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Analytical Methods

Determination of oligosaccharide contents in 19 cultivars of chickpea (*Cicer arietinum* L) seeds by high performance liquid chromatography

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ABSTRACT

The extraction procedure of oligosaccharides from chickpea seeds was optimized and sufficient extraction was achieved with 50% of ethanol-water in a ratio of 10:1 for solvent to defatted chickpea meal at 50 °C for 30 min. Under the optimal extraction conditions, the contents of oligosaccharides in 19 cultivars of chickpea seeds were determined by HPLC. The results showed that the main α-galactooligosaccharides (α-GOS) in chickpea seeds were raffinose, stachyose, verbascose and an unknown glycoside, which was isolated, purified, and identified as ciceritol. Ciceritol, the main sugar in all the chickpea samples, accounted for about 50% of the total α-GOS. There were considerable variations in the levels of α-GOS and sucrose between species, especially verbascose which could only be detected in 7 samples. With the highest amount of α-GOS and a low amount of sucrose, the 171 was the best choice for obtaining α-GOS for use as a prebiotic in functional foods.

1. Introduction

It is well known that balance of intestinal bacterial flora is important for human health, especially bifidobacterium, which could dominate pathogenic organisms and thus invigorate human health. It has been reported that the growth of intestinal bifidobacteria is facilitated by certain oligosaccharides such as galacto-, isomalto-, and xylo-oligosaccharides (Alles et al., 1999; Mussatto & Mancilha, 2007; Salminen et al., 1998). In fact, many prebiotic oligosaccharides are used as ingredients in various products, such as soft drinks, cookies, cereals, candies, and infant foods (Nakakuki, 2003; Tomomatsu, 1994). Due to the lack of α-galactosidase in the upper intestinal tract of humans, α-linked galactooligosaccharide (α-GOS) could avoid digestion and reach the colon as substrates for luminal fermentation by the microbial flora. These characteristics allow α-GOS to be considered as prebiotics (Gibson & Roberfroid, 1995).

Oligosaccharides with α-galactosidic linkages are widely distributed in the plant kingdom. An especially large amount of α-GOS occurs in generative parts of plants, where they perform protective physiological functions, act as germination inhibitors when there is not enough water available, and play a role in cold acclimation of many plants (Horbowicz & Obendorf, 1994; Kuo, Van Middlesworth, & Wolf, 1988; Larsson, Johansson, & Svenningsson, 1993). Therefore, α-GOS can be obtained by extraction from plants, mainly from legume seeds, such as soybean, lupin, lentil and pea seeds. α-GOS from soybean are the only legume oligosaccharides in the market, and the main producer is Japan (Crittenden & Playne, 1996). Unfortunately, α-GOS from soybean seeds contain a high amount of sucrose, which limits application. Chickpea is one of the oldest and most widely consumed legumes in the world (FAO, 1994; Zia-Ul-Haq et al., 2007). Chickpea seeds are usually grown for human consumption. They are a cheap source of high quality proteins in the diets of millions in developing countries. In addition, they are also a good source of carbohydrates, minerals and trace elements. It has been reported that ciceritol is a characteristic glycoside in chickpea seeds (Bernabe et al., 1993; Quemener & Brillouet, 1983; Sanchez-Mata, Penuela-Teruel, Camara-Hurtado, Diez-Marques, & Torija-Isasa, 1998). With the high content of α-GOS (5–10%), chickpea seeds should be a good source of α-GOS (Sanchez-Mata et al., 1998). Herein, we report, in detail, the analysis of oligosaccharides in 19 cultivars of chickpea seeds, with the aim of selecting cultivars with the highest amounts of α-GOS for use as ingredients in functional foods. First, we investigated the effect of extraction conditions (extraction solvent, temperature, time and solvent to defatted chickpea seeds ratio) on the extractability of oligosaccharides in chickpea seeds. Then, sugars in 19 cultivars of chickpea seeds were extracted using the optimal extraction conditions

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and determined by high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Samples and chemicals

Chickpea seeds were provided by the National Center for Soybean Improvement, Nanjing Agricultural University, and College of Agriculture, Xinjiang Agricultural University. The seeds were stored at 4 °C. Activated charcoal (Darco G-60, 100 mesh), fructose, glucose, maltose, ribose, sucrose, stachyose and xylose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Raffinose and Celite 535 were from Fluka. Millipore membranes (0.45 μm) were from Millipore. All other chemicals were of analytical grade.

2.2. Opitimization of extraction conditions for oligosachharides

The seeds were dried at 85 °C for 2 h and ground in an electric grinder (100-mesh sieve). Lipid was removed from the dry meal (500 g) with 2 l of petroleum ether (boiling range, 37–55 °C). The defatted chickpea meals (DCM) were stored at 4 °C for use. To study the effect of different variables on the extraction of oligosaccharides in chickpea seeds, extraction was carried out with five concentrations of ethanol (0%, 30%, 50%, 70% and 80%, v/v), four temperatures (room temperature, 50 °C, 70 °C and boiling temperature), three different extraction times (15, 30 and 60 min), and four different ratios of DCM to solvent (1:5, 1:10, 1:15, 1:20, w/v). The optimal conditions selected for extraction were as follows: triplicate samples (1.0 g) were extracted 3 times with 10 ml 50% ethanol–water at a ratio of 10:1 (solvent to DCM) in a water-bath at 50 °C for 30 min. After each extraction, the samples were centrifuged at 2500*g* for 20 min. Supernatants from three cycles of extraction were combined and concentrated by using a rotary vacuum evaporator (Heidolph, Germany), and then dissolved with 5.0 ml of the mobile phase of HPLC (acetonitrile–water 75:25, v/v, HPLC grade of acetonitrile). Before injection, all samples were filtered through a 0.45 μm millipore membrane.

2.3. Analysis of oligosaccharide contents in 19 cultivars of chickpea seeds

2.3.1. HPLC analysis of oligosaccharides

The separation and quantification of oligosaccharides from chickpea seeds were carried out by an Agilent 1100 series HPLC system (Agilent, USA), which consisted of a G1311A pump and a G1362A refraction index detector (Zeng et al., 2007). Both, sugar compounds in chickpea extractions and different standard sugars, were separated on a Sugar-D column (4.6 × 250 mm, Nacalai Tesque Inc., Japan), using acetonitrile–water (75:25, v/v) as the mobile phase at the flow rate of 1.0 ml/min. Injection volume was 20 μl. Sugar compounds in chickpea extractions were identified by comparing the retention times with those of standard sugars.

2.3.2. Quantification of oligosaccharides

Different amounts of sucrose, raffinose, ciceritol (obtained by isolation and purification from chickpea seeds) and stachyose were dissolved in distilled water. Acetonitrile was added to each solution to obtain a composition similar to that of the mobile phase. Quantification of each sugar was accomplished by comparing the peak areas of the samples with those of the standard solutions. As a commercial verbascose standard was not available, it was quantified using stachyose as the standard. A standard curve was plotted for each sugar and adjusted by least squares. The regression coefficients of the curves were always greater than 0.99.

2.4. Isolation of ciceritol from chickpea seed

The extract of chickpea seeds was loaded onto a column (3 × 50 cm) of charcoal–Celite (1:1, w/w) and successively eluted with water and increasing concentrations of ethanol (5–30%). The fractions were screened by reaction with naphthoresorcinol and those with sugars were further detected by HPLC. The fractions containing ciceritol were combined, concentrated *in vacuo*, and purified by gel permeation on a column (1.5 × 90 cm) of Biogel P-2. Degassed water was used as the eluent at a flow rate of 20 ml/h. The fractions containing pure ciceritol, analyzed by HPLC, were combined, concentrated, and freeze-dried. The structure was confirmed by ESI-TOF-MS (Applied Biosystems mass spectrometer) and NMR (Bruker DRX500) with D_2O as the solvent.

2.5. Statistical evaluation

All analyses were performed in triplicate. For all statistical evaluations of results, a general linear model (ANOVA) was used, followed by a pairwise comparison using the method of Tukey. Mean values being compared were considered to be significantly different at a *P*-value of less than 0.05. All statistical evaluation was done with the statistical software MINITAB 13 (Minitab, 2000; Ekvall, Stegmark, & Nyman, 2007).

3. Results and discussion

3.1. HPLC analysis of sugars in chickpea seeds

Fig. 1 shows the chromatographic profile of extract of chickpea seeds. Monosaccharides (xylose, fructose and glucose), disaccharides (sucrose and maltose) and oligosaccharides (raffinose and stachyose) were identified according to the retention time of the standard sugars. Three unknown compounds appeared in the chromatograms. Considering their elution orders, U1, U2 and U3 may be a disassharide, trisaccharide and pentasaccharide, individually. Due to the lack of commercial standards, U2 and U3 were speculated to be ciceritol and verbascose, respectively, by comparing their retention times with literature reports (Frias, Hedley, Price, Fenwick, & Vidal-Valverde, 1994; Ruperez, 1998). As the main component in chickpea extracts as shown in Fig. 1, U2 was isolated and purified for mass and ^{1}H-NMR analyses.

3.2. Isolation of ciceritol

Pure ciceritol was obtained from crude extract of chickpea seeds by separation on a charcoal–Celite column and a Biogel P-2 column. In the case of charcoal–Celite chromatography, almost all the monosaccharides were eluted with water. Ciceritol (possible) and sucrose were eluted with 15% ethanol. MS analysis showed that there was a molecular ion at *m/z* 541 for $[M+Na]^+$, which indicated that the molecular weight of this sugar was 518, corresponding to that of ciceritol (Fig. 2). ^{1}H-NMR spectrum gave characteristic signals at δ 3.65 ppm (OCH_3), δ 4.88 ppm (H-1") and δ 5.08 ppm (H-1′), similar to other reports for ciceritol (Bernabe et al., 1993; Quemener & Brillouet, 1983). Therefore, U2 was identified as ciceritol.

3.3. Effects of extraction conditions on oligosaccharides in chickpea seed

3.3.1. Extraction solvent

The effects of various concentrations of ethanol on extraction are shown in Fig. 3A. 50% ethanol was found to be the most effective of the various solvent systems investigated. The yield of oligosaccharides was much lower when extracted with water or 30%

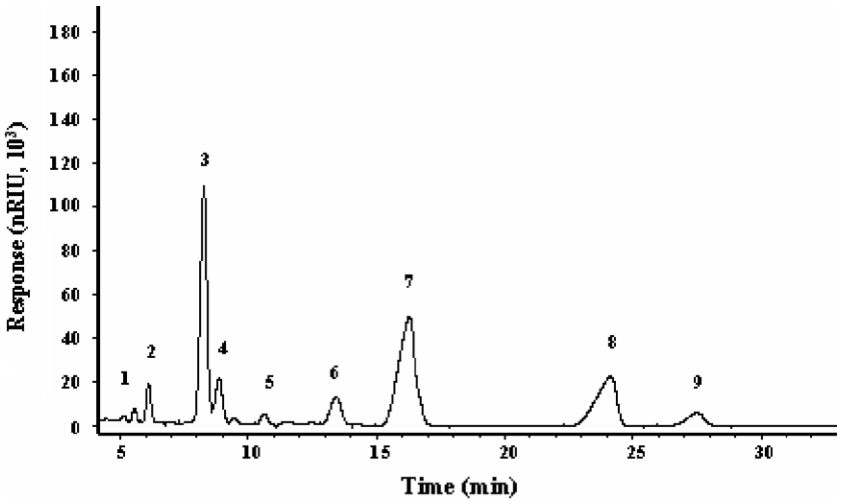

Fig. 1. Chromatographic profile of free sugars in extraction of chickpea seeds. 1, Ribose; 2, Fructose; 3, Sucrose; 4, Unknown compound; 5, Maltose; 6, Raffinose; 7, Ciceritol; 8, Stachyose; 9, Vebascose.

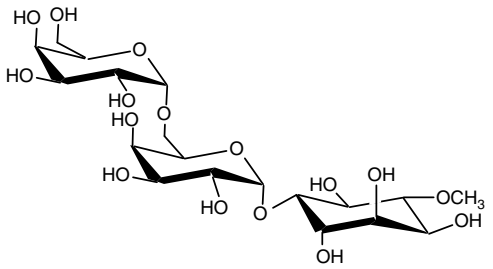

Fig. 2. The chemical structure of ciceritol.

ethanol. Water or lower concentrations of ethanol are supposed to be the optimal extraction solvent for the low molecular weight sugars. Unfortunately, they were also excellent solvents for interference between carbohydrates and hydrophilic components, such as polysaccharides and proteins. In addition, α-amylase and α-galactosidase, present in the plant material, may degrade starch and α-galactosides if not inactivated during or prior to extraction. When using solvents with concentrations of ethanol higher than 50%, the yield decreased sharply due to incomplete extraction. Meanwhile, certain proteins may be denatured by ethanol with higher concentrations, and it is possible that any precipitates formed might sterically hinder the diffusion of α-GOS into the

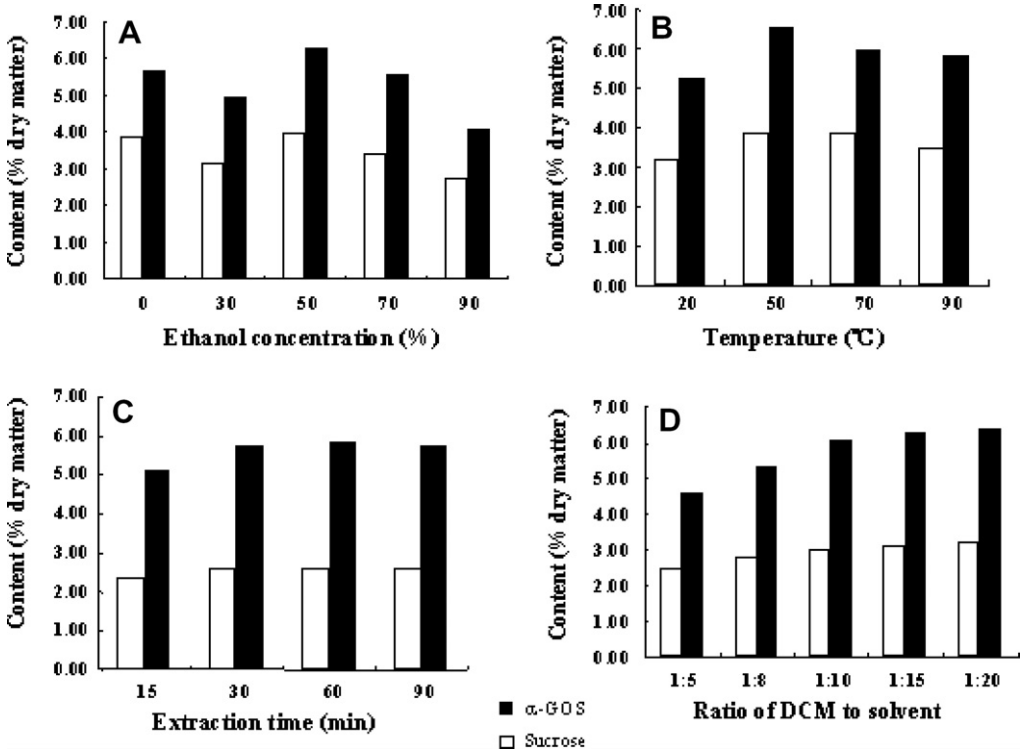

Fig. 3. The effects of extraction conditions on the contents of α-GOS and sucrose in a chickpea seeds. Concentrations of ethanol (A); temperature (B); extraction times (C); and ratio of DCM to solvent.

ethanol solutions. Based on the results of previous work (Johansen, Glitso, & Bach Knudsen, 1996), it is possible that the yield of extraction in 80% ethanol would be higher at boiling temperature. However, this process could only be carried out with great caution due to volume expansion. The effectiveness of 50% ethanol as an extraction solvent for oligosaccharides could most likely be ascribed to their higher polarity at lower concentration of ethanol; 50% ethanol was the optimal solvent for extraction.

3.3.2. Temperature

Concerning the effect of temperature on oligosaccharide extraction, it was found that the extractability of oligosaccharide increased at temperatures up to 50 °C, followed by a sudden decrease at higher temperature (Fig. 3B). At extraction temperatures of 10–50 °C, it has been reported that higher temperatures increase effectiveness. Nissreen and Mckenna (1997) also came to the same conclusion that high temperature soaking of soybean increased the hydration rate constant and decreased the soaking time necessary to achieve equilibrium. Upon increasing the temperature above 50 °C, the yield dropped. This may be due to the rapid heat-denaturation of chickpea soluble proteins, which thereafter entrapped soluble sugars and impaired their extraction (Kim, Kim, & Hwang, 2003).

3.3.3. Extraction time

The average amounts of each sugar were lower for 15 min of extraction than for 30 min or 60 min, while no difference was observed between 30 and 60 min (Fig. 3C). The results indicated that 30 min was enough for the extraction of oligosaccharides from chickpea seeds.

3.3.4. Solvent to DCM ratio

The extractability of oligosaccharides from chickpea seeds varied significantly in various solvent to DCM ratios (Fig. 3D). With increase in the volume of solvent added to the sample, more oligosaccharides were obtained. When increasing the solvent to DCM ratio above 10, there are no significant differences in the yields of oligosaccharides. Therefore, the solvent to DCM ratio of 10:1 was sufficient for complete extraction of oligosaccharide.

In conclusion, the optimal conditions for extraction of sugars from chickpea seeds were 50% ethanol–water as the extraction solvent with a 10:1 ratio of solvent to DCM at 50 °C for 30 min.

3.4. Oligosaccharides in 19 cultivars of chickpea seeds

The oligosaccharides in 19 chickpea seeds were extracted under the optimal extraction conditions and the amounts of the main sugars were determined by HPLC. The results showed that oligosaccharides in chickpea seeds were α-GOS, consisting of raffinose, ciceritol, stachyose and verbascose, and disaccharides were almost all sucrose, except for a small amount of U1 and a trace of maltose. The sucrose contents and α-GOS compositions are shown in Table 1 and Fig. 4. The total amounts of α-GOS, expressed as a percentage of dry matter, varied from 6.35% to 8.68%, and were similar to a

Table 1
The contents of α-galactooligosaccharides and sucrose in 19 cultivars of chickpea seeds[a]

Samples	Sucrose	Raffinose	Ciceritol	Stachyose	Verbascose	Total α-GOS
176	1.80 ± 0.04 l	0.57 ± 0.07^{g}	4.38 ± 0.06^{def}	2.19 ± 0.10^{hij}	0.70 ± 0.03^{b}	7.83 ± 0.04^{d}
224	4.97 ± 0.16^{c}	0.69 ± 0.05^{de}	3.08 ± 0.13^{l}	2.54 ± 0.12^{cd}	0.35 ± 0.02^{e}	6.66 ± 0.19^{gh}
185	2.61 ± 00.08^{hi}	0.91 ± 0.09^{b}	4.26 ± 0.11^{efg}	2.26 ± 0.06^{fghi}	–	7.43 ± 0.12^{ef}
221	5.22 ± 0.14^{b}	0.92 ± 0.03^{b}	3.39 ± 0.13^{k}	2.21 ± 0.14^{ghi}	–	6.52 ± 0.14^{hi}
Wushi	2.56 ± 0.03^{hi}	0.89 ± 0.02^{b}	4.24 ± 0.08^{efg}	2.38 ± 0.02^{defg}	0.42 ± 0.03^{d}	7.92 ± 0.10^{d}
193	3.94 ± 0.11^{ef}	0.74 ± 0.04^{cd}	3.43 ± 0.09^{k}	2.46 ± 0.09^{cde}	0.61 ± 0.03^{c}	7.26 ± 0.24^{f}
196	2.09 ± 0.10^{k}	0.46 ± 0.04^{h}	4.00 ± 0.14^{ghi}	1.96 ± 0.11^{k}	0.27 ± 0.02^{f}	6.68 ± 0.13^{gh}
2	2.15 ± 0.03^{k}	0.62 ± 0.02^{efg}	4.10 ± 0.18^{gh}	1.64 ± 0.10^{l}	–	6.35 ± 0.11^{i}
218	2.83 ± 0.12^{g}	0.61 ± 0.03^{fg}	4.20 ± 0.19^{fg}	2.57 ± 0.09^{c}	–	7.39 ± 0.24^{ef}
177	2.65 ± 0.10^{gh}	0.62 ± 0.02^{efg}	4.61 ± 0.19^{cd}	2.16 ± 0.06^{hij}	–	7.40 ± 0.24^{ef}
169	3.81 ± 0.15^{f}	0.81 ± 0.05^{c}	3.04 ± 0.10^{l}	3.06 ± 0.12^{b}	–	6.91 ± 0.14^{g}
166	4.09 ± 0.16^{de}	0.56±0.02^{g}	3.90 ± 0.17^{hij}	2.03 ± 0.05^{jk}	–	6.49 ± 0.16^{hi}
Xu	4.13 ± 0.12^{d}	0.62 ± 0.02^{efg}	3.80 ± 0.16^{ij}	2.42 ± 0.10^{cdef}	–	6.84 ± 0.24^{g}
216	2.69 ± 0.08^{gh}	0.65 ± 0.05^{ef}	4.37 ± 0.25^{def}	2.21 ± 0.12^{ghi}	–	7.24 ± 0.18^{f}
171	2.36 ± 0.05^{j}	0.78 ± 0.03^{c}	4.81 ± 0.16^{c}	3.09 ± 0.11^{b}	–	8.68 ± 0.16^{b}
209	4.07 ± 0.17^{de}	0.65 ± 0.02^{ef}	3.85 ± 0.11^{hij}	2.33 ± 0.09^{efgh}	0.68 ± 0.03^{b}	7.51 ± 0.22^{ef}
178	2.45 ± 0.07^{ij}	0.68 ± 0.04^{def}	4.50 ± 0.10^{de}	2.11 ± 0.06^{ijk}	0.36 ± 0.02^{e}	7.65 ± 0.09^{de}
174	2.71 ± 0.11^{gh}	0.79 ± 0.04^{c}	5.06 ± 0.22^{b}	2.44 ± 0.10^{cde}	–	8.30 ± 0.10^{c}
Mei 1	3.95 ± 0.03^{def}	0.58 ± 0.02^{g}	3.71 ± 0.11^{j}	2.56 ± 0.05^{c}	–	6.85 ± 0.06^{g}

[a] Data are expressed as% (w/w) dry matter (mean ± SD).
[b–l] Different superscripts in the same column denote a statistically significant difference ($P \leqslant 0.05$) for each chickpea variety.

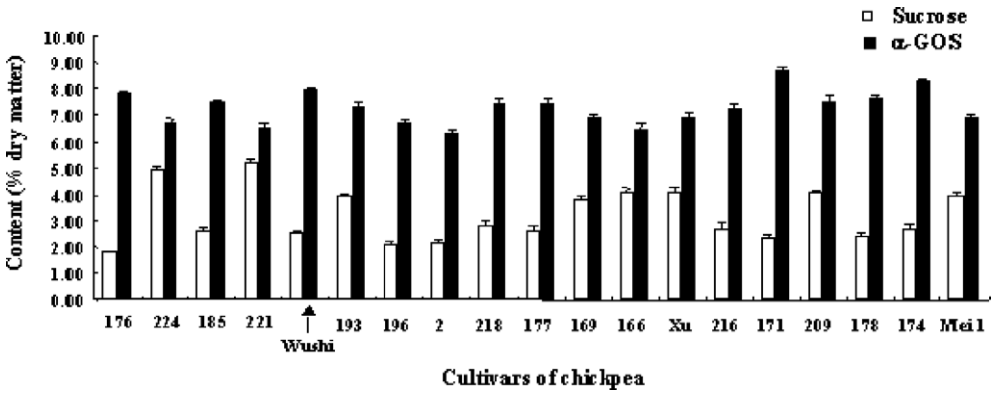

Fig. 4. The contents of α-GOS and sucrose in 19 cultivars of chickpea seeds.

previous report (Harpal & Edward, 1984). Ciceritol, the main glycoside in all the chickpea samples, accounted for about 50% of the total α-GOS, while stachyose accounted about 35%.

Discriminant analysis was applied in order to differentiate chickpea samples with different amounts of sugars. The results showed that there were considerable variations in the levels of sucrose between cultivars, which ranged from 1.80 to 5.22%. Substantial differences in α-GOS contents were also detected, but verbascose could only be detected in 7 samples by HPLC. The contents of raffinose, ciceritol, stachyose and verbascose were within the range of 0.46–0.92%, 3.00–5.10%, 1.60–3.10% and 0.27–0.70%, respectively. The considerable variation in compositions between different cultivars may be attributed to the genotypes and environmental factors. Trugo, Almeida, and Gross (1988) reported that sucrose and verbascose contents were genetically influenced whereas raffinose and stachyose were dependent on environment. A shorter vegetation period promotes a significant increase in stachyose and verbascose, and a decrease in the sucrose content. Furthermore, Gorecki, Brenac, Clapham, Willcott, and Obedorf (1996) described the effect of maturation temperature on the composition of soluble carbohydrates in yellow lupin seeds. In these species, seed matured at 18 °C had more than twice the amount of stachyose and verbascose than had seeds matured at 25 °C. However, similar information is not available for chickpea.

4. Conclusions

This work demonstrates the effects of extraction solvent, temperature, time and solvent to DCM ratio on extractability of oligosaccharides from chickpea seeds. The optimum conditions to extract oligosaccharides from chickpea seeds, based on the results from this study, were 50% ethanol-water as the solvent and a ratio of 10:1 of solvent to DCM at 50 °C for 30 min. Under the optimal extraction conditions, oligosaccharides in 19 cultivars of chickpea seeds were extracted and the contents of each sugar were analyzed by HPLC. The cultivar, 171, was characterized by the highest α-GOS content (8.68%) and low sucrose content (2.36%), which would be favourable for further preparation of high-purity α-GOS free of sucrose. Therefore, 171 can be a good source for obtaining pure α-GOS for use as a functional food ingredient, which can contribute to human health.

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