Kinematica polytron (Brinkman Instruments, Rexdale, ON) on dry ice. The homogenate was filtered and washed with an excess of chilled acetone. The retentate, which is referred to as "acetone powder", was spread on filter paper and air-dried for 1 h at room temperature. One gram of acetone powder was re-extracted with 15 mL of cold phosphate buffer (pH 7.0, 0.1 M). The slurry was then centrifuged (Beckman Instruments Inc., Mississauga, ON) at 28200*g* for 30 min at 4 °C, and the supernatant was used as the crude enzyme preparation.

Two milliliters of sinigrin (Sigma Chemical Co., St. Louis, MO) solution (1 mM in phosphate buffer) and 2 mL of crude enzyme solution were mixed on a vortex. The mixture was incubated at 35 °C for 1 h, followed by inactivation of the enzyme by boiling for 15 min. After cooling under tap water, a 100 μL aliquot was used for glucose analysis according to the *O*-toluidine procedure (glucose kit, catalog no. 635, Sigma). Glucose content of the enzyme solutions before and after substrate incubation period was determined for each sample. The difference between the glucose contents before and after the incubation was taken as the glucose converted by the enzyme in 1 h. Protein content of the enzyme solutions was determined using the phenol reagent method (protein kit, catalog no. 690-A, Sigma). Enzyme activity was reported as micrograms of glucose per microgram of protein per minute.

Glucosinolate Analyses. Intact glucosinolates in canola samples were isolated and purified on a DEAE ion-exchange column (DeClercq and Daun, 1989).

Extraction of Glucosinolates. Canola samples (5 g) were heated at 95 °C for 15 min for inactivation of enzymes. Four milliliters of boiling water was added into the ground sample (200 mg) and mixed, and then the mixture was heated at 95 °C for 5 min. After cooling and centrifugation at 900*g*, the supernatant was transferred into graduated centrifuge tubes containing 150 μL of 0.5 M barium/lead acetate solution. Residue from the first water extraction was re-extracted with 4 mL of boiling water for 5 min at 95 °C as above. Supernatants from the two extraction steps were combined, and the volume was adjusted to 10 mL. The mixture was centrifuged (900*g*), and the supernatant was used for glucosinolate purification.

Column Preparation. Dry DEAE-Sephadex A-25 (100 mg) in an 0.8 × 4 cm Econo-column (Bio-Rad Laboratories, Hercules, CA) was allowed to swell in deionized water. Air bubbles were removed by stirring. Prior to sample application, 5 mL of 0.5 N NaOH, followed by 10 mL of water to remove excess NaOH, was passed through the column. The eluate was monitored to ensure neutral pH. The resin was changed to acetate form by adding 5 mL of 0.5 M pyridine acetate solution followed by 10 mL of water.

Isolation of Glucosinolates. Three milliliters of crude glucosinolate extract was applied to the prepared column.

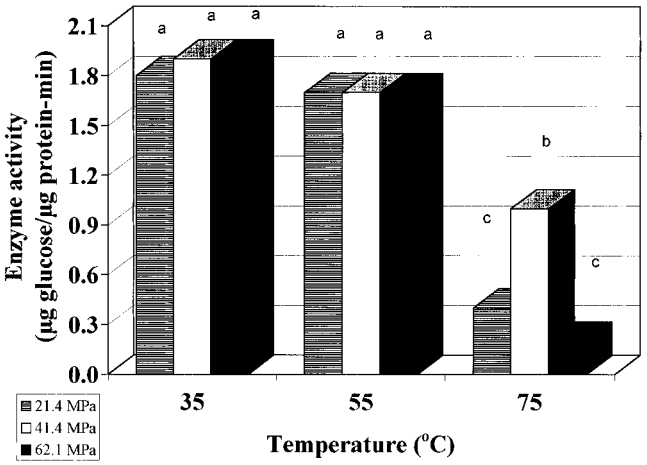

Figure 1. Effect of pressure and temperature on the myrosinase activity in canola flakes with 19% moisture content. Bars with the same letter are not significantly different ($p > 0.05$) (lsd = 0.39).

Then, the column was washed with 2 × 2 mL of water, 2 × 2 mL of 3% formic acid, and 2 × 2 mL of water; the eluate was discarded each time. Glucosinolates were eluted with 2 × 4.75 mL of 0.3 M K_2SO_4, and the volume was adjusted to 10 mL.

Measurement of Glucosinolate Content. A 1 mL aliquot of isolated glucosinolates was mixed with 7.0 mL of 80% H_2SO_4 and 1.0 mL of 1% thymol in ethanol. The mixture was incubated at 100 °C for 60 min, then cooled and mixed. Absorbance was measured against 0.3 M K_2SO_4 at 505 nm using a diode array spectrophotometer (Model 8452A, Hewlett-Packard Ltd., Orangeville, ON). Four blanks and four standards were prepared. Standards consisted of 1.0 mL of sinigrin solution (0.3 μmol/mL), 7.0 mL of 80% H_2SO_4, and 1.0 mL of 1% thymol solution, while blanks were prepared with 1 mL of 0.3 M K_2SO_4, 7.0 mL of 80% H_2SO_4, and 1.0 mL of 1% thymol solution. The glucosinolate content of the canola samples was reported as micromoles per gram of dry and oil-free sample.

Statistical Analysis. SC-CO_2 extractions at each condition were carried out in duplicate in randomized order. Duplicate analyses of the residue from each SC-CO_2 extraction were performed, and means were reported for each treatment. Analysis of variance of the results was carried out using the General Linear Model procedure of SAS Statistical Software, version 6 (SAS Institute, 1989). Multiple comparison of the means was carried out by least significant difference (lsd) test at $\alpha = 0.05$ level.

RESULTS AND DISCUSSION

Myrosinase Activity. Flaked canola seeds (7.7% moisture) had significantly ($p < 0.05$) lower enzyme activity than the whole seeds (8.5% moisture), 2.0 and 2.7 μg of glucose/(μg of protein·min), respectively, which might be due to the flaking and preheating processes.

When canola flakes (7.7% moisture) were exposed to SC-CO_2 at 21.4−62.1 MPa and 35−75 °C for 3 h, 85−100% of original myrosinase activity was retained (Dunford, 1995). The effects of temperature and pressure were not significant ($p > 0.05$), and the residual enzyme activities following SC-CO_2 treatment were similar to that in the feed material. These results were similar to those reported for mustard myrosinase activity following SC-CO_2 treatment at 30.0 MPa and 40 °C for 3 h (Taniguchi et al., 1987b). To assess the effect of moisture content of canola flakes on the myrosinase activity during SC-CO_2 processing, a series of experiments were carried out with flakes containing 19.0% moisture, which is similar to the moisture content of oil seeds at harvest (Figure 1).

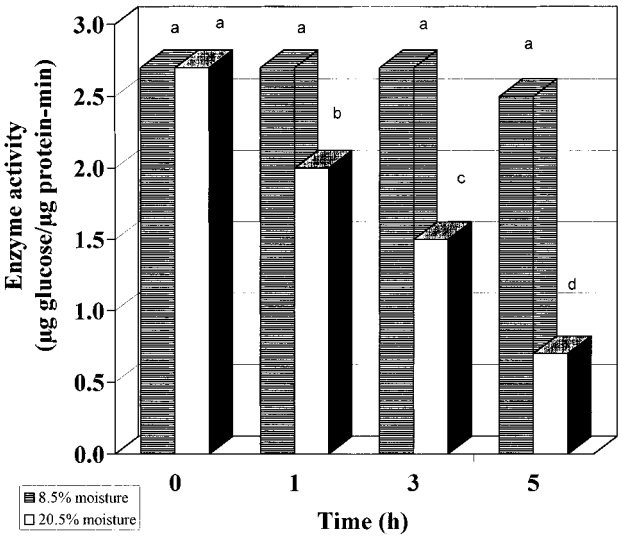

Figure 2. Effect of time and moisture content of the whole canola seeds on the myrosinase activity during SC-CO_2 treatment at 62.1 MPa and 75 °C. Bars with the same letter are not significantly different ($p > 0.05$) (lsd = 0.39).

At 35 °C, 85% of the original enzyme activity was retained in the canola flake residues, which was similar ($p > 0.05$) to that in the feed material. At 19% moisture level, there was a significant ($p < 0.05$) drop in enzyme activity with an increase in temperature up to 75 °C at all pressure levels studied (Figure 1).

Highest myrosinase inactivation was achieved at 62.1 MPa and 75 °C, at which only 10% of the original enzyme activity was retained after 3 h. In addition, this set of conditions resulted in the maximum oil yield (Dunford and Temelli, 1996). At 41.1, 21.4 MPa, and 75 °C residual enzyme activities were 50 and 20% of the original, respectively (Figure 1). The fact that the residual enzyme activity at 21.4 MPa was significantly ($p < 0.05$) lower than that at 41.4 MPa was unexpected. To our knowledge, the literature lacks information on the effect of SC-CO_2 treatment on enzyme activity as a function of pressure during oil extraction. However, Temelli et al. (1994) reported increased level of aggregation of sarcoplasmic proteins of Atlantic mackerel with presssure (34−62 MPa) during SC-CO_2 extraction of oil. Another factor contributing to the denaturation of proteins during SC-CO_2 treatment at high moisture levels is the formation of carbonic acid due to the equilibrium reached between CO_2 and water. This equilibrium is shifted toward carbonic acid with pressure. It was not possible to measure the pH of canola flakes during the SC-CO_2 extractions. Further research is needed to understand the mechanism of myrosinase inactivation under combined effects of pressure and temperature at high moisture levels in the SC-CO_2 environment.

The time dependence of myrosinase inactivation in whole canola seeds was examined at 62.1 MPa and 75 °C, since the highest enzyme inactivation was achieved under these conditions with flaked seeds. After 3 h of SC-CO_2 treatment of whole canola seeds (8.5% moisture), no enzyme inactivation was observed (Figure 2). Ninety-three percent of the original myrosinase activity was retained in the whole seeds even after 5 h of SC-CO_2 treatment. However, when the moisture content of the whole seeds was modified to 20.5%, there was significant ($p < 0.05$) enzyme inactivation (Figure 2). At this moisture level, residual myrosinase activities were 74, 56, and 26% after 1, 3, and 5 h of SC-CO_2

Table 1. Effect of Temperature, Pressure, and Moisture on the Glucosinolate Content (Micromoles per Gram) of Canola Flakes after SC-CO_2 Treatment[a]

	7.7% (w/w) moisture			19.0% (w/w) moisture		
temp (°C)	21.4 MPa	41.4 MPa	62.1 MPa	21.4 MPa	41.4 MPa	62.1 MPa
35	9.6^{a}	9.3^{ab}	9.5^{ab}	9.6^{a}	9.2^{ab}	9.4^{ab}
55	9.5^{ab}	9.2^{ab}	9.4^{ab}	9.5^{ab}	8.9^{ab}	8.6^{ab}
75	8.9^{ab}	9.1^{ab}	9.4^{ab}	8.9^{ab}	8.4^{b}	8.9^{ab}

[a] lsd = 1.15. Means with the same letter are not significantly different ($p > 0.05$).

treatment of canola seeds, respectively. Comparison of myrosinase inactivation in canola flakes and whole seeds under SC-CO_2 conditions indicates that enzyme inactivation was more difficult to achieve in whole seeds than in flaked seeds. After 3 h of SC-CO_2 processing at 62.1 MPa and 75 °C, residual myrosinase activity in canola flakes (7.7% moisture) was 85%, whereas in whole seeds (8.5% moisture) (Figure 2) no enzyme inactivation was detected. A similar trend was observed at the higher moisture level, but the residual enzyme activity measured in whole seeds, 56% (Figure 2), was significantly ($p < 0.05$) higher than that in the flaked seeds, 10% (Figure 1). A similar trend was observed during the heat inactivation of rapeseed (Kozlowska et al., 1983).

Glucosinolate Content. The glucosinolate content of canola flakes was slightly lower than that of the whole seeds, 9.6 and 10.2 μmol/g, respectively. This result was not unexpected since some enzymatic glucosinolate hydrolysis is inevitable due to the flaking process which brings myrosinase into contact with glucosinolates. Comparable glucosinolate levels in canola were reported by Owusu-Ansah and Marianchuk (1991). This level of glucosinolates in canola is well below the Canadian standard of 30 μmol/g of meal.

Reduction in glucosinolate content of canola flakes (7.7% moisture) was minimal after SC-CO_2 treatment under the temperature and pressure conditions studied (Table 1) despite the fact that there was no significant change in myrosinase activity. When the moisture content of the flakes was modified to 19.0%, slightly higher glucosinolate degradation was observed at 55 and 75 °C and 41.1−62.1 MPa. Although enzyme activity was significantly ($p < 0.05$) decreased at 75 °C (Figure 1), some glucosinolates were hydrolyzed during the inactivation process. This might be due to the faster glucosinolate hydrolysis rates compared to the enzyme inactivation rate at higher temperatures and higher moisture level. At the highest temperature studied, 75 °C, highest residual enzyme activity was observed at 41.4 MPa, which resulted in the largest glucosinolate degradation.

Figure 3 presents glucosinolate degradation in whole seeds with SC-CO_2 extraction time at different moisture levels. At 8.5% moisture level, there was no change in the glucosinolate content up to 3 h of SC-CO_2 extraction. Glucosinolate degradation reached a significant level ($p < 0.05$) during the final 2 h of SC-CO_2 extraction. At 20.5% moisture level, there was only a slight decrease in glucosinolate content during the first hour, which remained steady up to 5 h (Figure 3). This can be attributed to the retention of original activity at 8.5% moisture level, as opposed to the significantly ($p < 0.05$) reduced myrosinase activity of the samples containing 20.5% moisture (Figure 2). Enzyme inactivation in whole seeds with higher moisture content was much

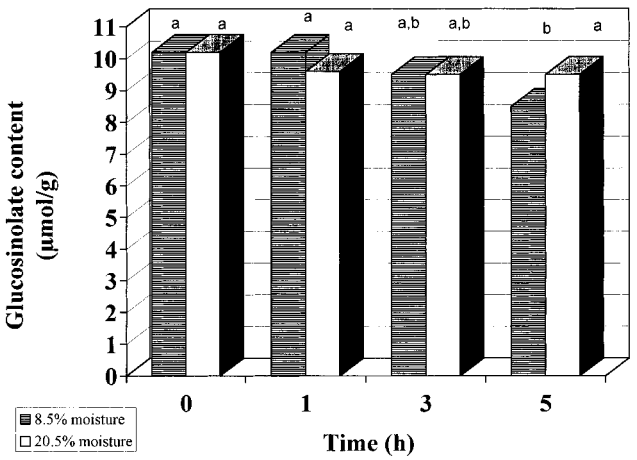

Figure 3. Effect of time and moisture on the glucosinolate content of whole canola seeds during the SC-CO_2 treatment at 62.1 MPa and 75 °C. Bars with the same letter are not significantly different ($p > 0.05$) (lsd = 1.15).

faster than that of the lower moisture content seeds (Figure 2). Therefore, seed samples with low moisture level had higher enzyme activity throughout the SC-CO_2 treatment. After 3 h of extraction, the glucosinolate contents of the residues were similar (Figure 3) for both moisture levels, even though there was a significant difference in their enzyme activities (Figure 2). This is probably due to the slower hydrolysis rate when the moisture content of the reaction medium is low.

Conclusions. This study demonstrated that the myrosinase enzyme was inactivated to different levels during canola oil extraction with SC-CO_2 depending on the extraction parameters. Myrosinase inactivation in flaked canola samples was minimal under SC-CO_2 conditions at low moisture levels. Combined effects of high temperature (75 °C), pressure (62.1 MPa), and moisture level (20%) were necessary to achieve 90% enzyme inactivation in canola flakes during 3 h of SC-CO_2 extraction. In whole canola seeds only 44% myrosinase inactivation could be detected under similar extraction conditions. Glucosinolate degradation in canola flakes was minimal at all moisture levels studied. Increasing the moisture content of the whole seeds reduced the glucosinolate degradation rate due to low level of residual myrosinase activity. In terms of SC-CO_2 processing of canola flakes, moisture modification prior to extraction is beneficial for myrosinase inactivation during oil extraction; however, it has a negative effect on extraction efficiency since some of the water is extracted along with the oil (Dunford and Temelli, 1996). When canola flakes are extracted at low moisture levels, myrosinase enzyme is not affected but glucosinolate degradation is minimal during the 3 h of extraction under the conditions studied. Thus, myrosinase inactivation prior to SC-CO_2 extraction may not be necessary. In this case, stability of the meal for its further utilization needs to be investigated due to the high enzyme activity levels involved.

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