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Elemental composition and microstructure analysis of a rabbit urolith

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Abstract Following physical examination and radiography analysis, cystotomy was performed to remove a rabbit's single bladder stone. This rabbit urolith was analyzed by X-ray fluorescence spectrometry (EDXRF) and scanning electron microscopy (SEM). The EDXRF technique was successful for the determination of major elements (Ca, Mg, P, K and S) and presented sufficient sensitivity to also trace elements (Sr, Fe, Cu, V, Cr, Mn, Zn and Pb) determination. The results showed significant quantitative and structural variations among the urolith regions. The EDXRF technique using the fundamental parameters method and SEM attend as complementary techniques that can be useful in the management of urinary stone analysis.

Keywords Urolith · Rabbit · X-ray fluorescence · Scanning electron microscopy

Introduction

Urolithiasis affects 5–15 % of the population worldwide and the recurrence rates are close to 50 % [1]. The pathogenesis of urolith formation is not completely understood, but at least three theories have been proposed to explain their development, including the super saturation/precipitation of urine crystalloids, nucleation by organic matrix substances and the absence of an inhibitor of stone formation [2]. The precipitation of inorganic or organic urinary solutes results in crystals or amorphous deposits, which depends on the characteristics of the urine when the urolith was initially formed. Moreover, the outer layers of the stone correspond to conditions that are more recent.

Uroliths may contain various combinations of chemicals compounds. The most typical stones calcium is presented in oxalate, phosphate and carbonate forms [3].

Rabbits are particularly susceptible to Ca-containing uroliths due to their metabolism of Ca. Compared to other species, their absorption of Ca from the gut and levels of Ca in the blood are very high. Consequently, the excretion

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Table 1 Experimental conditions for the EDXRF spectrometer

X-ray tube	Rh
Excitation	50 kV; 100 μA (variable)
Detector	Si(Li)
Atmosphere	Vacuum (<30 Pa)
Run analysis	Group 1: Na to Sc and Group 2: Ti to U
Irradiation area	3 mm^2 Ø
Time counting	100 s
Emission line	Kα for Ca, Mg, P, K, S, Sr, Fe, Cu, V, Cr, n and Zn determination Lβ1 for Pb determination.

of Ca via the urine contains a typical sludge-like substance [4].

There are many techniques available for identifying urolith chemical composition and structure, although no single method can provide all the required information. Therefore, a combination of elemental determination and morphological analysis is essential to understand the formation and growing of a urolith [5].

X-ray fluorescence spectrometry has been used for elemental determination in biological samples, where different corporal fluids such as cancerous tissue [6] hair [7], whole blood [8, 9], urine [10] and bones [11] have been analyzed from humans and laboratory animals. West and collaborators previously reviewed and evaluated diverse arrangements X-ray fluorescence spectrometries, such as energy disperse X-ray fluorescence (EDXRF), wavelength dispersive X-ray fluorescence (WDXRF), synchrotron radiation X-ray fluorescence (SRXRF), total reflection X-ray fluorescence (TXRF) and micro X-ray fluorescence spectrometry (μXRF) in different biological matrices [12]. In addition, Scanning electron microscopy (SEM) is a non-destructive and precise technique for studying the morphology of urinary stones [13]. This knowledge will not only help to elucidate factors contributing to the initiation and growth of stones but may also facilitate treatment protocols.

This report describes a study of a single urolith of a 7-year-old female rabbit. The calculus was analyzed using EDXRF and SEM techniques. Elemental composition determination and microstructure analysis were performed in the core as well as three different regions of the stone.

Materials and methods

Radiographic diagnosis

Abdomen radiographs were taken with the rabbit in lateral–lateral and ventral–dorsal positions in an X-ray machine (Tecno-design, São Paulo, Brazil).

Cross-section of the urolith

The urolith was cut in cross-section in four perpendicular cuts using a precision sectioning saw (IsoMet 1000, Buehler—Illinois, USA) equipped with a diamond blade.

EDXRF analysis

Four different regions (core, middle, border and outer) of sliced urolith were analyzed by a SHIMADZU Co. model Rany 720 X-ray fluorescence spectrometer; and the experimental measurement conditions are shown in Table 1. The fundamental parameters method [12, 14] was used and the mass was balanced in monohydrated oxalate ($C_2O_4H_2O$).

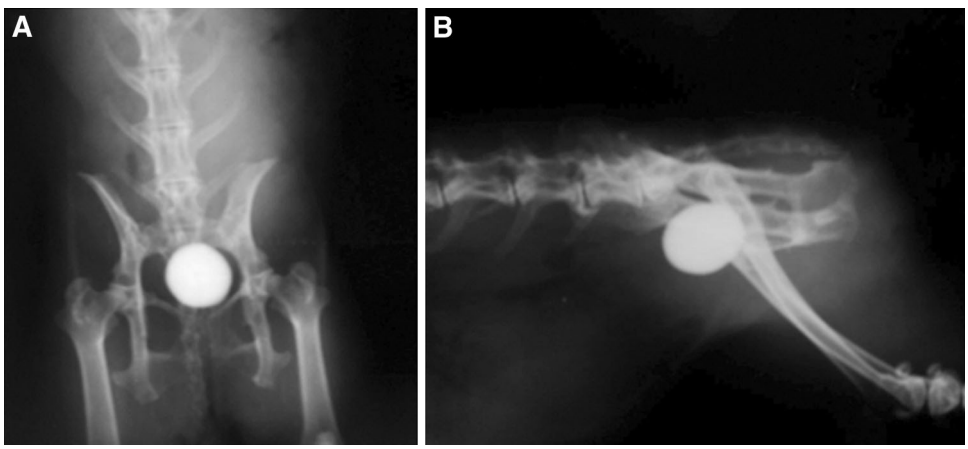

Fig. 1 Upon radiographic study, the oval calculus had an approximate diameter of 2 cm and a radio-opaque appearance

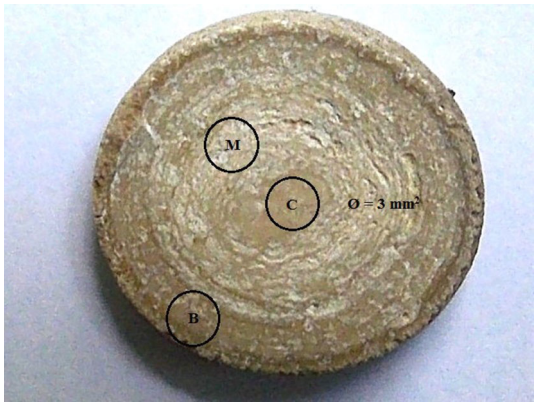

Fig. 2 The rabbit urolith was sliced opened, and the internal selected irradiation area—3 mm^2 Ø (*C* central, *M* middle, *B* border regions) was subjected to EDXRF analysis

SEM analysis

The sliced urolith was also analyzed by SEM (Philips XL 30, Philips, Eindhoven, The Netherlands). The samples were submitted to carbon coating and core, middle, border and outer regions were photographed.

Statistical analysis

The results are presented as the mean ± SE. Multiple comparisons of mean values were performed using one-way ANOVA followed by Bonferroni's test, using GraphPad Prism for Windows (version 4.0, GraphPad Software, San Diego, Calif., USA; http://www.graphpad.com). $P < 0.05$ was considered statistically significant.

Results and discussion

A 7-year-old mixed breed female rabbit, weighing 3.750 kg, presented dysuria, decreased appetite and prostration. The diagnosis was based on clinic examination and X-ray radiographies results. Radiograph of the abdomen was performed in positions lateral–lateral and ventral–dorsal and was identified a solitaire oval stone in

Table 2 Elemental composition of the rabbit bladder stone determined using the EDXRF technique

Element	Internal area			Outer layer	Literature/interval values
	Core	Middle layer	Border layer		
wt%					
Ca	25.30 ± 0.04	23.30 ± 0.04	26.30 ± 0.04	18.90 ± 0.03	2.645 ± 1.951–26.778 ± 1.747 [16] Qualitative analysis [9]
Mg	3.06 ± 0.06	2.98 ± 0.06	1.98 ± 0.06	0.65 ± 0.03	0.051 ± 0.023–9.629 ± 1.272 [15] Qualitative analysis [13]
P	0.997 ± 0.010	0.902 ± 0.008	0.753 ± 0.008	0.653 ± 0.008	Qualitative analysis [13] Qualitative analysis [12]
K	0.320 ± 0.006	0.266 ± 0.006	0.283 ± 0.006	0.238 ± 0.004	0.207 ± 0.037–2.145 ± 0.412 [16] Qualitative analysis [13]
S	0.146 ± 0.002	0.142 ± 0.002	0.215 ± 0.004	0.397 ± 0.004	–
mg kg^{-1}					
Sr	304 ± 5	276 ± 5	360 ± 6	283 ± 4	0.4–415 mg kg^{-1} [13]
Fe	33 ± 6	39 ± 5	35 ± 5	29 ± 4	0.216 ± 0.104–0.421 ± 0.349 wt% [16] 18.8–138.3 mg kg^{-1} [13]
Cu	30 ± 3	31 ± 3	50 ± 5	48 ± 4	0.338 ± 0.117–0.996 ± 0.333 wt% [16] 5.1–34.1 mg kg^{-1} [17]
V	27 ± 10	31 ± 9	27 ± 11	6 ± 6	4.1–166.3 mg kg^{-1} [18]
Cr	16 ± 8	31 ± 7	47 ± 8	11 ± 5	0.019 ± 0.003–0.051 ± 0.013 wt% [16]
Mn	8 ± 6	<5	19 ± 6	11 ± 5	0.063 ± 0.082–0.205 ± 0.273 wt% [15] 3–34 mg kg^{-1} [13]
Zn	11 ± 2	15 ± 2	15 ± 3	17 ± 2	0.021 ± 0.006–0.953 ± 0.364 wt% [16] 12.8–909.8 mg kg^{-1} [13]
Pb	10 ± 6	16 ± 6	15 ± 7	10 ± 5	0.005 ± 0.002–0.083 ± 0.040 wt% [16]

Number of repetitions: 3; 3 s: 95 % confidence level

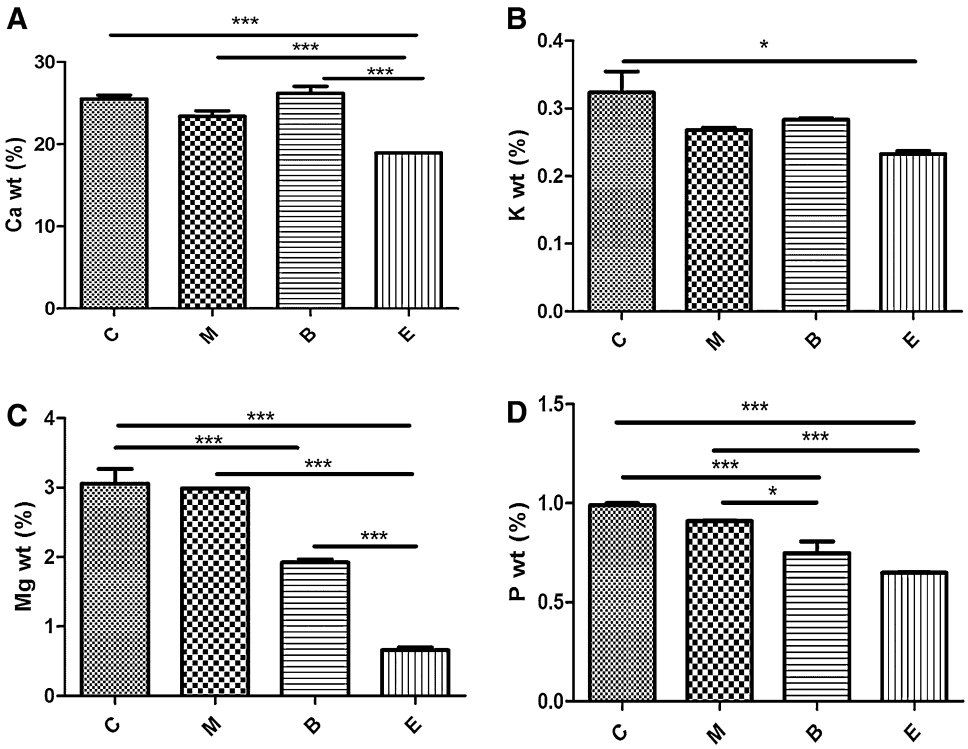

Fig. 3 Statistical analysis of the concentration of major elements in the layers of the rabbit urolith. **A** The Ca content in the outer layer was significantly lower in comparison to all three internal layers (E vs. C, E vs. M, E vs. B, $P < 0.001$). **B** The K concentration in the core was significantly lower than in the outer layer ($P < 0.05$). **C** The Mg concentration decreased from the core to the outer layer (C vs. B, C vs. E, M vs. E, B vs. E; $P < 0.001$). **D** The level of P also decreased from the core to the outer layer (C *vs*. B, C vs. E, M vs. E, B vs. E; $P < 0.001$)

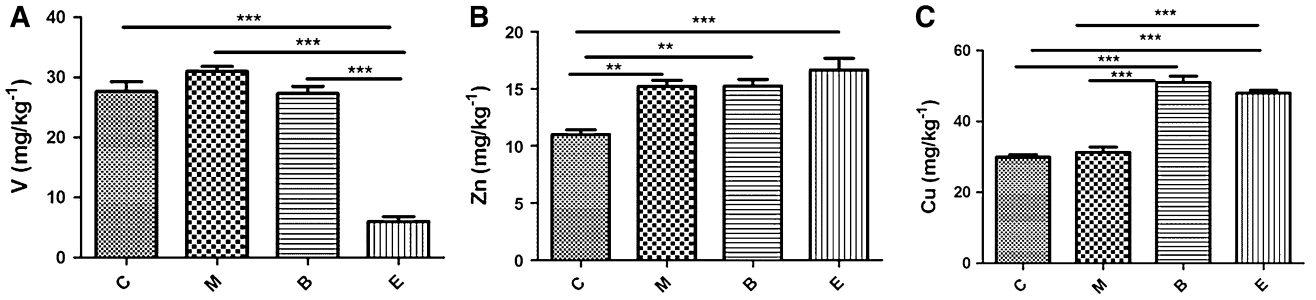

Fig. 4 Statistical analysis of the trace element concentrations in the layers of the rabbit bladder stone. **A** The V concentration was similar among the internal layers but was significantly reduced in the outer layer ($P < 0.001$). **B** The Zn concentration increased form the core to the outer layer (C vs. B, M vs. B, C vs. E; $P < 0.001$). **C** The Cu content was increased in the border ($P < 0.001$) and outer layers ($P < 0.001$)

the bladder of the animal (Fig. 1). The high radiopacity of the stone is the first indication of high calcium content [18].

The opened urolith revealed a single nucleus and two concentric layers within (Fig. 2). The different concentric zones surrounding the centre of the stone can be differentiated clearly and it is recognizable that these regions have been formed from different phases.

The results of the EDXRF analysis (mean ± SD), as well as the related literature values, are presented in Table 2. Ca, Mg, P and K are presented as major elements and Sr, Fe, Cu, V, Cr, Mn, Zn and Pb as trace elements. The results presented adequate precision and sensitivity for chemical characterization of urinary stones and are in agreement with the values from the literature.

Statistical analysis revealed that the Ca concentration did not fluctuate between the internal areas of the stone; however, the Ca content in the outer region was significantly lower in comparison to all three internal regions (E vs. C, E vs. M, E vs. B, $P < 0.001$) (Fig. 3A). Similarly, the K concentration did not change significantly between internal areas; although the level of K in the core was significantly lower than that in the outer region ($P < 0.05$) (Fig. 3B). In addition, the Mg concentration decreased from the core to the outer regions (C vs. B, C vs. E, M vs. E, B vs. E; $P < 0.001$) (Fig. 3C), and the same pattern was observed for P (C vs. B, C vs. C, M vs. E; $P < 0.001$ and M vs. B, $P < 0.05$) (Fig. 3D). The Ca, Mg, P and K concentrations were in agreement with values previously reported by Gionnossi and collaborators using the AAS and

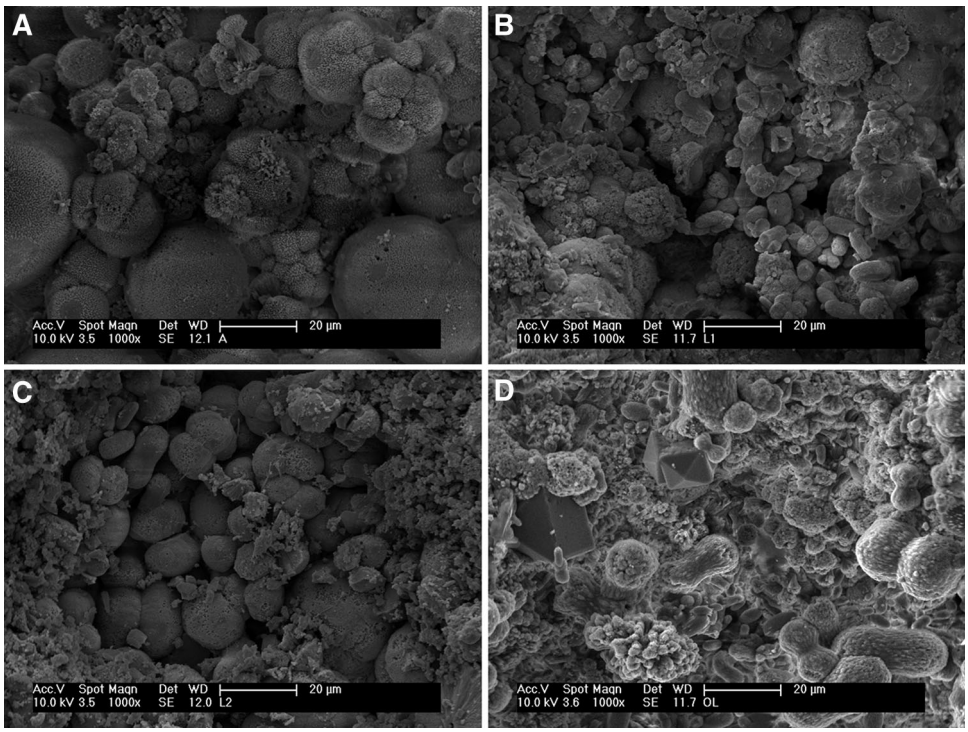

Fig. 5 Representatives scanning electron micrographs of the four different areas of the bladder stone. **A** Micrograph of the core region showing cracked spherules. **B**, **C** Micrographs of the middle and border layers showing small, scattered spherules and amorphous crystals. **D** Calcium oxalate crystals with a typical tetragonal bipyramidal shape (*asterisk*) and amorphous crystals. Magnification (×1,000)

ICP-OES techniques [17]. S content did not compare because quantitative analysis has been not reported in the literature.

The concentrations of the trace elements Sr, Fe, Cu, V, Cr, Mn, Zn and Pb (mg kg^{-1}) were in agreement with [19] values previously determined using the synchrotron X-ray fluorescence (SXRF) technique. Statistically significant differences were found for V, Zn and Cu. The concentration of V was similar among the internal regions, but it was significantly reduced in the outer region ($P < 0.001$) (Fig. 4A). In contrast, the Zn concentration increased from the core to the outer region (C vs. B, M vs. B, C vs. E; $P < 0.001$) (Fig. 4A). Finally, in comparison to the core, the Cu content was increased in the border ($P < 0.001$) (Fig. 4A) and outer regions ($P < 0.001$) (Fig. 4C). This study found elemental compositional differences among the different regions of the rabbit bladder stone to the major and trace elements, and this finding may explain the different morphological structures observed under electron microscopy. In the core, or nucleus, of the stone (Fig. 5A), cracked spherules with an irregular matrix were observed. In the middle and border regions, small, scattered spherules and amorphous crystals were observed (Fig. 5B, C). In the external area, calcium oxalate crystals and amorphous crystals were detected (Fig. 5D). The massive presence of spheres in the urolith nucleus indicates that lithogenic process was induced by high concentration of mucoprotein (consisting of mucus and inflammatory debris) in the rabbit urine. According to Kunchur, accumulation of mucoprotein lead to formation of fibrils which join together to form matrix spherules. Thus the calcium accretion converts the spherule into spheroliths [20]. The concentration of mucoprotein in the urine is increased by unbalanced diet (rich in protein, poor in crude fiber). On the periphery of the stone it was seen calcium oxalate crystals indicating crystallization of urinary salts.

The results presented in this study indicate that ED-XRF technique allowed elemental determination with good precision and accuracy. Furthermore, in comparison to other techniques such as AAS and ICPOES the advantages of the EDXR are related to its non-destructive, multi-elemental and rapid analysis, as well as no destructive chemical treatments for sample preparation are required.

Conclusions

EDXR and SEM are well known and established techniques used to analyze biological samples as tissues and blood. This report provided enough information to conclude that these techniques can also be applied for the purposes of multi-element determination and microstructure analysis of bladder stones, being able to help the illness diagnosis. In addition, both techniques can provide information to better understand the lithogenic process, which can be useful to implement a treatment to prevent the recurrence of the urolithiasis.

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Conflict of interest None.

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