Exploring possibilities of calcium isotopic analysis in aqueous humor using a fast and miniaturized calcium isolation procedure

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Abstract

The potential of isotope ratios to understand biochemical processes in the body has been investigated in the past years. In addition, to carry out a more comprehensive study on the etiology and evolution of diseases, analysis of microsamples has gained importance. The isotopic study of aqueous humor could contribute to elucidate the pathophysiological mechanisms that are produced inside the eye leading to the development of glaucoma. In such a way, it is known that proteins and essential elements such as calcium play important roles in the aqueous humor outflow dynamics and/or glaucoma disease due to their behavior as biological regulators. Thus, aiming at exploring possibilities of calcium isotopic analysis by multicollector ICP-MS in aqueous humor within the context of eye diseases, and more specifically glaucoma, an optimized Ca isolation purification procedure was developed. As aqueous humor volumes available are usually at the µL level, and considering that most of the procedures discussed in the literature require high volumes of eluents, we propose a fast and miniaturized procedure applicable to low sample volumes, combining a reduced amount of resin and low eluent consumption. In the selected protocol, about 0.25 mL of sample were purified, using 0.5 mL of cation exchange resin and requiring just 21 mL of 1 M HCl as eluent. Results showed the capabilities of the proposed approach for the analysis of Ca isotopic ratios in human aqueous humor. Further studies will be addressed using this novel procedure in larger cohorts of both glaucoma patients and controls subjects.

Introduction

Miniaturization strategies both for collection and analysis of biological fluids, in combination with improved performance of analytical techniques, for diagnosis purposes are current trends of research.[1] Due to the low limits of detection achieved with modern inductively coupled plasma-mass spectrometry (ICP-MS) systems, either having a low amount of sample and/or a low target element concentration should not be a major concern.[2] The situation turns different when dealing with high precision isotopic analysis by multi-collector (MC) ICP-MS, since the analysis of absolute concentrations of isotopes at low levels is not an easy task with available instrumentation.[3] For this reason, having either a low amount of sample and/or a low concentration of a target element can strongly compromise cutting edge applications focused on the analysis of biological fluids. The use of aerosol desolvating systems, high-efficiency Jet interface, high gain faraday cup amplifiers (10^{13} Ω respect to 10^{11} Ω conventional ones) and ion counter devices have improved performance in terms of sensitivity.[4,5] In addition, sample mixing of the analyte with a non-enriched in-house standard,[3] preconcentration procedures,[6] or the use of droplets of serum deposited on silicon (with laser ablation sample introduction),[7] have been among the strategies proposed to extend the applications of MC-ICP-MS within a clinical context.

Calcium (Ca) participates in numerous physiological and biochemical processes in the body (e.g. synaptic transmission, cell signaling, bone formation, blood coagulation, etc.) and its balance is strictly regulated to keep relatively constant concentration levels to assure proper homeostasis.[8] Despite being one of the most abundant mineral elements in the human body, Ca has not been much investigated regarding isotope ratio analysis in human samples. To our knowledge, whole blood,[9] serum,[10,11] and/or urine[12-17] are the main biological fluids in which Ca isotopic ratios were investigated, being mostly employed to monitor changes in bone mineral balance.[9,12,14,15]

Ca also plays an important role in the physiology of the eye and the visual cycle. In fact, several ocular pathologies, including different forms of retinal degeneration and the aberrant growth of retinal precursors or other cell types in the eye, are often caused by mutations in genes involved in Ca regulation.[18] Also, Ca-binding proteins participate in the control of oxidative stress, apoptosis, inflammation, or Ca^{2+} homeostasis.[19] Systematic differences in the isotopic composition of essential elements have been reported so far for different pathologies (healthy *versus* non healthy), being the potential of this tool widely demonstrated during the last decade.[20] Therefore, measurements of Ca isotopic fractionation, attributed to the different (bio)chemical reactions, could provide important information related to the status

of the eye, contributing to shed light in existing alterations related to the homeostasis of this element in the visual system. Dysregulation of Ca homeostasis has been implicated in glaucoma,[21] a neurodegenerative eye disease of multifactorial origin where dysfunction of the normal flow of the human aqueous humor is considered one of the main risk factors. This intraocular fluid is mainly derived from the blood plasma and is secreted at a rate of 2.0-2.5 μL min^{-1}. It contains proteins, metabolites and essential elements which may be altered during glaucoma disease.[22] Access to aqueous humor (*in vivo* sampling) requires of an invasive approach for its collection, and it is important to note that not all aqueous humor can be extracted from the patients' eyes, being the obtained volumes usually below 250 μL.[23] Within this context, aqueous humor can be considered an ideal biological fluid to further explore novel methodologies permitting isotopic Ca analysis in samples of very limited volume.

Over the last years, several studies describing Ca isolation procedures for subsequent isotopic analysis by MC-ICP-MS have been reported.[17,24,25] Such procedures generally make use of 1-2 mL of an ion-exchange resin, and sample loading volumes between 0.5 and 2 mL. High volumes of solvents (~60 mL or even higher) are usually needed to complete the purification procedure. However, those procedures can be considered as time consuming, thus reducing sample throughput. Also, in case of samples having low volume availability, such extended procedures do not provide advantages and there is indeed no need of using a high amount of chemical reagents.

Thus, aiming at exploring possibilities of Ca isotopic analysis in aqueous humor within the context of eye diseases, and more specifically glaucoma, an optimized Ca isolation purification procedure was developed in this work. As aqueous humor volumes available for analysis are usually at the μL level, and also considering that most of the procedures discussed in the literature for such aim require high volumes of solvents, we propose a fast and miniaturized novel procedure applicable to low sample volumes, combining a reduced amount of resin and low solvent consumption.

Experimental

Samples collection

This pilot work was designed according to the Declaration of Helsinki on biomedical research involving human subjects and full ethical approval was obtained from Clinical Research Ethics Committee at the Principality of Asturias (Oviedo, Spain). Specifically, 4 control donors (83 ±

7.26 years old) and 6 patients diagnosed with pseudoexfoliation glaucoma (75.5 ± 14.46) were recruited at the Institute of Ophthalmology Fernández-Vega (Oviedo, Spain) and aqueous humor was obtained after they signed an informed consent. All individuals underwent ophthalmic examination and glaucoma patients were also subjected to more specific ophthalmic tests. All of them suffered from cataracts and did not present relevant ocular pathologies such as retinopathies or maculopathies. The individuals diagnosed with pseudoexfoliation glaucoma had deposits of exfoliative material on the anterior lens surface and/or iris in one or both eyes, as revealed during slit-lamp examination. Aqueous humor (30-350 µL) was collected for both cohorts (control donors and glaucoma patients) at the beginning of cataract surgery, by placing a 27-gauge needle in the anterior chamber. Once collected, samples were stored at -80 ºC until analysis.

Optimization of the Ca isolation procedure was carried out by measuring human serum of a healthy Spanish volunteer. This sample is regularly used as quality control in our laboratory and we have a record on elemental concentrations to assure proper performance of the experimental work.

Reagents, materials and instrumentation.

9.8 M H_2O_2 (Sigma Aldrich, Spain) and 14 M HNO_3 (tracemetal™ grade, Fisher Scientific, USA) were used for mineralization of human serum and aqueous humor samples. The Ca isolation procedure was accomplished with an AG50W-X8 resin (200-400 mesh, hydrogen form, Bio-Rad, Spain). Polypropylene + polyethylene empty columns from Triskem International (France) with 7 mm of internal diameter were filled with 0.5 mL of the resin. Diluted HCl solutions used for the different isolation procedures tested were prepared starting from optima grade 12 M HCl (VWR International, Spain) by proper dilution with ultrapure water (Purelab flex dispenser, Elga Lab water, UK).

Elemental determinations and Ca recoveries after isolation were measured in a 7700 ICP-MS (Agilent, USA) equipped with a collision/reaction cell using He gas (4.3 mL min^{-1} flow rate) by means of external calibration. Ga was selected as internal standard for instrumental drift and matrix effect correction. Standard stock solutions with concentrations of 10,000 mg L^{-1} Ca (Lot # 1404943, High-Purity Standards, USA) and 1,000 mg L^{-1} Ga (Merck, Spain) were employed to prepare calibration standards. Also, standard stock solutions of P, Mg, K, Fe, Cu, Zn (1,000 mg L^{-1}) and Na (with 10,000 mg L^{-1}) from Merck were used to evaluate elution profiles of the tested protocols as well as the verification of the absence of matrix elements in the Ca purified fractions obtained. Elemental analysis was done in 0.12 M HCl.

Calcium isotope ratios were measured using Neptune MC-ICP-MS (Thermo Scientific, Germany) instrument in 0.14 M HNO_3 (an evaporation step of the 0.12 M HCl media purified Ca fraction was performed). Table 1 collects cup configuration and instrumental settings employed. Samples were introduced into the ICP with a PFA concentric nebulizer (100 $\mu L \cdot min^{-1}$ flow rate) and a Scott-type double pass spray chamber. The Neptune is equipped with 9 Faraday cups connected to 10^{11} Ω amplifiers. The ^{42}Ca, ^{43}Ca and ^{44}Ca isotopes were monitored following a sample-standard bracketing sequence and were measured in 0.14 M HNO_3. No contribution of $^{88}Sr^{2+}$ or $^{84}Sr^{2+}$ to $^{44}Ca^{+}$ and $^{42}Ca^{+}$ signal, which was accounted by monitoring the signal measured at 43.5 mass ($^{87}Sr^{2+}$), was. In fact, background levels below $6 \cdot 10^{-5}$ mV were measured for 43.5 m/z for samples and for the ICP std used for bracketing correction (prepared from Ca stock solution). Ca measurements were performed in pseudo-medium resolution after selecting a position of the magnetic field so that $^{42}Ca^{+}$ is free from $^{40}Ar^{1}H_2^{+}$ interference, that is, on the left flat shoulder of the spectral peak.[25] Ca concentration for both types of samples and the standard solution used for bracketing correction were adjusted to 10 mg L^{-1}. At these conditions, registered signals were at about 0.19 V, 0.04 V and 0.7 V for 42, 43 and 44 Ca isotopes, respectively. Due to the low signal for ^{43}Ca, the isotopic ratio $^{44}Ca/^{42}Ca$ was used. Isotopic composition, expressed as $\delta^{44/42}$ (see equation 1), was calculated respect to the ICP Ca standard solution that was also subjected to the optimized isolation procedure. Prior to δ calculation, removal of outliers based on a 2σ-test was processed on-line.

$$\delta^{44/42}Ca = \left[\frac{(Ca^{44/42})_{sample} - (Ca^{44/42})_{standard}}{(Ca^{44/42})_{standard}}\right] \times 1000 \text{ (equation 1)}$$

Sample preparation

Digestion of the samples (human serum and aqueous humor) was carried out in Savillex® beakers, previously cleaned with both HNO_3 and HCl of pro analysis purity grade (VWR, Spain) and subsequent repeated rinsing with ultrapure water. Samples of aqueous humor (volumes are collected in Table 1) were acid digested at 110 ºC overnight using 500 μL of 14 M HNO_3 and 125 μL of 9.8 M H_2O_2 (i.e. 4:1 v/v ratio). Aliquots of 1 mL of the serum sample were acid digested at 110 ºC overnight with 4 mL of 14 M HNO_3 and 1 mL of 9.8 M H_2O_2. Digested samples were then evaporated until dryness at 90 ºC and reconstituted in the medium required for the Ca isolation procedures assayed (1 M HCl was employed for the optimized procedure), namely 1 mL for serum and 0.30 mL for aqueous humor, respectively.

0.25 mL of the corresponding digested samples (serum and aqueous humor) were submitted to the purification process while the rest was used to determine the elemental Ca content.

Results and discussion

Determination of elemental calcium

In Table 2 it is collected the volume of aqueous humor available of each sample and the Ca concentrations determined for the investigated samples. As it can be seen, Ca concentration levels range from 43 to 220 $mg \cdot L^{-1}$. Due to the limited number of samples available no statistical analysis for groups comparison was performed. Only averaged levels of Ca for each group were examined, resulting in similar results for both cohorts: 135 ± 62 $mg \cdot L^{-1}$ (controls) and 114 ± 66 $mg \cdot L^{-1}$ (patients). The Ca levels in aqueous humor have been also examined in recent literature with different results. For instance, Zhou *et al*.[26] found on average 48.8 ± 5.5 $mg \cdot L^{-1}$ Ca (n = 24, age: 72.46 ± 11.83 years, senile cataract individuals). These results are in agreement with other publications presenting Ca concentrations in aqueous humor between 50-80 $mg \cdot L^{-1}$ Ca.[27] On the other hand, Dolar-Szczasny *et al.*[28] have published a work showing levels of trace elements in aqueous humor of patients with cataracts and they have reported averaged values (294.5 ± 162.3 $mg \cdot L^{-1}$ Ca), as well as minimum (98.98 $mg \cdot L^{-1}$ Ca) and maximum concentrations (1162 $mg \cdot L^{-1}$ Ca) found on 115 patients with cataracts of an average age of 74 ± 7.27 years. Therefore, the Ca levels obtained in our work, ranging from 53 to 220 $mg \cdot L^{-1}$, are within the range of published values. The concentrations of this element in aqueous humor was compared with values for individuals with distinct pathologies, including diabetes,[29] or cytomegalovirus retinitis,[23] being not observed significant differences in the levels of Ca as consequence of the disease, which may indicate a well-regulated homeostatic balance of this element in the body.

Optimization of calcium isolation procedure

Based on literature published on high-precision measurement of Ca isotope ratios in biological samples, and considering the limited volume of aqueous humor available, we selected just 0.5 mL of resin for Ca isolation. Then, the appropriateness of different procedures, all of them employing AGW-X8 cation exchange resin, was tested with human serum as model sample. The first one corresponded to a procedure using 2.5 M HCl as solvent for sample loading.[17] However, with such approach, we found that Ca was not retained in the resin, being directly eluted from the column.

A summary of the different steps involving the other two tested protocols (protocol 1 and protocol 2) has been collected in Table 3. In addition to Ca, also P, Mg and Na were initially monitored. Elution profiles (2 mL fractions) obtained for the investigated protocols are shown in Fig. 1. Fig. 1a corresponds to a protocol adapted from that proposed by Tacail *et al*.[24] (protocol 1). It can be seen that in our experiments, using 1 M HCl as eluent (protocol 1), Ca elutes between 9-21 mL after sample loading, a considerably lower volume as compared to that employed previously,[24] requiring 65 mL of eluent for 2 mL of sample and 3 mL of resin. Elution is also faster and simpler than following the protocol 2, as shown in Fig. 1b for the adapted protocol from Grigoryan *et al.*[25] . The differences of protocol 2 with respect to the previous one[25] was also the lower volume in our procedure, both of resin (0.5 mL *versus* 1 mL) and sample v (0.25 mL *versus* 1 mL). In this protocol 2, it was observed that Ca eluted in 0.8 M HCl, being not required 4 M HCl as expected,[25] probably due to wash effect. For both developed protocols the Ca recovery was quantitative: 95% for protocol 1 and 94.8% for protocol 2. For further experiments, protocol 1 was selected because of simplicity.

In order to assure proper purification of the target element from other matrix elements, the selected isolation protocol 1 was applied to five digestion replicates of the same serum sample (together with three procedural blanks), collecting all Ca in the appropriate single fraction. After evaporation and reconstitution of such fraction in 0.12 M HCl, were also measured by ICP-MS, assuring the absence of concomitant matrix elements Na, K, Mg, Fe, Cu, Zn and P.

Despite an acidic digestion process was performed prior to isolation, it was considered important to further check the suitability of the selected protocol 1 for aqueous humor samples. Among matrix elements present in aqueous humor, a recent publication [30] showed concentration ranges in individuals having cataracts (same case as the control group analyzed in this group) between 2070-3936 mg L^{-1} for Na; 13-30 mg L^{-1} for P and 5-17 mg L^{-1} for Mg. The trace elements Cu, Fe and Zn were found to be at the μg L^{-1} range.

In order to preserve as much sample as possible, Ca and elemental matrix components for investigated samples were only measured after the isolation procedure (Ca procedural blanks referred to the undiluted solutions were 2.3 $\pm$ 0.3 μg/g). Na, K, Mg, Fe, Cu, Zn and P were determined in the Ca purified fraction, being in all cases below detection limits. In the digested samples, only Ca elemental concentrations were measured. Ca recoveries obtained for aqueous humor samples were calculated after evaporation of the 16 mL of 1 M HCl where Ca was eluted, followed by reconstitution in 0.12 M HCl. Results are collected in Table 2. After isolation, Ca recoveries between 94 and 114% were obtained (Table 2), further demonstrating the suitability of the procedure used for calcium.

Calcium isotope ratios measurements by MC-ICP-MS.

With the aim of demonstrating the applicability of the fast and miniaturized Ca isolation procedure, possible alterations of the Ca homeostasis were investigated by performing isotopic analysis in the purified aqueous humor fractions. Isotopic analysis was carried out in 10 $mg \cdot L^{-1}$ Ca solutions. Bracketing correction was done respect to a Ca ICP standard that was also subjected to cation exchange procedure, thus avoiding procedural blanks subtraction (signal from samples and the standard used for correction are subtracted for delta calculation, therefore, as the ICP standard solution was subjected to the isolation procedure, procedural blank subtraction is not required). Fig. 2 collects the $\delta^{44/42}$Ca values obtained for the investigated samples (see numerical values in Table 2). It can be seen that $\delta^{44/42}$Ca range from -1.4 to 0.81 for the control group whereas for the cohort of patients with eye disease, a wider range was obtained, that is, $\delta^{44/42}$Ca varied from -2.4 to 0.68.

The results obtained in this work, showing a wider $\delta^{44/42}$Ca range in the aqueous humor of patients with a glaucoma disease than controls subjects, are in well agreement with the behaviour found for Ca isotopic analysis in serum of individuals with active myeloma respect to non-active myeloma.[10] The alterations of calcium isotopic composition could be directly related to alterations regarding S100 calcium binding proteins. Such family of proteins is involved in numerous cellular activities and, for instance, S100A8 is associated with the presence of ROS in ocular surface inflammation. In fact, levels of S100A8 have been found to be elevated in tear fluid from glaucoma patients under chronic medication.[19] Some calcium binding proteins, apart from Ca, may also be regulated by Zn or Cu.[19,31] Therefore, the results obtained here for Ca could also be reflected on Cu and Zn isotopic compositions. Indeed, a recent work has revealed significant differences in the isotopic composition of Zn (not for Cu) in the aqueous humor of glaucoma patients when compared to control subjects. In such work, Zn isotopic composition in the glaucoma cohort was found to be enriched in the lighter isotope, which may be associated with the dysregulation zinc-binding proteins synthesis or function, including metallothioneins, during glaucoma disease.[30] Therefore, larger cohorts must be considered in further experiments to address the role of Ca within the eye.

Conclusions

In this work, a fast Ca purification procedure was developed, avoiding the use of a high volume of both sample and eluents. The feasibility to measure Ca isotope ratios by MC-ICP-MS in aqueous humor using the developed protocol was demonstrated and analysis was carried out comparing glaucoma patients versus a group of controls. However, it should not be obviated the limited number of samples measured. Still, as aqueous humor is located in a rather watertight compartment, results might be easier for interpretation as compared to other body fluids such as blood. The preliminary results obtained in this work foster the study of isotopic alterations of Ca in the aqueous humor of large cohorts of glaucoma patients and their possible implications with dysregulation of the metabolism of Ca-binding proteins and/or Ca signalling. In addition, in the search of a less invasive approach, maybe tear fluid could be an interesting alternative to evaluate in future studies employing the methodological development proposed here.

Conflict of interest

There are no conflicts to declare.

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TABLES

Table 1. Instrument settings and acquisition parameters for MC-ICP-MS measurements.

Cup configuration	$^{42}Ca^{+}$: L4; $^{43}Ca^{+}$: L2; 43.5: L1; $^{44}Ca^{+}$: C
RF power (W)	1100
Ar flow-rates (L min^{-1})	Plasma gas: 15; Auxiliary gas: 0.8 Sample gas: 1.03
Resolution mode	Medium
Number of blocks	6
Number of cycles	8
Integration time (s)	4.194

Table 2. Elemental concentrations (uncertainty is estimated as 3%) of Ca measured in aqueous humor samples and Ca recoveries obtained after isolation *via* ICP-MS. Volume of aqueous humor sample available for analysis has been also indicated. $\delta^{44/42}$Ca values obtained have been also corrected. Uncertainties for CT2 and CT4 correspond to standard deviation obtained for two measurement replicates of the same sample. For the rest of the samples the uncertainty corresponds to RSD (expressed in ‰) obtained in the measurement of $^{44}Ca/^{42}Ca$ ratio for the standards in between each sample.

		Sample volume (μL)	**[Ca] (mg·L^{-1})**	**Recovery (%)**	$\delta^{44/42}$Ca
CONTROLS	CT1	180	67	110	0.77± 0.52
	CT2	60	173	114	0.81 ± 0.21
	CT3	80	200	94	-1.4 ± 0.4
	CT4	200	98	106	0.22 ± 0.16
PATIENTS	PEXG1	60	151	103	-2.4 ± 0.5
	PEXG2	30	220	97	-0.40 ± 0.20
	PEXG3	115	53	98	0.68 ± 0.17
	PEXG4	115	102	102	0.32 ± 0.23
	PEXG5	350	115	99	-1.6 ± 0.4
	PEXG6	160	43	100	-1.4 ± 0.5

Table 3. Experimental conditions of protocol 1 and protocol 2 tested for Ca purification. 0.5 mL of AG50W-X8 resin was used for both procedures.

	PROTOCOL 1	**PROTOCOL 2**
1. Resin cleaning	6 M HCl (10 mL) 2.5 M HCl (10 mL) Milli Q (10 mL)	
2. Resin conditioning	1 M HCl (10 mL)	0.4 M HCl (10 mL)
3. Sample loading	1 M HCl (0.25 mL)	0.4 M HCl (0.25 mL)
4. Matrix elution	1 M HCl (8 mL)	0.4 M HCl (34 mL) 0.5 M HCl + 12.94 M acetone (10 mL) 0.8 M HCl (7 mL)
5. Ca elution	**1 M HCl (13 mL)**	**0.8 M HCl (13 mL)**

FIGURES

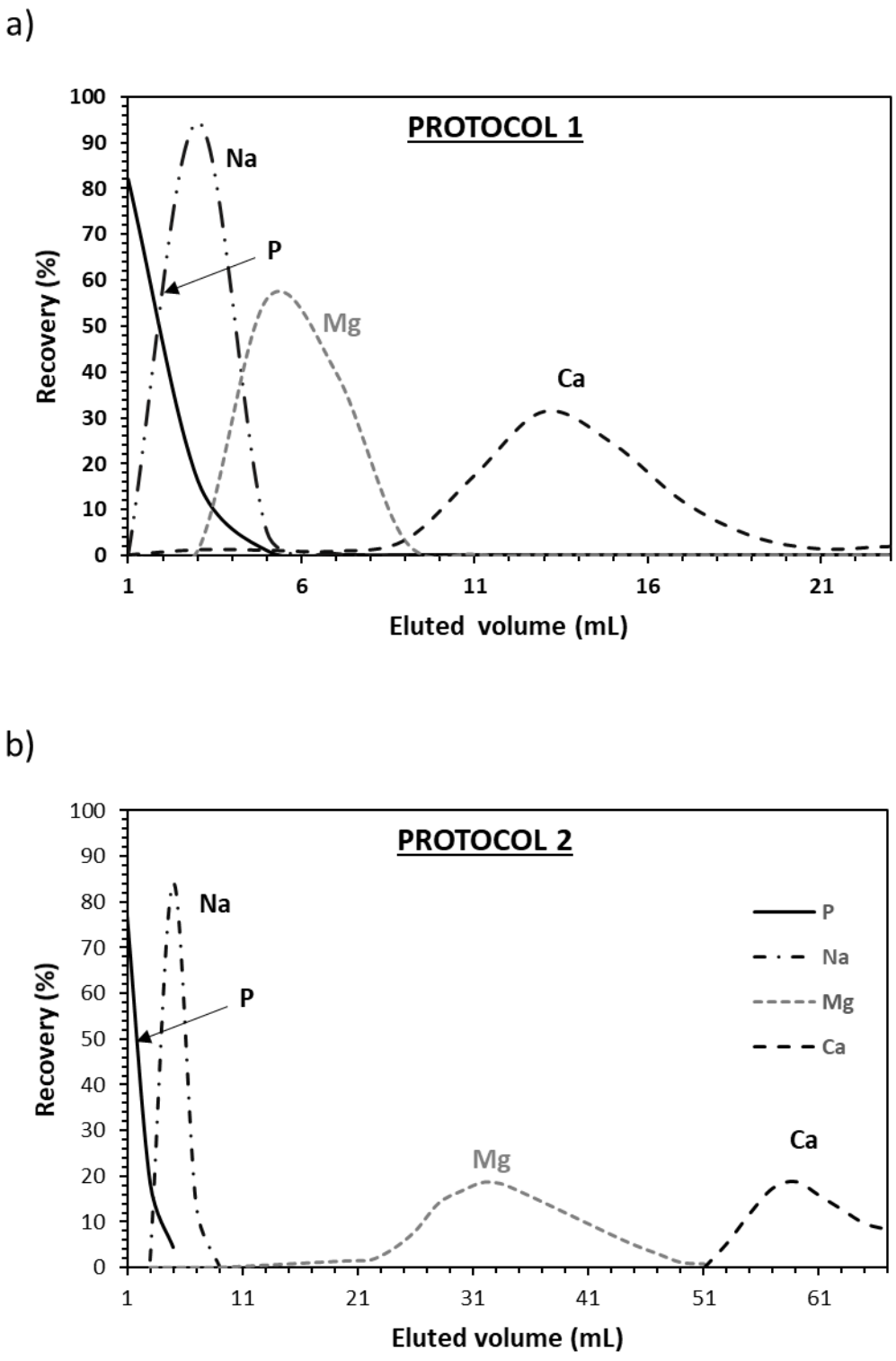

Fig. 1. Elution profiles obtained for PROTOCOL 1 (a), and PROTOCOL 2 (b) using 0.5 mL AG50W-X8 resin.

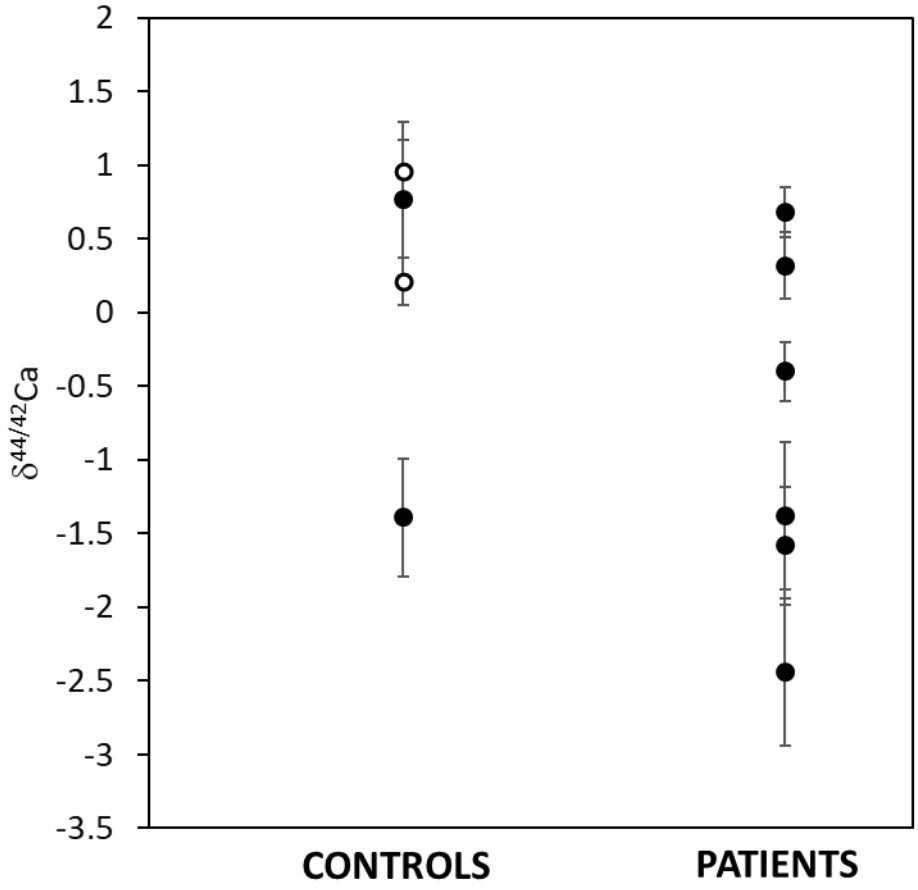

Fig. 2. $\delta^{44/42}$Ca values obtained for controls and patients after bracketing correction. Error bars correspond to the standard deviation of two measurement replicates for samples filled in white colour. Error bars for samples in black colour correspond to RSD (expressed in ‰) obtained in the measurement of ^{44}Ca/^{42}Ca ratio for the standards in between each aqueous humor sample.

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