

1     **A RELIABLE GAS CAPILLARY CHROMATOGRAPHIC DETERMINATION**  
2                                   **OF LACTULOSE IN DAIRY SAMPLES**

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14    *Key words*

15    Gas chromatography

16    Lactulose

17    Milk

18    Heat treatment

19

1 *Abstract*

2

3 A gas capillary chromatography method for the determination of lactulose has been  
4 developed. The method has been evaluated for precision and accuracy using phenyl- $\beta$ -  
5 D-glucoside as internal standard with satisfactory results and, then, applied to 27  
6 commercial milk samples (pasteurized, UHT, sterilized, powder, condensed and  
7 chocolate-based milks). Results showed that it was suitable for the determination of  
8 lactulose in milks subjected to heat treatments of different intensity, giving good  
9 chromatographic resolution, as well as precise and reproducible results. Thus, lactulose  
10 levels found in pasteurized, UHT, sterilized and reconstituted powder milks were  
11 similar to those previously reported. In addition, this method provided a good separation  
12 between sucrose and lactulose/lactose peaks which allowed the suitable quantification  
13 of lactulose in samples such as condensed and chocolate-based milks which contain a  
14 high concentration of sucrose.

15

1 **Introduction**

2

3 Lactulose (4-O- $\beta$ -D-galactopyranosyl-D-fructofuranose) is a disaccharide  
4 formed by isomerization of lactose in basic media and during heat treatment of milk [1].  
5 It has been proposed as a chemical indicator capable of distinguishing UHT and  
6 sterilized milks [2, 3]. Also, having a sensitive analytical method for its detection,  
7 lactulose could be used for differentiating between UHT and pasteurized milks [4-6], as  
8 well as within different types of UHT and pasteurized milks either in combination with  
9 other indicators [7] or by itself [8].

10 The main analytical methods used for determination of lactulose are based on  
11 gas [9] and liquid [10] chromatography and enzymatic procedures [11, 12]. The main  
12 drawback of the official enzymatic method [11] is the requirement of six different  
13 enzymes to avoid the effects of glucose interference making the assay very time-  
14 consuming and expensive. Recently, other enzymatic methods exhibiting a higher  
15 sensitivity have been developed [8, 12]. Nevertheless, the main difficulty for the  
16 accurate measurement of low levels of lactulose using these methods is the potential  
17 interference from any free fructose which might originally be present in some types of  
18 milk. This point is particular important in special milks such as chocolate-based milks  
19 or condensed milks which contain considerable amount of sucrose and, consequently,  
20 may contain fructose.

21 As carbohydrates have neither chromophore nor fluorophore groups, lactulose  
22 determination by liquid chromatography is normally attained by isocratic separations  
23 with refractive index detection giving, thus, a relatively high detection limit [10]. Also,  
24 very time-consuming HPLC methods using a post-column labelling of lactulose

1 compatible with gradient elution have been developed [13, 14]. Although derivatization  
2 is necessary, gas chromatography provides very good separation of lactulose from other  
3 carbohydrates and the sensitivity can be higher than in HPLC methods [15]. Despite  
4 capillary columns provide higher efficiency, the presence of huge amounts of lactose in  
5 milk can explain the fact that laboratory-prepared packed and micropacked columns,  
6 which have a larger sample capacity, have been widely used for lactulose determination  
7 [9, 16-19]. Nevertheless, the GC quantitative determination of low amounts of lactulose,  
8 as those present in pasteurized and powder milks, using these micropacked columns can  
9 be inaccurate as consequence of the high content of lactose which can led to an  
10 overlapping with the lactulose peaks. Thus, a 48 hours treatment with ethanol of milk  
11 samples for partial removal of lactose was proposed prior to analysis [20].

12 In this paper we describe a reliable, sensitive and highly selective gas  
13 chromatographic method using a commercial capillary column for lactulose  
14 determination in milk samples, including special milks, subjected to different heat  
15 treatments.

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1 **Experimental**

2

3 *Materials*

4 *Chemicals*

5 Reagents employed for GC analysis including sugar standards (lactose,  
6 lactulose, sucrose), internal standard ( $\beta$ -phenyl-glucoside) and derivatising reagent (*N*-  
7 trimethylsilylimidazole) were obtained from Sigma (St. Louis, USA). *N,N*-  
8 Dimethylformamide 99% was from Merck (Darmstadt, Germany). Ultrapure water  
9 quality with 1 – 5 ppb TOC and < 0.001 EU/mL pyrogen levels (Milli-Q) was produced  
10 in-house using a laboratory water purification Milli-Q Synthesis A10 system (Millipore,  
11 Bellerica, Mass., USA) and was used throughout.

12

13 *Milk samples*

14 A total of 27 commercial samples were purchased from local stores: 10 UHT, 6  
15 pasteurized, 3 chocolate-based, 3 condensed, 2 sterilized, 2 powder milks and 1 powder  
16 dairy product. All samples were analysed before the recommended expire date. Powder  
17 milk samples were reconstituted with deionised water to 10% total solids before  
18 treatment. In addition, two samples of bovine raw milk, obtained from a local farm,  
19 were also analysed. The pH of all samples was measured in a MP 225 pH meter with  
20 glass electrode (Mettler-Toledo GmbH, Schwerzenbach, Switzenbach, Switzerland).

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1 *GC analysis*

2 *Sample preparation*

3 1 mL of milk sample was added with methanol up to 10 mL in a volumetric  
4 flask to remove proteins and fats. Mixtures were gently stirred followed by standing for  
5 at least 1 hour at room temperature until the supernatant became transparent.  
6 Supernatants were employed for carbohydrate analysis and a solution of 0.01% (w:v)  
7 phenyl- $\beta$ -D-glucoside in methanol/water (70:30, v/v) was added as internal standard.  
8 Prior to derivatization equal volumes (0.5 mL) of supernatant and internal standard were  
9 mixed and dried at 38-40°C in a rotavapor.

10

11 *Derivatization and GC analysis*

12 0.1 mL of N,N-Dimethylformamide were added to the dried mixtures and held at  
13 65°C for 1 hour to obtain a constant anomeric composition. Then, 100  $\mu$ L of *N*-  
14 trimethylsilylimidazole were added to silylate the carbohydrates and the reaction was  
15 completed in 30 minutes at 65°C. The reaction is stopped by cooling and the silylated  
16 carbohydrates extracted with 0.1mL of hexane and 0.2 mL of water. Volumes in the  
17 range of 1-2  $\mu$ L of the organic phase containing silyl derivates were injected onto the  
18 column. To study the response factor relative to the internal standard, standard solutions  
19 containing lactulose and lactose were prepared. The identity of lactulose and lactose  
20 present in milk samples was confirmed by comparison with relative retention times of  
21 standard samples.

22 The trimethylsilyl ethers were separated using a commercial 30 m x 0.32 mm  
23 inside diameter, 0.5  $\mu$ m film fused silica capillary column SPB<sup>TM</sup>-17, bonded,  
24 crosslinked phase poly (50% diphenyl/50% dimethylsiloxane) (Supelco, 595 North

1 Harrison Road, Bellefonte, PA, USA). Separation was performed at 235°C for 9.5min,  
2 followed for an increase up to 270°C at rate of 20°C/min and keeping this temperature  
3 for 5 minutes. Temperature of injector and detector was 300°C during the analysis.  
4 Injections were carried out in split mode (1:50-1:60). Data was acquired by means of  
5 HP ChemStations (Hewlett Packard, Wilmington, DE. USA).

6

## 1 Results and Discussion

2  
3 Fig. 1 illustrates the GC profiles of raw, raw with added lactulose (20.8 mg L<sup>-1</sup>)  
4 and chocolate-based milks. In all samples analysed, the separation was accomplished in  
5 12 minutes with excellent chromatographic resolution of the lactulose and lactose peaks.  
6 The signal corresponding to lactulose comprised a narrow and symmetrical peak  
7 preceded by a minor and very broad peak (Fig. 1C), indicative of the presence of several  
8 anomeric forms not resolved by GC [6]. However, the equilibration with N,N-  
9 dimethylformamide before derivatization allowed to obtain a constant anomeric  
10 composition. Another advantage of this method is the separation of sucrose from  
11 lactulose and lactose peaks (Figs. 1C). The GC peaks of these carbohydrates frequently  
12 overlapped using the methods described in the literature [6, 9, 16, 21].

13 Prior to quantification of the lactulose and lactose in milk samples, the suitability  
14 of the method was evaluated. The response factors obtained for each disaccharide  
15 relative to the internal standard determined over the expected operating range of lactose  
16 (50 g L<sup>-1</sup>) and lactulose (range 10.4 mg L<sup>-1</sup> – 2080 mg L<sup>-1</sup>) were 0.86± 0.07 and 1.08± 0.09,  
17 respectively. The precision of the entire method was determined by analysing the  
18 lactulose content in the same UHT milk sample within the same day (*n*=12) and in 5  
19 different days (*n*=10), obtaining a relative standard deviation of 3.7% and 5.1%,  
20 respectively. Finally, to evaluate the accuracy of the method, known amounts of  
21 lactulose in the range of 10.4 to 520mg L<sup>-1</sup> were added to raw milk samples. As shown  
22 in Table 1, satisfactory recoveries were obtained for the whole range studied.

23 Table 2 summarises the concentration of lactulose and lactose found in the  
24 commercial samples analysed. This method was sensitive enough to detect and quantify



1 lactulose in all samples, including pasteurized milks. A clear differentiation was  
2 obtained among pasteurized, UHT and sterilized milks with levels ranged from 13.0 mg  
3 L<sup>-1</sup> to 32.1 mg L<sup>-1</sup>, 95.6 mg L<sup>-1</sup> to 437.1 mg L<sup>-1</sup> and above 622.7 mg L<sup>-1</sup>, respectively.  
4 These contents were within the ranges reported previously [4, 6, 20, 22-28].

5         The levels of lactulose found in powder milk samples were considerably lower  
6 than in UHT milks as previously reported [24] indicating the milder processing  
7 conditions to which powder milk is subjected in the industry. Moreover, the  
8 isomerization of lactose is relatively slow in the solid state [29], being the condensation  
9 with proteins via the Maillard reaction the predominating reaction of lactose during the  
10 storage of milk powders [24, 30, 31].

11         Regarding lactulose levels in condensed and chocolate-based milk samples, to  
12 our best knowledge, no data are available in the literature. This can be explained by the  
13 fact that sucrose and lactulose/lactose peaks were not resolved with the GC methods  
14 described for the lactulose determination [6, 9, 16, 21]. Thus, the lactulose levels  
15 determined for condensed milks were within the range observed for UHT milks,  
16 whereas the levels detected in chocolate-based milks were even higher than those found  
17 in the sterilized milks. This can suggest a very severe heat treatment, specially in  
18 samples no. 23 and 25 which are labelled as UHT products (Table 2). Interestingly, the  
19 pH of all milk samples was within the normal values (6.6-6.8) except for the sample no.  
20 25 which had a pH value of 7.3 and, consequently, unusually high levels of lactulose  
21 (1955.3 mg L<sup>-1</sup>). Martínez-Castro and Olano [18] indicated that at pH values above 7  
22 the formation of lactulose increased markedly during heat treatment of milk.

23         In view of these results, it can be concluded that the proposed method allows a  
24 reliable quantitative determination of lactulose by gas capillary chromatography in

1 commercial milk samples subjected to different heat treatments. In addition, the  
2 separation of sucrose from lactulose and lactose enables the correct determination of  
3 lactulose in milks which contain a high amount of sucrose such as condensed and  
4 chocolate-based milks.

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1 **References**

2

3 [1] Adachi S, Patton S (1961) J Dairy Sci 44: 1375-1393

4 [2] European Commission (EC) (1992) Dairy Chemists' Group-Doc VI/5726/92,  
5 Project de decision de la commission fixant les limites et les methods permettant de  
6 distinguer les differents types de laits de consumations traits thermiquement.

7 [3] International Dairy Federation (IDF) B-Doc 235, Influence of Technology on the  
8 quality of heated milk and fluid milk products, 1993.

9 [4] Geier H, Klostermeyer H (1983) Milchwissenschaft 38: 475-477

10 [5] Andrews GR (1984) J Soc Dairy Technol 37: 92-95

11 [6] Corzo N, Olano A, Martínez-Castro I (1986) Rev Agroquím Tecnol Aliment 26:  
12 565-570

13 [7] Villamiel M, Arias M, Corzo N, Olano A (1999) Z Lebensm Unters Forsch A 208:  
14 169-171

15 [8] Marconi E, Messia MC, Amine A, Moscone D, Vernazza F, Stocchi F, Palleschi G  
16 (2004) Food Chem 84: 447-450

17 [9] Olano A, Calvo MM, Reglero G (1986) Chromatographia 21: 538-540

18 [10] International Dairy Federation (IDF) Standard 147. Heat-treated milk.  
19 Determination of lactulose content, High Performance Liquid Chromatography  
20 (Reference Method), 1991

21 [11] International Dairy Federation (IDF) Standard 175. Determination of lactulose  
22 content, Enzymatic Method, 1995

23 [12] Amine A, Moscone D, Bernardo RA, Marconi E, Palleschi G (2000) Anal Chim  
24 Acta 406: 217-224

- 1 [13] Ersser RS, Mitchell JD (1984) *J Chromatogr* 307: 393-398
- 2 [14] Reimerdes EH, Rothkitt KD (1985) *Z Lebensm Unters Forsch* 181: 408-411
- 3 [15] Martínez-Castro I, Calvo MM, Olano A (1987) *Chromatographia* 23: 132-136
- 4 [16] Haverkamp J, Kamerling JP, Vliegthart JFG (1971) *J Chromatogr* 59: 281-287
- 5 [17] Martínez-Castro I, Olano A (1978) *Rev Esp Lechería* 110: 213-217
- 6 [18] Martínez-Castro I, Olano A (1980) *Milchwissenschaft* 35: 5-8
- 7 [19] Olano A, Martinez-Castro I (1981) *Milchwissenschaft* 36: 533-536
- 8 [20] de Rafael D, Calvo MM, Olano A (1996) *Milchwissenschaft* 51: 552-553
- 9 [21] Nikolov ZL, Reilly PJ (1983) *J Chromatogr* 254: 157-162
- 10 [22] Olano A, Calvo MM, Corzo N, (1989) *Food Chem* 31: 259-265
- 11 [23] Andrews G (1989) *Bulletin of the International Dairy Federation* 238: 45-52
- 12 [24] Corzo N, Delgado T, Troyano E, Olano A, (1994) *J Food Prot* 57: 737-739
- 13 [25] Pellegrino L, De Noni I, Resmini P, (1995) *Int Dairy J* 5: 647-659
- 14 [26] Akalin AS, Gönc S (1997) *Milchwissenschaft* 52: 377-380
- 15 [27] Valero E, Villamiel M, Miralles B, Sanz J, Martínez-Castro I. (2001) *Food Chem*
- 16 72: 51-58
- 17 [28] Rada Mendoza M, Olano A, Villamiel M, (2005) *J Agric Food Chem* 53: 2995-
- 18 2999
- 19 [29] Olano A, Martínez-Castro I (1989) *Bulletin of the International Dairy Federation*
- 20 238: 35-44
- 21 [30] Hurrell RF, Finot PA, Ford JE (1983) *Brit J Nutr* 49: 343-354
- 22 [31] Resmini P, Pellegrino L, Masotti F, Tirelli A, Prati F (1992) *Sci Tec Latt-Cas* 43:
- 23 169-186
- 24

1 **Table 1.** Recoveries (%) of different amounts of lactulose added to raw milk analysed  
2 by GC.  
3

Added lactulose (mg L <sup>-1</sup> )	Recovered lactulose (mg L <sup>-1</sup> )	Recovery (%)
10.4	11.5 (14.2%)*	110.6
20.8	20.3 (6.5%)	97.6
52	46.8 (3.5%)	90.0
104	94.6 (6.9%)	91.0
208	186.4 (2.8%)	89.6
520	480.5 (2.0%)	92.4

4 \* Relative standard deviation in brackets,  $n=4$

1 **Table 2.** Lactulose and lactose contents of different commercial milk samples.

Type of milk	Samples	Lactulose (mg L <sup>-1</sup> )	Lactose (g L <sup>-1</sup> )
Pasteurized	1	14.2 (9.9%)*	44.9 (6.6%)
	2	14.1 (9.4%)	51.3 (6.8%)
	3	13.0 (11.8%)	52.2 (11.8%)
	4	20.2 (8.0%)	44.0 (7.9%)
	5	32.1 (7.6%)	46.9 (2.5%)
	6	27.7 (13.1%)	53.1 (9.2%)
UHT	7	437.1 (5.9%)	52.0 (5.3%)
	8	162.6 (5.8%)	47.3 (1.5%)
	9	307.0 (9.4%)	46.9 (7.4%)
	10	384.1 (6.1%)	51.6 (7.7%)
	11	178.1 (7.6%)	53.6 (9.1%)
	12	186.6 (4.0%)	46.9 (1.3%)
	13	331.7 (2.9%)	48.7 (1.1%)
	14	95.6 (5.9%)	51.1 (4.5%)
	15	327.9 (5.4%)	49.2 (4.7%)
	16	171.9 (3.7%)	50.1 (5.6%)
Powder dairy products	17	37.4 (8.8%)	80.8 (3.8%)
Powder (reconstituted)	18	24.2 (13.2%)	63.1 (4.8%)
	19	48.8 (1.0%)	53.1 (5.6%)
Condensed	20	207.5 (9.8%)	78.0 (3.7%)
	21	301.4 (9.4%)	50.7 (6.9%)
	22	313.3 (6.3%)	88.2 (14.2%)
Chocolate-based	23	1064.7 (7.9%)	45.7 (6.2%)
	24	758.2 (7.0%)	44.1 (5.3%)
	25	1955.3 (4.5%)	46.0 (6.8%)
Sterilized	26	705.2 (8.2%)	47.7 (8.4%)
	27	622.7 (2.5%)	48.7 (2.3%)

2 \*Relative standard deviation in brackets, n=4

1 **Legends of the figures**

2

3 **Figure 1.** Gas chromatography profiles of trimethylsilyl derivatives of disaccharides  
4 from (A) raw milk, (B) raw milk with 20.8 mg L<sup>-1</sup> of added lactulose and (C) chocolate-  
5 based milk. 1= phenyl-β-D-glucoside (internal standard), 2= lactulose, 3= sucrose, 4  
6 and 5= lactose.

Figure 1. Montilla et al 2005

