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Determination of difloxacin and sarafloxacin in chicken muscle using solid-phase extraction and capillary electrophoresis☆

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Abstract

This paper describes a method for residue analysis of difloxacin and sarafloxacin in chicken muscle. Clean-up and preconcentration of the samples are effected by solid-phase extraction (C_{18}) and the determination is carried out by capillary electrophoresis using a photodiode array detection system. The method was validated with satisfying results. The calibration graphs are linear for difloxacin and sarafloxacin from 50 to 300 μg/kg. The limit of detection obtained for difloxacin and sarafloxacin are 10 and 25 μg/kg, respectively, which allows the detection of positive muscle samples at the required maximum residue limits of European Union.

Keywords: Difloxacin; Sarafloxacin; Quinolones

1. Introduction

Difloxacin is an antibacterial drug that belongs to the fluoroquinolone class [1]. These compounds display a wide antibacterial spectrum and act directly on bacterial DNA gyrase inhibiting cell reproduction that leads to cell death [2]. A significant and progressive increase in the use of quinolones in animal production was noted over the present decade [3]. Quinolones and antibiotics, in general, are both used for prophylaxis and the treatment of diseases and as feed additives for mass gain promotion [4,5]. There are concerns that the widespread usage of antibiotics may be responsible for the promotion of resistant stains of bacteria. There is now a strict legislative framework controlling the use of these substances, with the aim of minimising the risk to human health associated with consumption of their residues. Therefore, to ensure human food safety, the European Union (EU) has set maximum residue limits (MRL) of veterinary medicinal products in foodstuffs of animal origin at Community level under Council Regulation No. 2377/90 and its later modifications. In chicken muscle, the maximum residue limit for difloxacin is fixed at 300 μg/kg [6–8]. Difloxacin is metabolised as sarafloxacin, however there is no regulation, at the moment, for the metabolite in chicken muscle, but the MRLs in other biological materials are between 10 and 100 μg/kg for skin plus fat and liver, respectively.

Several methods have been reported for determination of residues of sarafloxacin and difloxacin in various biological matrices based on HPLC determi-

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nations with UV [9,10], fluorescence [11–14] and mass spectrometric detection [15–18]. However, the literature contains only a few methods using capillary electrophoresis (CE), to analyse quinolones in body fluids and pharmaceutical formulations [19–22].

CE has become a very useful tool for pharmaceutical analysis because of its high resolution, speed and the extremely small sample volume required. There are many applications for quality control of pharmaceuticals and their formulations. However, the use of CE in the bioanalysis of drugs and their metabolites is restricted due to the low concentration sensitivity of this technique. In order to determine analytes at a low concentration range some preconcentration procedures have proved useful. During the last decade, solid-phase extraction (SPE) has gained acceptance within the analytical community and is now rapidly replacing traditional liquid–liquid extraction. The combination of the tremendous separation power of the CE with a convenient and disposable cartridge in SPE, allows the improvement of the CE sensitivity and the reduction of the limits of detection and quantification.

In this work we have combined SPE with CE in order to establish a methodology that allows the determination of difloxacin and its metabolite sarafloxacin in chicken tissues at concentration below the EU permissible MRL, for animal products.

2. Experimental

2.1. Chemical and reagents

Sarafloxacin and difloxacin were obtained from Abbott (Barcelona, Spain) and marbofloxacin was supplied by Vetoquinol (Barcelona, Spain). The structures of the quinolones studied are shown in Fig. 1. All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Diethylmalonic acid was supplied by Aldrich (Madrid, Spain), potassium dihydrogenphosphate, sodium hydroxide, dichloromethane, trifluoroacetic acid, acetonitrile, methanol and hexane were supplied by Merck (Barcelona, Spain) and acetic acid was obtained from Carlo-Erba (Barcelona, Spain). Water, with a resistivity of 18.2 MΩcm, was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Bond Elut C_{18} cartridges (500 mg) were obtained from Varian (Harbor City, USA).

2.2. Standard solution

Individual stock standard solutions (50 μg/ml) were prepared by dissolving the appropriate amount of sarafloxacin, difloxacin and marbofloxacin, used as internal standard, in 25 ml of 50 m*M* acetic acid solution. The standard solutions were prepared monthly, and were stored at 4 °C in the dark.

2.3. Instrumentation

CZE was performed on a P/ACE System model 5500 (Beckman Instruments, Palo Alto, CA, USA) equipped with an autosampler, a fluid-cooled capillary cartridge, automatic injector and a photodiode array detector. Beckman SYSTEM GOLD software was used for system control, data collection and integration of the electropherograms. The pH of the buffer solutions was measured with a Crison 2002 poten-

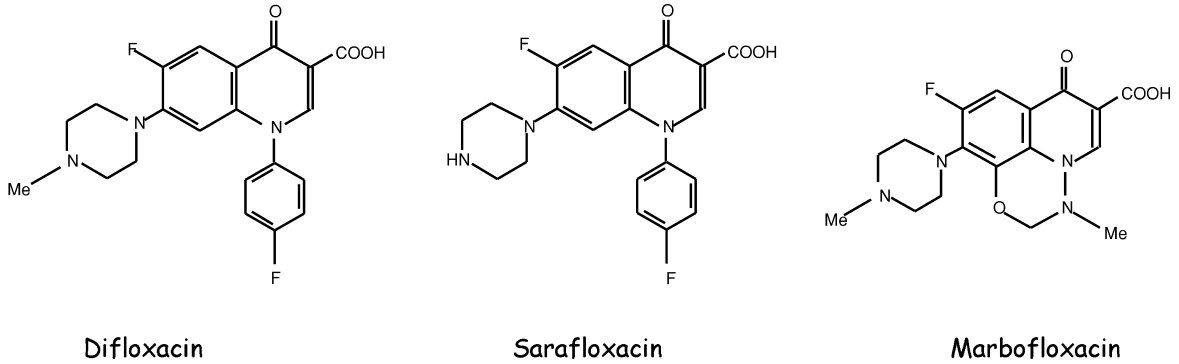

Fig. 1. Structure of the quinolones studied.

tiometer (Crison Instruments, Barcelona, Spain) using a Ross electrode 81-02 supplied by Orion Research (Boston, MA, USA). A Supelco (Madrid, Spain) vacuum tank was used in order to perform the SPE procedure. A SBS rotary Shaker (SBS Instruments, Barcelona, Spain), a helix stirrer (Euroestar Basic, IKA-Werke, Staufen, Germany) and a macrotronic Selecta (Barcelona, Spain) centrifuge were used to carry out extractions.

2.4. Electrophoretic conditions

The running buffer used was a 25 m*M* solution of diethylmalonic acid adjusted to pH 8.22 with 3 *M* NaOH. An untreated fused-silica capillary 57 cm (50 cm from inlet to detector)×75 μm I.D., (Polymicro Technologies Phoenix, AZ, USA) was also used. Before use, the capillary was rinsed with 1 *M* NaOH for 1 min, then with Milli-Q water for 1 min and finally flushed with running buffer for 3 min. The injection was hydrodynamic at a pressure of 0.5 p.s.i. for 2 s (1 p.s.i.=6894.76 Pa). The capillary temperature was 25 °C and separation voltage was 20 kV. The detection wavelength was set at 275 nm.

2.5. Spiking control sample

Fortified muscle samples were prepared by spiking 5 g of minced blank muscle tissue with the adequate volumes of standard solutions of sarafloxacin and difloxacin at a concentration of 50 ppm each. The concentrations of the fortified muscle samples were 10, 25, 50, 75, 100, 150, 200, 250 μg/kg, respectively, after adding water to obtain a final volume of 1 ml. Before the analysis, samples were allowed to stand for 20 min in the dark at room temperature.

2.6. Extraction procedure

A 5-g amount of thawed and minced chicken muscle tissue was accurately weighed and placed in a 50-ml centrifuge tube. A volume of 5 ml of phosphate buffer, prepared from 0.05 *M* solution of potassium dihydrogenphosphate adjusted to pH 7 with 3 *M* NaOH, was added to the sample and the mixture was mechanically homogenised in a helix stirrer for 5 min. The homogenate was extracted twice with 20 ml and 10 ml of dichloromethane, respectively, by means of a rotary shaker for 20 min, and after centrifugation (10 min, 2500 rpm, 1467 *g*), the organic extracts were collected into a 40-ml centrifuge tube. The organic phase was extracted twice with 5 ml of 0.5 *M* NaOH. The aqueous phase was separated from the dichloromethane by centrifugation (5 min, 2500 rpm, 1467 *g*) and was transferred to a clean vial. In order to neutralise the solution, a volume of 15 ml of 200 m*M* phosphoric acid was added. Then the extract was defatted, a volume of 10 ml of hexane was added. After centrifugation (5 min, 2500 rpm, 1467 *g*), the aqueous phase was passed through a C_{18} (500 mg) cartridge, previously activated with 2 ml of MeOH, 2 ml of water, and 2 ml of 50 m*M* phosphate buffer, pH 7. The cartridge was rinsed with 2 ml of water and 0.5 ml of MeCN. The quinolones were eluted with 2 ml of 4% trifluoroacetic acid in water–acetonitrile (25:75, v/v), followed by 1 ml of MeCN. A volume of 10 μl of the stock marbofloxacin solution was added as an internal standard. The collected eluate was evaporated to dryness under a stream of N_2 at 50 °C. The residue was redissolved in 100 μl of MeCN–water (1:1), and was injected into the capillary electrophoresis system.

3. Results and discussion

In order to predict the optimal pH for the separation of difloxacin and its metabolite sarafloxacin, we have applied a model of electrophoretic behaviour previously developed [23,24]. The method predicts the electrophoretic behaviour of substances in CZE, considering the expression that relates the electrophoretic mobility of quinolones, m_e, with the dissociation constants, pH, activity coefficients and mobilities of the acidic and basic species of the substance

$$m_e = \frac{a_{H+}^2 m_a + K_1 K_2 m_b}{a_{H+}^2 + K_1 a_{H+} + y K_1 K_2} \quad (1)$$

where m_a and m_b are the mobilities of the acidic and basic species, respectively, and y is the activity coefficient and K_1 and K_2 are the dissociation constants.

From this expression, if dissociation constants, m_a and m_b are known and taking into account the effect of activity coefficients, we can predict the electro-

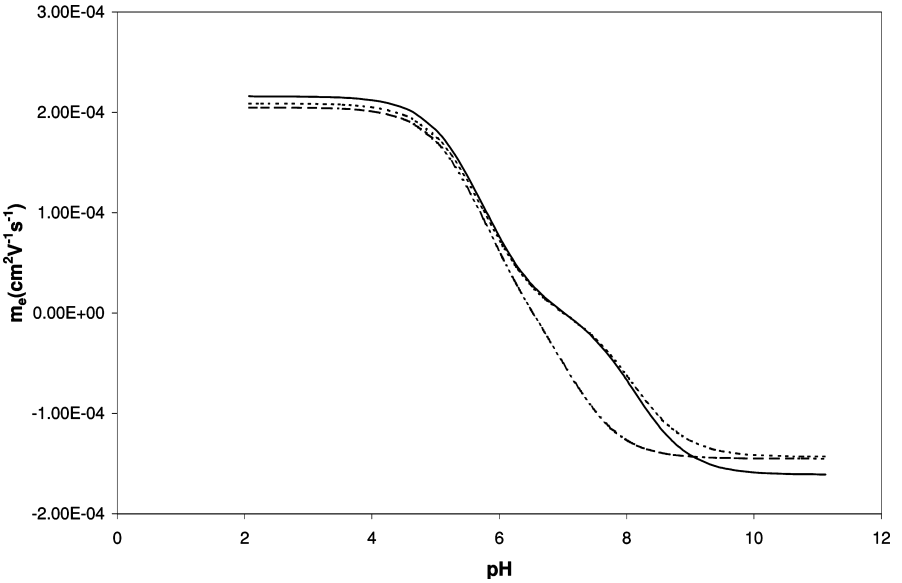

Fig. 2. Plot of the predicted electrophoretic mobilities vs. pH. Symbols: Sarafloxacin, - - -; Marbofloxacin, ———; Difloxacin, – - - –.

phoretic mobility of each compound over the whole pH range. Fig. 2 shows the electrophoretic mobilities vs. pH of the difloxacin and sarafloxacin obtained by using Eq. (1). In this figure we have also included the mobilities of marbofloxacin, which was used as the internal standard. Values of dissociation constants and mobilities of acidic and basic species of the three quinolones studied have been determined elsewhere [23,25,26]. Fig. 2 shows the migration order of the substances at each pH studied and allows the determination of the pH at which better separation is obtained. The best separation range of pH occurs when differences between mobilities of the substances studied are the biggest.

Fig. 3 shows the differences, in absolute values, between mobilities of the substances studied in this

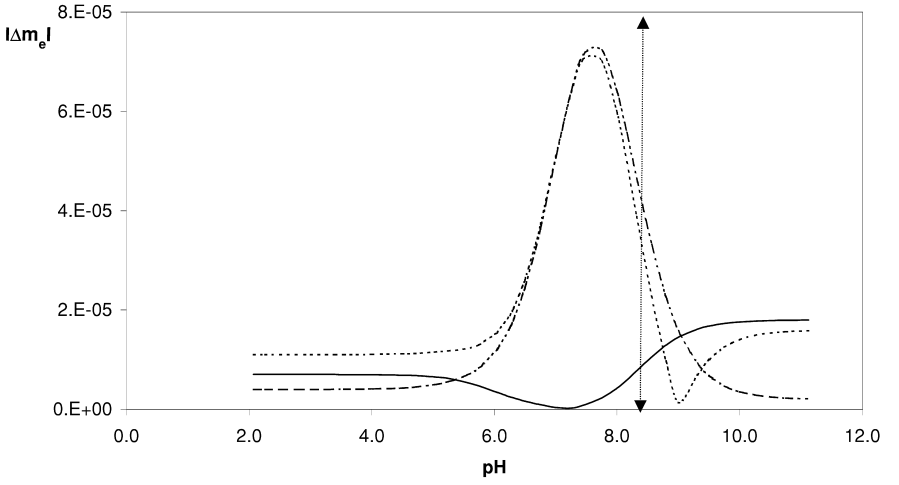

Fig. 3. Plot of the difference between the mobilities of the quinolones studied vs. pH. Symbols: Sarafloxacin–Difloxacin, - - -; Sarafloxacin–Marbofloxacin, ———; Difloxacin–Marbofloxacin, – - - –.

work and the internal standard. From this figure it is deduced that the best separation for sarafloxacin–difloxacin and marbofloxacin–difloxacin is expected to be at pH around 7.8. However, at this pH the sarafloxacin–marbofloxacin pair, shows a low $|\Delta m_e|$. In order to obtain a better separation for this pair of substances, a pH of 8.2 is proposed. A mixture of difloxacin, sarafloxacin and marbofloxacin was injected in diethylmalonic buffer at different pH values from pH 8.0 to pH 9.0 to verify this prediction.

Fig. 4 corresponds to an electropherogram of an extract of chicken muscle without fortification, obtained by the extraction procedure described previously. Fig. 5 shows the electropherogram obtained in the analysis of muscle tissue spiked with 100 μg/kg of sarafloxacin, difloxacin and marbofloxacin. The migration order for these substances is sarafloxacin, marbofloxacin and difloxacin. This order agrees with the prediction of the model used to optimised the pH of the separation, obtaining symmetric well resolved peaks.

The SPE–CE method proposed has been validated for the determination of sarafloxacin and difloxacin using marbofloxacin as the internal standard in muscle tissue of chicken.

The detector responses were found to be linear for the different components in the 25–300 μg/kg range. The linearity of the curves for sarafloxacin and difloxacin was tested using the peak-area ratios between each quinolone and the internal standard. The correlation coefficient, *r*, intercept and slope values for difloxacin and sarafloxacin are shown in Table 1.

To evaluate the recovery of the method, triplicates of drug-free samples spiked with difloxacin and sarafloxacin in the concentrations of 25, 50, 100 and 200 μg/kg were extracted following the proposed method. The recovery values for difloxacin and sarafloxacin for each spiked level studied and the standard deviation are shown in Table 1.

Intra- and inter-day accuracy for the method proposed were also evaluated. Data of intra-day accuracy were based on the analysis of three replicates of chicken tissue samples fortified at two levels

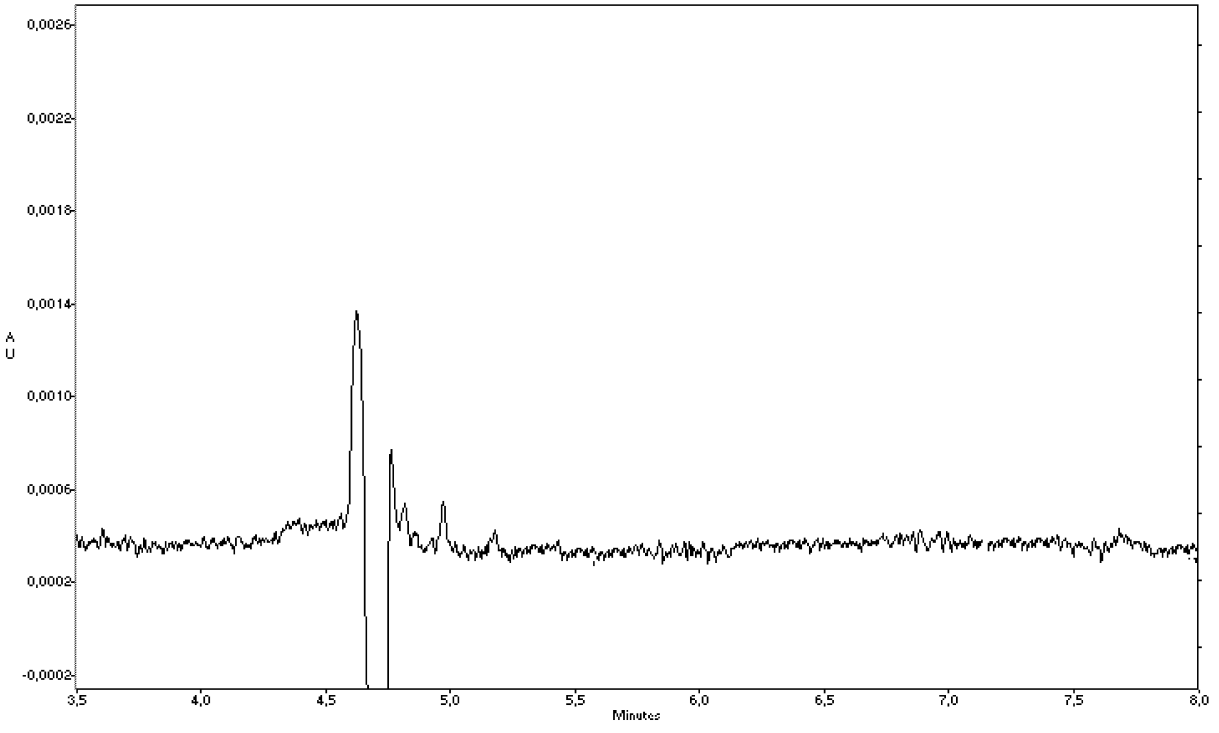

Fig. 4. Electropherogram of an extracted drug free chicken tissue.

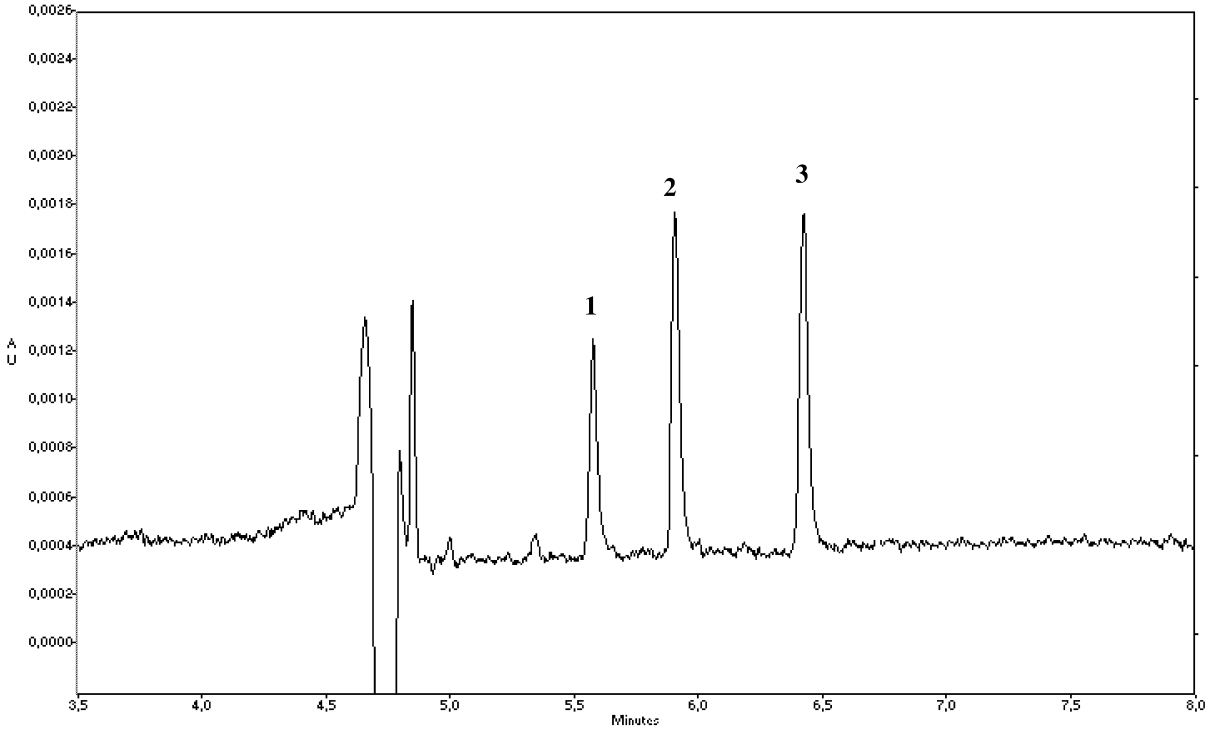

Fig. 5. Electropherogram of chicken muscle tissue spiked with 100 μg/kg for difloxacin, sarafloxacin and the internal standard, marbofloxacin. Diethylmalonic buffer 35 m*M* at pH 8.22. $\lambda = 275$ nm. Peaks: 1 = Sarafloxacin, 2 = Marbofloxacin, 3 = Difloxacin.

Table 1
Figures of merit of total procedure SPE–CE for the determination of difloxacin and sarafloxacin

Parameter	Difloxacin		Sarafloxacin	
Calibration curves ($n=7$)				
Intercept	0.00±0.01		0.13±0.04	
Slope	1.99±0.06		1.05±0.02	
Correlation coefficient	0.998		0.999	
Recovery				
Fortification level (μg/kg)	*R* (%)	RSD (%)	*R* (%)	RSD (%)
200 ($n=3$)	94.8	4.9	46.4	3.5
100 ($n=3$)	103.1	0.8	55.5	3.2
50 ($n=3$)	100.1	1.3	40.9	4.4
25 ($n=3$)	96.7	5.1	–	–
Intra-day reproducibility				
Fortification level (μg/kg)				
200 ($n=3$)	92.3	2.9	45.3	0.9
100 ($n=3$)	103.2	1.1	56.5	0.6
Inter-day reproducibility				
Fortification level (μg/kg)				
200 ($n=6$)	96.0	6.0	47.3	6.3
100 ($n=6$)	99.1	5.0	55.5	3.2
LOD (μg/kg)	10		25	
LOQ (μg/kg)	25		50	

of concentration (100 and 200 μg/kg). Inter-day accuracy data were based on the analysis of chicken tissue samples extracted on 3 consecutive days. The samples analysed were also fortified at two concentration levels (100 and 200 μg/kg).

The sample concentration that produces a peak height three times the noise baseline level has been considered the limit of detection (LOD) of the method, whereas the limit of quantification (LOQ) has been considered as the sample concentration that produces a peak height ten times the noise signal [27]. The LOD and LOQ determined for sarafloxacin are 25 and 50 μg/kg, while for difloxacin the LOD and LOQ are 10 and 25 μg/kg, respectively.

This study has shown that residues of the two antibacterial compounds, difloxacin and sarafloxacin, in chicken tissues can be analysed after some clean-up steps of the samples. Good recovery, precision and sensitivity were obtained. The LOD and LOQ values obtained are below the maximum residue limits established for these drugs in the Council Regulation 2377/90 of the EU.

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