JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of D-*chiro*-Inositol in Tartary Buckwheat Using High-Performance Liquid Chromatography with an Evaporative Light-Scattering Detector

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A simple method for rapid determination of D-*chiro*-inositol (DCI) (a potent mediator of insulin metabolism) contained in tartary buckwheat (*Fagopyrum tataricum* L. Gaench) was developed on the basis of high-performance liquid chromatography coupled to an evaporative light scattering detector (ELSD). DCI was separated from an extract of tartary buckwheat on an Alltech Prevail Carbohydrates ES 5 μm column. During postcolumn detection, DCI was detected by ELSD. The detection limit was 100 ng. This method was sensitive enough for determining DCI in tartary buckwheat and its related products.

KEYWORDS: D-*chiro*-Inositol; evaporative light-scattering detector; tartary buckwheat

INTRODUCTION

There has been a continued interest in inositols because of their high potency as insulin function enhancer for human body. Inositols have great potential as adjunctive drug for the treatment of insulin resistance diseases, such as type 2 diabete and polycystic ovary syndrome (PCOS) (*1, 2*). D-*chiro*-Inositol (DCI) is an epimer of *myo*-inositol, which is a coenzyme of glycosylphosphatidyl inositol protein that participates in the insulin signaling pathways and, thus, simulates glucose transport (*3–5*), so that DCI may be the main insulin mediator, which could work to increase the action of insulin and decrease blood pressure, plasma triglycerides, and glucose concentrations (*6, 7*). In addition, DCI was more effective than *myo*-inositol in preventing folate-resistant mouse neural tube defects (*8*).

Tartary buckwheat was an important natural source of DCI. Obendorf and Horbowicz (*9*) detected free DCI in lupines, pigeon peas, soybeans, chickpeas, mungbeans, and buckwheat. Among all of the seeds analyzed; only mungbean seeds contained higher levels of free DCI than buckwheat. It was also found that most DCI existed in the form of its galactosides, named fagopyritols (FP). Indeed, five different fagopyritols had been identified in buckwheat, more than in other plant materiasl such as soybeans, lupines, lentils, and chickpea seeds (*10*). Animal studies have demonstrated that the administration orally of the buckwheat extracts could lower serum glucose concentrations in rats. It was expected that DCI in the buckwheat extracts may be responsible for this action (*11*).

The fact that tartary buckwheat containing high level of DCI creates a large opportunity for developing new food products (both common foods and functional foods) from the functional components of buckwheat seeds, such as griddlecakes, wine, sauce, crackers, noodles, tea, and capsules (*12*). To date, the common method for detecting DCI in buckwheat has been based on a complicated gas chromatography (GC) method, in which a silylation process with trimethylsilylation imidazole (TMSI) is essential (*13, 14*). It is, thus, necessary to establish a simple way to detect DCI in buckwheat and related products. The aim of this work is to establish a rapid and convenient quantitative method for determining the total DCI present in buckwheat and DCI-rich products, such as noodles, tea, and crackers, so that a new way of DCI detection is introduced.

MATERIALS AND METHODS

Chemicals and Solvents. DCI standard (99%) and trifluoroacetic acid (TFA, 99%, bp 70 °C) were purchased from Sigma-Aldrich (Shanghai, China). Acetonitrile (HPLC) was purchased from Fisher Chemicals (Shanghai, China).

Plant Materials. Tartary buckwheat (*Fagopyrum tataricum* L. Gaench) seeds (Fenxi buckwheat) were obtained from Heshun Country in Shanxi Province, China. The other two cultivars supplied by National Center of Crop Germplasm Conservation(Beijing, China) were Jiujiang buckwheat (Jiangxi, China) and Gongshan buckwheat (Yunnan, China). The seeds were dried at 40 °C, ground with a laboratory mill, and passed through a 20 mesh screens sieve. Tartary buckwheat related products, including four brands noodles of tartary buckwheat flour, two brands tartary buckwheat tea powder, and three brands tartary buckwheat cracker, were purchased from local supermarkets.

Instrumentation and Chromatography. The high-performance liquid chromatography (HPLC) system consisted of two Shimadzu LC-20A pumps and a Shimadzu LC-20A autosampler (Kyoto, Japan). An Alltech Prevail Carbohydrates ES 5 μm column (4.6 × 250 mm, Alltech, Deerfield, IL) was used. The separation was

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10.1021/jf0717541 CCC: $40.75 © 2008 American Chemical Society
Published on Web 01/01/2008

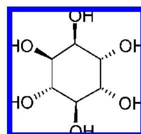

Figure 1. Chemical structure of D-*chiro*-inositol.

performed using a solution of acetonitrile/distilled water mixture with a gradient elution (0−20 min, acetonitrile concentration was 80%; 20−50 min, acetonitrile concentration was lowered to 65%). The flow rate was set at 1 mL/min. The eluent after the column was sent to an evaporative light scattering detector (ELSD) 2000ES (Alltech, Deerfield, IL). ELSD conditions were optimized to achieve maximum sensitivity. The temperature of the drift tube was set at 95 °C. The nebulizing gas (nitrogen gas) flow rate was set at 2.2 L/min, and the gain was set at 1.

Extraction and Quantification of DCI. The extraction was performed by mixing 1 g of sample with 20 mL of ethanol/water (1:1, v/v) solution in one conical flask. Two identical flasks were used, with one incubated in a water bath and the other in an ultrasonic bath. The extraction was performed at room temperature for 30 min, and then, the extract was filtered through Whatman No. 4 filter paper. A total of 1 mL of extract was put in a vial and dried in an oven. Until the solution was completely dried, 2.0 mL of 3 N trifluoroacetic acid (TFA) was added into the vial with the cap on to hydrolyze the sample. The hydrolysis was performed in a water bath. The hydrolysis condition was optimized through a factorial experiment design. After the hydrolysis, the sample was evaporated to dryness and then redissolved by 1 mL of methanol, filtered through 0.45 μm Millex-HN syringe filters (13 mm) (Bedford, MA) and then stored in a HPLC autosampler vial for immediate HPLC−ELSD analysis. The DCI standard solution was prepared by dissolving 0.1 mg DCI in methanol, to make a solution containing 0.1 mg/mL of DCI. Then, series of DCI solution were injected at 1, 2, 4, 6, 8, and 10 (100–1000 ng) to make the standard curve. During the experiment, all of the solvents were analytical-grade, unless otherwise noted.

A three-level full-factorial 3^4 was designed to make sure the best condition of TFA hydrolysis. The factors were A, hydrolysis temperature (50, 60, and 70 °C); B, TFA addition amount per 1 mL of extract (0.5, 1.0, and 2.0 mL); C, hydrolysis time (2, 3, and 4 h); and D, TFA concentration (1, 2, and 3 N).

RESULTS AND DISCUSSION

As a kind of cyclytol (**Figure 1**), DCI had no ultraviolet absorption and, thus, is difficult to be detected by usual detector such as UV−vis detector. To enable detection of DCI by HPLC, we chose the evaporative light-scattering detector (ELSD), a semi-universal detector that can detect any nonvolatile analyte. As shown in **Figure 2A**, ELSD was suitable for detecting DCI.

At the same time, we attempted to seek the most appropriate column. There were several HPLC columns available such as an Econosphere NH_2 5 μm column (*13*) and a Lichrosphere NH_2 column (*14*), the bulking agent of which were all with the amino group. However, DCI was not separated well, which was indicated by the asymmetrical peak and nonstraight baseline of chromatography. Considering the fact that DCI belongs to multicyclitol, a carbohydrates column might be a desirable column. We used a Prevail Carbohydrates ES 5 μm (Alltech, Deerfield, IL) column in the HPLC system. As shown in **Figure 2A**, a well-defined peak ascribed to DCI was detected at a suitable retention time when standard DCI (2000 ng) was subjected to HPLC coupled with ELSD.

The concentration of ethanol for DCI extraction usually is 50% (*13, 15*). In this work, we compared two different extractive ways: shaking extraction and ultrasonic extraction. Shaking extraction had a better reproducibility referring to yield (SD = 0.13%, n = 10) than ultrasonic extraction (SD = 3.2%, n = 10). Although ultrasonic had relatively high yield, its precision was low because the ultrasonic energy was not homogeneously distributed. Thus, shaking extraction was used in the following works.

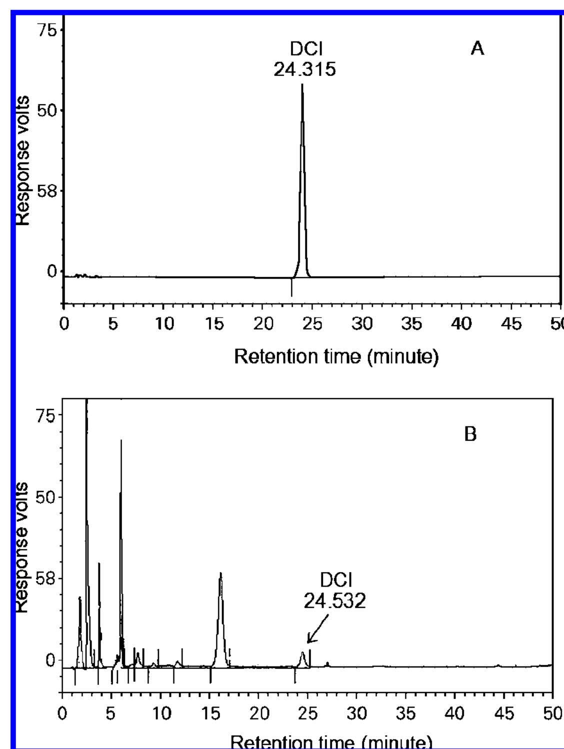

Figure 2. Separation and detection of D-*chiro*-inositol by HPLC−ELSD. (**A**) Chromatography of D-*chiro*-inositol standard. (**B**) Chromatography of D-*chiro*-inositol present in buckwheat seeds extracts.

Most DCI in buckwheat exists in the form of fagopyritols, which can be converted to DCI by acidic hydrolysis. In addition, Johnson et al. discovered that DCI was stable under acidic conditions, while other sugars were destroyed; thus, a high concentration DCI could be kept at this condition (*13*). For example, being hydrolyzed with 2 N TFA for 3 h at 70 °C (*10*) or 1 N TFA for 16 h at 80 °C (*16*), a high yield of DCI would be produced. In this work, a three-level factorial 3^4 design was conducted to optimize the hydrolysis conditions, including hydrolysis temperature (50, 60, and 70 °C), TFA addition amount per 1 mL of extract (0.5, 1.0, and 2.0 mL), hydrolysis time (2, 3, and 4 h), and TFA concentration (1, 2, and 3 N). **Table 1** showed the experimental design matrix and the DCI content for each run. The last three rows in **Table 1** gave the sum of yield of each level for the four factors. For example, for III, the value of 6.41 at column "B" was the sum of the yield at trials 3, 4, and 8, all of which chose level 3 (2.0 mL) for B. The results were analyzed by SASS 8.2 software. An F test was conducted. High values for the calculated F mean a greater influence of factor on the experimental results. As seen in **Table 2**, the calculated values of F_A, F_B, F_C, and F_D were 321.08, 9.82, 78.69, and 1.00, F_A and F_C were greater than the $F_{0.05}(3, 2)$ = 19.16, and the highest F_i value was F_A with the factor of hydrolysis temperature, which was the most influential factor. While factor D of the TFA concentration was the considered statistically as insignificant. In addition, for each level of a factor, the sum yield was calculated in the cases where the level occurred (see the last three rows in **Table 1**). The level corresponding to the maximum sum yield among the three levels would be chosen for the optimal set of factors. In summary, from the experimental design in **Table 1**, the optimal factors

Table 1. Factorial Design To Determine the Optimal Hydrolysis Condition

	factors				
run	*A* temperature[a] (°C)	*B* amount[b] (mL)	*C* time[c] (h)	*D* concentration[d] (N)	DCI content (mg/g)
1	50	0.5	2	1	1.66
2	50	1	3	2	1.89
3	50	2	4	3	1.98
4	60	2	2	2	2.28
5	60	0.5	3	3	2.30
6	60	1	4	1	2.14
7	70	1	2	3	2.31
8	70	2	3	1	2.15
9	70	0.5	4	2	2.38
I	5.53	6.34	6.25	5.95	
II	6.72	6.34	6.34	6.55	
III	6.84	6.41	6.50	6.59	

[a] Hydrolysis temperature; the oven was set at the certain temperature. [b] TFA addition amount of 0.5, 1.0, and 2.0 mL of TFA solvent was added in to 1 mL of buckwheat seeds extract, respectively. [c] Hydrolysis time. [d] TFA concentration of 1, 2, and 3 N.

Table 2. Results of Analysis of Variance

factor	SS[a]	df[b]	S[c]	*F* value	significance[d]
A	0.35	2	0.17	321.08	**
B	0.01	2	0.01	9.82	
C	0.09	2	0.04	78.69	*
D	0.00	2	0.00	1.00	

[a] Sum of square. [b] Degree of freedom. [c] Mean of square. [d] $F_{0.05}(3, 2) = 19.16$; $F_{0.01}(3, 2) = 99$.

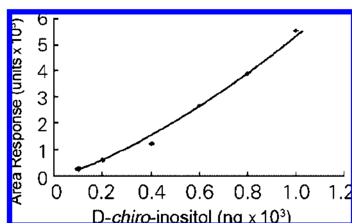

Figure 3. Standard curve of D-*chiro*-inositol by HPLC−ELSD.

were identified as $A_3B_3C_3D_3$; i.e., the optimal hydrolysis conditions were adding 2 mL of TFA into 1 mL of dried extract for hydrolysis in the water bath at 70 °C for 4 h, with TFA concentration being 3 N.

To apply the proposed method for determining DCI in buckwheat, the standard curve between DCI concentration and HPLC area response was made. As shown in **Figure 3**, the standard curve of DCI using ELSD was not linear and this was a common feature of this detector. The correlation between DCI concentrations and area response were best described by the following equation: $y = 32.559x^{1.4017}$ (y = area units; x = DCI amount), $r^2 = 0.9955$. The sensitivity of the HPLC−ELSD method for DCI determination was relatively high with a detection limit of 100 ng for DCI.

We further applied the optimized hydrolysis methods to three tartary buckwheat cultivars, using HPLC−ELSD to detect the DCI and other components, as shown in the chromatography in **Figure 2B**; a good separation between DCI and other components were obtained.

Using this standard curve, the concentration of DCI in three cultivars of buckwheat (**Table 3**) and related products were defined as 0.099−0.387% (99–387 mg/100 g of dry weight) (**Table 4**). *A*−*D* was noodles made of buckwheat flour, which had relatively low content of DCI compared to buckwheat seeds. Because certain ductility and viscoelasticity of dough for making

Table 3. DCI Concentration of Buckwheat in Three Cultivars

cultivars	DCI[a] (mg/g dry weight)
1[b]	0.212 ± 0.006
2[c]	0.228 ± 0.005
3[d]	0.178 ± 0.005

[a] Values were means ± SD ($n = 3$). [b] As Fenxi buckwheat (Shanxi, China). [c] As Jiujiang buckwheat (Jiangxi, China). [d] As Gongshan buckwheat (Yunnan, China).

Table 4. DCI Concentration of Buckwheat Products on the Market

product (form)	DCI[a] (mg/g)
A[b] (noodle)	0.112 ± 0.003
B[c] (noodle)	0.147 ± 0.006
C[d] (noodle)	0.106 ± 0.003
D[e] (noodle)	0.133 ± 0.002
E[f] (tea powder)	0.366 ± 0.006
F[g] (tea powder)	0.387 ± 0.007
G[h] (cracker)	0.099 ± 0.005
H[i] (cracker)	0.104 ± 0.002

[a] Values are means ± SD ($n = 3$). [b] As Fengda noodles of buckwheat powder (Anhui, China). [c] As Bailemai noodles of buckwheat powder (Shanxi, China). [d] As Zhanxiang noodles of buckwheat powder (Henan, China). [e] As Shengchu noodles of buckwheat powder (Beijing, China). [f] As Jiaxin buckwheat health tea (Shanxi, China). [g] As Guoweibuckwheat health tea (Shanxi, China). [h] As Alpha health cracker (Tianjin, China). [i] As Beiyinmeibuckwheat health cracker (Hangzhou, Zhejing).

noodle were essential and buckwheat contained low mucedin, it was impossible to make noodles with buckwheat flour alone, so that so-called buckwheat noodles were made of mixture of wheat flour and buckwheat flour actually (*17*). The same reason for cracker of *G* and *H* had even lower concentration of DCI. While as for *E* and *F*, because they belonged to health food, which had the license of health food, they must made DCI enriched by certain technology to ensure a high content of DCI.

To confirm complete recovery of DCI from buckwheat, we added standard DCI (100–500 μg, 0.1%) to 0.1% buckwheat extraction and determined DCI according to the proposed method. The recovery of DCI was calculated as >85%.

Several methods have been developed to determine DCI content. The common method was based on gas chromatography (*9, 10, 15*), in which a silylation process with trimethylsilylation imidazole (TMSI) was needed, making the detection complicated and time-consuming. A few researchers tried to use HPLC coupled with differential refractive index detector (RID) to detected DCI (*13, 14*); however, RID had a low sensitivity to DCI, with a poor separation. Given this situation, the detection of DCI was very limited. The method proposed in this work provides an efficient alternative for the common methods.

Horbowicz et al. did the detection of the DCI in buckwheat by gas chromatography–mass spectroscopy (GC−MS), which detected a total DCI up to 2.2 mg/g in embryo of buckwheat seeds, while 1.05 mg/g in mature seed (embryo and endosperm) (*10*). Steadman et al. reported a total content of 192 mg of DCI/100 g of tartary buckwheat (*15*). These results were consistent with our determined values; however, the determination procedures were relatively complicated as discussed before. The HPLC−ELSD method proposed in this work enables the simple and convenient determination of DCI. This will be a great advantage when multiple samples were analyzed.

In conclusion, we developed a simple quantitative method for determining DCI in buckwheat. Currently, although various DCI-supplemented food products are available for consumers in China, their DCI contents are not specified, because of the

lack of practical method to measure DCI. The method developed in this work is capable of measuring DCI present in various consumer products, such as noodles, tea, cracker, and buckwheat plant sources.

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Received for review June 13, 2007. Revised manuscript received December 12, 2007. Accepted December 13, 2007. The present study was supported by the Talent Fund (for Guixing Ren) of the Chinese Academy of Agricultural Science, Technology Transformation Fund (2005EFN211400040), Chest Fund (2005DIA4J01) and Supporting Fund (2006BAD02B06-03) from The Ministry of Sciences and Technology, People's Republic of China.

JF0717541