Title:

Development and validation of a Capillary Zone Electrophoresis method for the determination of calcium in composite biomaterials

Running title:

Ca^{2+} determination in biomaterials by CZE

Development and validation of a Capillary Zone Electrophoresis method for the determination of calcium in composite biomaterials

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Abstract

This work presents the development and validation of a capillary zone electrophoresis method for calcium ions release quantitation from composite biomaterials with potential application in bone tissue engineering. Calcium ions quantitation was made using a fused silica capillary (40 cm, 75 µm ID) and background electrolyte containing 5 mM imidazole, 6 mM α-hydroxyisobutyric acid, 1 mM 1,4,7,10,13,16-hexaoxacyclo-octadecane and 20 % w/v methanol, at pH 4.5. Indirect UV-detection mode at 214 nm was performed. The separations were achieved using normal polarity at 6 kV, a cartridge temperature of 25 °C and 0.5 psi for 5 s for sample introduction. Parameters of validation such as specificity, linearity, limit of detection and quantitation, accuracy, precision and robustness were evaluated according to the International Conference on Harmonization guidelines. The new developed method resulted to be suitable for calcium determination from composite biomaterials for potential application in bone tissue engineering.

Keywords

Calcium quantitation, Capillary Zone Electrophoresis, Composite materials, Indirect UV, Ions Release, Validation.

Introduction

The use of metallic ions as therapeutics agents (MITA) has acquired a great interest in the field of regenerative medicine [1]. Ions such as copper, calcium, cobalt, iron, gallium, magnesium, strontium and zinc can be considered in this regard; and most of them are essential cofactors of enzymes in the organism [1]. In tissue engineering (TE), dissolution products from scaffolds have an important role in the integration between biomaterial and tissue, and produce different intra and extracellular responses [2-4]. These considerations prompted the incorporation of MITA into different releasing systems, e.g. scaffolds, intended for therapeutic applications [1-2,5-6]. Particularly, a large amount of biomaterials employed in the elaboration of scaffolds for bone tissue engineering (BTE) contain calcium ions, Ca^{2+} [7-15]. In addition, and interestingly, calcium ions are involved in the stimulation of the differentiation of bone cells, osteoblasts proliferation, bone metabolism and bone mineralization [1,16-17]. Previous works reported that dissolution products from bioactive glass materials for BTE, e.g. *Bioglass*®, containing Ca^{2+} among other ions, are involved in osteogenic and bone forming responses [3-4]. Due to inorganic ions usually have little UV (ultraviolet) absorbance or no chromophores groups in their chemical structure, conventional quantitation methods such as high performance liquid chromatography (HPLC) are not possible to employ and more sophisticated techniques are usually required such as ion chromatography (IC) [18-23], flame atomic absorption spectrometry (FAAS) [24-25] and flame atomic emission spectrometry (FAES) [24-25]. In this way, there are reports in literature which showed that the release of several ions as copper [6], calcium [26], silicon [26] and gallium [5] from different biomaterials for BTE were quantified by using techniques as FAES, electron probe X-ray microanalysis (EPA) and mass spectrometry (MS). However, all these mentioned techniques are highly complex and very expensive, and in some cases qualified operators are required. Calcium ions are released in small amounts -ppm or ppb- ; thus it is clear the need to develop new efficient methods to quantify those ions release from the matrixes. As an alternative, capillary electrophoresis (CE) methods are gaining popularity as highly efficient separation methods for ions analysis in different matrices due to its high separation efficiency and resolution, versatility, low sample consumption, short analysis time and the possibility of automation [27-32]. In addition, an indirect UV-CE technique could be applied if an UV absorbing agent is incorporated into the electrolyte [33-40].

According to our knowledge, there is no report in literature about CE methods for Ca^{2+} quantitation from this kind of biomaterials. The aim of this work was to develop and validate a simple, sensitive and reliable indirect UV-CE method to quantify Ca^{2+} release from composite biomaterials to be used in BTE.

Materials and Methods

Reagents

Dihydrate calcium chloride ($CaCl_2.2H_2O$) and imidazole were purchased form MERCK (Darmstadt, Germany). α-hydroxyisobutyric acid (HIBA), 1,4,7,10,13,16-hexaoxacyclo-octadecane (18-CROWN-6), ammonium phosphate monobasic salt ($NH_4H_2PO_4$), sodium chloride and methanol were purchased from Sigma Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade and used without further purification. Ultrapure water was obtained from EASY pure RF equipment (Barnstead, Dudubuque, IA, USA). All solutions were filtered through 0.45 µm nylon membrane (Micron Separations, Westboro, MA, USA) before use.

Instrumentation

All separations were performed with a P/ACE ™ MDQ Capillary Electrophoresis system, equipped with diode array detector (190-600 nm) and data were processed by Karat V.8 software (Beckman, Fullerton, CA, USA). An uncoated fused-silica capillary of 40 cm length (30 cm to detector) and 75 µm i.d. (MicroSolv Technology, Eatontown, NJ, USA) was used.

Electrophoretic system and capillary conditioning

Ca^{2+} quantitation was performed using a background electrolyte (BGE) consisting of 5 mM imidazole, 6 mM HIBA, 1 mM 18-CROWN-6 and 20 % w/v methanol, at pH 4.5. All samples were introduced into the

capillary by pressure at 0.5 psi for 5 s. The instrument was operated under normal polarity mode with constant voltage of 6 kV and the detection was performed at 214 nm. Cartridge temperature was maintained at 25 °C. The capillary was rinsed initially with 0.5 mol L^{-1} potassium hydroxide for 3 min, then with 0.1 mol L^{-1} potassium hydroxide for 5 min, washed with deionized water for 5 min and then with BGE for 5 min. Between runs, the capillary was conditioned with 0.1 mol L^{-1} potassium hydroxide for 1 min, washed with deionized water for 1 min and then with BGE for 1 min. In all cases, 30 psi of pressure was applied.

Quantitation

Preparation of Ca^{2+} standard solution

A stock solution containing 1 mg mL^{-1} of Ca^{2+} was prepared in water. Solutions for the calibration curve were obtained by appropriate dilution with 10 mM ammonium phosphate buffer at pH 7.4 (diluent) in a range of Ca^{2+} concentration from 1 µg mL^{-1} to 50 µg mL^{-1}.

Preparation of sodium standard solution

Individual stock solutions containing 1 mg mL^{-1} of sodium (Na^{+}) were prepared in water.

Sample preparation

Calcium cross-linked alginate films containing bioactive glass nanoparticles intended for BTE applications were similarly prepared as mentioned in a previous work [5]. Different phosphate salts containing contra-ions such as Na^{+}, K^{+} and NH_4^{+} were tested to prepare buffers at different concentrations (from 5 to 20 mM) for Ca^{2+} release study. Five samples of 10 mm of diameter were tested and aliquots of them were withdrawn at regular time intervals (2, 4, 8, 24, 48, 72 and 96 h) and analysed for the amount of calcium release.

Results and Discussion

Optimization of BGE

For the analysis of inorganic cations by CE, a simple method is to add a compound to the BGE that absorbs in the UV spectrum in order to allow a negative signal from ions when they pass through the detector [37,41-42]. The absorbent molecule most applied is imidazole [42-43]. It is used at acidic pH values to allow a better UV response; in this case a pH value of 4.5 was chosen. HIBA, together with imidazole − an auxiliary complex agent −, was added to improve the separation of the metal ions as well as their quantitation. 18-CROWN-6 was introduced to allow a better resolution between ammonium (NH_4^+) and nearby ions peaks. Different concentrations of HIBA (2-10 mM) and 18-CROWN-6 (0.5-2 mM) were analysed, and the best results were obtained at 6 mM and 1 mM, respectively. Different percentages of organic solvents were added to the BGE to improve the peak shape (from 5-25 %), and 20 % v/v of methanol was found to be the best condition to allow symmetric peaks.

Instrumental parameters

Capillary temperature, applied voltage and hydrodynamic sample introduction were optimized to obtain the best conditions in terms of ions resolution and analysis time. Cartridge temperature of 25 °C was chosen as the optimum value. The applied voltage was found to be 6 kV for better resolution with suitable current inside the capillary. The current value during runs was 3 µA. Sample introduction time was tested in the 1 to 10 seconds range and different pressures were applied, being 0.5 psi at 5 seconds the best condition for obtaining adequate peak symmetry, peak area and resolution of the cations.

Sample preparation

For release study, the buffer solution used should be the one which resemble the physiologic ionic media, e.g. phosphate buffer saline solution (PBS). After testing different buffer solutions made from sodium, potassium or ammonium phosphate salts for the release study, the best resolution between Ca^{2+} and nearby peaks was achieved using ammonium phosphate buffer at pH 7.4 at 37°C. A concentration of 10 mM ammonium phosphate buffer was chosen for immersing samples to achieve a suitable ionic strength for ions separations and electrophoretic run conditions together with a good capacity buffer.

Validation

CE system validation was accomplished following the International Conference on Harmonization (ICH) guidelines with respect to the parameters such as specificity, precision, accuracy, linearity, limit of detection (LOD) and limit of quantitation (LOQ) and robustness [44].

Specificity

Specificity was evaluated by comparing migrations times between Na^+ and Ca^{2+}, because Na^+ may be present in the sample prepared from films (in Figure 1, Na^+ peak can be seen from a standard solution, and in Figure 2 the presence of Na^+ from films and release medium can be observed). Three replications were assayed and the resolution factor (Rs) between Ca^{2+} and Na^+ was calculated according to USP 32 [45], the value obtained was 1.5. In addition, the Rs between NH_4^+ peak (from buffer solution) and Ca^{2+} was higher than 1.5.

Linearity, LOQ and LOD

Linearity was evaluated at five different concentration levels using standard solutions of Ca^{2+} prepared in diluent (Figure 3). The linearity was evaluated in a range from 1.0 µg mL^{-1} to 50 µg mL^{-1}. The LOD (S/R=3) and LOQ (S/R=10) values were 0.03 µg mL^{-1} and 0.1 µg mL^{-1} respectively (Table **1**).

Precision

Precision was evaluated for intraday (n=4) and inter-day assay (n=4) and it was expressed as relative standard deviation percent -RSD(%)- values for migration times and peak areas (Table **1**). The RSD(%) values obtained in the study of precision were lower than 2.

Accuracy

Recovery studies were used to test accuracy. Three samples at different times from release study were spiked with enough concentration of Ca^{2+} standard to obtain a 30% higher response respect to the sample. Percentages of recovery values above 100 were obtained (Table **1**). The recovery values presented were good and they were obtained with high precision.

Robustness

Different parameters such as time of injection, cartridge temperature, run voltage and electrolyte pH value have been changed in ± 2% to study robustness. In all cases, three replications were made and no significant changes have been found. RSD(%) values for migration time and area of Ca^{2+} standard solution and Rs between Ca^{2+} and nearby peaks were suitable (RSD(%) values lower than 2 %).

Calcium cross-linked alginate films

Samples were analyzed to study the release rate of Ca^{2+} from films during the first days of the assay. Figure 4 shows the release profile, where values were in the range of 5-13 µg mL^{-1}. It is important to note that Ca^{2+} release follows a zero order kinetic reaction during the period studied. Figure 2 shows the electropherogram of a sample taken from release study, blank (ammonium phosphate buffer) and calcium standard solution of 5 µg mL^{-1}. Figure 5 shows electropherograms of the samples at different times from the release study.

Ca^{2+} quantitation: advantages over other reported methods

Reports show that there are several methods for Ca^{2+} quantitation by CE [38,46]. And most of them are methods applied for Ca^{2+} analysis from water samples [46-47]. However, this new proposed CZE method was optimized for Ca^{2+} quantitation released from biomaterials in a media which contains other ions and it resulted specific, sensitive, precise, accurate and robust, and it is important to notice that this is the first CE method applied to the quantitation of the release of Ca^{2+} from biomaterials for TE.

In addition, this method is simpler, cheaper and more sensitive than other methods by CE as isotachophoresis [48]. Furthermore, the use of CE methods which include simplicity, versatility, reliability, high sensitivity and low sample consumption, result more convenient in compare with sophisticated methods as FAAS [49-50] or FAES [24]. Even though very low levels for detection and quantitation limits are obtained, these methods result more expensive and highly complex. In addition, IC is used as a method for ions quantitation [27,51], and there is a method reported for Ca^{2+} release quantitation from bioactive glasses [51]. Although similar sensitivity levels were achieved, the high cost of consumables could limit its application, when faster and cheaper methods are required.

Conclusion

A development of an indirect UV-CE method for Ca^{2+} quantitation from biomaterials for BTE was achieved obtaining good separation between Ca^{2+} and nearby ions peaks and very low LOD and LOQ for Ca^{2+} ion.

In addition, this CE method has several advantages over traditional methods for Ca^{2+} or other ions quantitation such as IC, FAAS, FAES, EPA and MS in terms of methodology simplicity, low cost of operation and low sample consumption, which make this method an alternative to quantify Ca^{2+} from composite biomaterials with application in regenerative medicine. Moreover, the proposed capillary electrophoretic system could be applied to determine other ions incorporated into biomaterials for BTE such as magnesium and strontium, which have similar characteristics to Ca^{2+} in terms of mass and charge, but an adjustment on sample preparation and parameters of validation must be considered. In addition, according to results in terms of quantification and validation, the proposed method could be applied in stages of development, quality control and release and stability studies of biomaterials or pharmaceuticals.

Abbreviations

BGE: background electrolyte, BTE: bone tissue engineering, CE: capillary electrophoresis, 18-CROWN-6: 1,4,7,10,13,16-hexaoxacyclo-octadecane, CZE: capillary zone electrophoresis, EPA: electron probe X-ray microanalysis, HIBA: α-hydroxyisobutyric acid (HIBA), HPLC: high performance liquid chromatography, IC: ion chromatography, ICH: International Conference on Harmonization, FAAS: flame atomic absorption spectrometry, FAES: flame atomic emmision spectrometry, LOD: limit of detection, LOQ: limit of quantitation, MITA: metallic ions as therapeutic agents, MS: mass spectrometry, PBS: phosphate buffer saline, Rs: resolution factor, RSD(%): relative standard deviation percent, TE: tissue engineering, UV: ultraviolet.

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Legends for figures

Figure 1. Electropherogram of a standard solution of calcium and sodium containing 5 µg mL^{-1} of each one. a) NH_4^+ (from buffer solution); b) Na^+; c) Ca^{2+}.

Figure 2. a) Electropherogram of ammonium phosphate buffer, as blank; b) Electropherogram of calcium standard solution of 5 µg mL^{-1}; c) Electropherogram of a sample after 48 h from release study of calcium cross-linked alginate films; **Ca^{2+} peak; *Na^+ peak.

Figure 3. Linearity for Ca^{2+} from of 1.0-50.0 µg mL^{-1} (y= 5503.3x - 1887.6, R^2 = 0.999).

Figure 4. Calcium release from calcium cross-linked alginate films during the first days. A zero order kinetic release was observed. The graph shows the mean value of Ca^{2+} released and the standard error of the mean. R^2 = 0.968

Figure 5. Electropherograms for Ca^{2+} release from films at different times: 2, 4, 8, 24, 48 and 96 h (a, b, c, d, e and f, respectively). *Ca^{2+} peak.

Figure 1

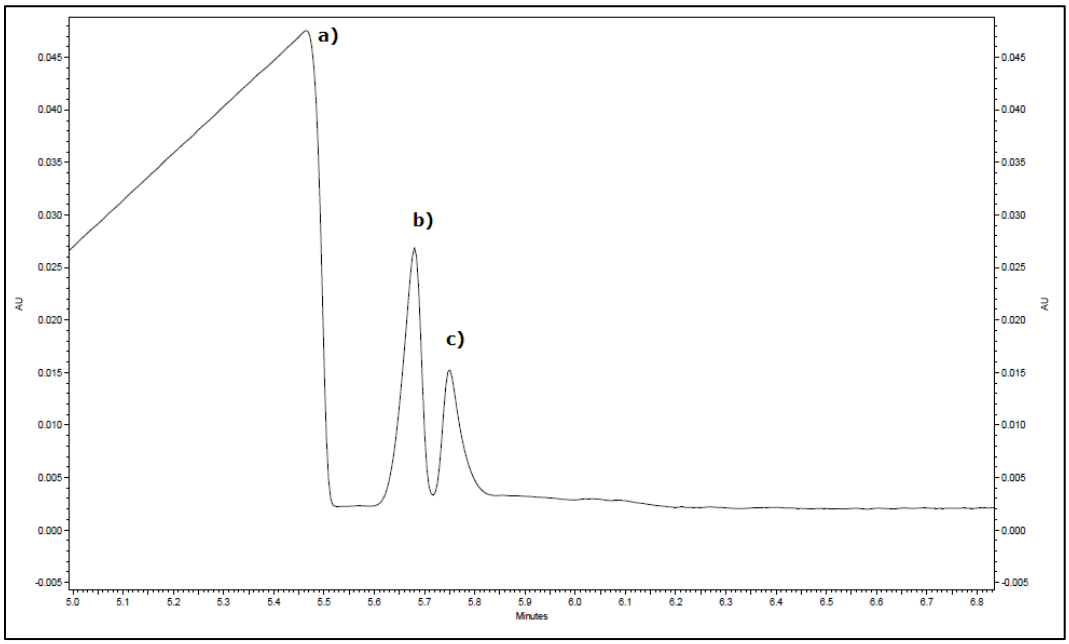

Figure 1. Electropherogram of a standard solution of calcium and sodium containing 5 µg mL^{-1} of each one. a) NH_4^+ (from buffer solution); b) Na^+; c) Ca^{2+}.

Figure 2

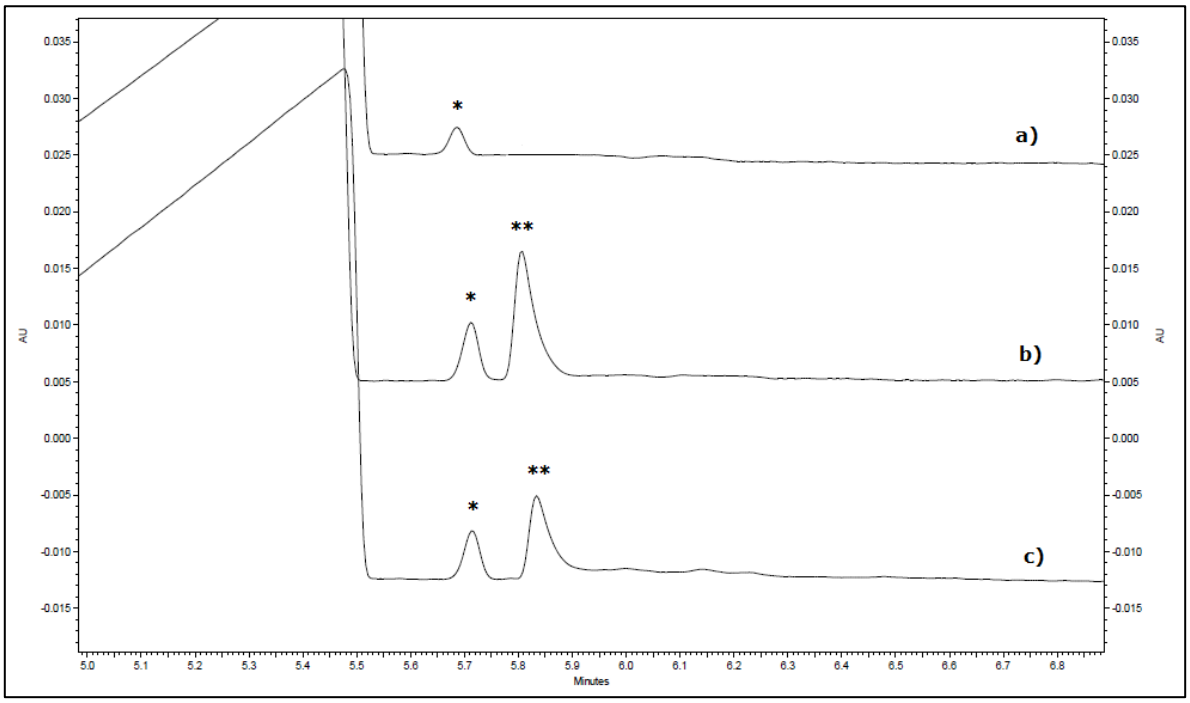

Figure 2. a) Electropherogram of ammonium phosphate buffer, as blank; b) Electropherogram of calcium standard solution of 5 µg mL^{-1}; c) Electropherogram of a sample after 48 h from release study of calcium cross-linked alginate films; **Ca^{2+} peak; *Na^{+} peak.

Figure 3

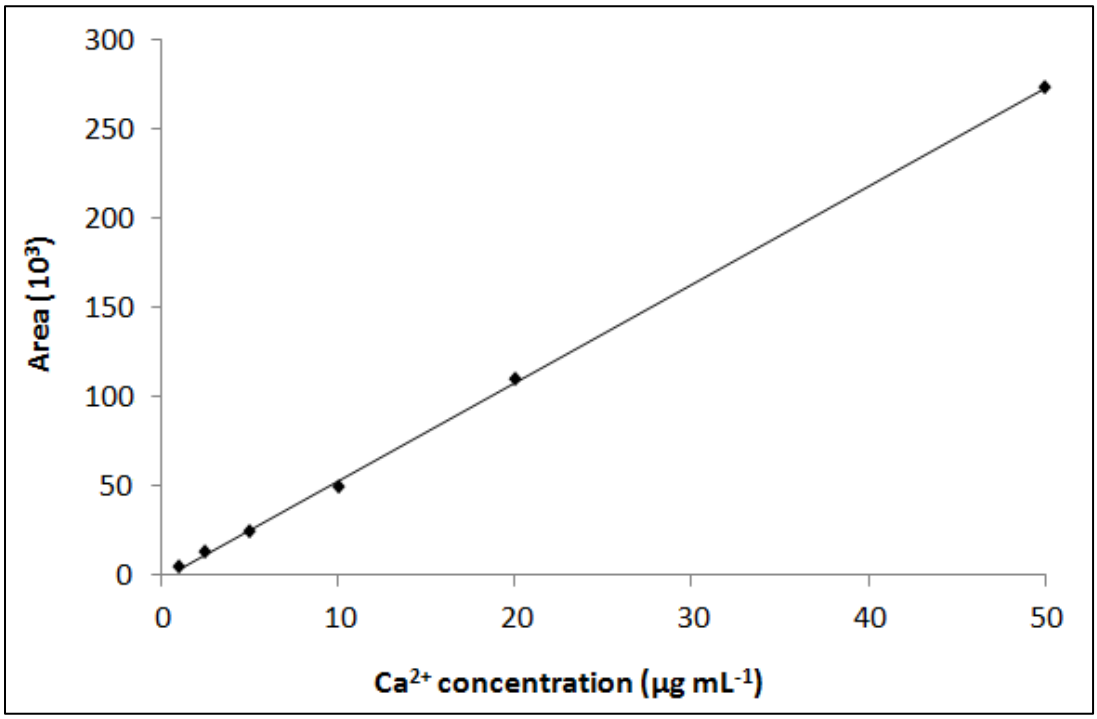

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Figure 4

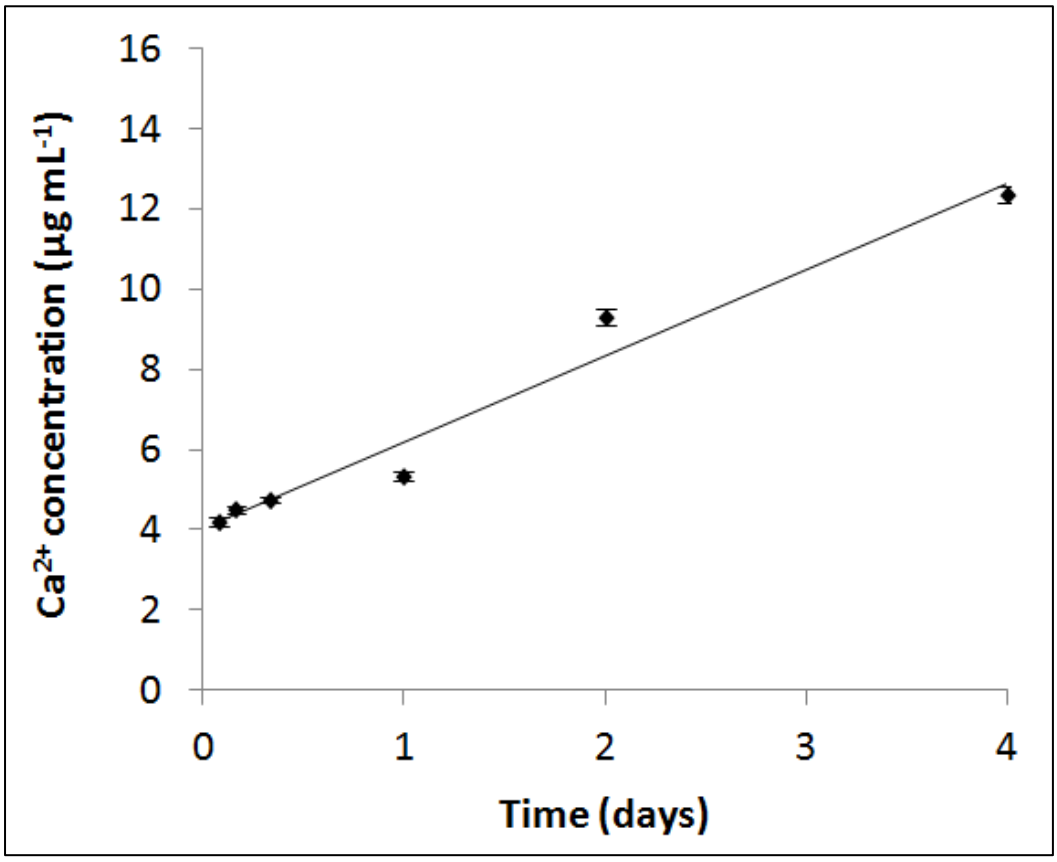

Figure 4. Calcium release from calcium cross-linked alginate films during the first days. A zero order kinetic release was observed. The graph shows the mean value of Ca^{2+} released and the standard error of the mean. $R^2 = 0.968$

Figure 5

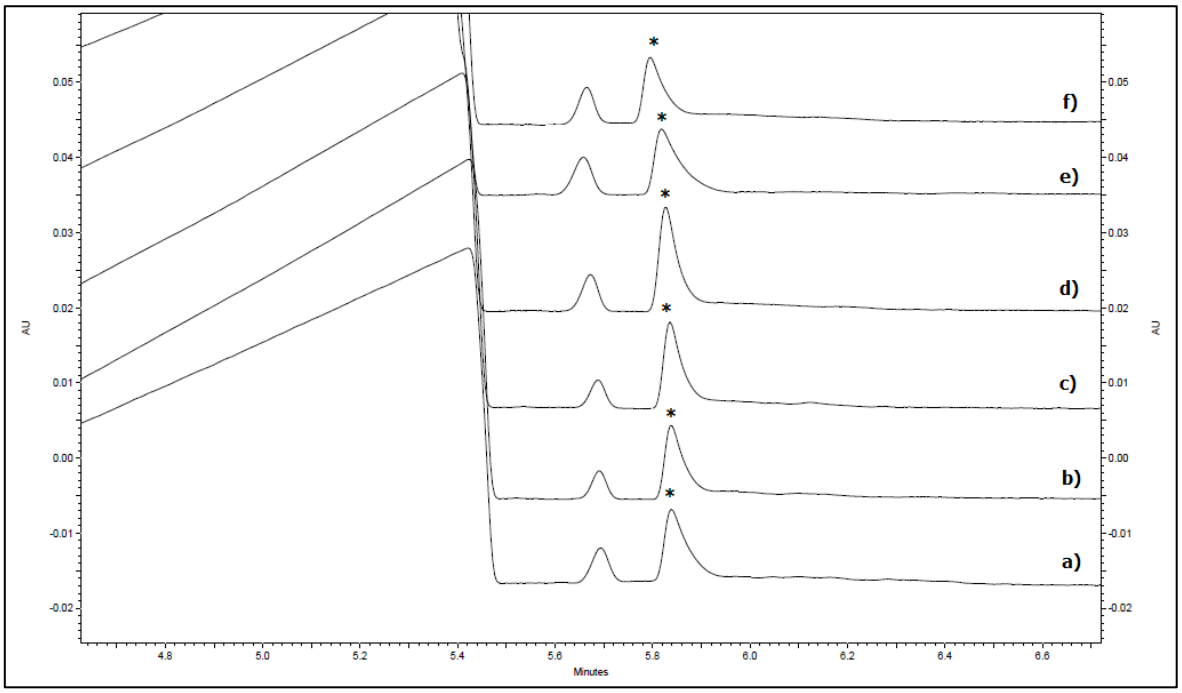

Figure 5. Electropherograms for Ca^{2+} release from films at different times: 2, 4, 8, 24, 48 and 96 h (a, b, c, d, e and f, respectively). *Ca^{2+} peak.

Table **1**. Parameter of validation of calcium analysis method

Parameter		**Value**	
Linearity range (µg mL^{-1})		1.0-50.0	y= 5503.3x - 1887.6
r^2		0.999	
LOD (µg mL^{-1})		0.03	
LOQ (µg mL^{-1})		0.1	
Precision			
Intraday (n=4)	RSD(%) of Migration Time	0.25	
	RSD(%) of Peak area	1.30	
Interday (n=4)	RSD(%) of Migration Time	0.34	
	RSD(%) of Peak area	1.47	
Accuracy [a] (n=3)		106.6 (1.5)	
		106.6 (0.2)	
		105.7 (1.6)	

[a] Percentage recovery mean values obtained from three samples on three different times from release study. RSD(%) values in parenthesis.