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Low Molecular Weight Carbohydrates in Pine Nuts from *Pinus pinea* L.

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ABSTRACT: Low molecular weight carbohydrates in pine nuts from *Pinus pinea* L. (n = 7) have been studied by gas chromatography−mass spectrometry as their trimethylsilyl oximes. Besides previously reported components, such as glucose, fructose, sucrose, and raffinose, several soluble carbohydrates have been identified for the first time in this product, including saccharides (galactose, maltose, and planteose) and cyclitols (pinitol, galactinol, galactopinitol A1, fagopyritol B1, and other glycosyl-inositols). Most abundant cyclitols were *chiro*-inositol, fagopyritol B1, and pinitol, with concentrations ranging from 126.7 to 222.1 mg (100 g)$^{-1}$, 94.2 to 177.1 mg (100 g)$^{-1}$, and 51.2 to 282.8 mg (100 g)$^{-1}$, respectively.

KEYWORDS: *soluble carbohydrates, cyclitols, fagopyritol, pine nut, raffinose, bioactive carbohydrates*

INTRODUCTION

The edible seeds of *Pinus pinea* L. (pine nuts, pine kernels) are traditionally consumed in the countries of the Mediterranean basin, Spain being the main producer (about a 45% of world production). Besides their organoleptic properties, pine nuts present a high nutritional value.

Several compositional studies about pine nuts have been carried out,[1,2] including determination of phenolic acids,[3] phytic acid,[4] minerals,[5] lipids,[6] and antioxidants.[7] These studies have shown that pine kernels possess several interesting nutritional properties.

On the contrary, soluble carbohydrates of pine nuts have been scarcely studied. Although ionic chromatography allowed the identification and determination of glucose, fructose, sucrose, raffinose, and stachyose,[8] these carbohydrates are usually determined as "total soluble sugars".[1,2] Moreover, scarce evidence about the presence of cyclitols in pine nuts has been reported. Cyclitols, such as *chiro*-inositol and pinitol (D-3-*O*-methyl-*chiro*-inositol), are considered bioactive carbohydrates, because they have been shown to exert an acute and sustained antihyperglycemic effect in a diabetic mouse model, where the effect may be an insulin-like effect on glucose transport that is independent of insulin.[9] To the best of our knowledge, only Kim et al.[10] have reported the content of *chiro*-inositol in a sample of pine nuts (without indication of the botanical species); in that work, all the potential derivatives of *chiro*-inositol present in the sample were converted into free *chiro*-inositol and determined by HPLC.

In this work the analysis of free soluble sugars in pine kernels has been carried out, paying special attention to the determination of bioactive cyclitols, most of them identified for the first time in this product.

MATERIALS AND METHODS

Standards. Galactose, glucose, glucose 6-phosphate, fructose, sucrose, maltose, raffinose, planteose, pinitol, phenyl β-D-glucoside, *myo*-inositol, *chiro*-inositol, and galactinol were acquired from Sigma Chemical Co. (St. Louis, MO). Planteose was kindly given by Dr. G. L. Côté (USDA, Peoria, USA).

Samples. Pine nuts from pines in Tres Cantos (Madrid) were manually collected by Dr. R. Morales (Real Jardín Botánico, CSIC, Madrid, Spain). Nuts-in-shell were removed from the cones and cracked; kernels were separated from external shell and inner seed coat.

Six commercial samples of peeled pine nuts, five of them of Spanish origin and one imported from Italy, and samples of buckwheat (*Fagopyrum esculentum*), soybeans (*Glycine maxima*), and chickpeas (*Cicer arietinum*) were purchased at local markets. Samples were stored properly in dry conditions, protected from direct sunlight until their analysis. All samples were analyzed before their shelf life date.

Extraction. Pine nuts (0.5 g) were crushed in a mortar and extracted with 5 mL of milli-Q water at controlled temperature (60 °C) during 2 h (first cycle) in crystal vessels. The solid residue was removed by centrifugation at 4400g at 10 °C, and the process was repeated twice (second and third cycles) under the same conditions to achieve an exhaustive extraction. A clear solution was obtained from each cycle, which was then independently derivatized.

GC−MS analysis. Trimethylsilyl oximes (TMSO) were prepared as previously described.[11] In brief, 0.5 mL of phenyl β-D-glucoside (1 mg mL^{-1}) was added to 1 mL of extract, and the solution was evaporated under vacuum. Then, samples were treated with 350 μL of 2.5% hydroxylamine chloride in pyridine (30 min at 75 °C), and 350 μL of hexamethyldisilazane plus 35 μL of trifluoroacetic acid (45 °C for 30 min). After centrifugation, 1 μL of supernatant was taken for injection.

Gas chromatography−mass spectrometry (GC−MS) analyses were carried out on a 7890 gas chromatograph coupled to a 5975 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using helium as the carrier gas. A 30 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column coated with TRB-1 (cross-linked methyl silicone) from Teknokroma (Barcelona, Spain) was used. The oven temperature was held at 200 °C for 15 min, then programmed to 270 at 15 °C min^{-1}, then programmed to 290 at 1 °C min^{-1}, and finally programmed to 300 °C at 15 °C min^{-1}. The final temperature was held for 30 min. The injector was at 300 °C, and injections were made in split mode with a split ratio of 1:20. The mass spectrometer was operated in electronic impact (EI) mode at 70 eV, scanning the 50−

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650 m/z range. The interface and source temperature were 280 and 230 °C, respectively. Acquisition was done using HP-ChemStation software (Hewlett-Packard, Palo Alto, CA, USA).

When commercial standards were not available, identification of low molecular weight carbohydrates (LMWC) has been carried out using linear retention indices (I^T), relative abundances of characteristic m/z fragments, and bibliographic data. I^T of each TMSO carbohydrate (i) was calculated as:

$$I^T = 100\left[\frac{t_{Ri} - t_{Rz}}{t_{R(z+1)} - t_{Rz}} + z\right]$$

where t_R was the total retention time measured at programmed temperature, z the number of carbon atoms of the n-alkane eluted before the target peak, and $(z + 1)$ the number of carbon atoms of the n-alkane eluted after the target peak, according to van den Dool and Kratz.[12] n-Alkanes from C_8 to C_{40} were considered for this study. Retention data and mass spectra for glycosyl cyclitols whose standards were not commercially available were obtained from those present in buckwheat, soybeans, and chickpeas.

Response factors (RF) relative to the internal standard were calculated over the expected concentration range. Due to the lack of commercial standards, the RF of galactinol was used for all glycosyl cyclitols. All analyses were carried out in duplicate. Precision was calculated from the results obtained for a standard mixture (glucose, fructose, *myo*-inositol, and sucrose), and a pine nut sample which was extracted, derivatized, and analyzed by quintuplicate. In all cases, relative standard deviations were lower than 10%.

RESULTS AND DISCUSSION

Figure 1 shows the GC profile of a pine nut sample. Several components with mass spectra characteristic of carbohydrates were detected, including 3 monosaccharides, 2 disaccharides, 2 trisaccharides, 3 free inositols, 6 glycosyl-inositols, and a sugar phosphate. Identification was carried out by matching retention time and mass spectral data with those of available standards. Besides the four saccharides (namely, glucose, fructose, sucrose, and raffinose) previously reported,[8] galactose, maltose, and planteose were also identified in the studied samples. Maltotriose was found as traces in several samples.

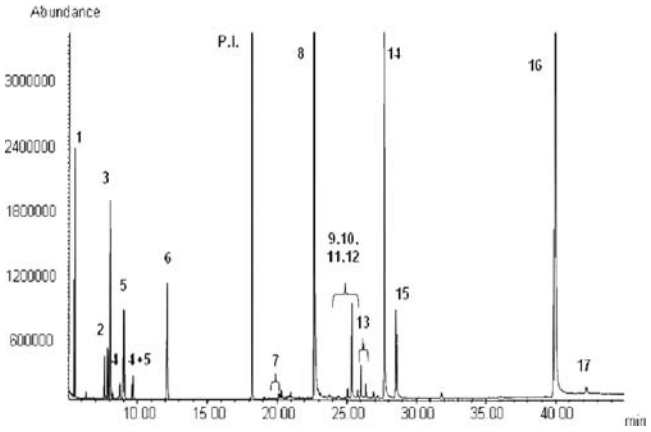

Figure 1. GC profile of low molecular weight carbohydrates in a pine nut sample as their TMSO: 1, pinitol; 2, fructose; 3, *chiro*-inositol; 4, galactose; 5, glucose; 6, *myo*-inositol; 7, glucose 6-phosphate; 8, sucrose; 9, 11, 12, unidentified glycosyl-inositols; 10, galactopinitol A1; 13, maltose; 14, fagopyritol B1; 15, galactinol; 16, raffinose; 17, planteose; PI, phenyl β-D-glucoside (internal standard).

To assess an exhaustive recovery of LMWC three different cycles of extraction were carried out. Percentages of extraction ranged 85−100% for the first cycle; 0−15% for the second cycle, and 0−1.5% for the third cycle. Table 1 shows the minimum, maximum, and average values of LMWC, expressed as mg (100 g)$^{-1}$, of the analyzed pine nut extracted within three cycles. Quantitative differences in LMWC composition found in these samples could not be attributed to a specific cause. Moreover, no real differences in the composition were observed between commercial and collected pine nut samples. I^T values have also been summarized in Table 1.

Table 1. Retention Indices (I^T) and Concentration (mg (100 g)$^{-1}$) of Low Molecular Weight Carbohydrates (as TMSO) in the Analyzed Samples of Pine Nuts (n = 7)

peak	compound	I^T	range	av[a]	SD[b]
1	pinitol	1896	51.2−282.8	146.9	92.2
2	fructose	2013, 2021	42.4−162.7	112.6	40.8
3	*chiro*-inositol	2029	126.7−222.1	176.3	39.5
4	galactose	2053, 2079	8.9−42.1	24.3	10.3
5	glucose	2061, 2079	54.9−172.7	112.9	37.1
6	*myo*-inositol	2139	45.8−89.0	61.5	15.2
7	glucose-6-phosphate	2471, 2493	traces[c]	traces	
8	sucrose	2736	1242.1−2146.0	1587.2	348.8
9	glycosyl-inositol	2882	traces−5.5	2.6	2.2
10	galactopinitol A1	2904	traces−2.1	0.9	0.7
11	glycosyl-inositol	2935	1.6−5.9	3.2	1.5
12	glycosyl-inositol	2954	19.3−39.4	27.6	6.9
13	maltose	2985, 2990	1.6−72.3	147.5	30.9
14	fagopyritol B1	3072	94.2−177.1	24.1	17.4
15	galactinol	3100	4.1−49.3	26.0	28.3
16	raffinose	3380	397.0−1067.7	815.1	216.0
17	planteose	3420	5.9−16.8	11.7	3.4

[a]av = average. [b]SD = standard deviation. [c]Limit of detection: 0.14 mg (100 g)$^{-1}$. Limit of quantitation: 0.46 mg (100 g)$^{-1}$.

Sucrose was the main sugar in all examined samples, as reported by Ruggeri et al.,[8] with amounts in the 1.2−2.1 g (100 g)$^{-1}$ range. The second most abundant sugar was raffinose, which averaged 815 mg (100 g)$^{-1}$. Monosaccharides varied in a broad concentration range, but glucose and fructose always showed similar concentrations, around 100 mg (100 g)$^{-1}$. Galactose, maltose, and planteose appeared at lower amounts.

Traces of several sugar phosphates were found in all samples, but only glucose 6-phosphate could be positively identified.

Chromatograms also displayed several cyclitols and derivatives: the sum of free inositols (pinitol, *chiro*-inositol, and *myo*-inositol) averaged 385 mg (100 g)$^{-1}$, *chiro*-inositol being, in general, the most abundant of them (127−222 mg (100 g)$^{-1}$). Up to six glycosylated cyclitols were detected. Among them, the most abundant peak was identified as fagopyritol B1 by comparison of its I^T (3072) and mass spectrum with that present in an extract of buckwheat (*Fagopyrum esculentum*).[13] The abundance ratios of m/z 305/318 and 318/319 fragments were very similar to those described by Obendorf et al.[14] as characteristics for this compound. Fagopyritol B1 ranged from 94 to 177 mg (100 g)$^{-1}$ in pine nut samples. However, fagopyritol A1 was not detected. Galactinol was identified by matching with the standard. A small peak with I^T of 2904 showed the characteristic spectrum of a galactopinitol. Its retention index and mass spectrum were identical to those of galactopinitol A1 present in soybeans and in chickpeas, as described by Schweizer et al.[15]

Three compounds marked as 9, 11, and 12 in Figure 1 showed mass spectra compatible with glycosyl-inositols, probably galactosides. They could not be identified since mass spectra of glycosyl-inositols are rather similar and, besides the lack of standards, the number of published GC and MS data for this type of compounds is very limited. Peaks 9 and 11 appeared in almost all samples at low levels (around 3 mg (100 g)$^{-1}$ each), whereas peak 12 was slightly more abundant (19–39 mg (100 g)$^{-1}$).

From the presented results, it can be concluded that pine kernels from *P. pinea* have a high content of bioactive carbohydrates. The amount of total *chiro*-inositol (sum of free compound and derivatives) found in the examined samples (as deduced from Table 1) is clearly higher than that reported by Kim et al.[10](1.74 g kg^{-1}). Moreover, the concentration of fagopyritol B1 in pine nuts is similar to that found in several buckwheat flours.[13] Inositols have been shown to have favorable properties in human health[10,16,17] and to present substantial beneficial effects for the treatment of certain diseases. *chiro*-Inositol, pinitol, and glycosyl-inositols appear to exert an insulin-like effect,[9] showing positive activity for type II diabetics and patients with polycystic ovary syndrome.[15] Moreover, α-galactoside sugars, such as raffinose, are currently considered as prebiotics, i.e. able to modify the composition of the colon microflora.[18]

In conclusion, several LMWC have been found in pine nuts from *P. pinea* for the first time. Noticeable amounts of *chiro*-inositol and several glycosyl cyclitols (especially *chiro*-inositol derivatives) with relevant bioactive properties have been detected. These results point out that probably pine kernels should be considered a healthy food component, not only for their composition in phenolic compounds and unsaturated lipids but also for their low molecular weight carbohydrate content.

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Notes

The authors declare no competing financial interest.

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