A new liquid chromatography method for the simultaneous and sensitive quantification of lactose and lactulose in milk

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ORIGINAL ARTICLE

A new liquid chromatography method for the simultaneous and sensitive quantification of lactose and lactulose in milk

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Abstract The disaccharide lactulose is known to have a large influence on human digestion, the effects of which range from prebiotic to laxative action. Lactulose can be derived from lactose by either alkaline isomerization or enzymatic transgalactosylation of fructose. The aim of this study was to establish a new analytical method for the quantification of both disaccharides lactose and lactulose using high performance liquid chromatography (HPLC) and evaporative light scattering detection (ELSD). The new method needs to provide good chromatographic separation of lactose and lactulose while being fast and reliable. Therefore, four analytical methods using different HPLC columns were developed and compared. A HPLC column with an amino-bonded polymeric matrix yielded better results compared to amino-bonded silica-phase resin or cation-exchange resin. The new method had detection limits for lactose and lactulose of 3.8 and 2.5 mg L^{-1} and was successfully applied to the quantification of lactulose and lactose in commercial milk products.

Keywords HPLC · Lactulose · Lactose · Evaporative light scattering detection · Method development

摘要 种新的液相色谱法同时检测乳中乳糖和乳酮糖。 乳酮糖为二糖，具有益生元的功能。乳酮糖除对人消化能力有较大的影响外，还具有对人体放松的作用。 乳酮糖的制备 可以采用乳糖在碱性条件下的异构化作用或者在酶催化下发生果糖的转半乳糖苷作用。 本文建立了配以蒸发光散射检测器(ELSD) 的高效液相色谱法(HPLC) 测定乳糖和乳酮糖的定量检 测方法。新的色谱条件必须

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保证乳糖和乳酮糖有较高的分离度，并且具有快速和准确的 特点。因此，选用不同的色谱柱用四种分析方法对测定结果进行了比较和分析。 与氨基 键合硅胶柱或阳离子交换树脂柱相比， 氨基键合柱可以将乳糖和乳酮糖较好地分离。所建立的方法对乳糖和乳酮糖的检测限分别为3.8 和 2.5 $mg \cdot L^{-1}$。 该方法可以成功地用于乳制品中乳糖和乳酮糖的定量测定。

关键词 HPLC · 乳酮糖 · 乳糖 · 蒸发光散射检测 · 方法的建立

1 Introduction

The prebiotic disaccharide lactulose has been subject to clinical trials for 50 years and is nowadays regarded as a valuable substance for both pharmaceutical and food applications (Ballongue et al. 1997; Bouhnik et al. 2004; Claeys et al. 2003; Mizota et al. 2002; Schuhmann 2002; Seki et al. 2007; Tuohy et al. 2002). Lactulose (4-O-β-D-galactopyranosyl-D-fructose) can be synthesized by different reactionswith the alkaline isomerization of lactose via *Lobry de Bruyn—Alberda van Ekenstein—* rearrangement being the only one applied in industrial scale (Aider and De Halleux 2007). An alternative way to produce lactulose is offered by a side reaction of lactose hydrolysis, the transgalactosylation of fructose (Kim et al. 2006; Lee et al. 2004; Mayer et al. 2004). Our current research focuses on this enzymatic transformation of milk-based lactose into lactulose and the precise determination of these sugars is a vital part of this work. Moreover, the precise analysis of lactose and lactulose in food or drug formulations is of special interest as both substances exert dose-dependent effects upon intake (lactose maldigestion, prebiotic or laxative action of lactulose).

The aim of the present work was to develop a sensitive yet fast and reliable method for the quantification of lactose and lactulose in milk-based products. A glance over available literature on lactulose demonstrates the broad diversity of applied analytical methods (Marconi et al. 2004; Martínez-Castro et al. 1987; Ruiz-Matute et al. 2007; Verhaar et al. 1979). Besides enzymatic assays lactulose is mainly analysed using chromatography, either thin-layer, liquid or gas chromatography. We decided to perform analysis via high performance liquid chromatography (HPLC) because it allows simultaneous and fast identification of mono-, di- and oligosaccharides. As for detection, we chose an evaporative light scattering detector (ELSD) which is commonly acknowledged for its sensitivity (Clement et al. 1992). Our method development is based on the comparison of four different HPLC columns in regard to their separation performance of lactose and lactulose.

2 Materials and methods

Materials. Carbohydrate standards lactose, lactulose, glucose, galactose and fructose of analytical grade were obtained from Sigma-Aldrich Inc. (Munich, Germany) and Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Distilled water (conductivity <4 $\mu s\ cm^{-1}$) was used for dilution. Commercially available lactose- and lactulose-containing products were: lactose-free yoghurt and sterilized milk (Breisgaumilch GmbH, Breisgau i.S., Germany). Skim milk was processed in the Dairy for

Research and Training at the University of Hohenheim by separation of raw milk (62 °C) and pasteurization (72–75 °C, 30 s).

Sample preparation. For chromatographical analysis, commercial samples were prepared by dilution with water, protein precipitation by addition of Carrez-solution and twofold filtration. Every sample was dissolved with distilled water, while the dilution factor was varied from 1:10 to 1:50, depending on the sample. Before the dilution was made up to its intended volume, 0.5 mL of each Carrez-solution I and II were added sequentially and the mixture was left for 10 min, which was found to be sufficient for complete protein precipitation. Carrez-solution I and II were prepared according to Indyk et al. (Indyk et al. 1996). Afterwards, the diluted milk product samples were made up to full volume and then filtered through filter paper (Whatman's No. 1, Ø=150 mm; Whatman GmbH, Dassel, Germany). The resulting permeate was further passed through a membrane with a pore size of 0.20 μm (Chromafil RC-20/25, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The extract was injected into the HPLC-system following a 1:2 dilution with acetonitrile (gradient grade, Merck KGaA, Darmstadt, Germany).

HPLC-system. Sugar separation was carried out using a chromatographic unit from Agilent Technologies Inc. (Santa Clara, CA, USA). The unit (Series Agilent 1100) consisted of a vacuum degasser, a quaternary pump, an autosampler and a column oven. Sugar detection resulted from evaporative light scattering (Alltech ELSD 2000 ES, Alltech Associates Inc., Deerfield, IL, USA).

Stationary phases. Four HPLC Columns with different stationary phases were tested. A detailed description of the employed columns is given in Table 1.

One hundred per cent distilled water was used as mobile phase for analysis with column Rezex RCM-Monosaccharide Ca^{+}, while the other columns were eluted with a mixture of distilled water and acetonitrile (CH_3CN). Composition of mobile phases and further analysis conditions such as flow rate and column temperature were optimized during this work. Original detector settings were: no Impactor, Gain 1, 80 °C tube temperature and 2.5 $L \cdot min^{-1}$ nitrogen flow.

Table 1 Specification of employed HPLC columns

Trade name and manufacturer	Column dimension in mm	Packaging properties[a]	Separation mode
Rezex RCM-Monosaccharide Ca^{+}, Phenomenex Inc., Torrance, CA, USA	300×7.8	Sulfonated crosslinked styrene divinylbenzene copolymer with calcium couterions	Ion exclusion
Prevail Carbohydrate ES, Alltech Associates Inc., Deerfield, IL, USA	250×4.6	Five micrometer spherical polymer beads coated with amino-based proprietary bonding material	Hydrogen bonding
Sphere Clone NH2, Phenomenex Inc., Torrance, CA, USA	250×4.6	Five micrometer Silica particels bonded with a monolayer of 3-aminopropylsilan	Hydrogen bonding
Zorbax Carbohydrate Analysis, Agilent Technologies Inc., Santa Clara, CA, USA	250×4.6	Five micrometer "Zorbax—silica" particels bonded with a monolayer of 3-aminopropylsilan	Hydrogen bonding

[a] According to supplier

Method development. Column performances were optimized with regard to retention time (t_R) and peak resolution (RS):

$$R_S = \frac{2(t_{R2} - t_{R1})}{w_{b1} + w_{b2}},$$

where w_{b1} is the peak width for substance 1 in minutes and w_{b2} is the peak width for substance 2 in minutes.

According to Ruecker et al. (2001) a $RS>1.4$ indicates the baseline separation of two substances.

Baseline noise (N). The value was calculated based on a sixfold measurement of maximum baseline amplitude by injection of 20 μL acetonitrile:

$$N = f_{max} - f_{min},$$

where f_{max} is the maximum baseline swing in mAU and f_{min} is the minimum baseline swing in mAU.

Limit of quantification (LOQ) and limit of detection (LOD). The values for LOQ and LOD with regard to lactose and lactulose were calculated based on the ratio of peak height (S) to the baseline noise (N) and the calibration curves for both disaccharides. The limit of sugar quantification (LOQ) is defined as $S/N=10$, while the limit of sugar detection (LOD) is $S/N=3$.

Recovery. The recovery of lactulose was measured by adding 0.4 g lactulose to 10 mL skim milk ($c=40\ mg{\cdot}mL^{-1}$). After sample preparation, milk was analysed along with a lactulose solution with the same concentration. Recovery of lactulose is expressed as the peak area of lactulose in the milk sample related to the peak area of lactulose solution.

Repeatability. The repeatability of peak areas of lactose and lactulose was determined with six consecutive chromatographic analyses of solutions with analyte concentration of 0.01 $g{\cdot}L^{-1}$.

Quantification. Two six-point calibration curves were made for sugar standards lactose, lactulose, glucose, galactose and fructose, ranging from 0.005 to 0.04 $g{\cdot}L^{-1}$ and from 0.04 to 0.3 gL^{-1}. Carbohydrates were identified by comparing retention times with sugar standards. Peak areas of sugar standards were used for quantification.

3 Results

3.1 Comparison of stationary phases

The objective of this work was to determine among four different columns the column with the best separation of lactose, lactulose, glucose, galactose and fructose, with our main focus being the separation and further quantification of the disaccharides. Therefore, chromatographic settings such as composition and flow

rate of the mobile phase and column temperature were varied from the original recommendations stated by producer. Retention time (t_R) and peak resolution (R_S) were calculated in order to compare the separation performance of the different stationary phases. While retention time should be short resulting in a fast analysis, R_S should be higher than 1.4. At this value, it is defined that the mirrored substance is clearly separated up to the baseline from the previous peak (Ruecker et al. 2001). Table 2 reflects a few steps of method optimization for the chosen columns, with the best settings for each column indicated in bold symbols.

Table 2 makes clear that column Rezex differs principally from the three other employed columns as it elutes the disaccharides lactose and lactulose prior to the monosaccharides, which can be explained by the size exclusion effect of cationic resins (Pedruzzi et al. 2008). The best peak resolution for lactose and lactulose was achieved at a 0.6 mL·min^{-1} flow rate of distilled water at 80 °C, when lactose and lactulose are clearly separated (R_S=1.46). Lactulose could not be fully separated from the following monosaccharide glucose, which elutes only 30 s after lactulose, resulting in a R_S of 0.59. The separation performance of column Rezex at optimized chromatographic conditions is depicted in Fig. 1.

Columns Zorbax and SphereClone generally showed the same elution order for the standard sugar solution, eluting the monosaccharides fructose, galactose and glucose prior to the disaccharides lactulose and lactose. Retention times of lactulose and lactose with column Zorbax under optimized conditions were 15.2 and 16.2 min and the subsequent R_S for these peaks 1.03, thus yielding in an insufficient separation. Disaccharide separation with column SphereClone was achieved with a broad separation of lactulose and lactose (R_S=2.02) and retention times of 20.4 and 22.6 min, respectively. Chromatograms with both columns are depicted in Fig. 1.

Separation performance of column Prevail is represented in Table 1: monosaccharides were eluted prior to lactulose and lactose and the difference to columns SphereClone and Zorbax is the altered elution of galactose and glucose. Optimization of column settings leads to a clear separation of lactose and lactulose up to the baseline (R_S=5.68) at relatively short retention times of 10.3 and 12.6 min. While the difference in optimized retention times of lactose and lactulose is nearly the same as with column SphereClone (~2.4 min), column Prevail shows a better peak resolution (R_S=5.68 compared to 2.02). This is mainly due to the smaller width of the peaks for lactose and lactulose obtained with column Prevail, shown in Fig. 1. Generally, chromatographic analysis with column Prevail had the best accordance with our requirements: a clear peak resolution for lactose and lactulose (R_S>1.4) and a short analysis time.

3.2 Method evaluation

Comparing four different stationary phases in regard to the separation of lactose and lactulose, we found that column Prevail featured the best peak resolution at a short analysis time. Optimized chromatographic settings were: isocratic elution with a mixture of acetonitrile and distilled water (70:30%), a mobile phase flow rate of 0.9 mL·min^{-1} at 25 °C. The new method was then characterized by determining the average noise, the LOD and quantification and the recovery of lactulose.

The baseline noise is influenced by both column resin and detector settings. In order to characterize the resin-associated noise of the chosen method, we compared

Table 2 Retention times (t_R) and resolution factor (R_S) of sugar standards for different chromatographic settings and columns Rezex RCM Monosaccharide, Zorbax Carbohydrate, SphereClone NH_2 and Prevail Carbohydrate ES. Bold: best chromatographic performance regarding both t_R and R_S for lactose and lactulose

Column	Column settings			Carbohydrates in order of elution									
	Mobile phase %ACN/%H_2O	Flow rate in mL·min^{-1}	Column temperature in °C	t_R in min	R_S	t_R in min	R_S	t_R in min	R_S	t_R in min	R_S	t_R in min	R_S
Rezex				Lactose		Lactulose		Glucose		Galactose		Fructose	
	0/100	0.7	80	9.13	–	9.88	1.36	10.3	0.30	11.9	1.41	12.8	2.31
	0/100	**0.6**	**80**	**9.56**	–	**10.4**	1.41	**10.9**	0.59	**12.1**	1.51	**13.8**	2.57
Zorbax				Fructose		Galactose		Glucose		Lactulose		Lactose	
	75/25	1.4	50	4.65	–	5.27	1.03	[a]		8.65	4.82	9.27	0.67
	80/20	1.4	40	6.37	–	7.63	1.73	[a]		16.4	7.80	18.0	0.98
	75/25	**1.0**	**30**	**7.71**	–	**8.60**	1.49	**9.26**	0.80	**15.2**	6.23	**16.2**	1.03
SphereClone	60/40	0.6	25	6.24	–	6.61	1.13	6.90	0.63	8.42	3.02	8.81	0.82
	70/30	0.8	25	8.96	–	10.1	2.41	10.7	0.94	16.1	6.61	17.6	1.79
	73/27	**0.8**	**25**	**10.2**	–	**11.7**	2.89	**12.6**	1.17	**20.4**	8.13	**22.6**	2.02
Prevail Carbohydrate				Fructose		Glucose		Galactose		Lactulose		Lactose	
	75/25	0.7	30	10.6	–	13.0	6.77	13.8	1.92	17.8	8.07	22.3	7.33
	75/25	0.9	30	8.23	–	10.1	6.32	10.8	1.89	13.8	7.37	17.4	6.81
	70/30	0.9	30	6.36	–	7.38	4.45	7.77	1.52	8.83	3.65	10.5	5.03
	70/30	**0.9**	**25**	**6.96**	–	**8.24**	5.12	**8.70**	1.64	**10.3**	4.91	**12.5**	5.68

[a] Substance eluted simultaneously with previous analyte; ACN acetonitrile; t_R, retention time; R_S, peak resolution

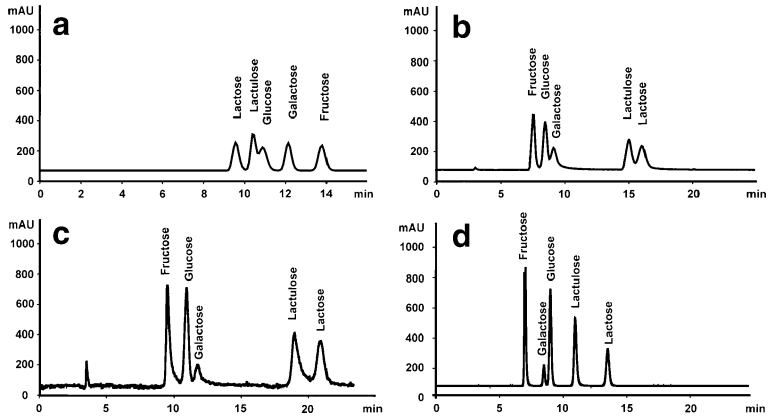

Fig. 1 Chromatogram of standard solution with lactose, lactulose, glucose, galactose and fructose (respective concentration=0.1 g·L^{-1}) with columns Rezex RCM monosaccharide (**a**), Zorbax carbohydrate (**b**), SphereClone NH2 (**c**) and prevail carbohydrate ES (**d**) under optimized chromatographic settings (see Table 2) with 20 μL injection. ELSD-settings: tube temperature 85 °C; nitrogen flow rate 2.5 L·min^{-1}

column Prevail to columns Zorbax and SphereClone with identical detector settings. The average noise, measured with the three columns, is displayed in Table 3.

Table 3 clarifies that column Prevail exerts with 0.3 mAU considerably less baseline noise than columns Zorbax and SphereClone. Especially the latter has a high baseline noise, which can also be seen in Fig. 1c. An intensive baseline noise of an amino-bonded resin can be caused by hydrolytic breakdown and subsequent detection of aminogroups (Herbreteau et al. 1995). This effect is enforced with increasing percentage of water in the mobile phase.

The determination of column noise (*N*) allows the calculation of the LOD and the LOQ for lactose and lactulose. Besides, repeatability and recovery of the evaluated method were determined for lactose and lactulose, respectively. Table 4 summarizes the evaluated parameters for the developed method with column Prevail. Both lactose and lactulose have low limits of detection with 3.8 and 2.5 mg·L^{-1}, respectively. Repeatability of lactose and lactulose detection was evaluated with relative standard deviations of 2.6% and 3.8%, while 99.5% of lactulose could be recovered after sample preparation.

3.3 Application to real samples

The developed method for simultaneous detection of lactose and lactulose was applied to the analysis of commercial samples which contain low amounts of lactose

Table 3 Detector noise for different columns. Column settings: see Table 2, indicated in bold letters; ELSD-settings: tube temperature 85 °C; nitrogen flow rate 2.5 L·min^{-1}

Column	Zorbax carbohydrate	SphereClone NH_2	Prevail carbohydrate
Noise in mAU±SD	2.6±0.8	34±9.9	0.33±0.09

n=6, SD standard deviation

Table 4 Limit of detection (LOD) and quantification (LOQ), repeatability and recovery of HPLC-Column Prevail Carbohydrate ES. Column settings: see Table 2, indicated in bold letters; ELSD-settings: tube temperature 85 °C; nitrogen flow rate 2.5 L·min^{-1}

	LOD in mg·L^{-1}	LOQ in mg·L^{-1}	Repeatability[a]		Recovery[b]
			Mean±SD in mAU	Rel. SD in %	Mean±SD in mAU
Lactulose	2.5	15.0	451±17	3.8	99.5±0.8
Lactose	3.8	17.3	496±13	2.6	n.d.

n.d. not determined, [a] n=6; [b] n=3, SD standard deviation

or lactulose. Figure 2 shows chromatograms of sterilized milk (a) and lactose-reduced yoghurt (b). Both demonstrate the methods' performance in regard to the detection of small amounts of disaccharides in milk matrices. The sample of sterilized milk contained (1.81+0.07) g·L^{-1} lactulose (n=6), while residual lactose concentration in lactose-reduced yoghurt was (0.72+0.09) g·kg^{-1} (n=3). Adachi (1958) firstly reported the presence of lactulose in intensively heat-treated milk, where it was formed due to an isomerization of lactose. Since then, the presence of lactulose in heated milk has been used as a parameter to differentiate between UHT-treatment and sterilization of milk (Marconi et al. 2004). The calculated lactulose concentration of 1.81 g·L^{-1} is relatively high for sterilized milk (Claeys et al. 2003), but may be caused by an additional heating of milk before sterilization. Note that the sample displayed in Fig. 2a was strongly diluted in order to quantify both disaccharides properly during one analytical run. This procedure triggered a lactulose peak which converges towards the LOQ of lactulose (see Table 4). Chromatographic analysis of lactose-reduced yoghurt shows that residual lactose concentration is <0.1%. This concentration is acceptable for consumers with lactose maldigestion. The reduction of lactose by enzymatic hydrolysis leads to the release of glucose and galactose, as can be seen in Fig. 2b. Both monosaccharides appear in such high concentration that they coelute as one peak. It has to be noted that chromatographic separation of glucose and galactose detoriates with increasing column usage (R_S<1 after 150 runs). This effect is probably caused by chemical

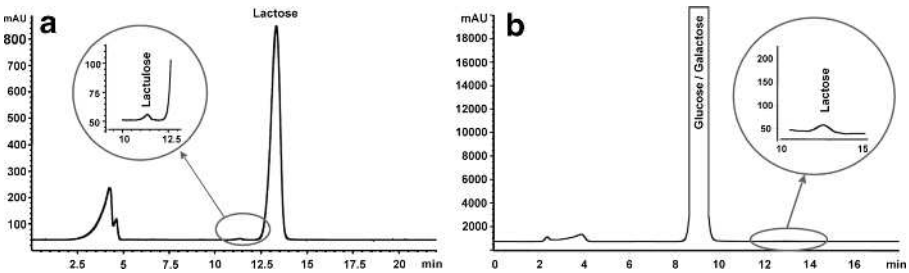

Fig. 2 HPLC-chromatogram of sterilized milk (**a**) and lactose-reduced yoghurt (**b**) with HPLC-column prevail carbohydrate ES. Column settings: see Table 2, indicated in *bold* letters; ELSD-settings: tube temperature 85 °C; nitrogen flow rate 2.5 L·min^{-1}

reactions between reducing sugars and the aminophase attached to the packaging material (Indyk et al. 1996; Porsch 1982).

4 Discussion

4.1 Comparison of stationary phases

Martínez-Castro et al. (1987) reviewed several chromatographical methods for analysis of lactulose without determining the analytical differences between different types of HPLC-columns. HPLC analysis of lactulose and/or lactose has been previously performed by cationic ion-exchange resins (Dendene et al. 1995; Mayer et al. 2004; Pedruzzi et al. 2007; Reimerdes and Rothkitt 1985; Ruecker et al. 2001; Verhaar et al. 1979) and amino-silica-based stationary phases (Chávez-Servin et al. 2004; Nikolov et al. 1985; Paseephol et al. 2008; Zokaee et al. 2002). Paseephol et al. (2008), who investigated the isomerization of lactose into lactulose, emphasized the difficulty to quantify lactulose in the presence of lactose. Facing the same problem, Kim et al. (2006) analysed their complex reaction mixture twofold: fructose and lactulose with an amino-modified silica packed column, while galactose and lactose were measured with a cation-exchange column. A comparison of a cation-exchange resin and an aminomodified phase in regard to the separation of lactose and lactulose has not been made yet. Nevertheless, comparative studies on the separation of mono- and disaccharides are provided by Indyk et al. (1996) and Muir et al. (2009). Both authors found that aminobonded columns are more suitable for a clear separation of di- and even oligosaccharides, while cation-exchange resins feature a better separation of monosaccharides. Considering the different elution order of these resins, it can be generally stated that early eluted compounds are likely to undergo coelution while longer retained substances becomemore separated from each other. Our data support this conclusion, as we achieved better peak separation for lactose and lactulose with the amino-based resin (Table 2). The three columns with amino-bonded resins employed in this study differ in regard to the packaging material: while columns Zorbax and Sphere-Clone consist of a silica gel, column Prevail is packed with a polymeric gel. This column has been previously shown to exert efficient separation of mono- and disaccharides in wine within a single run (La Pera et al. 2007).

4.2 Method development

Previous reports on evaporative light scattering detection of mono- and disaccharides have already proven the great sensitivity of this detection mode. Wei and Ding (2000) found a LODfor lactose of 60 $mg \cdot L^{-1}$, while La Pera et al. (2007) reported a LOD of 30 $mg \cdot L^{-1}$ and a LOQ of 100 $mg \cdot L^{-1}$. On the contrary, Chávez-Servin et al. (2004) who used refractive index detection achieved a LOD for lactose of 250 $mg \cdot L^{-1}$. In terms of repeatability and recovery of lactose and lactulose the comparisonwith published data on (Chávez-Servin et al. 2004; Indyk et al. 1996; La Pera et al. 2007) additionally validates the suitability of the developed method for reliable and sensitive detection as well as for quantification of lactose and lactulose.

5 Conclusion

We developed a HPLC method for simultaneous detection and quantification of lactose and lactulose. Among four different HPLC columns a column with an amino-modified polymeric resin showed the best peak resolution (R_S) for the disaccharides lactose and lactulose (R_S=5.68) within a short retention time. The developed HPLC method featured a high sensitivity (LOD<3 mg·L^{-1}) and precision (recovery>99%). Analysis of lactulose-containing sterilized milk and lactose-reduced yoghurt confirmed the method's suitability for detection and quantification of lactulose and lactulose.

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