TITLE

Comparison between Capillary Electrophoresis and High Performance Liquid Chromatography for the Study of the Occurrence of Patulin in Apple Juice Intended for Infants

Running title

Patulin in apple juice for infants

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Abbreviations: CE, capillary electrophoresis; CEC, capillary electrochromatography ; DAD, diode array detector; HMF, 5-hydroxymethylfurfural; HPLC, high performance liquid chromatography; LOD, limit of detection; MEKC, micellar electrokinetic chromatography; MES, 4-morpholino-ethyl-sulfonic-acid; PAT, patulin; PMTDI, provisional maximum tolerable daily intake; SDS, sodium dodecylsulphate; TLC, thin layer chromatography ; TRIS,

trihydroxymethyl-amoniomethane ; TFA, trifluoroacetic acid ; RSD, relative standard deviation.

Abstract

Apple juice samples intended for infants purchased in Navarra (Spain) have been analyzed for PAT occurrence. Two capillary electrophoresis methods, based on a MEKC and a CEC system, and an HPLC method were evaluated for the aforementioned study. The CEC system gave less satisfying separations and several practical problems, so samples have been analyzed by MEKC and HPLC. Both methods have been comparable in terms of recovery, precision, limits of detection, volume of organic solvents used and adequate selectivity with regard to PAT and HMF. The analysis time in HPLC has been slightly lower than in the MEKC methodology. The PAT levels obtained in apple juice by both validated methods showed a strong correlation ($p<0.001$). Therefore, both methodologies are useful for the accurate quantification of patulin in this matrix.

The PAT levels obtained in the 20 infant apple juices samples were in a range between <LOD and 29.6 μg L^{-1}, with a mean concentration of 8.0 μg L^{-1} which implies a dietary intake estimation of 104 ng kg^{-1} b.w. day^{-1} considering a body weight of 10 kg and an apple juice consumption of 130 mL day^{-1}, 26 % of the PMTDI recommended by JECFA.

1. Introduction

Patulin (4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one) (PAT) is produced by filamentous fungi of the genera *Penicillium, Aspergillus* and *Byssochlamys* present in a wide variety of foodstuffs: mainly apples but also pears, grapes, apricots, strawberries, blueberries, peaches, vegetables, berries, bread, and meat products (Rychlik and Schieberle, 2001; Ritieni, 2003; Majerus and Kapp, 2002). PAT is very soluble in water, very stable in aqueous acid mediums and there are contradictory results concerning its stability after heat treatment (Kadakal and Nas, 2003); therefore, the use of unsound apples can result in the presence of patulin in derived products such as apple juice, puree, pies, jam and foodstuff intended for infants. Also, patulin can be used for quality control purposes of foods elaborated with apples, indicating the quality of raw materials employed, mainly the presence of rotten apples (Prieta et al., 1994).

The PAT toxic effects have been studied in different animal species (Mahfoud et al., 2002). PAT has mutagenic properties and can cause neurotoxic effects in rodents (Hopkins, 1993). The International Agency for Research on Cancer has classified patulin in group 3 (not classifiable regarding its carcinogenicity to humans and with no adequate evidence in experimental animals) (IARC, 1986).

In 1995, the Joint Expert Committee on Food Additives of the World Health Organization (JEFCA, 1995), recommended that human exposure to PAT should be reduced to less than 0.4 μg kg^{-1} b.w. day^{-1}. Taking into consideration this recommendation and the data related to the occurrence of PAT in food, the European Commission approved the Directive (CE) nº 1881/2006 which established the PAT limits for different apple derivative commodities (Commision Regulation (EC) No. 1881/2006 of December 19, 2006). Taking into account that young children have a higher consumption of apple-based food compared to adults

(Piemontese et al, 2005; Barreira et al., 2010) and in order to protect infants, this directive indicates a lower permitted level in the case of food for young children, infants, and baby foods (10 μg L^{-1}) than that permitted for apple juices not intended for infants (50 μg L^{-1}). In order to evaluate the exposure to patulin, different studies have been conducted (Beretta et al, 2000; Ritieni 2003; Piemontese et al., 2005; Legarda and Burdaspal, 2005; Cano-Sancho et al., 2009; Murillo-Arbizu et al., 2009). A Scientific Cooperation Study (Majerus and Kapp, 2002) showed that apple juice and apple nectar are the main sources of patulin intake in most countries, particularly for young children; patulin can be considered a typical mycotoxin, with baby and children as the target (Ritieni, 2003). Some studies on the occurrence of patulin in baby food products have been reported (Prieta et al., 1994; Cano-Sancho et al., 2009; Mhadhbi et al., 2007; Legarda and Burdaspal, 2005; Plessi et al., 1998; Ritieni, 2003), but there is less data for baby food products than for adults products (Cano-Sancho et al., 2009).

Several studies have been performed to develop sensitive and selective methods for the determination of patulin in food. At first, patulin was semi-quantitatively analyzed using TLC methods (Scott and Kennedy, 1973; Stinson et al., 1977). Currently, the analysis of patulin is generally carried out using HPLC with diode array detection (Gökmen et al., 2005, Spadaro et al., 2007; González-Osnaya et al., 2007; Valle-Algarra et al., 2009). In comparison with HPLC methods, capillary electrophoresis (CE) methods have some advantages, such as being able to use a smaller volume of organic solvents, highly efficient separation and producing less waste volumes. Indeed, micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) methods have become powerful separation techniques for both neutral and charged compounds. Few studies on the use of capillary electrophoresis for the analysis of patulin have been conducted (Tsao and Zhou, 2000; Murillo et al., 2008; Murillo-Arbizu et al., 2008). To date, there have been no reported attempts to compare the CE methodology with the HPLC one in the determination of this mycotoxin.

The objectives of this study have been: a) to report the results of the analysis of patulin in apple juice intended for infants purchased in Spain, in view of the adverse effects caused by this mycotoxin, and the continuous need of exposure data for risk assessment. b) To compare the HPLC and CE techniques for PAT analysis in this matrix. In order to do that, samples have been analyzed by two validated methods, one of them being a previously reported method using MEKC (Murillo et al., 2008) and the second one being an HPLC method that has been developed and validated for PAT quantification in this type of matrix. Linearity, recovery, LOD, selectivity, time of analysis, cost and their applicability in real apple juice samples intended for infants have been compared.

2. Material and methods

2.1. Reagents

Pure crystalline patulin (CAS No. 149-29-1, >98 %), 5-hydroxymethylfurfural, acetic acid, trihydroxymethyl-amoniomethane (TRIS), 4-morpholino-ethyl-sulfonic-acid (MES), trifluoroacetic acid, anhydrous sodium bisulphite and anhydrous sodium carbonate were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Sodium tetraborate 100 mM, sodium tetraborate 50 mM / sodium dodecylsulphate (SDS) 100 mM solution were obtained from Agilent Technologies (Waldbronn, Germany). Ethylacetate, acetonitrile, methanol and ethanol HPLC grade were purchased from Riedel-de Haen (Seelze, Germany). Millipore type I water was used to prepare all of the aqueous solutions and it was obtained daily from a Milli-Q water-purifying system.

2.2. Apparatus

The electrophoretic separations were performed in an Agilent Technologies capillary electrophoresis system (model G1602A) controlled by Chemstation 3D software. DAD detection was used at a wavelength of 276 nm.

The MEKC method used in this study was previously described (Murillo et al. 2008). Briefly, an extended light path capillary of 56 cm and 64.5 cm for effective and total length, respectively, and 75 μm I.D was used; the electrolyte composition was sodium tetraborate 33.3 mM/ 66.6 mM of SDS/ 5 % acetonitrile, analysis temperature 35ºC, run voltage +15 kV, and hydrodynamic injection (50 mbar for 15 s).

In previous studies, we have obtained CE methods (using MEKC and MEEKC) useful in the determination of PAT levels in apple juices, and so CEC has been proved in this study as a possible alternative technique to the analysis of patulin. However, several technical problems arose. The excessive time and labor required to obtain stable current/baseline and reproducible retention times, the long time necessary for conditioning the column between runs, the lack of reliably and reproducibility, the fragility of the column affecting the method robustness, the bubble formation during analysis found during the optimization steps as well as the overall analysis price, and the fact that no improvements were found with regard to the previously MEKC method developed, led us to discard the CEC method for further investigation, and MEKC was chosen as the CE method to be used for the comparison with the HPLC system.

The HPLC instrument used was an Agilent Technologies 1100 liquid chromatographic system equipped with a diode array detector, controlled by Chemstation 3D software. Separations were obtained by using a 5 μm (15 cm x 0.46 cm) Zorbax Eclipse XDB-C18 column with a Tracer Extrasil ODS-2 precolumn, from Agilent Technologies (Waldbronn, Germany) and Teknokroma (Barcelona, Spain), respectively. The injection volume was 50 μL.

Chromatography was performed at 40 ºC, and a detection wavelength of 276 nm was used. The composition and proportion (aqueous and organic percentage) of the mobile phase were studied in order to obtain a good resolution between PAT and HMF.

2.3. Samples

Twenty apple juice samples, labeled as intended for infants older than four months were obtained from different supermarkets within Navarra (Spain) during 2008. Different brand names were selected in order to have a market-representative sampling. The volume of the samples was between 130 mL and 260 mL. They were stored in their original packets (glass bottles) at room temperature (18-25 ºC) until analysis was carried out (no longer than six months), always before the sample expiration dates indicated on the containers.

2.4. Standard sample preparation

Stock solutions of patulin and HMF standards were prepared in ethyl acetate (10 mg in 50 mL and 5 mg in 25 mL, respectively). Aliquots of the PAT and HMF solutions were stored at -20 ºC due to the fact that PAT is stable in ethyl acetate for several months under these storage conditions (MacDonald and Felguieras, 1997).

A working solution of PAT (10 μg mL^{-1} approximately) was prepared after evaporating 1 mL of the corresponding stock solution to dryness under a stream of nitrogen at 40 ºC and redissolving the residue in 20 mL of ethanol. The accurate concentration of PAT was determined by UV spectrophotometry at 276 nm (MW = 154.12; ε = 14600 L mol^{-1} cm^{-1}) (MacDonald and Felguieras, 1997). A working solution of HMF was prepared by evaporating 7 mL of its stock solution to dryness under a stream of nitrogen at 40 °C and then by dissolving the residue in 20 mL of ethanol, obtaining an HMF solution of 70 μg mL^{-1}. These solutions were stored at 0-4 °C. They were stable at this temperature for at least six months

(data not shown). Calibration samples were prepared by evaporating the adequate volumes of PAT and HMF working solutions, after having been temperated at room temperature for 30 min, under a nitrogen stream at 40 ºC, followed by dissolution in 200 µL of water at pH=4.0 acidified with 0.1 % acetic acid and filtering through a 0.45 µm filter (Syringe Driven Filter, Millipore Corporation, MA Bedford USA).

2.5. Sample preparation

The extraction of PAT for MEKC analysis was the same as that previously described (Murillo et al., 2008). Briefly, twenty milliliters of apple juice were extracted with 20 mL of ethylacetate. Ten milliliters of the organic phase were evaporated under a nitrogen stream in a water bath at 40 ºC. The residue was then dissolved in 200 µL of water acidified at pH=4.0 with a 0.1 % solution of acetic acid; afterwards, it was filtered through a 0.45 µm filter before analysis.

The method used in PAT extraction from apple juice samples for the HPLC analysis was based on the method developed by Yurdun et al., (2001), with some modifications: PAT was extracted from 20 mL of apple juice samples with 20 mL of ethylacetate in a vertical shaker model ABT4 (SBS®) for 10 min. After letting the mixture stand for 10 minutes, the two phases were segregated and the organic phase was mixed with anhydrous sodium carbonate at 1.5 % and shaken for 30 seconds. Then, it was left standing for 1 minute. The organic phase was filtered through a filter containing 10 g of anhydrous sodium bisulphite. Eight mL of the filtrate was evaporated to dryness under a weak stream of nitrogen at 40 ºC. The residue was redissolved in 0.2 mL of water acidified at pH=4.0 with a 0.1 % solution of acetic acid, and then filtered through a 0.45 µm filter.

2.6. Quantification and method evaluation

The PAT levels in the samples were determined by using a calibration graph of concentration versus response achieved by injection of patulin standard solutions and subsequent extrapolation. In the HPLC method, the response was measured as peak area. However, since peak area is related to migration time (Goodall et al., 1991), the normalized peak areas were used in the CE systems. All data obtained from the samples analyzed in this work was corrected by the recovery factor.

The optimization and validation of the MEKC method for the analysis of patulin in apple juice samples were previously reported (Murillo et al., 2008) (see table 1 for validation results).

In the case of the HPLC method, selectivity was studied by adding PAT and/or HMF to apple juice samples and then by observing the increase of the peak area of the corresponding compound that was added. Furthermore, the PAT identity confirmation was made by measuring and storing the DAD spectrum (range 200–600 nm) throughout the entire analysis process. In the assessment of linearity for HPLC, two calibration curves were plotted in the ranges of 0.1–1.0 μg mL^{-1} and 1.0–8.0 μg mL^{-1}. Three replicates of six calibration standards were analyzed for each range. Criteria used to verify linearity were: correlation coefficient (r) > 0.99, relative standard deviation (RSD) between response factors <5 %, confidence limits of the mean value of the calibration slope (p = 95 %) without including zero, confidence limits of the mean value of the calibration intercept interval (p = 95 %) including zero and lastly, when plotting the PAT concentration versus the residual points, a random distribution must be achieved. The limit of detection (LOD) and limit of quantification (LOQ) were established by analyzing three spiked apple juice samples at three different concentrations (7, 9, and 10 μg L^{-1}) and by using a method based on the calibration curve extrapolation at zero concentration (Murillo-Arbizu et al., 2008).

Within- and between-day precision and recovery of the HPLC method were established by making 27 determinations, covering the range of the method (three concentrations of 10.0, 60.0, and 120.0 μg L^{-1}/three replicates each one/three different days). Adequate volumes (between 23.5 μL and 281.4 μL) of the PAT working solutions in ethanol were added so as to reach these PAT levels in apple juice samples (20 mL). Recovery was determined by comparing the absolute responses of PAT obtained from the apple juice samples with the absolute responses of calibration standards. Where relevant, measured PAT levels were corrected for any natural contamination, as indicated by the analysis of the non-spiked material.

There are some authors (Boonzaaijer et al., 2005; Murillo-Arbizu et al., 2008) who freeze the apple juice samples as a way of storage before the analysis is carried out. To the best of our knowledge, there is no evidence which confirms that PAT levels of samples undergoing this procedure remain unaltered. So we considered it to be of interest, as part of the method validation studies, to determine whether or not the frozen procedure of apple juice has any influence on the PAT measured levels.

Three apple juice aliquots of 300 mL were spiked with patulin at three different concentration levels (20, 50 and 100 μg L^{-1}). The spiked samples and a similar aliquot of a non-spiked apple juice were aliquoted in 50 mL tubes. One of the tubes for each level was analyzed by the validated MEKC method (time zero) and afterwards, the remaining tubes were frozen at -20 ºC. At 1, 3 and 6 months, one of the aliquots from each level, which did not suffer more than one freeze-thaw cycle, was assayed.

Those samples with a PAT level between the LOQ and the LOD were considered to be positive and their levels were included in the statistical analysis; the samples not

contaminated with PAT (levels below the LOD) were considered to have a level of LOD/2 and were also included in the statistics

3. Results and discussion

The optimum HPLC conditions were: mobile phase consisting of trifluoroacetic acid 0.1 % / acetonitrile (94:6), flow of 0.6 mL min^{-1}, temperature at 40 ºC and 50 µL as injection volume. Under the aforementioned conditions, a good resolution among PAT and co-extractants was obtained when 20 different apple juice samples were analyzed. All of the requirements for assuring the correct validation of the method were fulfilled (see table 1).

This HPLC method gave recovery values similar to those obtained by other authors who have developed chromatographic methods for the determination of patulin in apple juices using different sample treatments (Brause et al., 1996; Herry and Lemetayer, 1996; Prado et al., 200; Legarda and Burdaspal, 2005; Valle-Algarra, 2009). However, the LOD achieved is lower than in other HPLC published methods, as can be observed in table 2.

The results obtained in the stability study of PAT in apple juice frozen at -20ºC were treated statistically; it was determined that there were no statistically significant differences ($p>0.05$) among the PAT concentrations analyzed at different times after a frozen-thawed cycle (see figure 1). This indicates that the mycotoxin is stable over a period of six months under these conditions and that no protein denaturalization effect on the PAT concentration was observed. It has been described that patulin is able to interact with proteins (Baert et al., 2007) and therefore, the possible denaturalization of these macromolecules during freezing could raise different patulin levels in samples after having been frozen. It must be pointed out that the samples studied were clear apple juices with low protein content. However, apple juice matrixes, such as cloudy apple juice or apple purees, in which the protein content is higher,

should also be monitored in order to evaluate this effect and preclude an underestimation of PAT levels (Baert et al., 2007).

3.1. Comparison of the MEKC method and the HPLC system

Both HPLC and MEKC methods used for analysis of patulin in apple juices produced adequate resolution of the mycotoxin and HMF and were specific, precise, and accurate. The limits of detection (LOD) of the methods were 0.68 and 0.70 μg L^{-1}, respectively, adequate enough since the maximum PAT level allowed by the European Commission in apple juice intended for infants is 10 μg L^{-1}. The LOQ were 1.6 and 2.5 μg L^{-1} for the HPLC and MEKC systems, respectively. A significant difference in favour of HPLC in regard to this parameter was obtained mainly because the injection volume in the HPLC system is considerably higher than in the MEKC method (50 μl versus nL, respectively). The precision for MEKC (RSD 4.0) was higher than for HPLC (RSD 7.8). Normally, CE repeatability is not as good as HPLC repeatability (Mayer, 2001). Our results can be explained due to different factors: the reconditioning of the capillary after each run, the use of normalized areas in order to evaluate the results in the MEKC system, and also due to the tedious extraction procedure used in the HPLC method. The linearity for the HPLC and MEKC methods is comparable. The recovery for both techniques (80.2 % for MEKC and 78.0 % for HPLC) is in the range established by the European Commission (Commission Regulation (EC) No. 401/2006). The selectivity, with regard to PAT and HMF, does not change when comparing the two systems, although it changes for other sample components due to the different principles of separation between MEKC and HPLC (see figure 2). The analysis time in HPLC (13 min) is slightly lower than in the MEKC methodology (15 min), and the volume of organic solvents used in the extraction and analytical procedure in HPLC and in MEKC can be considered to be similar. Figure 2 shows the chromatogram and the electropherogram obtained for the same apple juice sample.

The proposed methods (MEKC and HPLC) were applied to 20 apple juice samples intended for infants obtained from different supermarkets and pharmacies in Pamplona (Navarra, Spain) over a period of a year. Table 3 shows the patulin values obtained from the samples under study and analyzed by both methodologies.

A correlation study showed that the results obtained by the two systems are strongly correlated after determining that both variables followed a normal distribution. Figure 3 shows a graphical representation of the result obtained for the twenty apple juice samples studied by the two techniques.

Therefore, both methods are useful for the accurate quantification of patulin in this type of sample matrix.

Up until now, there have not been many surveys related to the levels of PAT in apple juice for infants (see table 2). In our study, the mean and median PAT concentration obtained (using the MEKC results) were 8.0 and 4.1 μg L^{-1}, respectively, in a range between < LOD and 29.6 μg L^{-1}. A total of 35 % of the samples exceeded the proposed European regulatory limit for products intended for infants of 10 μg L^{-1} but none of them had PAT values over 50 μg L^{-1} for foodstuff destined to general consumers.

In order to approximately evaluate the daily PAT intake of infants, an apple juice consumption of 130 mL (common volume of a recipient sold in the market) and a body weight of 10 kg (which corresponds approximately to a one-year-old infant) was assumed, thus obtaining a PAT daily ingestion of 104 ng kg^{-1} b.w. day^{-1}. This value represents 26 % of the PMTDI recommended by JECFA (400 ng kg^{-1} b.w. day^{-1}) (JEFCA, 1995). Considering the low levels of mycotoxin found, the risk for children due to patulin exposure through apple juice consumption does not appear to be of great concern. Nevertheless, other aspects, such as specific differences between adult and child vulnerability, should also be taken into account;

in addition, production methods should be controlled as much as possible in order to reduce patulin levels in apple juice intended for infants. Indeed, further studies should include screening for this patulin in other foodstuffs and research regarding its toxic effects in conjunction with other mycotoxins.

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1 **Tables and figures**

2

3 **Table 1**. Validation parameters for the MEKC and HPLC methods for patulin quantification in

4 apple juice samples.

5

Analytical method	Linearity			Recovery (%) (RSD %) (n=27)	t_m or t_r of PAT (min)	LOD/LOQ ($\mu g\ L^{-1}$)
	Range ($\mu g\ mL^{-1}$)	Calibration curves	r			
MEKC	0.08-1.0	y = 134.39x+1.55	0.997	80.2 % (4.0 %)	9.0	0.7 / 2.5
	1.0-8.0	y = 117.36x+15.34	0.998			
HPLC	0.1 - 1.0	y = 345.87x+1.90	0.999	78.0 % (7.8 %)	7.8	0.6 / 1.6
	1.0 - 8.0	y = 379.41x-31.75	0.999			

6

7 **Table 2:** PAT occurrence in apple products intended for infants

Matrix	Analytical method	n	Positive	Range (μg L^{-1} or μg kg^{-1})	LOD (μg L^{-1} or μg kg^{-1})	Recovery (%)	Reference
Apple foods for children	HPLC	12	0	--	1	85	Prieta et al., 1994
Infant apple juices	HPLC	3	2	<LOD- 5	5	--	Leggott and Shephard, 2001
Infant mixed fruit juices		7	4	<LOD- 20		--	
Foods for young children	HPLC	11	0	--	1.4	69.3 - 74.3	Valle-Algarra et al., 2009
Apple-based products for young children	HPLC	161	3	<LOD- 7.5	4	95.5	Legarda and Burdaspal, 2005
Apple juice for infants	HPLC	12	3	<LOD- 9.2	2.08	72.7 – 96.2	Cano-Sancho et al., 2009
Apple compote		36	15	<LOD- 9.6			
Multi-fruit compote		76	24	<LOD- 8.6			
Infant fruit purees	HPLC	21	0	--	5	>96%	Mhadhbi et al., 2007
Apple-based infant foods	HPLC	--	--	<LOD- 4.96	3.1	--	Plessi et al., 1998
Apple-based baby foods	HPLC	10	2	<LOD- 17.7	5	79.2 – 82.5	Ritieni, 2003
Infant clear juice	HPLC	10	0	--	1.2	53 – 74	Barreira et al., 2010
Baby-foods (purees)		76	5	<LOD – 5.7			
Fruit juice	HPLC	90	90	0.9 – 36.8	0.23	99.5	Moukas et al., 2008
Apple juice for infants	MEKC	20	14	< LOD - 29.6	0.7	80.2	This study

8 -- Unavailable

9

Table 3. Patulin concentration (μg L^{-1}) in the 20 apple juice samples analyzed by MEKC and HPLC.

Sample number	HPLC	MEKC	Sample number	HPLC	MEKC
1	<LOD	<LOD	**11**	<LOD	<LOD
2	<LOD	<LOD	**12**	27.09	24.27
3	15.72	14.34	**13**	<LOD	<LOD
4	<LOD	<LOD	**14**	13.75	13.73
5	4.16	3.50	**15**	7.14	6.91
6	<LOD	<LOD	**16**	25.63	29.61
7	4.35	4.59	**17**	2.50	2.76
8	17.80	18.85	**18**	9.61	9.38
9	14.33	12.53	**19**	11.93	10.58
10	3.18	2.88	**20**	3.76	3.71

Figure 1. PAT stability in apple juice under frozen conditions (-20ºC).

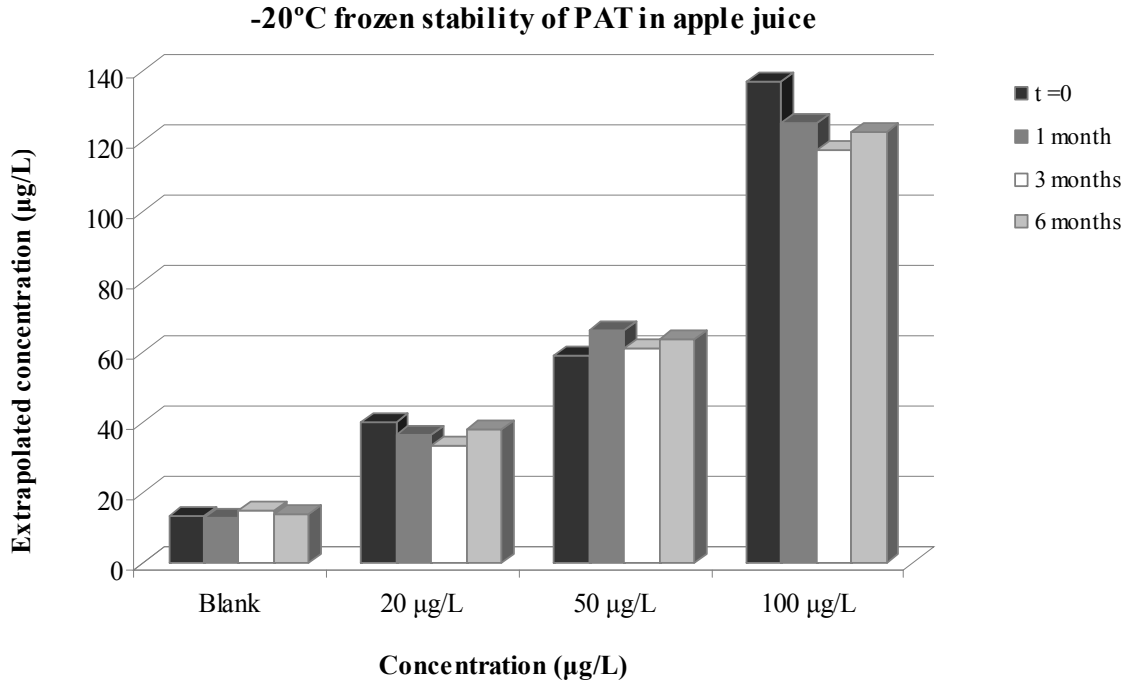

Figure 2. Chromatogram and electropherogram of an apple juice sample analyzed by HPLC and MEKC methods. MEKC system: sodium borate 33.3 mM / SDS 66.6 mM / 5 % acetonitrile; bubble cell capillary: 75 mm I.D., 64.5 cm of total length, and 56 cm effective length; injection 50 mbar 15 s; applied voltage 15 kV; cassette temperature 35ºC; UV detection at 276 nm. HPLC system: mobile phase trifluoroacetic acid 0.1 %:acetonitrile (94:6) in isocratic mode; column ZORBAX Eclipse XDB-C18 5 µm (15 cm x 0.46 cm); injection 50 µL; flow 0.6 mL min^{-1}; column temperature 40ºC pressurized applying 5 bar; UV detection at 276 nm.

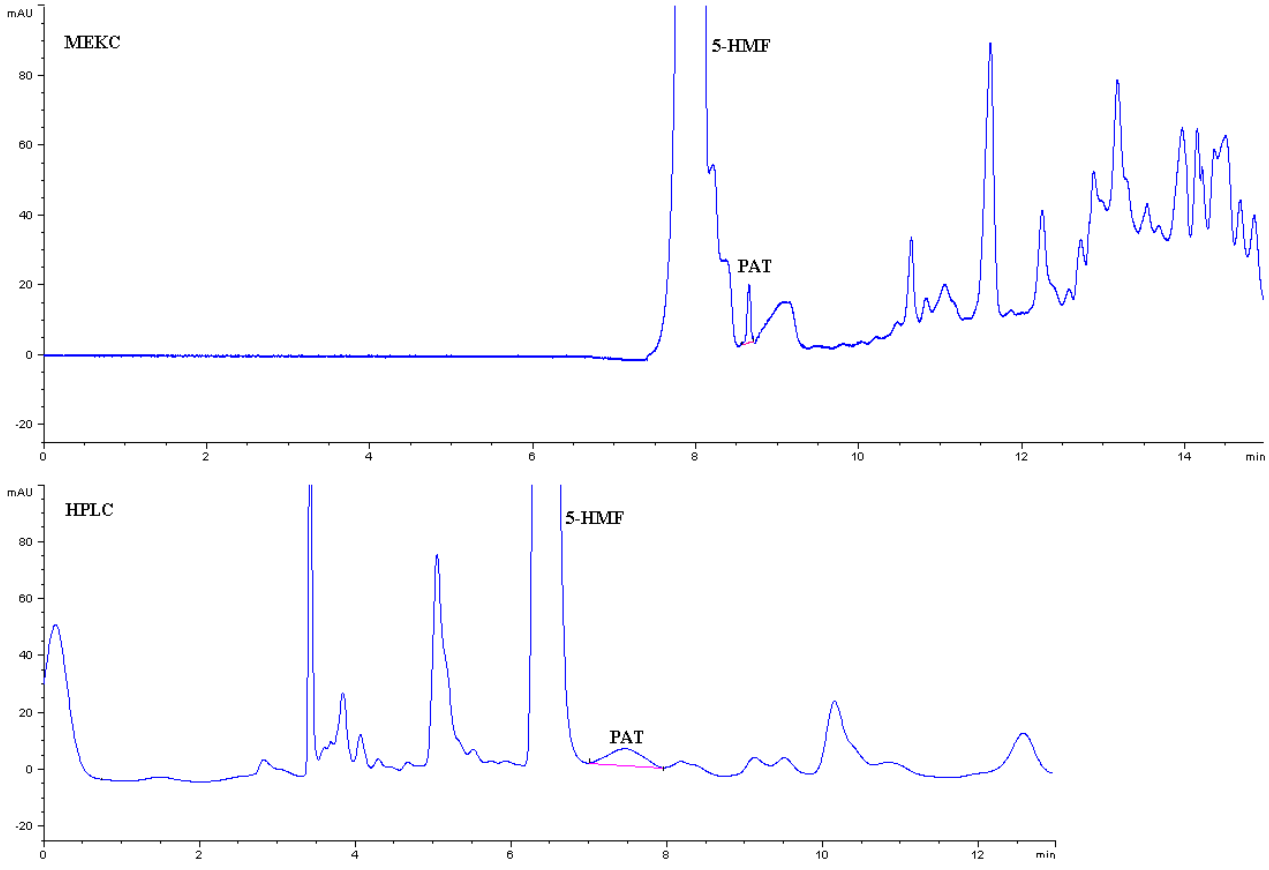

Figure 3. Comparison between the results obtained by HPLC versus the concentrations found by MEKC.