JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification of Lactulose from Mixtures with Lactose Using Pressurized Liquid Extraction with Ethanol–Water at Different Temperatures

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The viability of the purification of lactulose from a mixture with lactose [70:30 (w/w)] using pressurized liquid extraction (PLE) at 1500 psi for 30 min was studied. Different temperatures (from 40 to 130 °C) and proportions of ethanol:water (70:30, 80:20, 90:10, 95:5, and 100:0) as the extraction solvent were assayed. Lactose and lactulose were measured by gas chromatographic analysis as their trimethylsilyl derivatives. Data were fitted through multiple linear regressions to different quadratic models to describe both the extraction yield (in terms of mg of lactulose) and the purity of the lactulose extracted. The optimum extraction conditions provided by the model were as follows: extraction temperature, 40 °C; and solvent composition, 70:30 ethanol:water. PLE extraction under the optimized conditions was also applied to purify lactulose from lactose in a synthesis mixture. To our knowledge, this is the first time that PLE has been tested for extraction and purification of lactulose from its mixture with lactose; this technique showed several advantages over classical methods such as the short extraction time and the low solvent consumption.

KEYWORDS: Lactulose; lactose; pressurized liquid extraction; fractionation; purification

INTRODUCTION

Since the bifidogenic activity of lactulose (Lu; 4-O-β-D-galactopyranosyl-D-fructose) was first reported (*1*), the use of this disaccharide as a food additive and for medical purposes has received considerable research attention (*2*−*4*). Lu is used in medicine as a syrup to treat constipation and hepatic encephalopathy, and it has also been used in the diagnosis of gastrointestinal disorders (*2*−*4*). These properties have been mainly attributed to the low absorption of Lu in the upper digestive tract and its selective metabolism by bifidobacteria in the colon.

Lu, because of its health-promoting prebiotic effects, will be increasingly used in the food industry as a functional ingredient (*5*, *6*). Prebiotics are nondigestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of a limited number of bacterial species in the colon (*7*). A large amount of literature on this topic has been published, and the use of prebiotic carbohydrates as food ingredients has become a subject of great interest (*8*, *9*).

Lu is obtained from lactose (La; 4-O-β-D-galactopyranosyl-D-glucose) by isomerization in basic media. The conversion of La to Lu mainly depends on the catalytic systems and may vary from 20 to 80% (*10*). Lu is commercially available as a syrup containing about 80% solids, which has a Lu content of 66%, including variable amounts of La and small contents of other sugars such as galactose, epilactose, tagatose, and fructose. The presence of La in this product may not be desirable when dietary restrictions of this carbohydrate are prescribed (*11*, *12*).

Although published data on the solubility of Lu in alcohols are limited, in a previous study, we reported the difference on La and Lu solubilities in alcohols at room temperature (*13*) observing that Lu solubility was remarkably higher than that of La.

Several new extraction procedures, such as supercritical fluid extraction (SFE), solid-phase extraction (SPE), and pressurized liquid extraction (PLE), have been developed in the past few years to replace traditional processes. SFE has been recently used to separate Lu from La using different mixtures of ethanol:water as cosolvents (*14*) providing Lu purities higher than 95% with extraction yields around 45%. PLE (*15*) is based on conventional heating at elevated temperatures and at pressures up to 200 bar, enough to keep the solvent in liquid state. Therefore, dynamic extraction is run in a very short time with small volumes of organic solvent.

In this work, new experimental data have been obtained for extraction and selective recovery of Lu from mixtures with La using PLE. The effect of two factors, extraction temperature and solvent composition, in the extraction efficiency has been tested.

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10.1021/jf070018u CCC: $37.00
Published on Web 03/30/2007

MATERIALS AND METHODS

Reagents and Samples. La, Lu, phenyl-β-D-glucoside, and *N*-trimethyl-silyl-imidazol (TMSI) were acquired to Sigma (St. Louis, MO). Sea sand was obtained from Panreac (Barcelona, Spain). Absolute ethanol was from Prolabo (Fontenay-sous-Bois, France). High-purity water was produced in-house using a Milli-Q Synthesis A10 system (Millipore, Billerica, MA) and was used throughout. A mixture of Lu and La (70:30; w:w) was obtained from crystalline standards using a laboratory mill.

Synthesis of Lu. The synthesis of Lu was carried out following the method of Zokaee et al. (*10*), slightly modified. Four grams of La was dissolved in 40 mL of deionized water and mixed with 2 g of sodium aluminate. The mixture was kept under stirring at 40 °C taking sample at different times: 0, 1, 3, 5, 7, 9, 10, and 11 h. The reaction was stopped using a few drops of 25% sulfuric acid until pH 7. Samples were diluted with 10 mL of deionized water and centrifuged at 7000*g* for 5 min. The supernatant was collected and analyzed by gas chromatography (GC) as indicated below. The selected sample was freeze-dried previous to the PLE extraction.

PLE System. PLE was performed on a Dionex ASE 200 (Dionex, Sunnyvale, CA) system. Samples containing La and Lu were mixed at a proportion 1:9 with sea sand, which was selected as an inert material to hold the sample inside the extraction cell and to avoid the formation of preferential flow paths. The sample was placed in the extraction cell always in excess. At the bottom of the extraction cell, a stainless steel frit and a cellulose filter (Dionex) were placed in order to avoid the collection of suspended particles in the extraction vial (*16*). The extraction cell containing the sample was prefilled with solvent, heated, and pressurized; extraction was performed statically for 30 min. Fresh solvent was conducted through the cell after the static period, drawing the extract into the collector vial.

The extraction conditions were 1500 psi of pressure at constant temperature ranging from 40 to 130 °C using different ethanol:water proportions (from 100:0 to 70:30). Different extraction times (10, 20, 30, 45, and 60 min) were also assayed, and 30 min was selected to achieve a compromise between speed and equilibrium. The extraction pressure was kept constant because its influence on extraction efficiency is not a determinant factor (*15*, *17*). Pressurizing the extraction cell prevented the solvent from boiling at the extraction temperature and ensured that the solvent remained in intimate contact with the sample.

All experiments were carried out in duplicate. Repeatability of the process was also evaluated submitting one sample of Lu:La (70:30 w:w) to the PLE extraction five different times. The relative standard deviation was 0.9% in terms of the purity of Lu.

GC Analysis. Samples were diluted 1:10 (v/v) with 70% methanol: water, and 1 mL of these solutions was added to 0.3 mL of 1 mg/mL phenyl-β-D-glucoside as an internal standard. These samples were evaporated under vacuum at 38−40 °C. Derivatization was carried out using 150 μL of TMSI at 65 °C for 30 min. Silylated carbohydrates were extracted with 100 μL of hexane and 200 μL of water. One microliter of organic phase was injected onto the column.

GC was performed with a Carlo Erba HRGC 5160 Mega series gas chromatograph (Milan, Italy) equipped with a flame ionization detector. A 30 m × 0.25 mm i.d. × 0.25 μm fused silica column coated with DB-17 (50% phenyl silicone from J & W scientific, Folsom, United States) was used. The carrier gas (nitrogen) flow rate was 1.2 mL/min. Injector and detector temperatures were 300 °C. The oven temperature was programmed as follows: ramp from 250 to 270 °C at 2 °C/min and hold for 10 min. Samples were injected in split mode (split ratio 33:1). Chromatographic peaks were measured using a Chrom-Card 1.20 acquisition system (CE Instruments, Milan, Italy). Quantitative analysis of La and Lu was carried out using the response factor relative to phenyl-β-D-glucoside (internal standard) over the expected range.

Statistical Analysis. Statistical analysis was used to fit the experimental data. Two factors were considered in the study: extraction temperature (T) and solvent composition (S) considered as the % of ethanol in the solvent mixture. Different responses were selected as follows: the amount of Lu extracted (as mg Lu) and the purity of Lu (as % Lu). The quadratic polynomial model proposed for each response variable (Y_i) was

$$Y_i = \beta_0 + \beta_1 T + \beta_2 S + \beta_{1,1} T^2 + \beta_{2,2} S^2 + \beta_{1,2} TS \qquad (1)$$

where β_0 was the intercept, β_1 and β_2 were the linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ were the squared coefficients, and $\beta_{1,2}$ was the interaction coefficient. The parameters of the model were estimated by multiple linear regression (MLR) using the Statgraphics Plus v.5.1 program (Statistical Graphics Corp., Manugistics Inc., MD).

The effect of each term in the model and their statistical significance, for each of the response variables, was analyzed. The terms not significantly different from zero ($P > 0.1$) were excluded from the model, and the mathematical model was refitted by MLR. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the residual standard deviation (RSD). From the new fitted models, response surfaces and the contour plots were obtained.

RESULTS AND DISCUSSION

Table 1 shows the mean values obtained to selectively recover Lu from its mixture with La (70:30, w:w) using PLE as indicated above. As expected, under the assayed conditions, the total carbohydrate extracted increased with both factors: the water content of the solvent and the extraction temperature; this behavior has been widely reported for different sugars at atmospheric pressure (*13*, *17*, *18*). Regarding the amounts of extracted La, in general, these results are in agreement with those data reported by Machado et al. (*17*) about solubility of La in water−ethanol at 40 and 60 °C at atmospheric pressure. As can be seen in **Table 1**, the amount of Lu extracted increases with the temperature while the selectivity of the extraction decreases. In fact, the highest Lu purity was obtained at 40 °C for all of the solvent compositions, if water was in the solvent mixture. Different behavior was observed when 100% ethanol was used as the extraction solvent.

To perform an in-depth study on the effect of the two factors involved in the PLE process, that is, extraction temperature and solvent composition, in the recovery and purity of Lu, statistical analysis was carried out. By using a MLR, experimental data were fitted to the quadratic model described above (*1*). **Table 2** shows the summary of the regression results for both, % Lu (purity) and mg of Lu. Only the significant terms of the model (at 90%) are presented, and the coefficients have been recalculated after removing the nonsignificant terms. As for the response mg of Lu, S (solvent composition accounting for the amount of ethanol in the mixture with water), T, S × S, and S × T were the significant terms of the model. As can be observed (**Table 2**), the determination coefficient was 0.96 while the estimated error was 180.7. In terms of % Lu, T × T was the most significant term (at 95% confidence level); the rest of the terms of the model were only significant at the 90% level.

The graphical representation of the quadratic function along with the experimental data and the regions of maximum response are shown in **Figure 1**. For mg of Lu, it is easily seen that the yield increases when decreasing the percentage of ethanol in the solvent mixture and when increasing the extraction temperature. As for Lu purity (% Lu), the behavior is just the opposite, maximizing the purity at low temperatures while the response is not being significantly affected by the percentage of ethanol used in the solvent mixture.

Figure 2 shows the graphs of observed vs predicted values from the model for the two responses studied in the present work. As can be seen, the fitting is excellent, thus indicating the possibility of using the mathematical model to predict the behavior of the system outside the experimental domain. Therefore, results were extrapolated to obtain a reasonable characterization of the expected behavior in the region of lower ethanol content. **Table 3** shows the predicted values of mg of

Table 1. Mean Values ($n = 2$) of Experimental Data Obtained for the Extraction of Lu from a Mixture with La (70:30 w/w) Using PLE for 30 min with Different Ethanol:Water Mixtures [from 100:0 to 70:30 (v/v)] and Extraction Temperatures (from 40 to 130 °C)

initial amount of sample (g)	*T* (°C)	ethanol: water (v/v)	Amount (mg)		Purity %	
			Lu	La	Lu	La
1	40	100:0	17.13	4.22	80.23	19.77
1	60	100:0	40.17	6.50	86.07	13.93
1	80	100:0	90.70	24.84	78.50	21.50
1	90	100:0	89.34	20.11	81.62	18.38
1	95	100:0	91.62	22.65	80.18	19.82
1	100	100:0	108.27	27.93	79.49	20.51
1	110	100:0	110.63	28.15	79.72	20.28
1	130	100:0	150.62	85.61	63.76	36.24
1	40	95:5	84.68	4.28	95.19	4.81
1	60	95:5	129.67	8.67	93.73	6.27
1	80	95:5	123.76	29.92	80.53	19.47
1	90	95:5	251.50	41.92	85.71	14.29
1	95	95:5	230.85	73.36	75.88	24.12
1	100	95:5	243.41	91.49	72.68	27.32
1	110	95:5	223.18	111.07	66.77	33.23
1	40	90:10	145.85	6.55	95.70	4.30
1	60	90:10	220.06	21.10	91.25	8.75
1	80	90:10	534.48	40.99	92.88	7.12
1	90	90:10	597.43	133.03	81.79	18.21
2	40	80:20	888.44	30.4	96.69	3.31
3	60	80:20	2345.72	130.96	94.71	5.29
3	80	80:20	2228.94	382.97	85.34	14.66
6	40	70:30	3543.32	242.83	93.59	6.41

Table 2. Regression Coefficients for Factors and Statistics for the Fit Obtained from MLR

	response variables	
	Lu (mg)	% Lu
intercept	49775.21	–46.2359
S	–1115.08	4.3202
T	133.77	–1.2390
S × T	–1.35	0.0155
S × S	6.19	–0.0304
T × T		–0.0035
R^2	0.964	0.900
RSD	180.7	4.2

Lu and % Lu at different temperatures and solvent compositions. From these data, it can be inferred that by increasing the extraction temperature at a solvent composition equal to 70:30 (ethanol:water), the purity of the Lu decreases while the yield of Lu increase. As expected, a further decrease in the percentage of ethanol (to 60%) increases the amount of sugar extracted, but an important decrease in selectivity is also observed.

Thus, from the data presented in this study, the optimum conditions to extract Lu from mixtures with La are those providing maximum yield and purity; that means working at 40 °C with 70:30 (v/v ethanol:water) extraction solvent. This effect has not been previously observed by other authors, since studies on comparative solubilities of La and Lu in alcohols are scarce and most of them are performed in ethanol containing not more than 5% water at atmospheric pressure (*13, 19*). Other studies carried out in our laboratory to separate Lu from La using supercritical CO_2 extraction showed a considerably decreased Lu purity when 92.5:7.5 ethanol:water was used as cosolvent instead of 95:5 ethanol:water (*14*).

To confirm the utility of the PLE method for the separation of both carbohydrates, Lu was synthesized as indicated in the Materials and Methods. **Figure 3** shows the evolution of Lu, La, and galactose during the synthesis. The Lu concentration increased until 9 h of reaction while La decreased during the whole process. Galactose was observed from 1 h of reaction and increased with time, although only small amounts were detected at the end of the process. Only traces of tagatose could be observed at the end of the reaction time. At 9 h of treatment, 75.5% of Lu was obtained whereas 18.4% of La was already present (ratio Lu:La 80:20). Considering that no more Lu was obtained after this time and galactose continued increasing, this sample was selected for further experiments.

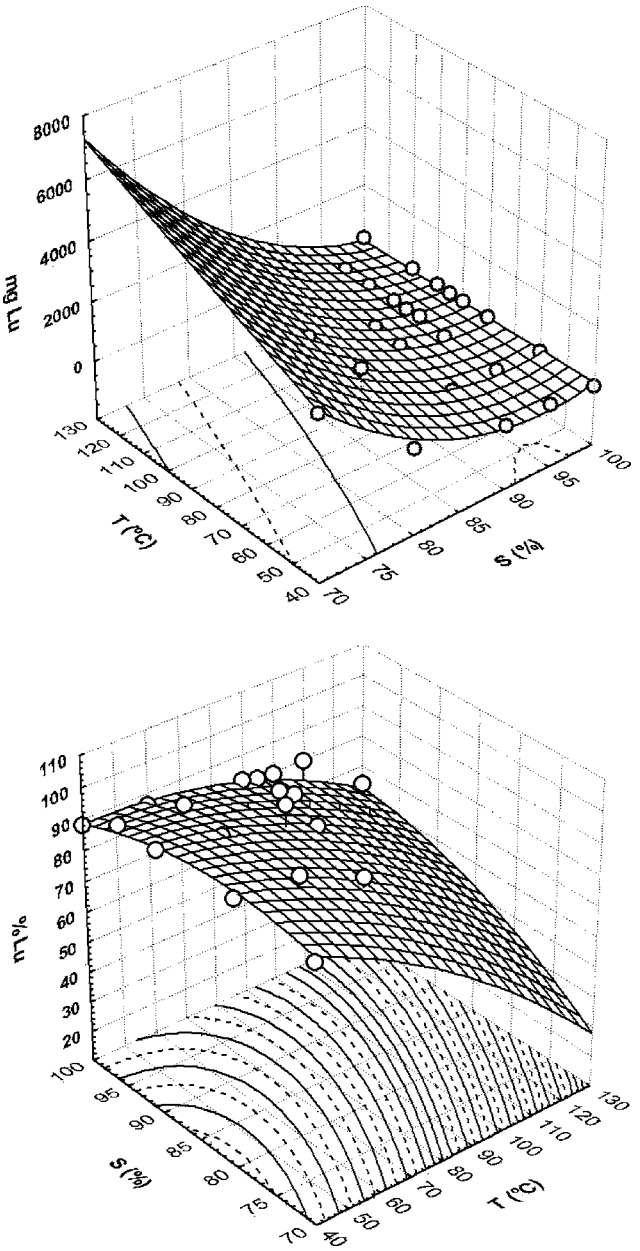

Figure 1. Surface and contour plots for the response variables studied (temperature and percentage of ethanol in the solvent): (**top**) mg of Lu extracted (mg Lu) and (**bottom**) Lu purity (% Lu).

PLE treatment at the selected optimum conditions (ethanol: water 70:30 and 40 °C) was applied to the mixture of synthesis. The purity of Lu was enhanced to 86.6% after the process; 9.0% of La and 4.4% of galactose were also extracted. The Lu:La ratio in this sample was 91:9, similar to data obtained with standards in **Table 1**.

The proposed extraction procedure is more rapid and involves less solvent consumption than other methods suggested in the literature to separate Lu from La such as the use of fractional

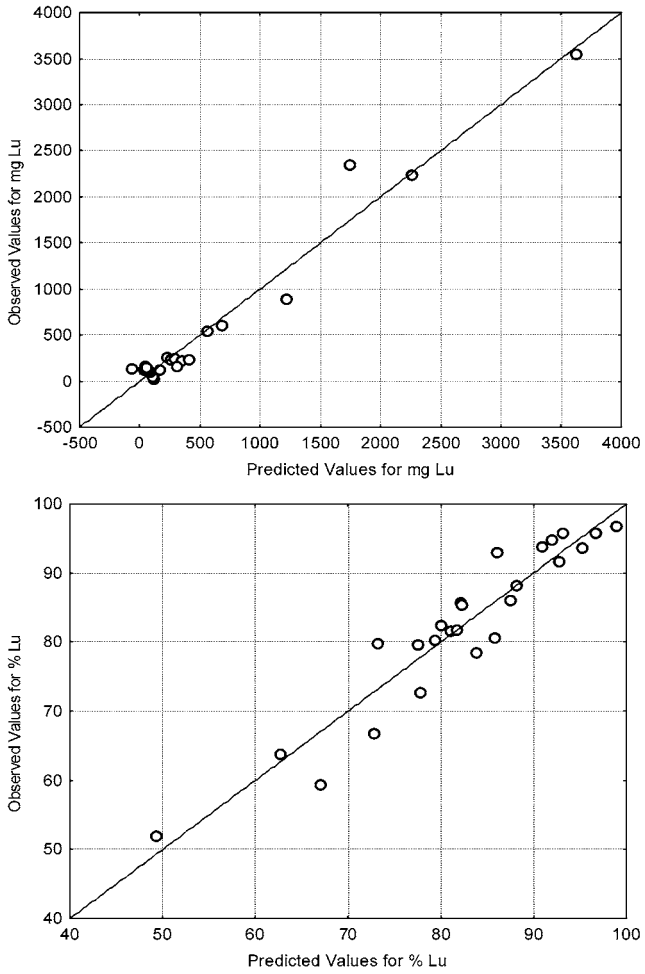

Figure 2. Predicted vs observed values for (**top**) mg of Lu extracted (mg Lu), and (**bottom**) Lu purity (% Lu) using temperature and percentage of ethanol in the solvent as response variables.

Table 3. Predicted Values for the Responses mg of Lu and % Lu Based on the Mathematical Model

		predicted	
temperature (°C)	ethanol:water (v/v)	mg of Lu	% Lu
40	70:30	3626.4	95.3
50	70:30	4022.3	90.6
60	70:30	4418.1	85.2
70	70:30	4813.9	79.1
40	60:40	7270.0	85.4
50	60:40	7800.8	79.2
60	60:40	8331.0	72.3
40	50:50	12152.0	69.5

crystallization (*20*, *21*), fractional adsorption on solid materials (*22*−*24*), or electrodialysis in the presence of weak acids (*25*).

Therefore, PLE has been shown to be a good time-saving technique for extraction and purification of Lu from a La mixture and it could be applied to other carbohydrate mixtures to acquire a whole knowledge about the effect of temperature and alcohol concentration in their separation processes. From data analysis, it seemed reasonable to lower the ethanol content in the mixture; unfortunately, the system could not hold these large proportions of water able to dissolve large amounts of carbohydrates that, in fact, can clogged the automatic system employed. Because the commercial equipment available is not specially designed for this purpose, modifications are being introduced in the system to optimize both the extraction yield and the selectivity

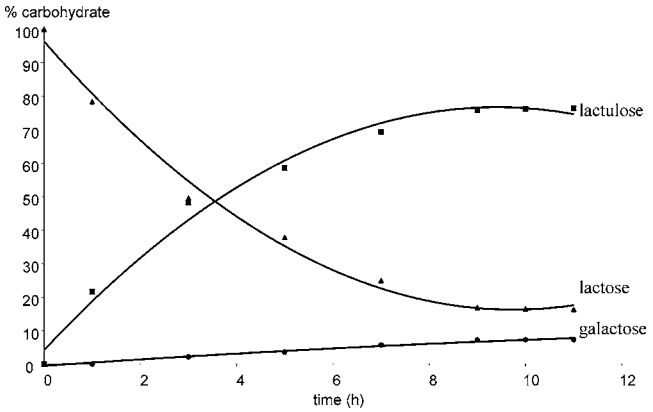

Figure 3. Percentage of La, Lu, and galactose obtained during the synthesis of Lu at 40 °C with sodium aluminate at different times.

of the process, to purify specific carbohydrates to be used as prebiotic ingredients.

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Received for review January 3, 2007. Revised manuscript received February 27, 2007. Accepted February 27, 2007. This work was financed by Programa Apícola Nacional (API03-007) supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and by the project AGL2004-07227-CO2-02 supported by the Ministerio de Educación y Ciencia of Spain.

JF070018U