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Hydroxymethylfurfural determination in infant milk-based formulas by micellar electrokinetic capillary chromatography☆

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This work is dedicated to the memory of Professor A. Moral who died 11 October 2000.

Abstract

A procedure for determining hydroxymethylfurfural (HMF) in milk-based products by micellar electrokinetic capillary chromatography (MECC) was settled on. The effect of the trichloroacetic acid on the migration time of HMF and presence of interference peaks were investigated. MECC procedure was applied on commercial liquid infant formulas and compared with the classical reversed-phase high performance liquid chromatography method, giving similar values of repeatability and recovery. HMF peak was well-resolved by using an uncoated fused-silica capillary (48.5 cm×50 μm i.d.) with 50 mM phosphate buffer (pH 7.5) containing 100 mM SDS as electrolyte, voltage at 20 kV (25°C). Sample injection was by pressure (50 mbar, hydrodynamic) for 2.5 s. HMF analysis by MECC was suitable for routine analysis since separation was completely achieved at 5 min.

Keywords: HMF; Capillary electrophoresis; Milk; Infant formula; Heat treatment

1. Introduction

The nutritional, organoleptic, and technological effect of a wide range of technological processes used in the milk industry may be evaluated by determining several bio/chemical compounds specially related to such processes, either through degradation of original milk compounds or a result of reactions at the high temperatures used. In this sense, whey protein denaturation, protein aggregation, Maillard reaction and sugar isomerisation has been studied during the heating of milk as sources of heat-induced markers (Pellegrino, Resmini & Luf, 1995). Keeney and Bassette (1959) developed a colorimetric method of determining hydroxymethylfurfural (HMF) in dairy products. Since then, a large amount of literature has appeared on the application of HMF as a heat-induced marker in milk and milk products. HMF is formed by the dehydration from hexoses (free, or linked to protein, e.g. Amadori products) via 1,2 enolisation with loss of three molecules of water in boiling oxalic acid (Gottschalk, 1952). In the past, the usefulness of HMF as a process-control index has begun to come into question; this, however, is due to the sensitivity of the method used to detect it rather than to the compounds itself. The interaction of 2-thiobarbituric acid and HMF to form a coloured compound measurable at 443 nm is not specific, given that other aldehyde compounds may take part in the reaction, and besides, the yellow complex is unstable over time (Morales, Romero & Jiménez-Pérez, 1996). Later, van Boekel and Zia-Ur-Rehman (1987), and Morales, Romero and Jiménez-Pérez (1992) applied rp-HPLC methodologies for measuring HMF directly in processed heated milk by its absorption at 280 nm after separation on a C18 reversed-phase silica column. At present, HMF is a widely applied parameter to asses the heat load of a thermal process in dairy industry (Morales, Romero & Jiménez-Pérez, 2000).

Nowadays capillary electrophoresis (CE) has been proved as a powerful and very promising technique for food analysis, because of its high efficiency and resolving power, small sample and buffer requirements.

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Moreover CE is ease of automation, and less rigorous requirements for sample cleanup, which gives considerable advantages over traditional separation techniques such as high perfomance liquid chromatography (HPLC) or gel electrophoresis separations (Dong, 1999). For instance, CE has been applied in the dairy field to the analysis of caseins and whey proteins, to study of polymorphism of milk caseins from caprine and ovine sources, and to study of proteolysis of milk proteins during cheese ripening (Recio, Amigo & Lopez-Fandiño, 1997). Micellar electrokinetic capillary chromatography (MECC) is one of the most widely used CE modes.

The literature is scarce on the determination of heat-induced markers by CE, mainly in milk products. Corradini and Corradini (1992) determined HMF in fruit juices by MECC but procedures for analysis of HMF in heat-treated milk has not been specifically developed. The aim of our work is to set up a suitable and fast procedure for the determination and separation of HMF by CE in heat-treated milk samples starting from the experience of the work of Corradini and Corradini. Moreover MECC analysis is compared with those obtained with the rp-HPLC method for HMF determination in liquid infant milk-based products.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulphate (SDS) of electrophoresis purity reagent grade and HMF were purchased from Sigma (St. Louis, MO). Analytical-reagent grade phosphoric acid, boric acid, and sodium hydroxide were all purchased from Panreac (Barcelona, Spain). Other reagents were purchased from Merck (Darmstadt, Germany). Milli-Q water (Millipore Corp., Madrid, Spain) was used.

2.2. Sample preparation

Milk (2 ml) was digested with 1 ml of 0.3 N oxalic acid solution in tightly stoppered Pyrex tubes for 1 h at 100°C. After a rapid cooling in ice, the mixtures were slowly deproteinised with 1 mL of trichloroacetic acetic acid (TCA) solution (40%, w/v) and centrifuged at $11\,000\times\boldsymbol{g}$ for 12 min at 4°C. After filtration through a 0.45-μm acetate filter (13 mm, MSI Inc., Westboro, MA), the sample was ready for both CE and HPLC analysis.

2.3. HPLC determination

Using the rerversed-phase HPLC (rp-HPLC) method of Morales et al. (1992) with some minor modifications, a degassed mobile phase was prepared with sodium acetate buffer (0.08 M), and the pH was adjusted to 3.6 with acetic acid. A Extrasyl-ODS2 analytical column (25×0.40 cm, 5-μm particle size, Analytical Tracer, Barcelona, Spain) was used at 32°C. The injection volume was 20 μl after appropriate dilution of sample, and detection at 280 nm (0.1 aufs and 0.5 s response time) was selected. HMF was quantified by the external standard method within the range 2–100 μM. A Kontron Instruments (Milan, Italy) chromatographic system was used.

2.4. CE determination

Electromigration was carried out with a HP^{3D} system equipped with built-in diode-array detector and HP ChemStation for system control, data collection, and data analysis from Hewlett-Packard (Madrid, Spain). MECC were performed on an uncoated fused silica capillary with a 48.5 cm of total length (40 cm of effective length), 50 μm internal diameter, and an extended light path (bubble factor of 3) supplied by Hewlett-Packard (Madrid, Spain). The new capillary was conditioned by washing with 1 M NaOH for 20 min, wait for 5 min, water for 5 min, and then buffer for 20 min. Micellar solutions were prepared by dissolving SDS in filtered phosphate buffer, which was prepared by titrating a solution of 50 or 100 mM phosphoric acid with 50 or 100 mM sodium hydroxide to pH 7.5. Running buffer was daily prepared, degassed by sonication and kept at ambient temperature before use. Samples were hydrodynamically injected by applying 50 mbar up to 8 s at the anionic end of the capillary. After every run capillary was hiflushed (7 bar) with 0.1 M NaOH (0.2 min), 0.2 M sodium phosphate buffer pH 7.5 (0.2 min), and then running buffer (0.4 min). Electrode buffers were renewed every five runs with 0.5 ml per pot. The temperature of the cartridge was maintained at the experimental value by means of an high-velocity air forced system. A standard electrophoretic run was performed at a constant voltage of 20 kV with the anode at the inlet side. Electropherograms were monitored at 280 nm wavelength (0.01 aufs and 0.3 s of response time), with raw spectral data collection between 190 and 500 nm. Calibration (0–100 μmol/l) was obtained from standard of HMF diluted in 10% TCA on the linear regression analysis of the peak-area ratios giving a correlation coefficient (r^2) of 0.9995.

2.5. Infant milk formulas

Six different commercial infant liquid milks of three different companies were purchased from Spanish local markets. Samples were divided in starter infant formula (up to 4–6 months, sample 2 and 4), follow-on infant formula (4–6 to 12 months, sample 3 and 5), and toddler or junior formulas (1 to 3 year, sample 1 and 6).

3. Results and discussion

The quantitative determination of HMF using the conditions described by Corradini and Corradini (1992) for fruit juices was hampered by the complexity of the heat-treated milk samples. During the heating of milk and milk-products not only HMF is formed, a myriad of compounds are formed which could to interfere with the analysis techniques (Morales et al., 1996). This is specially dramatic for the rp-HPLC separation of HMF in severely heated milk samples where two different peaks could to coelute with HMF (Morales & Arnoldi, 1999; Morales, Romero & Jiménez-Pérez, 1995). One of the advantages offered by CE in food analysis is the speed in the analysis time, hence running time (nearly 15 min) applied in the procedure of Corradini and Corradini was not acceptable for a routine analysis. Our aim was to optimise the electrophoretic procedure described by Corradini and Corradini for analysis of HMF in milk and milk-based products.

According to the information described by Royle, Bailey and Ames (1998) for separation of Maillard reaction products, capillary zone electrophoresis was developed with 50 mM borate buffer (pH 9.3) as electrolyte. At this running conditions, HMF was not separated at baseline as a unique compound in severely heated samples and migrate to the cathodic end with the electroosmotic flow (EOF) as a nonionic compound. In a second step, MECC was applied with borate buffer (pH 9.3) as well as phosphate buffer (pH 7.5) as running buffer, and SDS was also dissolved as a micelles forming substance.

In order to select the most appropriate conditions, it was used a severely heated milk sample (100°C for 3 h), and a HMF standard solution (26 μM) in water. Fig. 1 depicted the effect of ionic strength of electrolyte as affected by the anionic surfactant concentration in the running buffer. SDS micellar systems are reported to be similar to an octadecylsilane stationary phase in HPLC for moderately water-soluble compounds (Burton, Sepaniak & Mascarinec, 1987). During the separation micelles of SDS migrate toward the anode and can interact with solutes in a chromatographic manner through both hydrophobic and electrostatic interactions. Migration time increased almost linearly with the concentration of phosphate buffer and concentration of SDS dissolved in the buffer solution without affecting the separation of HMF. When borate buffer was used as electrolyte, HMF peak not interference free but baseline resolved. Furthermore, the migration time were increased when using borate buffer. It was selected the low-concentration phosphate buffer containing 100m M SDS due to a compromise between analysis time and low current.

But comparing the migration times of HMF in standard solution dissolved in water with digested milk samples, significant differences were observed. HMF in the water solutions migrated faster than in milk samples treated with TCA and oxalic acid, and those differences keep constant through all the analysis. Furthermore migration times were retarded when increased amounts of sample treated with TCA were injected into the system. On the other hand, the shape of the HMF peak in the milk sample was more broad than the standard in water. This effect is probably due to the pH differences between the running buffer and the sample zone. Moreover, this effect was more prominent at injection times closed to 8 s. The effect of the TCA concentration in the sample on the migration time of HMF was studied. Fig. 2 depicted the change of the migration time of HMF peak according the presence of TCA in the solution. It was observed that migration time of the HMF peak could be increased about 1 min if dissolved in water or treated with TCA (for protein precipitation).

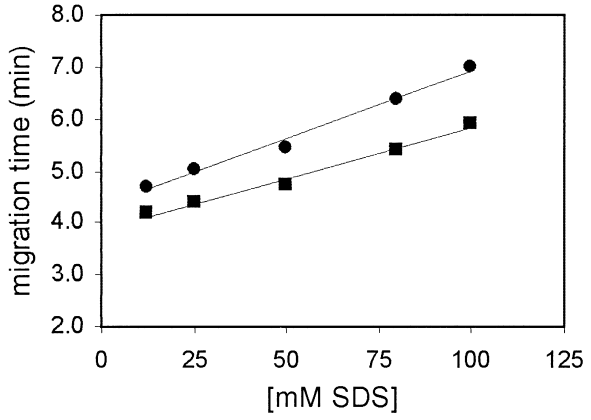

Fig. 1. Effect of phosphate buffer (pH 7.5) and sodium dodecyl sulphate (SDS) concentration on migration time of hydroxymethylfurfural (HMF). Separation of HMF standard (26 μM) applied at 15 kV (30°C). Phosphate buffer (100mM; ●), and 50 mM phosphate buffer (■).

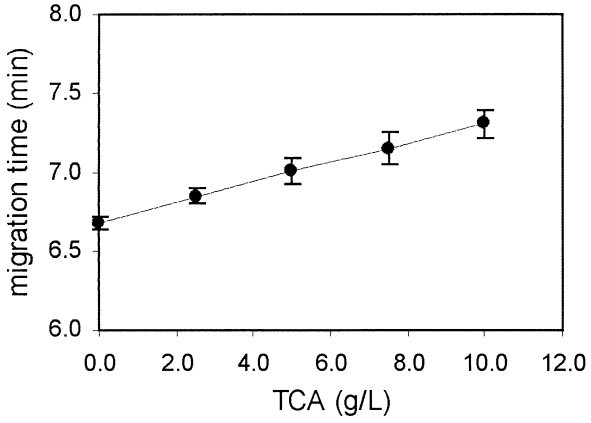

Fig. 2. Effect of trichloroacetic acetic acid (TCA) concentration (g/l) in sample on migration time of hydroxymethylfurfural. Separation applied at 15 kV (25°C) with a 50 mM phosphate buffer (pH 7.5) containing 100 mM sodium dodecyl sulphate. Hydrodynamic injection at 50 mbar for 5 s.

This observation was taken into consideration for further analysis in order to set the best condition for separation and determination of HMF in milk.

Fig. 3 depicted the effect of temperature (20–35°C) on the migration time of HMF. The temperature has an influence on the separation in the sense that micelles are only formed if a critical micelle temperature is exceeded. Therefore, compounds which have greater affinity for the SDS micelles have longer migration times, as compared to compounds that spend more time in the running electrolyte. Increasing the temperature up to 35°C is observed a proportional reduction of the migration times, whereas the resolution slightly decreased above 30°C as described by Corradini and Corradini (1992) for fruit juices. This was also observed in the decreasing of the capacity factor (k') value. Methanol was used as EOF marker in order to calculate the capacity factor since it is a solute that does not interact with the micelle. According these results it was settled a capillary temperature of 25°C. Finally, the effect of the electric field applied on the migration time of HMF was studied on a milk sample and a standard solution. Fig. 4 shows the results obtained for a HMF standard solution where higher capacity factor value was reached about 20 kV.

Overall, most appropriate electrophoretic conditions for HMF analysis in milk-based products by MECC are described by using 100 mM SDS in 50 mM phosphate buffer (pH 7.5) as running electrolyte at 20 kV (25°C) for 5 min of running time. Sample is hydrodynamically injected for 2.5 s. The repeatability of the migration time was performed at two different injection times. An average of 4.12±0.06 min ($n=6$), and 4.00±0.05 min ($n=6$) was obtained at 50 mbar for 5 and 2.5 s, respectively. Again, the effect of the TCA could be observed in the reduction of the migration times at lower injection times. Fig. 5 (a) shows the effect of the presence of 10% of TCA solution in a standard of HMF. But, it compared samples with equal TCA amount the migration times slightly decreased at lower injection times, Fig.5 (a, b). It was not able to detect a significant improvement in the reproducibility of the migration times with keeping the uncoated capillary rinsed with 50 mM phosphate buffer overnight. On the other hand, if electric field applied is ramped up over 0.3 min, is not observed an improvement in the reproducibility but a more stable baseline at the beginning of CE analysis was detected. A previous step of ramping the electric field was not take into consideration in order to make more reproducible the procedure among other CE equipments.

MECC procedure for determining HMF was applied on liquid infant formulas. In the recent years, liquid infant formulas have become to the European market. It is a product with a great acceptation by the consumer because of ready-to-use. Liquid infant formulas are a complex recombination of nutrients which are not necessary from a milk source. Hence interference of ingredients or newly formed substances with the procedure should be take into consideration. Data of HMF by MECC was compared with obtained by the rp-HPLC procedure. Results show that levels of HMF obtained by both metrologies are similar, Table 1. Junior Infant milks showed the highest HMF levels which agrees with the more drastic heat-load applied. These products has a shelf-life over 6 months since conventional sterilisation processes (120°C for 10–15 min) are applied to guaranty the microbiological safety. Rest of the samples are mostly UHT treated and the shelf-life is lower than 6 months. Repeatability of the MECC analysis as compared with the HPLC procedure was evaluated on sample 6, Table 2. Sample was prepared three times on four consecutive days. Results obtained by both methodologies are not significantly different. On the other hand, limit of detection was

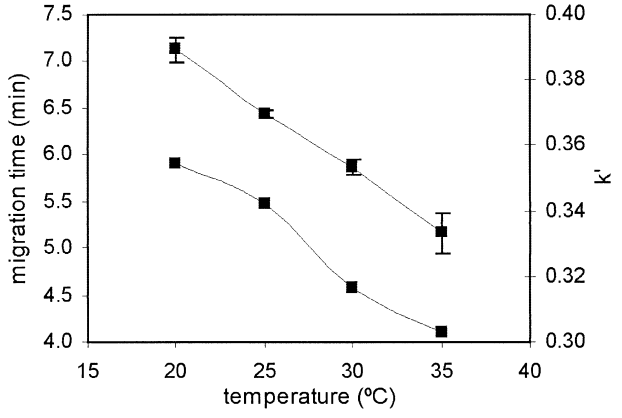

Fig. 3. Effect of temperature on migration time of hydroxymethylfurfural from a milk sample heated at 100°C for 3 h. Migration time (solid line), and capacity factor (dotted line). Separation applied at 15 kV with a 50 mM phosphate buffer (pH 7.5) containing 100 mM sodium dodecyl sulphate.

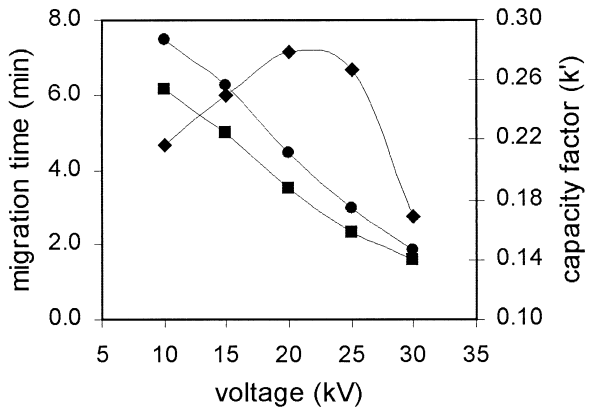

Fig. 4. Effect of electric field (kV) on the migration time of a standard of HMF (26μM) containing 10% trichloroacetic acetic acid. Methanol (■), hydroxymethylfurfural (●), and capacity factor (◆). Conditions are 50 mM phosphate buffer (7.5) containing 100 mM sodium dodecyl sulphate, 25°C.

settled about 2.5 μmol/l for the MECC procedure, being higher than 0.2 μmol/l as described by the HPLC procedure (e.g. Morales et al., 2000). This fact could be improved if a freeze-dried step is applied to the sample before MECC analysis or by a z-shaped longitudinal capillary flow cell (Heiger, 1992). An average recovery of 98.1% was obtained for the analysis of HMF by MECC (Table 3) which is similar that 99.5% described by Morales et al. (2000) for the chromatographic procedure.

Fig. 5 (b) shows HMF analysis by MECC of a commercial junior milk (sample 1), and a starter infant formula (sample 2). HMF peak appeared well resolved and free of interference as probed by diode-array detection (DAD) scan as compared a standard at the same buffer conditions. Fig. 6 shows the classical chromatographic determination of HMF in milk-based products by using isocratic elution. The analysis time is 15 min since non-polar compounds have to be allowed to elute. Otherwise, clarification of sample thought sep-pak C18 cartridges is needed which increases the working time. By applying MECC analysis saturation of the column is skipped since capillary is completely rinsed after each separation.

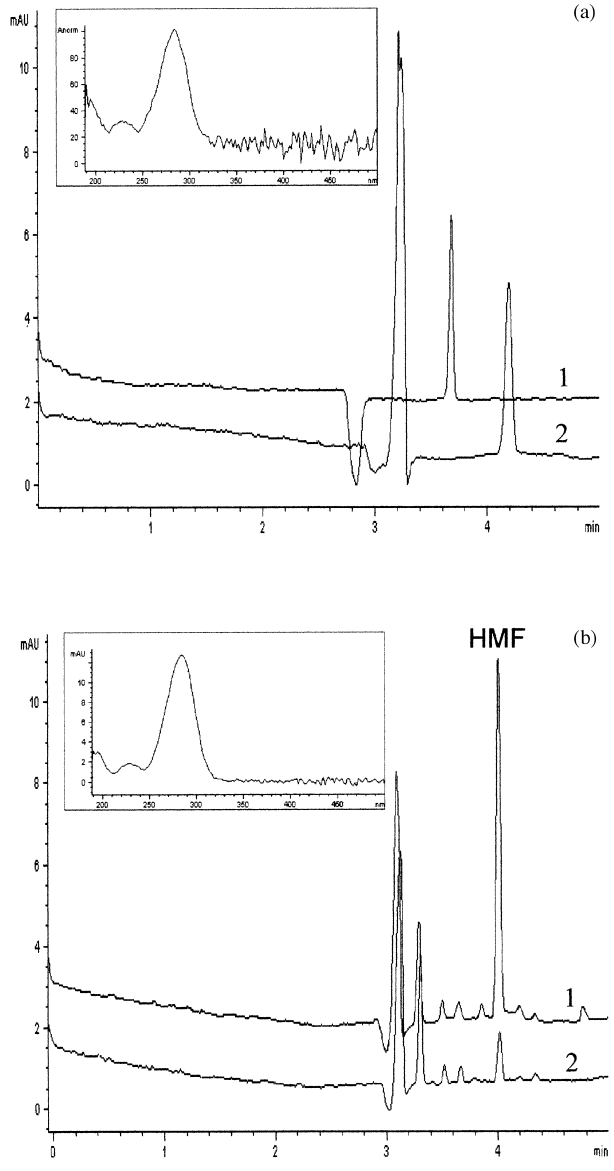

Fig. 5. Electropherograms of HMF standards dissolved in water (a1), and 10% trichloroacetic acetic acid solution (a2). Electropherograms of liquid infant sample number 1 (b1) and number 2 (b2). Subgraphs plots spectrum profile of a standard of hydroxymethylfurfural (HMF) (a) and HMF peak in sample 1 (b). Conditions are 100 mM sodium dodecyl sulphate in 50 mM phosphate buffer (pH 7.5), 20 kV, 25°C. Capillary electrophoresis analysis a and b injected hydrodynamically for 5 and 2.5 s, respectively.

Table 1
Comparison of hydroxymethylfurfural (HMF) data (with standard deviation) obtained by micellar electrokinetic capillary chromatography (MECC) and high performance liquid chromatography (HPLC) on different infant liquid formulas[a]

Sample	HMF (μmol/L)	
	MECC	HPLC
1	294.4±3.4	296.6±2.9
2	34.9±1.0	35.2±1.2
3	26.7±1.9	25.5±1.4
4	37.4±1.4	32.7±1.8
5	27.4±0.6	24.5±0.5
6	244.5±3.2	247.2±3.2

[a] Results are average of three independent measurements.

Table 2
Day-to-day analysis of hydroxymethylfurfural (HMF) content in a commercial infant liquid formula by micellar electrokinetic capillary chromatography (MECC) and high performance liquid chromatography (HPLC)[a]

Day	HMF (μmol/L)	
	MECC	HPLC
1	244.5±3.2	247.2±3.2
2	246.0±1.6	251.5±1.1
3	242.8±1.7	255.7±1.6
4	250.3±2.9	249.9±1.1

[a] Results are average of three independent measurements.

Table 3
Recovery study of hydroxymethylfurfural (HMF) analysis by micellar electrokinetic capillary chromatography (MECC) in a UHT milk sample (9.4 μmol/L) added with pure HMF

Sample	HMF expected (μmol/l)	HMF found (μmol/l)	Recovery (%)
1	21.2	21.3±1.2	100.5
2	33.0	32.8±2.6	99.2
3	56.7	54.3±1.8	95.7
4	80.3	77.8±1.9	96.9

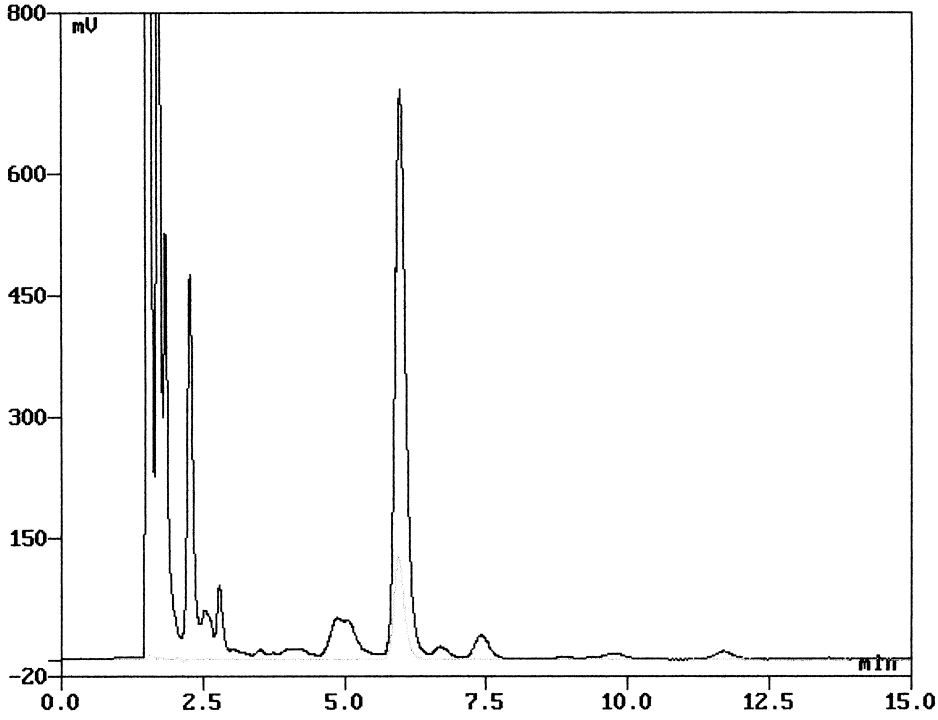

Fig. 6. A classical high performance liquid chromatography chromatograms from a standard of hydroxymethylfurfural (dotted line) and sample 1. Conditions as described in the text.

4. Conclusions

HMF, a classical heat-induced marker, is able to be analysed and determined in milk-based products by MECC. The procedure has been updated from described by Corradini and Corradini (1992) for fruit juices since the special hydrolysis conditions, fat and protein precipitation, and presence of interfering peaks in heated milk make it necessary. Again, advances in the field of fruit juices has been useful to be applied in the dairy science as happened with the chromatographic determination of HMF (e.g. Lee, Rouseff & Nagy, 1986). HMF peak is resolved by using 50 mM phosphate buffer (pH 7.5) containing 100 m SDS as electrolytes, at 20 kV and a temperature of the uncoated capillary of 25°C. MECC procedure has been evaluated, and it is efficient, economic and fast which could be applied as routine analysis for controlling heat-load of milk-based products.

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References

Burton, D. E., Sepaniak, M. J., & Mascarinec, M. P. (1987). Evaluation of the use of various surfactants in micellar electrokinetic capillary chromatography. *Journal of Chromatography Science, 25*, 514–518.

Corradini, D., & Corradini, C. (1992). Separation and determination of 5-hydroxymethyl-2-furfuraldehyde and 2-furaldehyde in fruit juices by micellar electrokinetic capillary chromatography with direct sample injection. *Journal of Chromatography, 624*, 503–509.

Dong, Y. (1999). Capillary electrophoresis in food analysis. *Trends in Food Science & Technology, 10*, 87–93.

Gottschalk, A. (1952). Some biochemically revelant properties of N-subtituted fructosamines derived from N-Glucosylamino-acids and N-Arylglucosylamines. *Biochemistry Journal, 52*, 455–460.

Heiger, D. N. (1992). *High performance capillary electrophoresis. An introduction*. Germany: Hewlett-Packard Company.

Keeney, M., & Bassette, R. (1959). Detection of intermediate compounds in the early stages of browning reaction in milk products. *Journal of Dairy Science, 42*, 945–960.

Lee, H. S., Rouseff, R. L., & Nagy, S. (1986). HPLC determination of furfural an 5-HMF in citrus juices. *Journal of Food Science, 51*, 1075–1076.

Morales, F. J., & Arnoldi, A. (1999). Analysis of galactosylisomaltol in milk systems using HPLC. *Food Chemistry, 67*, 185–191.

Morales, F. J., Romero, C., & Jimenez-Perez, S. (1992). An enhanced liquid chromatographic method for 5-hydroxymethylfurfural determination in UHT milk. *Chromatographia, 33*, 45–48.

Morales, F. J., Romero, C., & Jiménez-Pérez, S. (1995). New methodologies for kinetic study of 5-(hydroxymethyl)-furfural formation and reactive lysine blockage in heat-treated milk and model systems. *Journal of Food Protection, 58*, 310–315.

Morales, F. J., Romero, C. & Jiménez-Pérez, S. (1996). Study on 5-hydroxymethylfurfural formation during UHT treatment measured by two analytical procedures. In *Heat treatments and alternative methods*; (pp. 354–357). Brussels: International Dairy Federation.

Morales, F. J., Romero, C., & Jiménez-Pérez, S. (2000). Characterization of industrial processed milk by analysis of heat-induced changes. *International Journal of Food Science and Technology, 35*, 193–200.

Pellegrino, L., Resmini, P. & Luf, W. (1995). Assessment (indices) of heat treatment of milk, In: P. F. Fox, *Heat-induced changes in milk*. (pp. 409–453). Brussels: International Dairy Federation.

Recio, I., Amigo, L., & Lopez-Fandiño, R. (1997). Assessment of the quality of dairy products by capillary electrophoresis of milk proteins. *Journal of Chromatography B., 697*, 231–242.

Royle, L., Bailey, R. G., & Ames, J. M. (1998). Separation of Maillard reaction products from xylose-glycine and glucose-glycine model systems by capillary electrophoresis and comparison to reverse phase HPLC. *Food Chemistry, 62*, 425–430.

van Boekel, M. A. J. S., & Zia-Ur-Rehman (1987). Determination of HMF in heated milk by HPLC. *Netherland Milk and Dairy Journal, 41*, 297–306.