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Alternative reversed-phase high-performance liquid chromatography method to analyse organic acids in dairy products

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Abstract

A RP-HPLC method for the analysis of oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic and butyric acids in dairy products with a simple treatment of the sample has been developed. A gradient programme pumping phosphate buffer at pH 2.20 and acetonitrile was used to separate the compounds on a C_{18} column. Various parameters affecting analysis have been optimised to take <18 min with an excellent linearity ($R > 0.999$). The precision was good (R.S.D. < 5%) and the recovery found close to 100%. Its application to analyse the quality of some dairy products has been investigated.

Keywords: Validation; Milk; Cheese; Yoghurt; Organic acids

1. Introduction

Organic acids appear in dairy products as a result of hydrolysis of milk fat (free fatty acids such as acetic or butyric), direct addition as acidulants (citric and lactic), normal bovine biochemical metabolism (citric, orotic and uric) or bacterial growth (lactic, acetic, pyruvic, propionic and formic). Also, they are the major products of carbohydrate catabolism of lactic acid bacteria (LAB). Their ability to produce acids with resulting pH reduction is the major factor in milk fermentation [1]. The resulting acidity prevents the development of spoilage and pathogenic microorganisms, improving the hygienic quality of dairy products. However, it is important to note that the ability of LAB to inhibit undesirable bacteria depends not only on the reduction of pH, but also on the sort of organic acids they produce [1]. Quantitative determination of organic acids is important to monitor bacterial growth and activity and for nutritional reasons. Organic acids are also important because they contribute to the flavour and aroma characteristics of dairy products [2].

Although some CE methods have been developed to analyse organic acids in dairy products [3,4], these compounds have been commonly analysed by chromatographic techniques. Most methods developed to analyse organic acids in dairy products are HPLC methods that utilise ion-exchange columns [2,5,6]. Most of the ion-exchange methods use dilute sulphuric acid as the mobile phase, high operating temperatures at about 60 °C, expensive ion-exchange columns [2]; also co-elution of some of the organic acids and overlapping peaks have been frequently reported [7]. Our RP-HPLC method is advantageous in some of these respects: the use of more inexpensive columns, easier manipulation of the analytical parameters to optimise the separation, and the analyses were carry out at room temperature. Use of high temperatures would require a temperature control module for the column and would shorten the life of the column. RP-HPLC has been successfully used to measure simultaneously a variety of organic acids and phenolic compounds in fruit juice [8], although a run time longer than 75 min was needed.

The aim of this work was to develop a RP-HPLC technique alternative to the ion-exchange methods for the simultaneous determination of 11 organic acids metabolically important in dairy products and most commonly cited in the literature, including oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic and butyric acids. Various parameters affecting the analysis, including composition and pH of the buffer, flow and temperature, have been optimised and the validation of the developed method has been performed. Its application to the analysis of the quality of some dairy products has been investigated.

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2. Experimental

2.1. Chemicals

Oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic, butyric, maleic and phosphoric acids, and sodium phosphate were purchased from Sigma (St. Louis, MO, USA), and gradient HPLC-grade acetonitrile from Panreac Química (Barcelona, Spain). Milli-Q water (Bedford, MA, USA) was used to prepare buffers, stock solutions of each standard compound and the samples.

2.2. Sample preparation

Raw milk was obtained from the Centro de Investigaciones Agrarias de Mabegondo (A Coruña, Spain) and the commercial samples of yoghurt and cheese were purchased at local stores. One gram of sample was diluted to 10 ml (5 ml in the case of cheese) with water containing 2 μg ml^{-1} of maleic acid as I.S. and the preparation was vigorously shaken and blended with a vortex. In the case of cheese, the mixture was homogenised with a high sear blender (Ultra Turrax). The samples were centrifuged at 3000 × *g* (14,000 × *g* in the case of milk) for 15 min and 1 ml of the supernatant was filtered through 0.45 μm poly(vinylidene difluoride) (PVDF) membranes (Waters) before injecting. When testing sulphuric acid to extract the organic acids, 4.5 mM H_2SO_4 was used instead of water to dilute the sample.

2.3. Equipment and operating conditions

The analysis was carried out on a Breeze System (Waters, Mildford, MA, USA) consisting of a 1525 binary HPLC pump, a 717 plus autosampler and a 2487 two-channel UV detector set at 210 nm, operated using a Breeze software. The separation was performed on a Atlantis dC_{18} column (Waters) 250 mm × 4.6 mm, 5 μm. Twenty millimoles of NaH_2PO_4 adjusted to pH 2.20 with phosphoric acid was prepared daily and filtered through 0.2 μm hydrophilic polyethersulfone Supor 200 membranes (Pall Gelman Laboratory, Ann Arbor, MI, USA). Unless otherwise stated, the solvent programme utilised two reservoirs containing 1% of acetonitrile in 20 mM phosphate buffer adjusted at pH 2.20 with phosphoric acid (Solvent A) and acetonitrile (Solvent B); the flow rate was set at 1.5 ml min^{-1} at room temperature. The gradient programme started with 100% of Solvent A and after 7 min Solvent B was increased linearly to reach 7% in 5 min. From 12 to 19 min the rate was kept at 93% of Solvent A and 7% of Solvent B. After that the rate was changed to the starting conditions to equilibrate the column for 15 min before injecting again 10 μl of the next sample.

2.4. Validation parameters and statistical treatment

For the determination of linearity, regression lines were calculated as $y = a + bx$, where x was concentration, and y the response. Five concentration points in triplicate were used to prepare the calibration curves. The concentrations of each compound were prepared from stock solutions by dissolving the proper quantity in 10 ml of water containing 0.017 mM maleic acid as an I.S. For each compound, the coefficients of determination (R^2) were calculated and the linearity was analysed on the basis of the relative standard deviation (R.S.D.) values for the corresponding response factors. Detection limits were estimated as $(3a/b) \times 1/\sqrt{n}$, where a is the independent term of the curve, b the slope and n the number of replicates. Intra-day repeatability of the method was analysed by calculating the R.S.D. values for the responses and retention times of six replications. Day-to-day repeatability was estimated by calculating the R.S.D. values of a standard mixture analysed in triplicate during 3 consecutive days. Accuracy was determined using an added external standard. A sample of yoghurt was spiked in triplicate with known quantities of five of the organic acids migrating along the chromatogram and the percentage of recovery was calculated. The percentage of recovery rate was established from the experimental response values [(blank + standard) − blank] obtained according to the calibration curves and the real concentration of the standard added.

3. Results and discussion

3.1. Optimisation of the method

Several parameters such as flow, pH and concentration of the buffer, percentage of organic solvent in the buffer or temperature of the column were tested in order to obtain the best resolution of the analytes. Table 1 shows the effect of such parameters on the t_R of the acids. A 250 mm L column was used to work with the maximum efficiency available. Buffer at several pH values lower than the pK_a of the compounds were used to ensure all the acids were not ionised when testing the effect of the pH on the separation.

At pH 3.0, a co-elution of formic–pyruvic and of lactic–orotic was observed (Table 1). By decreasing to pH 2.5 the resolution of lactic acid with respect to orotic and acetic was complete. The best results were obtained at pH 2.2, with all the compounds resolved in <17 min except butyric acid, which was strongly retained in the column, and the order of elution for orotic and acetic was changed.

By increasing the flow to 1.5 ml min^{-1} we shortened the migration time without affecting considerably the resolution. However, the width of propionic acid was not improved and butyric acid did not appear on the chromatogram. Addition of organic solvents was tested to help the latest peaks to migrate faster through the column. Instead of methanol, acetonitrile was used because the background absorbance at 210 nm of acetonitrile is much lower. By adding 1% acetonitrile the t_R was shortened and the resolution remained similar. Also, the shape of the peaks was improved noticeably.

Table 1
Retention time (t_R) for the organic acids at the flow (F), pH and percentage of acetonitrile (% Acn) indicated[a]

F	pH	Conc.	Acn (%)	Oxalic	Formic	Pyruvic	Lactic	Acetic	Orotic	Citric	Succinic	Uric	Propionic	Butyric
1	3.0	0.02	–	2.91	**3.89**	**3.89**	5.6	6.29	5.61	7.90	11.26	12.5	16.41	–
1	2.7	0.02	–	2.97	3.85	4.18	**5.86**	6.37	**6.09**	9.47	11.71	12.42	16.84	–
1	2.7	0.05	–	2.93	3.85	4.07	**5.78**	6.33	**5.94**	9.24	11.61	12.67	16.58	–
0.5	2.7	0.02	–	5.80	7.50	8.22	11.57	12.63	12.03	18.83	23.28	24.66	33.34	–
1	2.5	0.02	–	3.02	3.90	4.34	5.91	**6.58**	**6.41**	10.27	11.82	12.67	16.70	–
1	2.2	0.02	–	3.06	3.89	4.58	5.93	6.32	7.5	10.75	11.61	12.44	16.29	–
1.5	2.2	0.02	–	2.06	2.61	3.04	3.92	4.17	4.95	6.96	7.50	10.60	13.66	–
1.5	2.2	0.02	1	2.06	2.56	2.77	3.57	3.76	4.26	5.09	5.68	5.97	8.08	23.55
1.5	2.2	0.02	2	2.06	**2.65**	**2.76**	3.35	3.56	3.78	4.67	5.28	5.18	7.76	22.26

Bold or underlined values in the same row mean co-migration or change of the order of elution of the corresponding peaks as mentioned in the text.
[a] F (ml min^{-1}); concentration (M); r_T (min).

However, butyric acid migrated after 23.5 min with a long tail making its quantification very difficult. Increasing to 2% acetonitrile a co-migration pyruvic–formic and uric–succinic was observed and the selectivity for uric and succinic was changed. Since the selectivity of the middle peaks is greatly affected by the presence of acetonitrile in the buffer, we could only work with increasing gradients after the 6th min. The minimum concentration of acetonitrile needed to obtain an acceptable peak shape for butyric acid to allow its quantification was 8% (Fig. 1). Heating the column reduced the analysis time but the resolution was sacrificed. The optimum temperature for this analysis was room temperature (24 ± 1 °C) and small variations within this range did not affect significantly the results.

3.2. Validation of the method

Calculation of the validation parameters was based on the indications suggested by Castro et al. [9].

Table 2 shows the results of the analysis of the linearity and detection limits for the 11 organic acids analysed using maleic acid as an I.S. Five levels in triplicate within the range of concentration indicated in the table were used to build the calibration curves. The data points from calibration curves were subjected to a least square regression analysis. The slope (a), intercept (b) and coefficient of determination (R^2) were calculated. Detection limits were estimated as $(3a/b) \times 1/\sqrt{n}$, where a is the independent term of the curve, b the slope and n the number of replicates. The coefficients of determination (R^2) obtained were excellent with values better than 0.999, except for butyric acid (0.9986). To verify the linearity of the method, the response factor (f) were calculated by dividing the area under the peak obtained in the chromatogram and the corresponding concentration. The R.S.D. values of f were in the range of 0–5% considered adequate to verify the linearity of the regression lines for analytical methods [9].

Table 3 shows the results obtained when studying the precision of the method. As the high sensitivity of the organic acids migrating between acetic and propionic in the presence of acetonitrile, the R.S.D. values for the t_R for these acids were slightly higher than the others, although all of them were lower than 1%. That high sensitivity is the reason why 15 min were required to recondition the column between runs in order to ensure the repeatability of the analysis. A standard mixture ($n = 6$) was used to calculate the precision of the analytical technique. Furthermore, another similar standard mixture analysed in triplicate during three

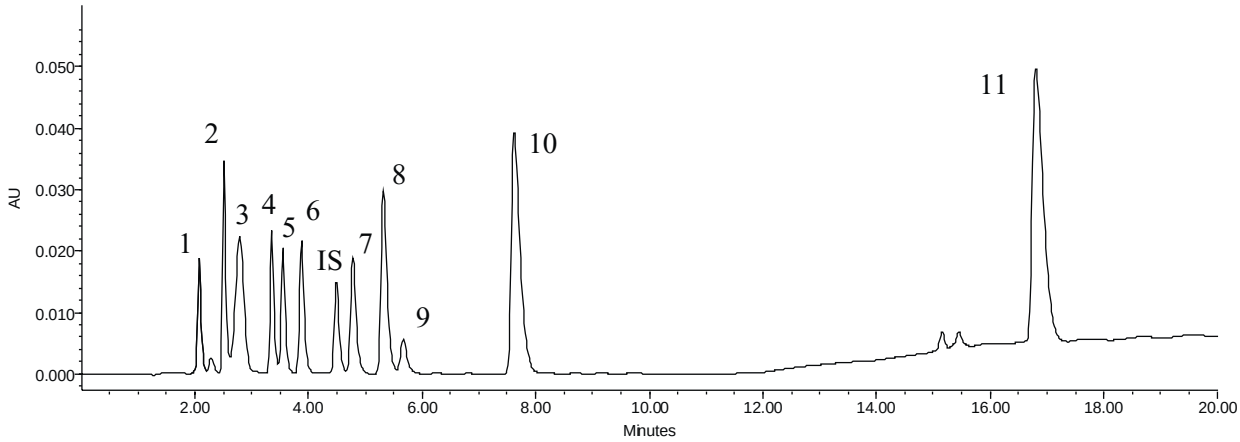

Fig. 1. Chromatogram of a standard mixture of: (1) 0.159 mM oxalic acid, (2) 8.472 mM formic acid, (3) 0.602 mM pyruvic acid, (4) 6.561 mM lactic acid, (5) 7.526 mM acetic acid, (6) 0.032 mM orotic acid, (I.S.) 0.017 mM maleic acid, (7) 0.157 mM citric acid, (8) 8.510 mM succinic acid, (9) 0.119 mM uric acid, (10) 26.998 mM propionic acid, and (11) 32.471 mM butyric acid.

Table 2
Regression equations for the calibration curves and analysis of the linearity

Acid	Range (mM)	Regression equation	R^2	R.S.D. of f (%)	Limit of detection (mM)
Oxalic	0.008–0.402	$y = 5.9641x + 0.0169$	0.9999	2.39	0.005
Formic	0.423–21.142	$y = 0.2009x - 0.0043$	0.9999	1.24	0.037
Pyruvic	0.030–1.514	$y = 4.3819x + 0.0137$	0.9996	2.16	0.005
Lactic	0.328–16.405	$y = 0.1931x + 0.0029$	0.9999	0.91	0.026
Acetic	0.376–188.15	$y = 0.156x - 0.0045$	0.9999	1.87	0.050
Orotic	0.002–0.081	$y = 41.890x - 0.00104$	0.9999	1.85	0.0004
Citric	0.078–3.923	$y = 0.9149x + 0.0075$	0.9999	2.63	0.014
Succinic	0.423–21.276	$y = 0.2894x + 0.0088$	0.9999	0.44	0.053
Uric	0.117–0.588	$y = 19.937x + 0.1379$	0.9994	1.95	0.012
Propinic	1.357–67.832	$y = 0.1728x + 0.0133$	0.9999	0.50	0.133
Butyric	1.623–81.175	$y = 0.2086x + 0.055$	0.9986	4.35	0.457

straight days ($n = 9$) was used to calculate the day-to-day repeatability. As expected, the R.S.D. values obtained when studying the intra-day repeatability were lower than those obtained day-to-day, although the deviation was <5%.

The recovery for the extraction of organic acids from milk and cheese with 4.5 mM H_2SO_4 or water has been reported in the literature [2,6,8], thus we did not intend to validate the method of extraction. However, we compared both methods to establish the efficiency for each solvent. As shown in the Table 4, a Student t-test was carried out and no significant differences ($P < 0.05$) were found between both methods when extracting the organic acids from milk, consequently the use of diluted acids could be avoided. In this case, the milk sample should be centrifuged at 14,000 × g for 15 min in order to obtain a supernatant clear enough to be injected in the HPLC. In fact, the use of sulphuric acid precipitates the proteins and seems to improve the extraction of some organic acids such as citric, succinic and uric in cheese, although the extraction of acetic and orotic is poorer (data not shown).

Yoghurt spiked with five organic acids (pyruvic, acetic, citric, propionic and butyric) appearing at different zones along the chromatogram was used to calculate the recovery of the method. One gram of yoghurt was extracted in triplicate with water containing the I.S. Additionally, yoghurt spiked with known concentrations of the five organic acids mentioned was extracted similarly. The recovery was calculated from the concentration quantified using the calibration curves versus the concentration added, obtaining values close to 100% for all of them (Table 5). These results were similar to the recovery values recorded by some authors [2,8].

3.3. Application for commercial dairy samples

Fig. 2 shows the chromatogram obtained when analysing milk, yoghurt and cheese. As shown in figure some unknown peaks appeared on the chromatogram in addition to the compounds under study, especially in the case of cheese.

Citric acid was the most abundant organic acid present in raw milk (Table 6). The quantities recorded were slightly lower (6.5 mM) than the normal range (7–11 mM) for total concentration of citric acid in milk [11]. In milk, most of the citrate (90%) is present in the serum, forming relative soluble complexes with Ca and Mg, and practically the rest is contained in the micelles of casein as colloidal particles [11]. It may be possible that some of the citrate present is in an insoluble form in the sample after it is dissolved in water. However, once the sample was injected and mixed with the acid buffer (pH 2.20), all the citrate seemed to have completely dissolved and recovered for its detection. This could explain the fact that no differences were found when

Table 3
Results of the analysis of intra-day ($n = 6$) and day-to-day ($n = 9$) repeatability

Acid	Concentration (mM)	R.S.D. for t_R (%)	R.S.D. for A (intra-day) (%)	R.S.D. for A (day-to-day) (%)
Oxalic	0.322	0.08	0.04	0.56
Formic	16.914	0.09	0.11	0.92
Pyruvic	1.211	0.03	0.33	3.45
Lactic	13.124	0.06	0.31	0.55
Acetic	15.052	0.11	0.46	1.26
Orotic	0.065	0.45	0.32	0.45
Citric	3.138	0.47	0.64	1.06
Succinic	17.021	0.34	0.42	1.24
Uric	0.469	0.67	0.17	0.92
Propionic	54.266	0.14	0.24	0.34
Butyric	64.940	0.15	0.79	1.50

Table 4
Analysis of milk extracted (1 g/10 ml) with water or 4.5 mM H_2SO_4

Acid	Milk + water	Milk + 4.5 mM H_2SO_4	Difference	Student *t*-test
Oxalic	–	–		
Formic	–	–		
Pyruvic	0.6 ± 0.1[a]	0.7 ± 0.2	0.1	2.8×10^{-1}
Lactic	14.6 ± 1.9	14.2 ± 3.7	−0.3	4.6×10^{-1}
Acetic	8.7 ± 0.5	5.5 ± 2.1	−0.3	8.9×10^{-2}
Orotic	8.7 ± 0.2	8.4 ± 0.3	−0.1	1.4×10^{-1}
Citric	123.5 ± 2.1	124.0 ± 6.2	0.5	4.6×10^{-1}
Succinic	–	–		
Uric	6.8 ± 0.1	6.7 ± 0.2	−0.1	4.5×10^{-1}
Propionic	–	–		
Butyric	–	–		

[a] Standard deviation ($n = 3$). All the concentrations are expressed in ppm (w/w) and represent the concentration of the sample once dissolved with 10 ml of solvent.

Table 5
Percentage recovery of the organic acids added to yoghurt ($n = 3$)

Acid	Yoghurt[a]	Spiked yoghurt[a]	Spiked yoghurt − yoghurt[a]	Std. added[a]	Recovery (%)
Pyruvic	5.6	18.6	13.0	13	97
Acetic	50.0	169.0	119.0	110	105
Citric	2570.0	3290.0	720.0	800	96
Propionic	–	568.8	568.8	503	113
Butyric	–	767.0	767.0	720	107

[a] All the concentrations are expressed in ppm (w/w) and represent the concentration of the sample once dissolved with 10 ml of water.

using H_2SO_4 or H_2O as solvent to extract the organic acids from milk (Table 4).

Lactic acid was the most abundant organic acid found in yoghurt (14 509.8 mg/100 g dry matter) and in cheese (14 601.5 mg/100 g dry matter) (Table 6). This acid is the major end product derived from the fermentation of the carbohydrate (lactose in the case of milk) by LAB [2]. Concentration of orotic acid found in yoghurt (∼80 mg/100 g dry matter) was equivalent to the values found when analysing orotic acid by CE [3] and slightly higher than the values recorded by Fernández-García and McGregor [10] when using HPLC. The quantity of orotic acid in milk depends on the cow's origin, diet and lactation [12]. It is an intermediate product in the synthesis of nucleotides and a growth factor

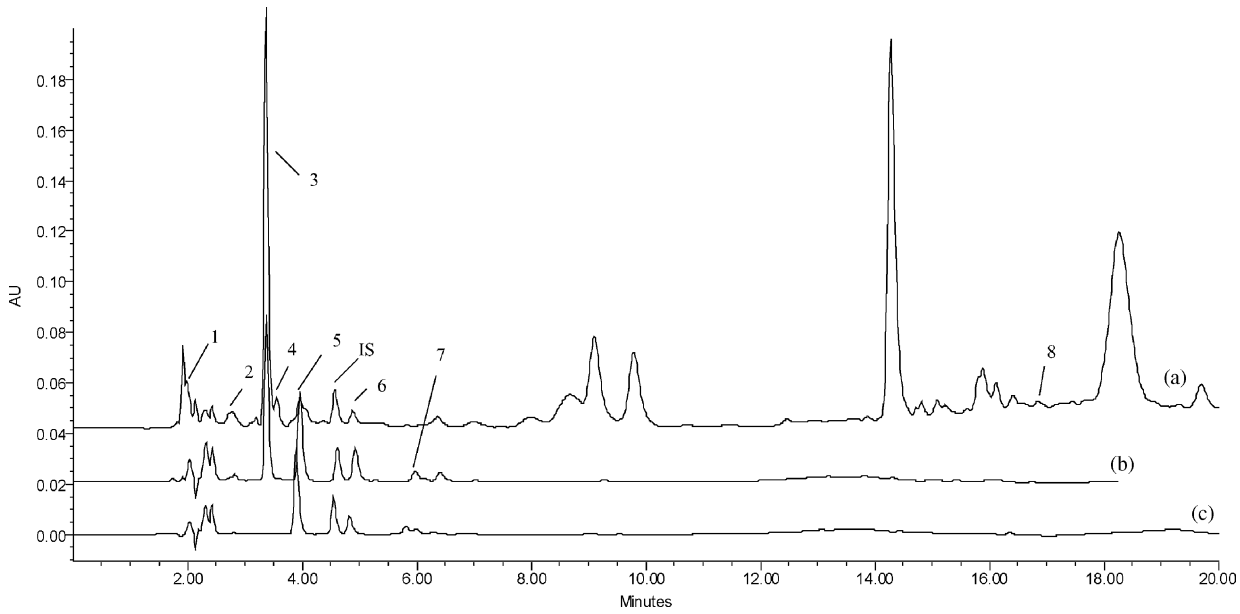

Fig. 2. Chromatogram of: (a) Arzua-Ulloa cheese, (b) plain yoghurt, and (c) raw milk. Peaks: (1) oxalic acid, (2) pyruvic acid, (3) lactic acid, (4) acetic acid, (5) orotic acid, IS: internal standard, (6) citric acid, (7) uric acid, (8) butyric acid.

Table 6
Concentration (mg 100 g^{-1} dry matter) of the organic acids in raw milk and in some commercial samples (plain yoghurt at 1 week before expiring, plain yoghurt at 1 week after expiring and Arzúa-Ulloa cheese)

Acid	Raw milk	Not expired yoghurt	Expired yoghurt	Arzúa-Ulloa cheese
Oxalic	–	73.0 ± 22.7	78.6 ± 1.9	22.2 ± 1.4
Formic	–	–	–	–
Pyruvic	6.4 ± 0.6	52.5 ± 10.2	48.0 ± 2.8	46.0 ± 4.6
Lactic	145.2 ± 19.1	14509.8 ± 234.8	15411.7 ± 23.2	14601.5 ± 1731.2
Acetic	86.0 ± 5.0	469.0 ± 122.0	430.0 ± 44.1	1006.0 ± 172.0
Orotic	87.0 ± 2.0	76.1 ± 1.1	82.0 ± 1.1	11.1 ± 1.1
Citric	1233.0 ± 21.0	1938.1 ± 22.1	2102.0 ± 22.0	392.1 ± 51.1
Succinic	–	–	–	182.0 ± 51.1
Uric	67.4 ± 0.7	67.1 ± 7.9	69.3 ± 0.4	14.4 ± 1.0
Propionic	–	–	–	–
Butyric	–	–	–	226.0 ± 26.0

for yoghurt starter cultures; a decrease up to 48% in orotic acid content during manufacturing and storage of yoghurt has been reported [13]. Concentration of citric acid remaining in yoghurt (∼2000 mg/100 g dry matter) was similar to other results (2.3 mg/g) recorded in the literature [10]. Acetic acid is another important organic acid detected in yoghurt and cheese, probably formed as product from the fermentation of lactose and citric acids.

In order to test the sensitivity of this method, the same yoghurt was stored at 4 °C for 2 weeks. This length of time was enough to detect clearly an increase of 10% of lactic acid caused by bacteria growth.

Citric acid is not the first energy source of bacteria, but can be metabolised very rapidly by *Lactococcus lactis* subsp. *diacetylactis* or *Leuconostoc* spp. in Cheddar cheese. Depending on the starter used, citrate can remain constant at 2% (w/w) up to 3 months of ripening, and decrease to 0.1% (w/w) at 6 months [1]. Citrate in cheese presumably reflects the concentration of colloidal citrate in milk. The concentration of citric acid in the sample of cheese was 392.1 mg/100 g dry matter, which is into the normal range (0.2–0.5% (w/w)) of citrate content in Cheddar cheese [1].

4. Conclusion

An alternative RP-HPLC method has been optimised and found to be well suited for the analysis of 11 organic acids metabolically important in dairy products and most commonly cited in the literature. It has been shown to achieve adequate separation in <18 min and the suitability of the technique has been verified by the analysis of the linearity, precision and accuracy.

This method appears to be an alternative to other analytical HPLC methods that use very expensive ion-exchange columns. The new method is fast and accurate and no loss of efficiency of the column has been observed during the course of this study. However, several unknown compounds appear on the chromatogram when analysing dairy samples, especially in the case of cheese. A clean-up procedure would be recommended, e.g. with solid-phase extraction C_{18} cartridges, to remove some possible interference and to longer the life of the column.

References

[1] P.F. Fox, J. Law, P.L.H. McSweeney, J. Wallace, in: P.F. Fox (Ed.), Cheese: Chemistry, Physics and Microbiology, Chapman & Hall, London, UK, 1993, p. 389.
[2] D. González de Llano, A. Rodríguez, P. Cuesta, J. Appl. Biotechnol. 80 (1996) 570.
[3] J.M. Izco, M. Tormo, R. Jimenez-Flores, J. Agric. Food Chem. 50 (2002) 1765.
[4] J.M. Izco, M. Tormo, R. Jimenez-Flores, J. Dairy Sci. 85 (2002) 2122.
[5] R.T. Marsili, H. Ostapenko, R.E. Simmons, D.E. Green, J. Food Sci. 46 (1981) 52.
[6] W.J. Mullin, D.B. Emmons, Food Res. Int. 30 (1997) 147.
[7] J.F.R. Lues, W.C. Botha, E.J. Smit, Int. Dairy J. 8 (1998) 959.
[8] G. Shui, L.P. Leong, J. Chroma. A 977 (2002) 89.
[9] M. Castro, S. Gascon, M. Pujol, J.M. Sans, P. Vicente, in: Monografía de Asociación Española de Farmacéuticos de la Industria, Sección Catalana; Comisión de Buenas Prácticas y Control de Calidad, Hewlett-Packard, Barcelona, 1989.
[10] E. Fernández-García, J.U. McGregor, J. Dairy Sci. 77 (1994) 2934.
[11] P. Walstra, T.J. Geurts, A. Noomen, A. Jellema, M.A.J.S. van Boekel, Dairy Technology: Principles of Milk Properties and Processes, Marcel Dekker, New York, 1999.
[12] G. Anastasi, M.L. Antonelli, A. Biondi, G. Vinci, Talanta 52 (2000) 947.
[13] L.A. Arla, J. Dairy Sci. 65 (1982) 353.