JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

New Methodologies for the Extraction and Fractionation of Bioactive Carbohydrates from Mulberry (*Morus alba*) Leaves

Sonia Rodríguez-Sánchez, Laura Ruiz-Aceituno, María L. Sanz,* and Ana C. Soria

Instituto de Química Orgánica General (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Supporting Information

ABSTRACT: Pressurized liquid extraction (PLE) was applied for the first time to extract bioactive low molecular weight carbohydrates (iminosugars and inositols) from mulberry (*Morus alba*) leaves. Under optimized conditions, PLE provided a similar yield to the conventional process used to extract these bioactives, but in less time (5 vs 90 min). To remove carbohydrates that interfere with the bioactivity of iminosugars from PLE extracts, two fractionation treatments were evaluated: yeast (*Saccharomyces cerevisiae*) incubation and cation-exchange chromatography (CEC). Both methods allowed complete removal of major soluble carbohydrates (fructose, glucose, galactose, and sucrose), without affecting the content of mulberry bioactives. As an advantage over CEC, the yeast treatment preserves bioactive inositols, and it is an affordable methodology that employs food grade solvents. This work found PLE followed by yeast treatment to be an easily scalable and automatable procedure that can be implemented in the food industry.

KEYWORDS: *mulberry (Morus alba), bioactives, iminosugars, inositols, pressurized liquid extraction (PLE), yeast, Saccharomyces cerevisiae, cation-exchange chromatography (CEC), GC-MS*

INTRODUCTION

The increasing trend of using functional foods or supplements containing bioactive compounds from natural sources has promoted the search for strategies to obtain plant extracts enriched with these compounds. Among bioactive low molecular weight carbohydrates (LMWC), it is worth noting iminosugars (also called polyhydroxyalkaloids), which have α-glucosidase inhibition activity, as they contribute to the regulation of body weight and the prevention of obesity,[1,2] and inositols, which have been described as useful for treating conditions associated with insulin resistance, among others.[3−5]

Over a hundred polyhydroxyalkaloids have been isolated from plants (mainly from Leguminosae, Hyacinthaceae, Solanaceae, Convulaceae, and Moraceae families) and from microorganisms (*Streptomyces* sp.).[6] Among them, the *Morus* genus draws special attention because different alkaloids, such as 1-deoxynojirimycin (DNJ, Figure 1), *N*-methyl-DNJ, 2-*O*-D-galactopyranosyl-DNJ, and fagomine (Figure 1), have been found in their leaves, roots, branches, and/or fruits. The amount and type of these polyhydroxyalkaloids vary depending on the species, the harvesting time, plant part, leaf size, etc.[7] In general, DNJ and fagomine are the most abundant (0.1−0.21% dry weight), and the highest glucosidase inhibitory activity has been attributed to these two compounds.[7−9] Different food grade products based on mulberry extracts are commercialized in Asia, and mulberry leaf infusions are consumed by diabetes mellitus patients due to their antihyperglycemic properties, which have been mainly attributed to their high polyhydroxyalkaloid content.[7,8]

With regard to bioactive inositols, few studies have addressed their content in mulberry extracts. Rodríguez-Sánchez et al.[9] reported the presence of *myo*-inositol and glycosyl-inositols (Figure 1) in the ranges of 0.75−12.34 and 0.01−3.37 mg/g, respectively, in leaves, branches, and fruits of different *Morus* species.

Pressurized liquid extraction (PLE) has recently emerged as an alternative to conventional solvent extraction techniques, providing faster extractions, lower solvent consumption, and higher selectivity. PLE also helps preserve labile bioactives by maintaining the sample in an oxygen- and light-free environment during extraction. In this respect, there are several studies on the application of PLE to obtain antioxidants from algae and microalgae,[10,11] pomace,[12] and birch bark.[13] With regard to carbohydrates, PLE has been mainly used for the fractionation of honey sugars to obtain fractions enriched in di- and trisaccharides,[14] to purify lactulose from mixtures with lactose,[15] and to extract monosaccharides and polyalcohols from woods used in wine aging.[16] However, no study has reported the application of this technique to extract iminosugars or any other mulberry bioactives.

The low amount of bioactives (iminosugars and inositols) present in *Morus* extracts, along with the unavoidable coextraction of other mulberry carbohydrates (glucose, fructose, sucrose, etc.) that can interfere with the bioactivity of mulberry extracts, makes the removal of these soluble sugars a requirement. Among the different procedures described in the literature for iminosugar fractionation, ion-exchange chromatography is one of the most commonly applied. Commercially available anionic and cationic resins with different functionalities, pore sizes, load capacities, etc., have been used; Dowex 50 and Amberlite GC120 in their NH_4^+ or H^+ forms have generally been employed for both in-column and in-batch

Received: November 27, 2012
Revised: April 2, 2013
Accepted: April 4, 2013
Published: April 4, 2013

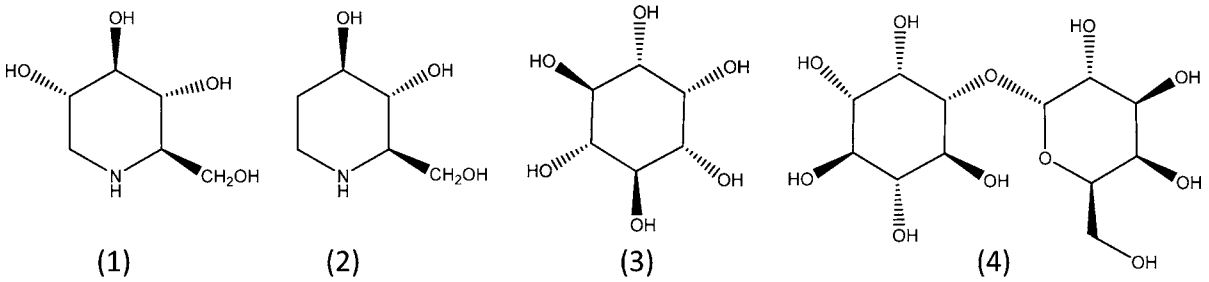

Figure 1. Structures of mulberry bioactive LMWC under study: DNJ (**1**); fagomine (**2**); *myo*-inositol (**3**); galactinol (**4**).

cation-exchange chromatography (CEC).[17−19] Furthermore, plant or food extracts may also be subjected to several consecutive treatments with different ion-exchange resins depending on the degree of enrichment or fractionation required.[20−25] With regard to inositols, papers[26,27] describe their purification using ion-exchange resins (Amberjet 4200, Dowex 50, or Dowex 1). Microbiological treatments such as yeast (*Saccharomyces bayanus* and *Saccharomyces cerevisiae*, among others) incubation have also been described to separate interfering carbohydrates (sucrose, fructose, and glucose) from cyclitols present in powdered carob[28] and legume[29] extracts. *S. cerevisiae* has also been used for the selective separation of monosaccharides and disaccharides with different glycosidic linkages.[30] The enrichment obtained by treating honey with *S. cerevisiae* has also been used for different applications, such as the determination of minor honey oligosaccharides[31] or the detection of difructose anhydrides (markers of adulteration with high fructose content syrups or invert syrups) in honey.[32]

Therefore, the aims of this work were to optimize the PLE process and compare it with the solid−liquid extraction of bioactive carbohydrates (iminosugars and inositols) from mulberry leaves and to evaluate yeast (*S. cerevisiae*) treatment as an alternative to fractionation by CEC for the selective removal of interfering soluble carbohydrates in PLE mulberry extracts.

MATERIALS AND METHODS

Samples and Standards. Samples of *Morus alba* leaves M1L and M2L were collected under botanical surveillance in Madrid (Spain) in May and August 2010, respectively. Mulberry leaves were air-dried at a controlled temperature (25 °C) and in the absence of light for 3 days.

Carbohydrate standards (fructose, glucose, sucrose, galactose, trehalose, *myo*-inositol, galactinol (*O*-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol), and phenyl-β-D-glucoside) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DNJ was purchased from Toronto Research Chemicals Inc. (Ontario, Canada).

Extraction of Low Molecular Weight Carbohydrates. *Solid−Liquid Extraction.* One gram of dried mulberry leaves (M1L) was ground in a domestic mill (Moulinex) and sieved (500 μm) before extraction at ambient temperature with 10 mL of different solvents (water, 0.1% HCl (v/v), methanol, ethanol, and mixtures of H_2O/methanol and H_2O/ethanol (50%, v/v)) for 30 min under stirring. Extracts were passed through Whatman no. 4 filters and kept at −20 °C until analysis. For recovery studies, mulberry samples were subjected to four consecutive extraction cycles with the selected solvent. The optimized conditions were further applied to the M2L sample. All extracts were prepared in triplicate.

Pressurized Liquid Extraction. Extractions were carried out in a laboratory-made miniaturized PLE system designed and patented by Ramos et al.[33] This instrument consists of an oven equipped with temperature control and regulation, in which a stainless steel extraction cell (99 mm × 4.4 mm i.d, total volume = 1.5 mL) was placed. This extraction cell was coupled to a model 1050 series isocratic pump (Hewlett-Packard, Palo Alto, CA, USA), which was used to deliver and pressurize the solvent, via a model 7000 six-port Rheodyne valve (Rheodyne L.P., Rohnert Park, CA, USA). Another valve of the same characteristics was connected to the outlet end of the extraction cell to deliver the extract to the collection vial.

Prior to loading the sample into the extraction cell, mulberry (M2L) powders were mixed with sea sand (Panreac, Barcelona, Spain) previously washed with different solvents, to hold the sample in place and to prevent the formation of preferential flow paths. Additional layers of sea sand were also placed at the bottom and at the upper part of the extraction cell to avoid the presence of dissolved gas and to compensate for the differences in sample amount when this parameter was optimized. The extraction cell was sealed with 5 μm stainless steel frits (Supelco, Bellefonte, PA, USA) at its lower and upper ends to avoid blockage of the stainless steel tubing and/or valve by suspended particles from the matrix or sea sand.

PLE was optimized for sample amount (0.05 and 0.10 g), extraction temperature (50−150 °C), time (2−30 min), and number of consecutive extraction cycles (1−2 cycles). All experiments were carried out in triplicate.

Carbohydrate Fractionation. *Yeast Treatment.* Aqueous PLE mulberry extracts (10 mL) were incubated with 0.125 g of yeast (*S. cerevisiae* type II) (Sigma Chemical Co.) at 37 °C under stirring. Aliquots were taken after 0 (just after adding the yeast), 3, 5, 8, and 12 h of treatment. Yeast removal was immediately carried out by centrifugation at 4400*g* for 10 min at 4 °C followed by filtration through Whatman no. 4 paper filters. A PLE mulberry extract sample that had not previously undergone yeast treatment was used as a control in this study. All assays were carried out in triplicate.

Cation-Exchange Chromatography (CEC). A glass column filled with a strong acid cation-exchange resin, Dowex 50 W X4 20/50 mesh, sulfonic acid in H^+ form (Fluka, Sigma Chemical Co.), was used. The resin was conditioned by washing with 3 column volumes of 1 N NaOH, followed by subsequent washes with Milli-Q water until neutral pH. Later, 3 column volumes of 1 N HCl was used and conditioning was finished after washing with Milli-Q water until pH 7.

After resin conditioning, aqueous PLE mulberry extract, previously freeze-dried and redissolved in acidic Milli-Q water (pH 4), was loaded into the column. Interfering LMWC were separated by consecutive elutions with Milli-Q water, whereas iminosugars were eluted with 2 M NH_4OH. Resin and sample amount and elution volumes were optimized to obtain extracts with the highest recovery and purity of selected iminosugars: DNJ and fagomine. All assays were done in triplicate.

Chromatographic Analysis. *Derivatization.* One milliliter of mulberry extract was mixed with 0.5 mL of internal standard (phenyl-β-D-glucoside, 1 mg/mL) and evaporated under vacuum prior to carbohydrate derivatization. Trimethylsilyl oximes (TMSO) were prepared according to the method of Sanz et al.[34] Oximes were obtained by adding 350 μL of a 2.5% solution of hydroxylamine hydrochloride in pyridine, which was then heated for 30 min at 75 °C. These derivatives were then silylated with hexamethyldisilazane (350 μL) and trifluoroacetic acid (35 μL) at 45 °C for 30 min. After reaction, samples were centrifuged at 4400*g* for 10 min, and 1 μL of supernatant was injected into the GC injection port.

Table 1. Contents (Average for $n = 3$) of Iminosugars, Inositols, and Other LMWC in Mulberry (M1L) Extracts Obtained by Using Different Solvents[a]

	mg/g dry mulberry							
solvent	fagomine	DNJ	fructose	glucose	galactose	*myo*-inositol	glycosyl-inositols	disaccharides
H_2O	0.33 (0.04) a	1.25 (0.01) b	16.29 (0.22) b	6.38 (0.04) b	0.43 (0.03) b	2.64 (0.13) b	0.32 (0.05) a	3.55 (0.10) e
0.1% HCl	0.34 (0.01) a	1.35 (0.02) a	20.23 (0.69) a	14.15 (0.12) a	0.58 (0.05) a	3.45 (0.03) a	0.28 (0.02) a	0.58 (0.37) f
50% MeOH	0.23 (0.04) b	1.26 (0.06) b	1.43 (0.04) d	1.60 (0.07) c	0.05 (0.01) c	2.33 (0.29) c	0.05 (0.01) b	23.59 (0.29) a
MeOH	0.10 (0.01) d	0.59 (0.01) d	0.81 (0.03) e	0.49 (0.03) d	0.03 (0.01) d	1.35 (0.03) e	0.01 (0.01) d	17.31 (0.40) c
50% EtOH	0.13 (0.01) c	0.80 (0.02) c	1.70 (0.08) c	1.62 (0.12) c	0.06 (0.01) c	1.84 (0.01) d	0.03 (0.01) c	22.09 (0.17) b
EtOH	tr e	tr e	0.35 (0.03) f	0.16 (0.03) e	tr e	0.11 (0.01) f	tr e	4.39 (0.35) d
	mg/g dry extract							
solvent	fagomine	DNJ	fructose	glucose	galactose	*myo*-inositol	glycosyl-inositols	disaccharides
H_2O	2.52 (0.02) b	10.97 (1.23) ab	131.94 (14.12) b	55.87 (4.70) b	3.52 (0.52) b	22.37 (0.75) b	2.77 (0.27) a	32.35 (0.71) e
0.1% HCl	2.83 (0.09) a	11.34 (0.34) a	152.80 (5.74) a	107.09 (10.87) a	4.22 (0.38) a	24.44 (1.05) a	2.17 (0.08) b	3.42 (0.39) f
50% MeOH	2.16 (0.19) c	10.28 (0.36) b	13.19 (1.00) d	12.51 (1.14) c	0.60 (0.07) c	22.24 (0.86) b	0.43 (0.03) c	284.04 (16.67) a
MeOH	1.53 (0.19) d	9.65 (0.15) c	11.70 (1.56) e	8.73 (0.33) d	0.39 (0.02) d	18.11 (0.37) d	0.23 (0.02) d	176.98 (10.49) d
50% EtOH	1.28 (0.19) e	7.53 (1.53) d	16.17 (0.45) c	16.18 (1.58) e	0.58 (0.08) c	18.76 (0.54) c	0.25 (0.03) d	221.67 (1.81) c
EtOH	tr f	tr e	9.29 (0.45) f	7.77 (0.44) f	tr e	6.66 (0.24) e	tr e	262.96 (8.10) b

[a]Standard deviation is given in parentheses. Entries followed by the same letter (a–f) in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level. tr, traces.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. GC-MS analyses ($n = 3$ replicates) were carried out following the validated method of Rodríguez-Sánchez et al.,[9] using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA). Analyses were carried out on a 30 m × 0.25 mm i.d., 0.25 μm df, methylpolysiloxane TBR-1 capillary column (Teknokroma, Barcelona, Spain), using helium at ∼1 mL/min as carrier gas. The oven temperature was programmed from 100 to 200 °C (15 min) at a heating rate of 15 °C/min and finally programmed to 300 °C (15 min) at 15 °C/min. Injections were carried out in split mode (1:20) at 240 °C. The transfer line and ionization source were thermostated at 280 and 230 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV within the mass range m/z 35–650. Acquisition was done using HPChem Station software (Agilent Technologies).

Qualitative analysis was based on the comparison of the obtained spectra with those of the Wiley mass spectral library[35] and with published data and was confirmed, when possible, by using linear retention indices (I^T). Available standard compounds were also used for further confirmation.

Quantitative data were obtained by the internal standard method. Standard solutions were prepared over the expected concentration range in mulberry extracts to calculate the response factor relative to phenyl-β-D-glucoside: fructose and glucose (0.1–4.0 mg/mL); sucrose (0.02–2.00 mg/mL); galactose, trehalose, *myo*-inositol (0.05–1.00 mg/mL); galactinol (0.01–1.00 mg/mL); and DNJ (0.05–0.50 mg/mL). For those that had no commercially available standards, such as fagomine and galactinol isomers, response factors were assumed to be those of DNJ and galactinol, respectively. Results were expressed in milligrams per gram of dry mulberry leaves and in milligrams per gram of dry extract. Dry matter content of extracts was gravimetrically determined at 102 °C until constant weight.

Statistical Analysis. Data were subjected to analysis of variance (simple linear model, ANOVA) by applying Fischer's least significant difference (LSD) test. The software used was Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA) for Windows. The significance of differences was defined as $P < 0.05$.

RESULTS AND DISCUSION

Extraction of Bioactives. *Solid–Liquid Extraction.* The first step in optimizing conventional extraction was to carry out a comparative study using different solvents. Table 1 lists the content of iminosugars, inositols, and other LMWC determined in mulberry (M1L) extracts after one cycle of extraction (30 min) using water, 0.1% HCl, methanol, ethanol, 50% methanol, and 50% ethanol as solvents. On the basis of previously described interference of several coextracted LMWC on the bioactivity of mulberry extracts, optimal conditions were chosen as a trade-off between a high extraction yield of iminosugars and inositols (data in mg/g of dry mulberry leaves) and a low content of interfering compounds (mono- and disaccharides) (data in mg/g dry extract).

With regard to the efficiency of extraction, the highest contents of DNJ and *myo*-inositol (1.35 and 3.45 mg/g dry mulberry) were extracted by using acidified water (0.1% HCl). There were no significant differences between the amounts of fagomine and glycosyl-inositols extracted by using water or 0.1% HCl; the contents of both compounds extracted by the remaining solvents were lower. Whereas significantly higher amounts of monosaccharides (fructose, glucose, and galactose) were extracted with 0.1% HCl followed by water, the highest dissacharide content was extracted by using hydroalcoholic mixtures and methanol (17.31–23.59 mg/g dry mulberry).

Water, acidified water, and the water–methanol mixture provided extracts with a similar composition in DNJ (10–11 mg/g dry extract). Whereas acidic water showed significantly higher contents of fagomine and *myo*-inositol, water extracted the highest amount of glycosyl-inositols (2.77 mg/g dry extract). Interfering carbohydrates, fructose and glucose, were also higher for 0.1% HCl. As expected, the amount of disaccharides was the highest when extracted by hydroalcoholic mixtures and alcohols.

On the basis of the results listed in Table 1, both water and 0.1% HCl were the best solvents for extraction of iminosugars and inositols. However, as the total amount of interfering sugars was higher for 0.1% HCl, water was selected as the optimal solvent for further studies.

Data obtained in four consecutive extraction cycles of 30 min were also evaluated to optimize PLE of target compounds. As expected, percentages relative to the total amount of selected bioactives extracted noticeably decreased with the cycle number (64, 22, 12, and 2% on average). The amount extracted in the fourth cycle was considered to be negligible, and thus the combination of extracts 1–3 was chosen for further studies.

Table 2. Content of Iminosugars and Inositols in Extracts of Mulberry (M2L) Leaves Obtained by Solid−Liquid Extraction and by PLE[a]

	mg/g dry mulberry			
extraction procedure	fagomine	DNJ	*myo*-inositol	glycosyl-inositols
solid−liquid extraction (three cycles)	0.47 (0.01) a	2.44 (0.04) a	7.88 (0.15) a	0.30 (0.07) a
PLE (one cycle)	0.48 (0.15) a	2.44 (0.50) a	8.90 (1.88) a	0.35 (0.06) a

[a]Standard deviation is given in parenentheses. Entries followed by the same letter (a) in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

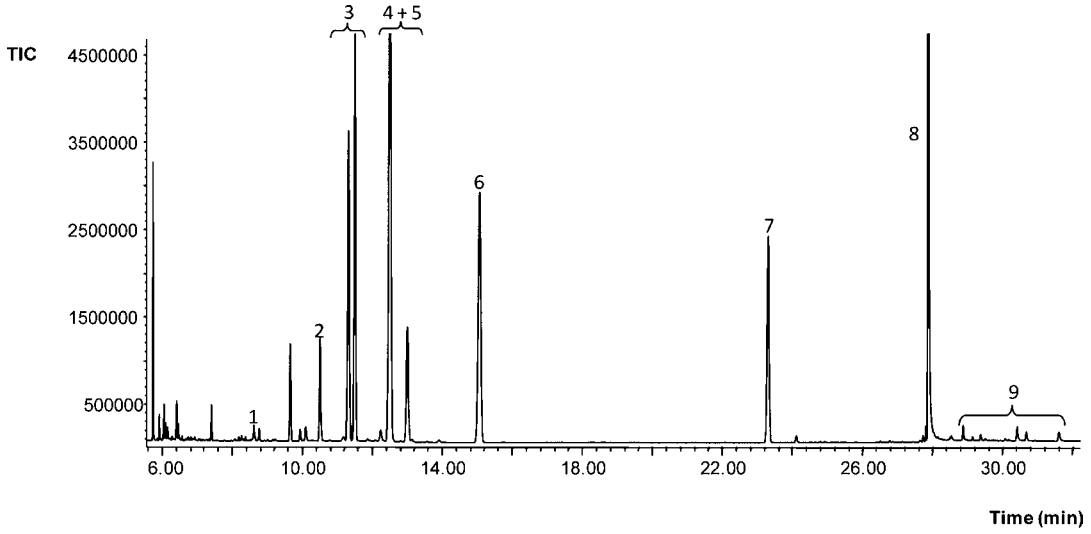

Figure 2. Chromatographic profile of a PLE extract of *Morus alba* (M2L) under optimized conditions. Peaks: 1, fagomine; 2, DNJ; 3, fructose, 4, glucose; 5, galactose, 6, *myo*-inositol; 7, phenyl-β-D-glucoside (I.S.); 8, sucrose; 9, glycosyl-inositols.

Pressurized Liquid Extraction. PLE temperature, time, and sample amount were optimized for extraction of mulberry bioactives. For comparative purposes, and according to the results previously described for conventional extraction, water was selected as the optimal solvent for PLE.

PLE of 0.1 g of mulberry leaves (M2L) was assayed at different temperatures (50, 100, and 150 °C) for 2 min. According to the ANOVA test (Fischer's LSD) for comparison of mean values, no significant differences were found for the amount of iminosugars extracted or their preferential extraction at the different extraction temperatures assayed. However, 50 °C was chosen as optimal because it yielded similar or even higher recoveries of *myo*-inositol and glycosyl-inositols compared to higher extraction temperatures.

Extraction time was not a determinant parameter when using PLE to extract iminosugars and inositols from mulberry leaves (0.1 g of sample extracted at 50 °C). As significant differences were found in the preferential extraction of DNJ and fagomine only at 5 min, this time was considered to be the most favorable.

To optimize the sample amount, 0.05 and 0.10 g of sample were considered. To keep the solvent volume within the extraction cell constant (1.3 mL), a higher amount of sea sand was used to compensate for the smaller sample amount. No significant differences were observed either in the amount extracted per sample amount (mg/g dry mulberry) or in the concentration of iminosugars in the mulberry dry extract (mg/g dry extract). However, because *myo*-inositol and glycosyl-inositol contents extracted were significantly higher for 0.05 g of mulberry leaves, this sample amount was used in subsequent experiments.

Under the selected PLE conditions, a second extraction cycle was also carried out. Because the only compounds extracted in variable percentages, ranging from 1 to 2%, were *myo*-inositol and sucrose, a single extraction cycle was chosen for PLE of mulberry bioactives.

For comparative purposes, Table 2 summarizes the amount of iminosugars/inositols extracted from mulberry leaves (M2L) by conventional solid−liquid extraction (three cycles) and by PLE (one cycle) under optimized conditions (Figure 2). For a similar sample amount/total solvent volume ratio (approximately 1:30 (w/v)), both extraction procedures gave rise to similar yields of DNJ, fagomine, *myo*-inositol, and glycosyl-inositols. However, extraction of these compounds required three cycles of 30 min in the case of conventional extraction, whereas similar results were obtained by PLE after only one cycle of 5 min. Moreover, total recovery of mulberry bioactives by PLE required a slightly lower volume of solvent (1.39 mL for 0.05 g of sample) as compared to conventional extraction (30 mL for 1 g of sample). Further advantages of PLE for obtaining extracts rich in bioactives that can be applied in the food industry include the possibility of using automated equipment that requires less sample manipulation and is easily scalable.

Fractionation Procedures. *Yeast Treatment.* The *S. cerevisiae* amount (yeast/carbohydrate ratio (w/w) 2:1) was selected by considering the previously determined carbohydrate content in mulberry extracts and in agreement with data reported in the literature.[29,31,32,36] As indicated by Ruiz-Aceituno et al.,[29] 37 °C was selected as an appropriate temperature for yeast incubations.

With regard to optimization of incubation time, Figure 3 shows the evolution over time of iminosugars and inositols

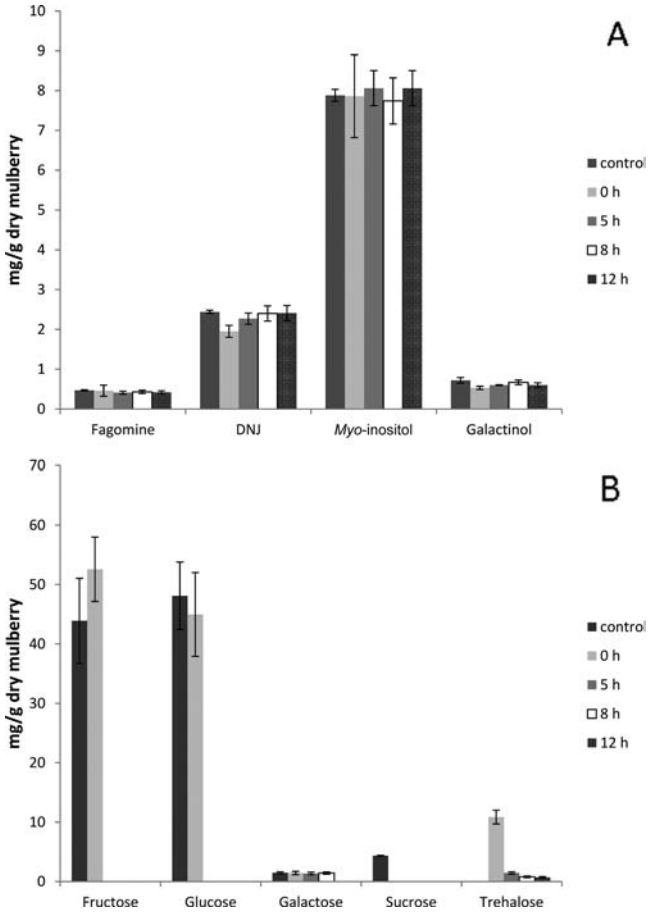

Figure 3. Evolution of iminosugar and inositol content (A) and of other LMWC (B) with time (0–12 h) of *S. cerevisiae* treatment at 37 °C of PLE mulberry leaf (M2L) extract. Data labeled as control correspond to the extract not subjected to incubation with yeasts.

(Figure 3A) and of other LMWC (glucose, fructose, galactose, sucrose, and trehalose) (Figure 3B) during fermentation with *S. cerevisiae* at 37 °C of the PLE extract from mulberry leaves (ML2). In these figures, data for control (extract not treated with yeast) and of extract taken just after the addition of yeast (0 h) are also included for comparison.

Iminosugar and inositol contents (Figure 3A) remained practically invariable irrespective of incubation time. However, noticeable differences were observed for the remaining carbohydrates (Figure 3B). Sucrose was digested at the beginning of the treatment due to the action of the extracellular invertase (*β*-fructosidase) present in yeast, as has been previously described by Ruiz-Matute et al.[15] The main action of this enzyme is to break the glycosidic linkage of sucrose into glucose and fructose; this explains the increase in fructose with respect to the control extract. A similar trend should be expected for glucose. However, this monosaccharide decreases from the beginning of the treatment. Prior studies have shown that glucose is metabolized more quickly than fructose by this yeast.[32] Although both sugars are linked to the same transporters, they present a higher affinity for glucose than fructose.[37] In addition, it has been reported that glucose can suppress the expression of several transporters specific for fructose.[38]

After 5 h of yeast treatment, nearly all glucose and fructose are removed from the extract. However, low amounts of galactose (1.47 mg/g dry mulberry) remain constant after 12 h of treatment. As a consequence of yeast metabolism, the appearance of trehalose was also observed; however, concentration of this carbohydrate decreased with digestion time. After 5 h of yeast treatment, very low levels of trehalose and galactose were detected. Galactose was completely fermented after 12 h, whereas a negligible amount of trehalose (0.79 mg/g dry mulberry) was determined at this time in mulberry extract.

These results indicate that incubating mulberry extracts with *S. cerevisiae* for 12 h at 37 °C allows the selective fractionation of interfering carbohydrates while preserving the contents of bioactive iminosugars and inositols.

Ion-Exchange Chromatography. The PLE extract of mulberry M2L was subjected to ion-exchange chromatography to remove interfering carbohydrates, according to the method of Nash et al.[24] with slight modifications. For a fixed Dowex 50 resin weight (3 g), the amount of extract to be loaded into the column and the volume of eluents (water for elution of carbohydrates and NH_4OH for iminosugars) were optimized.

Several volumes of PLE extract, corresponding to 90, 180, 300, 600, and 800 mg of mulberry leaves, were evaluated, and 600 mg was found to be the optimal amount to avoid low recoveries or resin saturation. Water volume (12–42 mL) was also evaluated for the removal of carbohydrates. Whereas complete elution of fructose, glucose, and galactose was obtained after two to three washing stages (12–18 mL), total removal of sucrose required the number of column volumes to be increased to 6 (total volume = 36 mL of H_2O).

For iminosugar elution, volumes of NH_4OH ranging from 12 to 24 mL were tested. Eighteen milliliters (3 column volumes) allowed total recovery of the DNJ and fagomine present in the control extract. The highest concentrations of these iminosugars were recovered after the second elution stage, 82 and 86% for fagomine and DNJ, respectively. In this fraction, removal of interfering carbohydrates was practically complete. However, *myo*-inositol and glycosyl-inositols were not recovered in these fractions, and they eluted at the same time as the other LMWC.

Comparison of yeast and CEC treatments (Table 3) shows that both procedures preserved the initial content of iminosugars in mulberry extract; the goal of removing interfering carbohydrates (fructose, glucose, galactose, and sucrose) was also achieved. Under optimized conditions, yeast treatment provided minimal amounts of trehalose, whereas CEC treatment gave rise to the loss of inositols and derivatives; this is a disadvantage of CEC compared to yeast treatment, as inositols also play a role in the control of diseases related to carbohydrate metabolism, such as diabetes mellitus. However, more pure iminosugars were obtained by CEC, although their elution was achieved using a nonfood grade solvent (NH_4OH). Further research should be carried out to find an alternative solvent that allows the implementation of this procedure in the food industry.

In conclusion, once temperature, time, and sample amount are optimized, PLE has proved to be an appropriate technique for the extraction of bioactive carbohydrates (inositols and iminosugars) from plant tissues (e.g., mulberry leaves). This work also demonstrates that both yeast treatment and CEC are useful procedures for removing interfering carbohydrates; the

Table 3. Content of Iminosugars, Inositols, and Other LMWC in the Mulberry (M2L) Extract Obtained by Conventional Extraction before (Control) and after Yeast/CEC Treatment[a]

sample	mg/g dry mulberry								
	fagomine	DNJ	fructose	glucose	galactose	*myo*-inositol	sucrose	trehalose	glycosyl-inositols
control	0.47 (0.01) a	2.44 (0.04) a	43.89 (7.17) a	48.10 (5.69) a	1.47 (0.16) a	7.88 (0.15) a	4.36 (0.04) a		0.32 (0.07) a
yeast (12 h)	0.41 (0.04) b	2.27 (0.14) a				8.06 (0.44) a		0.79 (0.08) a	0.30 (0.01) a
CEC (NH_4OH fraction)	0.48 (0.03) a	2.36 (0.19) a					tr b		
	mg/g dry extract								
sample	fagomine	DNJ	fructose	glucose	galactose	*myo*-inositol	sucrose	trehalose	glycosyl-inositols
control	2.55 (0.05) b	13.80 (0.60) b	279.69 (19.88) a	308.16 (35.92) a	9.40 (1.16) a	46.08 (3.91) a	28.13 (6.32) a		1.87 (0.16) a
yeast (12 h)	2.58 (0.06) b	14.28 (0.60) b				50.42 (7.35) a		5.18 (0.29) a	1.79 (0.06) a
CEC (NH_4OH fraction)	11.36 (1.17) a	53.84 (5.97) a							

[a]Standard deviation is given in parentheses. Entries followed by the same letter (a, b) in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level. tr, traces.

selection of one or the other depends on the required composition of the plant extracts.

■ ASSOCIATED CONTENT

Supporting Information

Data on optimization of temperature (Table S1), time (Table S2), and sample amount (Table S3) in the pressurized liquid extraction of iminosugars and inositols from mulberry leaves. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mlsanz@iqog.csic.es. Fax: 0034 91 564 48 53. Phone: 0034 91 258 75 59.

Funding

This work has been funded by Ministerio de Ciencia e Innovación (Project AGL2009-11909), Ministerio de Economía y Competitividad (Project CTQ2012-32957), and the Comunidad Autónoma of Madrid (Spain) and European funding from FEDER program (Project S2009/AGR-1464, ANALISYC-II). S.R.-S. and A.C.S. thank Ministerio de Economía y Competitividad of Spain for a predoctoral grant and a Ramón y Cajal contract, respectively. L.R.-A. is supported by CSIC through a JAE-Pre grant, cofinanced by the European Social Fund (ESF).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Lourdes Ramos for the use of PLE instrumentation.

■ ABBREVIATIONS USED

CEC, cation-exchange chromatography; LMWC, low molecular weight carbohydrates; PLE, pressurized liquid extraction

■ REFERENCES

(1) Asano, N. Naturally occurring iminosugars and related compounds: structure, distribution, and biological activity. *Curr. Top. Med. Chem.* **2003**, *3*, 471−484.

(2) Asano, N.; Kato, A.; Miyauchi, M.; Kizu, H.; Kameda, Y.; Watson, A. A.; Nash, R. J.; Fleet, G. W. J. Nitrogen-containing furanose and pyranose analogues from *Hyacinthus orientalis*. *J. Nat. Prod.* **1998**, *61*, 625−628.

(3) Clements, R. S.; Darnell, B. *Myo*-inositol content of common foods − development of a high-*myo*-inositol diet. *Am. J. Clin. Nutr.* **1980**, *33*, 1954−1967.

(4) Ueda, T.; Coseo, M. P.; Harrell, T. J.; Obendorf, R. L. A multifunctional galactinol synthase catalyzes the synthesis of fagopyritol A1 and fagopyritol B1 in buckwheat seed. *Plant Sci.* **2005**, *168*, 681−690.

(5) Ruiz-Aceituno, L.; Ramos, L.; Martinez-Castro, I.; Sanz, M. L. Low molecular weight carbohydrates in pine nuts from *Pinus pinea* L. *J. Agric. Food Chem.* **2012**, *60*, 4957−4959.

(6) Watson, A. A.; Fleet, G. W. J.; Asano, N.; Molyneux, R. J.; Nash, R. J. Polyhydroxylated alkaloids - natural occurrence and therapeutic applications. *Phytochemistry* **2001**, *56*, 265−295.

(7) Kim, J. W.; Kim, S. U.; Lee, H. S.; Kim, I.; Ahn, M. Y.; Ryu, K. S. Determination of 1-deoxynojirimycin in *Morus alba* L. leaves by derivatization with 9-fluorenylmethyl chloroformate followed by reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* **2003**, *1002*, 93−99.

(8) Kimura, T.; Nakagawa, K.; Kubota, H.; Kojima, Y.; Goto, Y.; Yamagishi, K.; Oita, S.; Oikawa, S.; Miyazawa, T. Food-grade mulberry powder enriched with 1-deoxynojirimycin suppresses the elevation of postprandial blood glucose in humans. *J. Agric. Food Chem.* **2007**, *55*, 5869−5874.
(9) Rodriguez-Sanchez, S.; Hernandez-Hernandez, O.; Ruiz-Matute, A. I.; Sanz, M. L. A derivatization procedure for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates by GC-MS in mulberry (*Morus* sp.). *Food Chem.* **2011**, *126*, 353−359.
(10) Herrero, M.; Cifuentes, A.; Ibanez, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae − a review. *Food Chem.* **2006**, *98*, 136−148.
(11) Plaza, M.; Herrero, M.; Cifuentes, A.; Ibanez, E. Innovative natural functional ingredients from microalgae. *J. Agric. Food Chem.* **2009**, *57*, 7159−7170.
(12) Wijngaard, H.; Brunton, N. The optimization of extraction of antioxidants from apple pomace by pressurized liquids. *J. Agric. Food Chem.* **2009**, *57*, 10625−10631.
(13) Co, M.; Koskela, P.; Eklund-Akergren, P.; Srinivas, K.; King, J. W.; Sjoberg, P. J. R.; Turner, C. Pressurized liquid extraction of betulin and antioxidants from birch bark. *Green Chem.* **2009**, *11*, 668−674.
(14) Ruiz-Matute, A. I.; Ramos, L.; Martinez-Castro, I.; Sanz, M. L. Fractionation of honey carbohydrates using pressurized liquid extraction with activated charcoal. *J. Agric. Food Chem.* **2008**, *56*, 8309−8313.
(15) Ruiz-Matute, A. I.; Sanz, M. L.; Corzo, N.; Martin-Alvarez, P. J.; Ibanez, E.; Martinez-Castro, I.; Olano, A. Purification of lactulose from mixtures with lactose using pressurized liquid extraction with ethanol-water at different temperatures. *J. Agric. Food Chem.* **2007**, *55*, 3346−3350.
(16) Alañon, M. E.; Ruiz-Matute, A. I.; Martinez-Castro, I.; Diaz-Maroto, M. C.; Perez-Coello, M. S. Optimisation of pressurised liquid extraction for the determination of monosaccharides and polyalcohols in woods used in wine aging. *J. Sci. Food Agric.* **2009**, *89*, 2558−2564.
(17) Kite, G. C.; Porter, E. A.; Egan, M. J.; Simmonds, M. S. J. Rapid detection of polyhydroxyalkaloid mono- and diglycosides in crude plant extracts by direct quadrupole ion trap mass spectrometry. *Phytochem. Anal.* **1999**, *10*, 259−263.
(18) Dräger, B. Chemistry and biology of calystegines. *Nat. Prod. Rep.* **2004**, *21*, 211−223.
(19) Asano, N.; Yamauchi, T.; Kagamifuchi, K.; Shimizu, N.; Takahashi, S.; Takatsuka, H.; Ikeda, K.; Kizu, H.; Chuakul, W.; Kettawan, A.; Okamoto, T. Iminosugar-producing Thai medicinal plants. *J. Nat. Prod.* **2005**, *68*, 1238−1242.
(20) Amezqueta, S.; Galan, E.; Fuguet, E.; Carrascal, M.; Abian, J.; Torres, J. L. Determination of D-fagomine in buckwheat and mulberry by cation exchange HPLC/ESI-Q-MS. *Anal. Bioanal. Chem.* **2012**, *402*, 1953−1960.
(21) Asano, N.; Yamashita, T.; Yasuda, K.; Ikeda, K.; Kizu, H.; Kameda, Y.; Kato, A.; Nash, R. J.; Lee, H. S.; Ryu, K. S. Polyhydroxylated alkaloids isolated from mulberry trees (*Morus alba* L.) and silkworms (*Bombyx mori* L.). *J. Agric. Food Chem.* **2001**, *49*, 4208−4213.
(22) Asano, N.; Tomioka, E.; Kizu, H.; Matsui, K. Sugars with nitrogen in the ring isolated from the leaves of *Morus bombycis*. *Carbohydr. Res.* **1994**, *253*, 235−245.
(23) Chen, T. M.; George, R. C.; Weir, J. L.; Leapheart, T. Thermospray liquid-chromatographic mass-spectrometric analysis of castanospermine-related alkaloids in *Castanospermum australe*. *J. Nat. Prod.* **1990**, *53*, 359−365.
(24) Nash, R. J.; Bell, E. A.; Fleet, G. W. J.; Jones, R. H.; Williams, J. M. The identification of a hydroxylated pyrrolidine derivative from *Castanospermum australe*. *J. Chem. Soc. D, Chem. Commum.* **1985**, 738−740.
(25) Molyneux, R. J. Isolation, characterization and analysis of polyhydroxy alkaloids. *Phytochem. Anal.* **1993**, *4*, 193−204.
(26) Saska, M.; Diack, M. Separation of inositols from sugars and sugars alcohols. U.S. Patent 5,482,631, Jan 9, 1996.
(27) Streeter, J. G. Simple partial purification of D-pinitol from soybean leaves. *Crop Sci.* **2001**, *41*, 1985−1987.
(28) Baumgartner, S.; Gennerritzmann, R.; Haas, J.; Amado, R.; Neukom, H. Isolation and identification of cyclitols in carob pods (*Ceratonia siliqua* L). *J. Agric. Food Chem.* **1986**, *34*, 827−829.
(29) Ruiz-Aceituno, L.; Rodríguez-Sánchez, S.; Soria, A. C.; Ramos, L.; Sanz, M. L. Optimisation of a biotechnological procedure for selective fractionation of bioactive inositols in edible legume extracts. *J. Sci. Food Agric.* **2013**, DOI: 10.1002/jsfa.6103.
(30) Yoon, S. H.; Mukerjea, R.; Robyt, J. F. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. *Carbohydr. Res.* **2003**, *338*, 1127−1132.
(31) Sanz, M. L.; Polemis, N.; Morales, V.; Corzo, N.; Drakoularakou, A.; Gibson, G. R.; Rastall, R. A. In vitro investigation into the potential prebiotic activity of honey oligosaccharides. *J. Agric. Food Chem.* **2005**, *53*, 2914−2921.
(32) Ruiz-Matute, A. I.; Soria, A. C.; Martínez-Castro, I.; Sanz, M. L. A new methodology based on GC-MS to detect honey adulteration with commercial syrups. *J. Agric. Food Chem.* **2007**, *55*, 7264−7269.
(33) Ramos, J. J.; Díez, C.; Cámara, C.; Ramos, L. Dispositivo miniaturizado de extracción con líquidos a presión. Universidad Complutense de Madrid-CSIC, Spain, 2006; PTC-ES 2006/000311.
(34) Sanz, M. L.; Sanz, J.; Martínez-Castro, I. Characterization of *O*-trimethylsilyl oximes of disaccharides by gas chromatography-mass spectrometry. *Chromatographia* **2002**, *56*, 617−622.
(35) *WILEY/NBS Registry of Mass Spectral Data*; Stauffe, M. F., Ed.; Wiley: New York, 1989.
(36) Goulas, A.; Tzortzis, G.; Gibson, G. R. Development of a process for the production and purification of α- and β-galactooligosaccharides from *Bifobacterium bifidum* NCIMB 41171. *Int. Dairy J.* **2007**, *17*, 648−656.
(37) D'amore, T.; Russell, I.; Stewart, G. G. Sugar utilization by yeast during fermentation. *J. Ind. Microbiol.* **1989**, *4*, 315−324.
(38) Verstrepen, K. J.; Iserentant, D.; Malcorps, P.; Derdelinckx, G.; Van Dijck, P.; Winderickx, J.; Pretorius, I. S.; Thevelein, J. M.; Delvaux, F. R. Glucose and sucrose: hazardous fast-food for industrial yeast? *Trends Biotechnol.* **2004**, *22*, 531−537.