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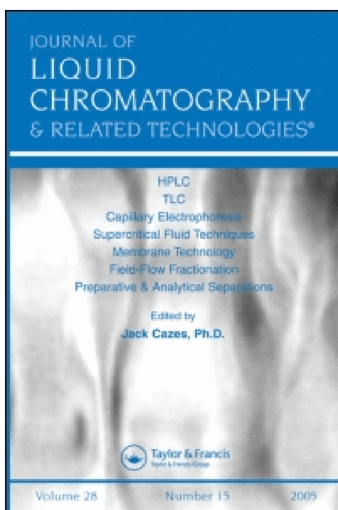

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SIMULTANEOUS DETERMINATION OF SUGARS, URIC AND OROTIC ACIDS IN INFANT FORMULAE BY HPLC-UV/RI

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ABSTRACT

In a simple, rapid isocratic HPLC method sugars (glucose, galactose, saccarose, maltose, lactose), uric and orotic acids were separated on a Spherisorb NH2, 5 μm Chromatographic column and detected using refractive index and ultraviolet (λ = 280 nm) detectors in series. The identification was made by comparison of the retention times with those of the corresponding standards; xilose and melizitose were used as internal standards. The determinations were performed in the linear range of 0.5-30.0 g/L for sugars, 1.0-18.0 mg/L and 0.5-20.0 mg/L for uric acid and orotic acid, respectively. The detection limits were 0.20 g/L for xilose, glucose, galactose, and lactose and 0.10 g/L, 0.35 g/L, 0.40 g/L for saccarose, maltose, and melizitose, respectively. For uric and orotic acids the detection limits were 0.5 mg/L and 0.1 mg/L, respectively. The validity of the method was verified. For recovery studies of internal standards (I.S.), several determinations were conducted, using the standard addition method at three specific concentrations (1.0, 5.0, and 10.0 g/l). The recoveries ranged from 95 to 101%.

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The precision of the method was also evaluated, the %CV being 1.01, 0.51, 0.45, 0.73, 0.82, 0.82, and 0.74 for xilose, saccarose, maltose, lactose, melizitose, uric acid, and orotic acid, respectively. The sample pre-treatment was simple with a single extraction. The good precision and accuracy obtained proved that this method is suitable for routine analysis.

INTRODUCTION

The infant formulae industry tries to obtain powdered formulae that upon reconstitution have a composition similar to that of the human milk, thus, their composition relatively to some constituents, namely the sugars, is not the same as the cow's milk, from which they are originally prepared. Regulation establishes which sugars can be added and their limits.[1] Other constituents such as uric and orotic acids are endogenous in milk, consequently, they appear naturally in infant formulae. Uric acid is an undesirable constituent as opposed to orotic acid whose presence is important, however the suplementation of the infant formulae with orotic acid is still a controversial subject.[2]

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The determination of sugars (glucose, galactose, saccarose, maltose, lactose), uric, and orotic acids on infant formulae takes on considerable importance with respect to the quality control of those products, for both labelling and nutritional reasons. To this end, a precise, reproducible, rapid, and economic analytical procedure is required for routine quality control in infant formulae industry. Owing to the good characteristics of separation, identification, and evaluation of different components in complex matrices showed by the HPLC methodology, alied to a good precision and accuracy, HPLC methods have been employed to analyze uric and orotic acids[3-4] and sugars[5-6] in dairy products. An attempt for simultaneous determination of some sugars and organic acids on cheese,[7] using an ion-exchange column has been reported.

The objective of our research was the development of a rapid, simple, and specific method for simultaneous determination of sugars, uric, and orotic acids in infant formulae by HPLC - UV/RI. Xilose and melizitose were used as internal standards. With the HPLC chromatographic column used (Spherisorb NH_2) and according to the literature,[8] the separation between glucose and galactose is difficult, but the separation of saccarose, maltose, lactose, and melizitose is good. The ion-exchange columns are considered preferential on the separation of monossacarides, but they present difficulties on the separation of some disaccharides.[6,9]

The separation of uric and orotic acids on ion exchange columns[4] has proved to be well reproducible, but owing to the column characteristics and slow elution, the peaks were broad and the sensitivity of the method correspondingly low. In order to retain the selectivity of ion-exchange chromatography, but improve the sensitivity, we attempted to use ion-pair formation of uric and orotic acids and separation on a Spherisorb NH_2 column.

The objective of our research was the development of a rapid, simple, and specific method for simultaneous determination of sugars, uric, and orotic acids in infant formulae by HPLC - UV/RI. Xilose and melizitose were used as internal standards.

MATERIALS AND METHODS

Reagents and Solutions

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All reagents used were (p.a) from Merck. The standards for the orotic and uric acids were from Sigma Chemicals Co. The water used for chromatography analysis had a resistance greater than 15 MΩ. This was filtered through a membrane of 0.45 μm porosity and subsequently degassed. The acetonitrile (Lichrosolv) used was, Merck "gradient grade". The mobile phase used was acetonitrile/HCL 0.01M (84:16).

Apparatus

The chromatographic analyses were carried out in a Gilson, high performance liquid chromatograph equipped with a type 305 pump and a type 7125 Rheodyne Injector with a 20 μl loop. A Gilson variable wavelength UV/VIS detector, a 132 RI detector and a Gilson 712 HPLC System Controller Software were also used.

The detectors were connected in series. The UV detector, set at 280 nm, was used for quantification of uric and orotic acids. The RI detector was used for quantification of sugars. The chromatographic separation was achieved with a Spherisorb NH_2 chromatography column, 5 μm, 250 mm x 4.6 mm i.d.. The analyses were performed isocratically at a flow rate of 1.0 mL/min and at room temperature.

Calibration and Calculations

Single standard solutions of sugars and acids were prepared to establish elution times. Quantification was based on the internal standard method, using xilose and melizitose as internal standards (I.S.). Five aqueous mixed standards for sugars (xilose, glucose, galactose, saccarose, maltose, lactose, melizitose), uric, and orotic acids covering a broad concentration range were prepared to establish calibration curves. Resulting peak heights were determined for duplicate 20 µl injections. Standard curves for each component were prepared by linear regression of peak height vs concentration. Afterwards, individual standard solutions were added one by one to infant formulae samples to verify correct identification of peaks using elution times.

Sample Preparation

Approximately 1.5 g of previously homogenized infant formulae powder was dissolved in 2 mL of warm distilled water. 0.5 mL of 5% oxalic acid (w/v) and 5 mL of 95% ethanol were then added. The mixture was homogenizeded for 5 min. The volume was adjusted to 20 mL with deionized water and allowed to stand for 10 min. The supernant was injected into the 20 µL loop with a 5 mL syringe fitted with a syringe filter holder containing 0.2 µm membranes filters.

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Precision of the Method

Complete duplicate analyses were performed on all samples to enable calculation of average deviations which were useful as a mesure of the extraction and chromatographic reproducibility. The precision of the analytical method was evaluated by measuring the peak height 10 times on the same sample.

Recovery Study

The recovery of internal standards added to infant formulae was evaluated to determine the accuracy of the test. Infant formulae were analyzed in duplicate before and after the addition of known amounts of I.S. Three different concentration levels of internal standards were analyzed, complete duplicate analyses were performed.

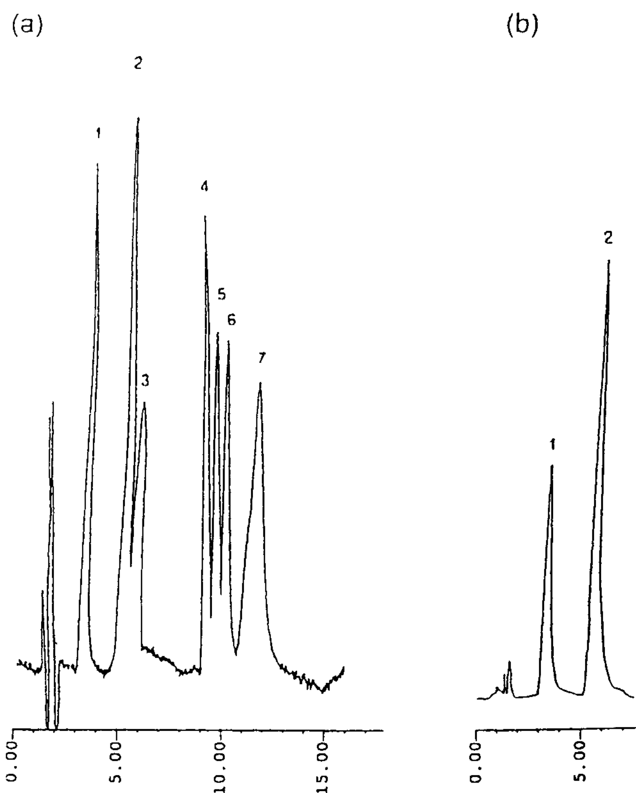

Figure 1. Typical chromatograms of aqueous standard solutions. A - sugars from RI detector. Sugar standards: (1) xilose, (2) glucose, (3) galactose, (4) saccarose, (5) maltose, (6) lactose, (7) melizitose. B - Acids from UV detector at 280 nm. Acid standards: (1) uric, (2) orotic.

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RESULTS AND DISCUSSION

Standard Calibration Curves

Simultaneous determination of sugars, uric, and orotic acids in infant formulae samples was made possible by using the UV and RI detectors connected in series. Typical chromatograms depicting separation of aqueous standard solutions of sugars and organic acids are shown in Fig. 1.

Table 1

Sugars, Uric andOrotic Acids Composition of Infant Formulae Samples[a]

Samples	Glucose (g/100g)	Galactose (g/100g)	Saccarose (g/100g)	Maltose (g/100g)	Lactose (g/100g)	Uric Acid (mg/100g)	Orotic Acid (mg/100g)
1	N.D.	N.D.	N.D.	N.D.	54.2 ± 2.3	6.80 ± 0.11	26.7 ± 1.0
2	N.D.	N.D.	N.D.	N.D.	58.2 ± 1.1	8.02 ± 0.07	25.1 ± 0.9
3	N.D.	N.D.	N.D.	N.D.	45.6 ± 2.2	6.10 ± 0.09	21.8 ± 1.1
4	N.D.	N.D.	N.D.	N.D.	54.4 ± 0.8	10.8 ± 0.10	37.6 ± 1.7
5	N.D.	N.D.	N.D.	N.D.	55.8 ± 0.9	5.12 ± 0.09	15.1 ± 0.8
6	0.51 ± 0.03	<0.3	7.12 ± 0.24	3.40 ± 0.10	47.8 ± 1.2	8.67 ± 0.13	23.1 ± 0.3
7	N.D.	N.D.	5.18 ± 0.71	N.D.	46.7 ± 1.3	4.17 ± 0.09	14.2 ± 0.7
8	0.53 ± 0.4	N.D.	N.D.	0.61 ± 0.08	26.7 ± 0.5	10.9 ± 0.20	34.1 ± 1.1

[a] Values are expressed as mean ± standard deviation of two determinations.
N.D. - not detected.

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Under the assay conditions described, a linear relationship between the concentration of sugars and the refractive index was obtained. The same happened between the concentration of uric and orotic acids and the UV absorbance at 280 nm. This linearity was maintained over the concentration range of 0.5-30.0 g/L for sugars, 1.0-18.0 mg/L for uric acid, and 0.5-20.0 mg/L for orotic acid.

The correlation coefficient for each standard curve invariably exceeded 0.99 for all compounds. The calibration curves for sugars, uric, and orotic acids were obtained by duplicate determinations of each of the calibration standards and the peak height values (arbitrary units) were plotted as average values.

The relative percent average deviations of duplicates were less than 2 % in all cases. The average regression equation for xilose, glucose, galactose, saccarose, maltose, lactose, melizitose, uric, and orotic acids were $y = 147.77 x + 0.981$; $y = 135.64 x + 0.331$; $y = 82.704 x + 0.355$; $y = 81.393 x + 0.859$; $y = 187.51 x + 5.10$; $y = 31.761 x + 1.75$; $y = 20.592 x + 2.106$; $y = 25.331 x + 7.1$; $y = 18.852 x + 5.1$, respectively.

The detection limit was calculated as the concentration corresponding to three times the SD of the background noise, obtained with 10 determinations. The detection limits were 0.20 g/L for xilose, glucose, galactose, and lactose and 0.10 g/L, 0.35 g/L, 0.40 g/L for saccarose, maltose, and melizitose, respectively. For uric and orotic acids the detection limits were 0.5 mg/L and 0.1 mg/L, respectively.

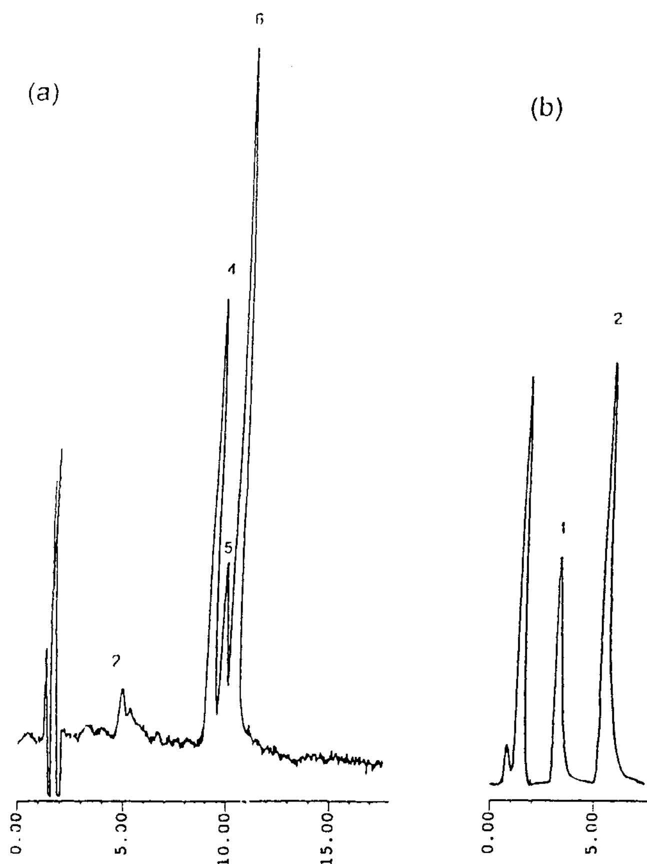

Figure 2. Typical chromatograms of infant formulae (chromatographic conditions described in the text). The letters and the numbers correspond to the letters and numbers in Fig. 1 with respect to peak identification.

Typical Analytical Results and Chromatograms for Infant Formulae

Typical results and average deviations of duplicate determinations for a variety of commercial infant formulae are presented in Table 1. Figure 2 (a and b) shows the typical chromatograms for infant formulae. Figure 3 (a and b) shows the chromatograms for infant formulae with internal standards addition, after having been subjected to identical treatment.

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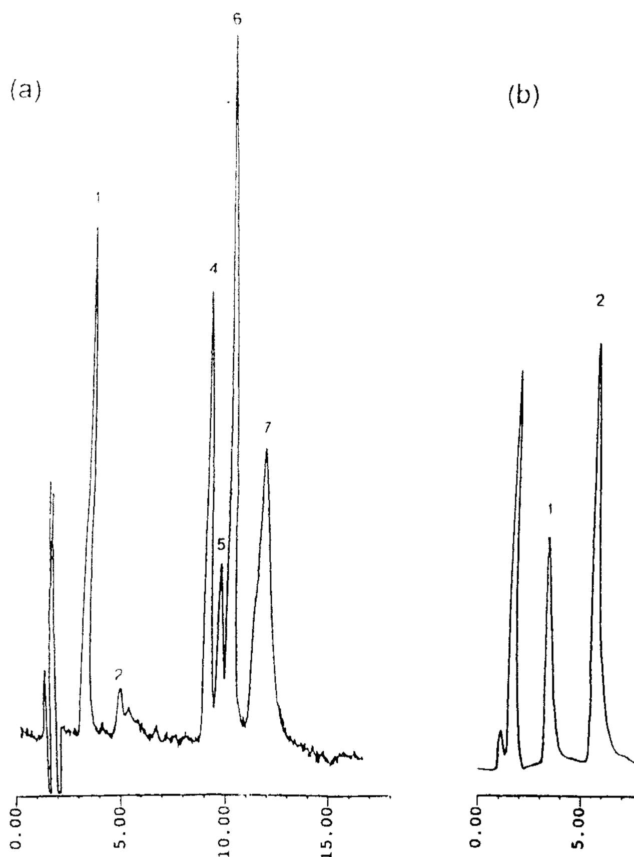

Figure 3. Typical chromatograms of infant formulae spiked with I.S. (chromatographic conditions described in the text). The letters and the numbers correspond to the letters and numbers in Fig. 1 with respect to peak identification.

The initial solvent peaks for all chromatograms occur between 1 and 2 min and result from water and other unretained components. The precision of the method was also evaluated by measuring the peak height of each compound, 10 times on the same sample. The %CV being 1.01, 0.51, 0.45, 0.73, 0.82, 0.82, and 0.74 for xilose, glucose, galactose, saccarose, maltose, lactose, melizitose, uric acid, and orotic acid, respectively (concentration of saccarose, maltose, lactose, uric, and orotic acids in infant formula were 7.12 g/100 g, 3.40 g/100g, 47.8 g/100g, 8.67 mg/100g, and 23.1 mg/100g respectively).

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Table 2

Recovery Study of Internal Standards Added to Homogenized Infant Formulae Powder

Xilose

Added (g/L)	Found (g/L)	Standard	CV%	Recovery
1.00	0.953	0.006	0.63	95.3
5.00	4.95	0.087	1.75	99.0
10.0	9.51	0.006	0.063	95.1

Mean value 96.5 ± 2.2%

Melizitose

Added (g/L)	Found (g/L)	Standard	CV%	Recovery
1.00	0.953	0.006	0.63	95.3
5.00	4.92	0.029	0.59	98.4
10.0	9.51	0.012	0.13	95.1

Mean value 96.2 ± 1.9%

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Recovery Study

As can be seen from Figure 2 (b), there is no chromatographic indication of interfering endogenous compounds during the retention time relating to xilose and melizitose. Table 2 presents the results obtained from the study of internal standards recovery. Internal standard recovery percentages ranged between 95 and 101% for the three concentrations studied, the average percent recovery was 96.5 ± 2.2 % for xilose and 96.2 ± 1.9 % for melizitose. These two findings highlight the appropriateness of the chosen internal standards and can be taken as a guarantee of the accuracy of the method developed.

CONCLUSIONS

The described procedure seems to fulfill the criteria of selectivity, sensitivity, reproducibility, and convenience of a method suited for routine assay of various sugars, uric acid, and orotic acid in infant formulae. The main

advantages of this method are simple sample preparation (a single extraction) and the use of a single NH_2 HPLC column which does not need to be regenerated and has a lower cost compared with ion-exchange columns. Furthermore, the method is rapid and easy to carry out. The method, herein, presented, possesses the demonstrable simplicity of allowing simultaneous analysis of five sugars and two organic acids in less than 15 minutes.

The results obtained allow us to foresee that the developed methodology could be extended to monitoring the levels of these compounds in other types of milk produts. The merging of all these qualities makes the method extremely useful for routine analysis and, especially so, after its automation.

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