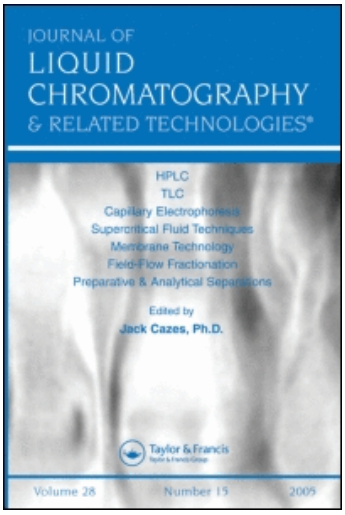

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Highly Sensitive Methods for Determination of Fluoride in Biological Samples

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ABSTRACT

A total of 40 samples of shell ashes from chicken eggs and 15 samples of chicken plasma have been investigated for amounts of fluoride. Two different methods have been applied. Egg samples were analysed with a spectrophotometric method (Alizarin : cerium : fluoride complex with detection at $\lambda = 620$ nm), following prior distillation of the samples in sulfuric media. Plasma samples were analysed by suppressed ion chromatography (IC) with conductivity detection. In this case, it was necessary to develop a cleaning method for the samples before their injection into the chromatographic system. The detection limits obtained were 25 ng mL^{-1} for the colorimetric method and 50 ng mL^{-1} in the case of the chromatographic method. Both methods were successfully applied to

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the analysis of the samples, and the obtained results were in accordance with the results obtained with the ion selective electrode (ISE) method. On the other hand, the proposed methods turn out to be a sensitive alternative when problems with sample size or cationic interferences are present.

Key Words: Fluoride; High performance liquid chromatography; Plasma; Egg shells.

INTRODUCTION

The determination of fluoride in different biological samples has been extensively investigated because of clinical and environmental interest.[1–3] Since the study by Singer and Armstrong,[4] increasing attention has been given to the analysis of blood, serum, and urine.[5–7]

Fluoride ion concentrations in serum are very low; therefore, a very sensitive method for determining fluoride is needed. Ion chromatography (IC) is widely employed for the determination of inorganic anions, including fluoride, in samples such as environmental waters or drinking water.[8] Gas chromatography methods have also been developed,[9] but detection limits reported are higher than normal levels of fluoride ion in biological samples, such as serum or plasma.[10] In order to decrease detection limit values, sensitive methods, such as fluorimetric high performance liquid chromatography (HPLC), have been developed.[11] The Ion Selective Electrode (ISE) for the fluoride ion has been extensively used for the quantification of free fluoride;[12–17] moreover, the fluoride selective membrane electrode is an ideal detection device for the determination of fluoride using a flow injection system because, by using FIA systems, the selectivity of the electrode is improved, due to the very short contact time of the interfering ions with the electrode membrane. So, extensive studies concerning the use of FIA–ISE, have been developed.[7,18–20]

Although less extensively applied, spectrophotometric methods have been also performed but, in all cases, for the estimation of total fluoride, the same sample pretreatment (alkaline fusion, dry ashing) should be carried out.[19,21]

The aim of the present work is the development of methodologies with enough sensitivity and selectivity for the analysis of fluoride in several types of biological matrices. The application of these methodologies will allow the study of the bioaccumulation and biodispersibility of the fluoride anion spiked to the diet of laying hens subsequent to the addition of sepiolite in their feed.[22] The use of sepiolite as an agglomerater of feed, or to prevent excess humidity in dregs in the poultry farm, is very widespread. However, sepiolite contains fluoride in variable concentrations.[23] For laying hens, the level of

tolerance to fluoride is 200–400 mg/kg. In these animals, the fluoride is bioaccumulated in bones being liberated at the moment in which the shell of the egg is fabricated.[24,25] In this paper, two different methods for fluoride determination, involving suppressed IC and spectrophotometry, have been proposed as alternatives to selective ion electrode in order to follow the free fluoride biological route in several matrices of laying hens.

EXPERIMENTAL

Reagents

All reagents were of analytical reagent grade, obtained from commercial sources. The standard of fluoride ($1000\,\mu g\,mL^{-1}$) was obtained from the sodium salt (Panreac, Spain). The standard injected solutions for HPLC were prepared every day in the mobile phase. The HPLC solvents were of HPLC grade (Sharlau, Spain).

Apparatus

A glass distillation system; Hitachi U-2000 Spectrophotometer; Microcom YM-3; centrifugal filter devices; (Millipore Corporation); centrifuge Mikro 12-24 (Hettich) were used. An ion chromatograph, Metrohm 790 Personal IC, equipped with a 80 μL loop and Personal IC 790 software (from Metrohm), was used to plot, acquire, and analyse chromatographic data.

Chromatographic Conditions

The column was a Metrohm Metrosep Anion Dual 1 (3×150 mm). The mobile phase was HCO_3/CO_3^{2-} (2.5 mM/2.4 mM). These solutions were filtered through a Millipore Durapore filter (0.45 μm pore size) and deareated by stirring under vacuum for 15 min before use. The flow rate was $0.5\,mL\,min^{-1}$ and a conductivity detector was used.

Samples Preparation

Shells Eggs Pretreatment

The samples were reduced to ashes (at 500°C) and distilled in sulfuric acid media. Next, the usual method was applied[26] and the total fluoride amount was obtained.

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Plasma Sample Pretreatment

Samples were kept frozen until they were analysed. In order to eliminate proteins and the heparin from the matrix, samples were centrifuged during 1 h at 1500 r.p.m. in a Centrifuge Mikro using microcom YM-3 filters. Working in this way, molecules of more than 5000 Daltons were removed from the media. However, the use of ion chromatography to analyze fluoride in samples with a high amount of Cl^- requires a previous step of eliminating that interference. Commercial systems for eliminating chloride in samples, such as seawater exist. They are based on the use of ion exchange resins; however, their use is limited at acid pH's. Because the best mobile phase that had been found for the analysis by IC was an alkaline phase, in this work, the elimination of the chlorides was accomplished by passing the samples, dropwise, into a polypropylene syringe filled with silver oxide that was obtained by precipitation from a $AgNO_3$ solution at basic pH. The silver oxide obtained was carefully washed with distilled water during the process of filtration in order to eliminate all the NO_3^- and OH^- residues. Figure 1 shows the chromatogram obtained when a mixture of $0.4\,\mu g\,mL^{-1}$ of fluoride and 0.1 M chloride solution is passed through the syringe and injected into the chromatographic system. It is possible to observe that most of the chloride has been eliminated from the matrix, by precipitation as silver chloride in the syringe.

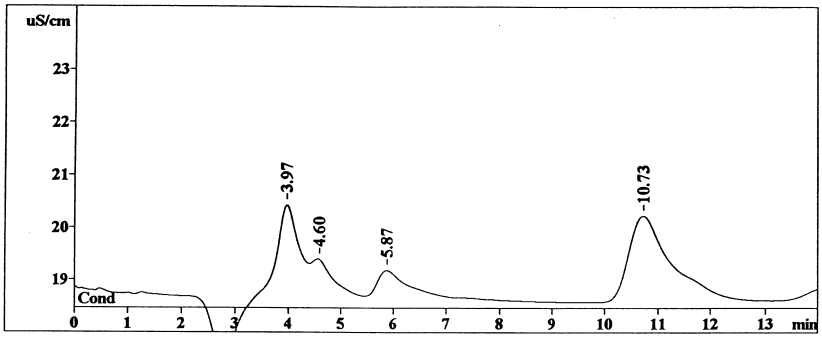

Figure 1. Chromatogram of a mixture of F^- $0.3\,\mu g\,mL^{-1}$ and Cl^- 0.1 M after passing through silver oxide. Mobile phase: HCO_3/CO_3^{2-} (2.5 mM/2.4 mM; $Q = 0.5\,mL\,min^{-1}$; $V_{inj.} = 80\,\mu L$).

RESULTS AND DISCUSSION

All the experimental parameters involved in the chromatographic system were optimized. Obtaining a good resolution between F^- and Cl^- signals was an important aspect to consider. Several mobile phases were studied. Working in acid conditions (such as benzoic acid, pH = 4.7), a decrease in retention times is caused for both analytes (retention time for F^- and Cl^- of 1.97 min and 2.1 min, respectively), so higher pH values of mobile phase were studied. HCO_3/CO_3^{2-} (2.5 mM/2.4 mM) was an adequate buffer in order to get a good resolution with respect to the chloride signal. On the other hand, a flow rate of 0.5 mL min^{-1} was chosen as optimal for the determination. Working in these conditions, the retention time of fluoride was 4.01 min, and the retention time of chloride was 6.00 min.

Calibration Graphs, Sensibility, and Precision

The precision and accuracy of both methods, the linearities of calibration graphs, and detection limits ($x_b + 3\sigma_b$), as well as determination limits ($x_b + 10\sigma_b$) were calculated. Table 1 shows the results obtained for each of the proposed methods. The linearity of the calibration plots is shown by the correlation coefficients, which are very close to unity in both cases.

Application of the Proposed Methods

Shell Ashes

About 1.0 g of sample is placed in a 100 mL still, 25 mL of water, a few fragments of glass pearls, and a little amount of silver nitrate(s) are added and stirred. After this, 25 mL of sulfuric acid (c) are added and the still connected to the condenser until 25 mL are distilled. The temperature of solution in the still cannot be higher than 150°C and the end of the condenser must be inmersed in water (5 mL maintained alkaline to phenolphthalein with ammonia during the entire distillation). The 25 mL collected must contain the fluoride from the sample. So, it was quantified in the following step, by the complex Al : Ce : F method.[26]

The method was applied to samples from chickens that have been fed with controlled fluoride amounts. Quantification of F^- was carried out by a calibration curve method. Figure 2 shows the absorption spectrum of the alizarin : cerium : fluoride complex. A total of 40 samples were analyzed and the results obtained were in accord with those that have been obtained from their analysis with the ISE method. The recovery of the complete process

Table 1. Calibration and statistical data.

Method	Calibration curve	Detection limit ($ng\,mL^{-1}$)	Determination limit ($ng\,mL^{-1}$)	Er (%)	C.V. (%)
Ionic chromatography	$Y = -7.28 + 131.39C$ ($\mu g\,mL^{-1}$); $r = 0.9993$	25	50	2.3	0.8
Spectrophotometry (Al : Ce : F)	$A = 4 \times 10^{-3} + 0.735C$ ($\mu g\,mL^{-1}$); $r = 0.9997$	50	65	2.1	0.9

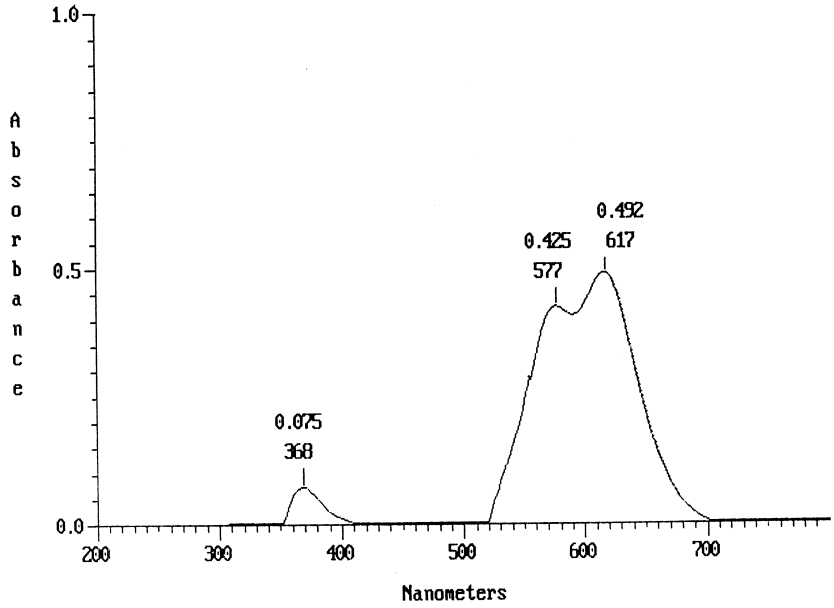

Figure 2. Absorption spectrum of Alizarin : Cerium : Fluoride complex. Fluoride concentration $0.7\,\mu g\,mL^{-1}$, pH = 5.0.

was 96% and the % coefficients of variation (C.V.) obtained ($n = 5$) less than 3%.

Plasma Samples

Quantification of F^- was carried out using the standard addition method. To this end, 0.5 mL aliquots of plasma, unspiked and spiked with increasing amounts of fluoride, are subjected to the procedure described previously (filtration by centrifugation and elimination of chlorides). Next, samples were diluted to 1.0 mL with mobile phase and 80 μL injected in the chromatograph under the optimal conditions described previously.

Figure 3 shows the chromatograms obtained. It is possible to observe the fluoride signal and how the peak intensity increased in the samples that have been spiked with fluoride.

A total of 15 samples were analysed in this way. The recovery of the method was 87% and the C.V. obtained were less than 4.2% ($n = 3$).

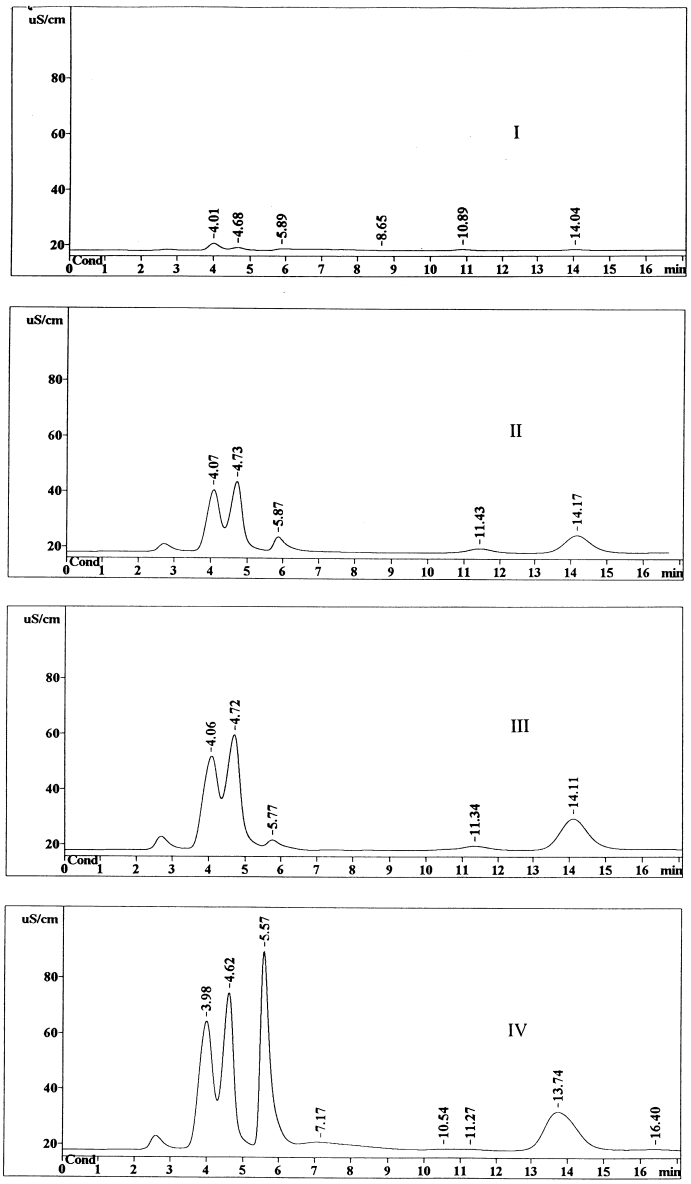

Figure 3. Chromatograms of plasma samples. Mobile phase: HCO_3/CO_3^{2-} (2.5 mM/2.4 mM; $Q = 0.5\,\text{mL min}^{-1}$ $V_{\text{inj.}} = 80\,\mu\text{L}$). (I) sample (II) sample + 0.5 μg of F^- (III) sample + 1.0 μg of F^- (IV) sample + 2 μg of F^-.

CONCLUSIONS

Both methods were successfully applied to fluoride quantification in real samples, and detection limits obtained are sensitive enough to be used as an alternative to selective ion electrode when significant interferences of cations are expected to be present.

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