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# Characterization and validation of candidate reference methods for the determination of calcium and magnesium in biological fluids

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ABSTRACT

Biological fluids monitoring is one of the important tools in prevention of diseases. Mineral elements in these media are essential factors to collaborate biological monitoring, disease conditions of humans, and maintenance of good health. These minerals are usually measured in laboratories using complicated and expensive techniques as ion chromatography (IC), atomic absorption spectroscopy (AAS), and inductive coupled plasma (ICP). The current work tries to validate alternative simpler and cheaper methods for the measurement of calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) in the biological medium. Therefore, we had optimized EDTA titration, flame-atomic emission spectroscopy (FAES), and ion-selective electrode (ISE) to measure biological calcium. In addition, EDTA titration and ISE used to measure magnesium in biological samples. In fact, we are the first who totally validated cheapest analytical methods for the analyses of these cations in biological fluids. The influence of standard calibration curve on method imprecision and inaccuracy was examined using external and internal quality control materials. In general, the recoveries of the optimized methods were sufficiently good [92–103%]. Good stable reproducibility was registered especially with the lowest values of the relative standard deviation (RSD) of the ISE. Besides, good readabilities expressed as the limits of quantitation (LOQs) for $Ca^{2+}$ measurements using FAES, ISE, and EDTA were (2.1–3.0 mg/L), (3.1–6.1 mg/L), and (3.9–7.7 mg/L), respectively. In addition, the LOQs for $Mg^{2+}$ measurements using ISE and EDTA were (1.2–4.5 mg/L) and (2.0–5.7 mg/L), respectively. Difference (%) of the reproducibility of the measurements in 5 days and the uncertainty was in the following range: FAES (just for $Ca^{2+}$) ISE EDTA. The validated methods showed acceptable correlations (0.9734–0.9990). They provided rapid, less tedious, practical and more accurate alternatives for the quantification of those bivalent cations in human fluids. Consequently, this research supports the implementation of cheap techniques and provides accurate analytical methods for the measurements of biological calcium and magnesium.



## 1. Introduction

Determination of the electrolytes in human fluids is one of the most important functions in the clinical laboratory. Electrolytes affect most metabolic processes. They serve to maintain osmotic pressure and hydration of various body fluid compartments, proper body pH, and the regulation of appropriate heart and muscle functions [1].

Bivalent cations are usually involved in acute and chronic inflammatory pathology. Alkaline-earth cations (i.e. $Ca^{2+}$, $Mg^{2+}$) play complex roles in human organism. They are found both intracellularly and extracellularly and consider crucial for many typical processes within the normal physiology [2]. $Ca^{2+}$ and $Mg^{2+}$ work also as catalysts or activators in many enzymatic reactions [3].

$Ca^{2+}$ and $Mg^{2+}$ distributions in human body are controlled by the performances of the human systems and hormones functionalities [4]. There are also other factors fluctuate the availabilities of the two elements in human fluids as the stress, surgery and chronic diseases [4, 5]. The sharp deviations of the concentrations of $Ca^{2+}$ and $Mg^{2+}$ from the normal ranges in biological fluids are responsible factors for host of symptoms affecting the quality of life. However, it is ironic in the face of the important roles of these ions in our lives; doctors are seldom ordering $Ca^{2+}$ and $Mg^{2+}$ tests in biological fluids even the two elements can employ in emergency medical situations. In fact, it is wise idea to

*Abbreviations:* CPC, *o*-cresolphthalein complexone; ISE, ion-selective electrode; FAAS, flame-atomic absorption spectroscopy; FAES, flame-atomic emission spectroscopy; IC, ion chromatography; ICP-MS, inductively coupled plasma-mass spectrometry; CSF, cerebrospinal fluid; ISO, International Organization for Standardization; IUPAC, International Union of Pure and Applied Chemistry; AOAC, Association of Official Analytical Chemists; EDTA, ethylenediaminetetraacetic acid; dd-DI $H_2O$, doubly distilled deionized water; PE, polyethylene; PP, polypropylene; SRM, Standard Reference Material; EBT, Eriochrome Black T; DSM-III, third edition of Diagnostic and Statistical Manual of Mental Disorders; PAR, physical activity recall; FIA, flow injection analysis; SED, standard error difference; CRMs, certified reference materials; CITAC, Cooperation on International Traceability in Analytical Chemistry.

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test $Ca^{2+}$ and $Mg^{2+}$ in biological fluids as blood, cerebrospinal (CSF), saliva, sweat, and urine. For example, the increasing ratio of salivary magnesium can be an indicator for the parotid malignant tumor [6] and of digitalis toxicity [7].

Different methods used to measure calcium and magnesium in biological fluids. Total calcium was determined in biological specimens using the *o*-cresolphthalein complexone (CPC) photometric method [8,9]. $Mg^{2+}$ concentrations in different biological materials were determined by calmagite method [10,11]. $Ca^{2+}$ and $Mg^{2+}$ were assessed in biological matrices using ion-selective electrode (ISE) methods [12]. The two bivalent elements were measured in biological samples by flame-atomic absorption spectroscopy (FAAS) [6] and assessed by flame-atomic emission spectroscopy (FAES) [7,8]. $Ca^{2+}$ and $Mg^{2+}$ were also tested in patients with chronic periodontitis by ion chromatography (IC) [13]. Furthermore, these ions were detected in body fluids by sophisticated and very expensive techniques as inductively coupled plasma-mass spectrometry (ICP-MS) [14]. With the absence of clear reliabilities with lack of verified performances of the implemented analytical methods in wide array of human fluids, we may find these methods were not properly validated before their introductions into the clinical application which apparently identified limitations also in the optimized procedures.

ISO, IUPAC and AOAC International have co-operated to issue agreed procedures or guidelines 'Design, Conduct and Interpretation of Method Performance Studies' [15] on the 'Proficiency Testing of (Chemical) Analytical Laboratories' and on the 'Internal Quality Control in Analytical Chemistry Laboratories' [16]. The working group that developed these procedures/guidelines had been asked to prepare guidelines on the use of recovery information in analytical measurement. Such guidelines would have to outline minimum recommendations to laboratories producing analytical data on the internal quality control (QC) procedures. A draft of the guidelines was discussed at the Seventh International Symposium on the Harmonization of Quality Assurance Systems in Chemical Laboratories, sponsored by IUPAC/ISO/AOAC International, held in Orlando, USA, 4–5 September 1996. Regarding these guidelines, the goal of the current work is assigned to validate the analytical performance of the measurement system developed for the determination of the ionized (free) calcium and magnesium ($iCa^{2+}$ and $iMg^{2+}$) in biological aqueous media and to characterize the contents of these cations in some biological fluids. Hence, the analyses of these cations by the complexometric titration using ethylenediaminetetraacetic acid (EDTA) as a titrant and by ISE are validated. Contrary to many spectroscopists who analyzed calcium using calcium hydroxide ($Ca(OH)_2$) band emission at 622 nm [17], we preferred to measure this element at the main atomic emission by low temperature (1300–1900 °C) FAES using a calcium interference filter. This selection would be a good choice that avoids the use of didymium glass (more information about this filter can be found at: http://www.hellma-analytics.com/text/273/en/didymium-glass-filter.html) which reduces the excess of sodium (Na) light that emits at 589 nm. FAES has also considerable appeal in the clinical chemistry as it provides a rapid and reliable means of estimating rubidium (Rb) (780 nm), potassium (K) (766 nm), lithium (670 nm), Na (589 nm) and strontium (Sr) (461) in body fluids. Beryllium (Be) can be determined by virtue of emission at 471 nm due to the molecular band emission from its monoxide (BeO). Unlike all the alkaline and alkaline-earth ions, the main emission peak ($\lambda_{em}$) for Mg is far from the visible spectrum (285 nm) which needs a higher flame temperature, a prism monochromator, and a sensitive detector. This instrument which incorporates these features produces a weak emission line of Mg. Therefore, Mg can be measured by FAAS with the use of grating spectrometer equipped with photomultiplier detector. As a result, in this study, only calcium is analyzed by FAES method has been completely validated.

The samples used in this study are biological fluids free of anticoagulants which unlikely create potential sources of interferences to $Ca^{2+}$ and $Mg^{2+}$ signals [18]. To control the source of the biological $Ca^{2+}$ and $Mg^{2+}$ in the collected samples, the volunteers are chosen on the basis that all of them are living far from plaster dust sources of any possible adjacent construction sites and the non-smoker volunteers are selected from non-smoker families.

## 2. Experimental

### 2.1. Chemicals and reagents

All chemicals and solvents were of analytical reagent grade or of highest purity available. High quality source of doubly distilled deionized water (dd-DI $H_2O$) with resistivity >18 MΩ cm was used for the preparation of all aqueous solutions. Additionally, in the case of insoluble substances, a special dissolution method was adopted.

More details about the source of purchased reagents and the methods of the preparations of solutions can be found in the Supplementary material (SM).

### 2.2. Materials

Various filtration techniques and disposable filtration devices, clean lab tools as pipette tips, $SO_4^{2-}$-free plastic disposable tubes, microfuge tubes, and collection tubes were equipped. Most of the vials and containers used were of polyethylene (PE) and polypropylene (PP) (The comparison of the laboratory collection materials effect on $Ca^{2+}$ and $Mg^{2+}$ elution in aqueous solution was presented in Fig. 1S in the SM). They washed with tap water, immersed in 5% v/v Detertec neutral for 48 h, rinsed with tap water then by dd-DI $H_2O$, immersed in 15% v/v $HNO_3$ for at least 24 h, and dried before use. Materials used in this study are listed in the SM.

### 2.3. Instrumentations

The FAES apparatus used is Jenway PFP7 flame photometer (Jenway Gransmore Green Felsted, Essex, UK) and the ISE devise is Microlyte 6 analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland). Moreover, the other techniques are listed in the SM.

### 2.4. Statistical analysis program

The statistical analysis was accomplished with SPSS, version 6.1.3 (SPSS Benelux BV, Gorinchem, The Netherlands) and Evalkit, version 3.1 (Tilburg, The Netherlands).

### 2.5. Analytical procedures

The following sections will describe the analytical procedures used to determine $iCa^{2+}$ and $iMg^{2+}$ in the biological fluids. However, pH method validation, pH method of analysis of biological fluids, EDTA standardization, and the two alkaline-earth calibration series are illustrated in the SM.

#### 2.5.1. Matrix recognition of some biological fluids

In order to define the quantities of the possible interfering agents to the signals of the biological $Ca^{2+}$ and $Mg^{2+}$ and due to the very little information of these components found in the literature, relative measurements ($K^+$, $Na^+$, sulfate ($SO_4^{2-}$), inorganic phosphate ($PO_4^{3-}$), total proteins, viscosity, and surface tension) of some constituents in some biological fluids where performed and the results were listed in Table 1S.

#### 2.5.2. Implementation of control samples

Before launching the laboratory measurements, three control samples are: artificial saliva, SRM-2670, and SRM-2694 available in our laboratory and suitable for biological fluids investigation at the initial stage before sample analyses are implemented as prime standards. SRM-2694