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High-throughput powder X-ray diffraction, IR-spectroscopy and ion chromatography analysis of urinary stones: A comparative study

Research Article

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Abstract: The instrumental qualitative analysis of urinary stones is a critical step in clinical practice and urological research. A powder X-ray diffraction, IR-spectroscopy and ion chromatography have been applied for the qualitative analysis of 20 urinary stones. Suggestions for a sample preparation and an optimal measurement strategy were formulated. The main difficulties for the powder X-ray diffraction qualitative analysis are a limiting amount of the sample and a preferential orientation of crystals, both issues should be minimized by the special sample preparation. Urinary stones samples have been clustered into four groups using different sets of numerical input data (cation and anion content, phase composition). At the same time a high-throughput multivariate clustering has been applied for powder X-ray diffraction and IR-spectroscopy data. The multivariate whole-profile approach can be used as a tool for a high-throughput time reducing technique for clinical practice, when a quick and stable classification of samples is required. All three sets of the data can be automatically separated into three clusters: oxalate-reach, oxalate-pure and non-oxalate samples. Uricite-pure and uricite-rich samples can be easily clustered.

Keywords: *Urinary stones composition • Ion chromatography • Powder X-ray diffraction • IR-spectroscopy • Hierarchical clustering*

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1. Introduction

The formation of kidney and urinary stones is one of the common urinary diseases in the society with increasing prevalence [1,2]. The appearance of stones in human urinary tract depends on the food and drink habits, however it also can be provoked by medical treatment and infection [3] or genetic pathologies [4]. Nowadays, the determination of the mineral composition and structure of urinary stones is a standard examination of patients suffering from kidney stone disease. The study of urinary stones including a stereomicroscopic examination as well as chemical analysis [5] is required since it can give important information about a type of metabolism problems. The results of corresponding tests are useful for choosing an optimal treatment strategy for existing stones dissolution and developing an individual program of preventive supervision of patients with stone disease.

Historically, different qualitative chemical tests were firstly applied for calculi analysis in clinical practice. Nevertheless, qualitative tests provide only a rough indication of relative amounts of different constituents in a mixed stone and the results possible misleading. It is always important to classify urinary and kidney stones based on the complete characterization of each individual sample. A phase composition and structure as well as thermal and mechanical properties of calculi can be examined by powder X-ray diffraction (PXRD), polarized light and scanning electron microscopies [6], IR- and Raman-spectroscopy as well as by thermal analysis [7-9]. Synchrotron-based XRD especially micro-focus diffraction as well as high-resolution transmission electron diffraction can be also utilised for the characterisation of single samples but without any opportunities for the daily routine analysis [10-12]. Elemental and chemical composition can be analysed using X-ray fluorescence spectroscopy [13], atomic absorption spectroscopy and secondary ion mass spectrometry as well as ion chromatography, which is the most convenient method for ionic content analysis. The minimal amount of individual sample and therefore minimal calculi for characterization can be reduced to 3-5 mg. All methods of analysis have merits and demerits as well as strong limitations. Only complex investigation may lead to the complete picture and to classify every unique sample of calculi. Recently, the corresponding classification has been developed based on the pathogenesis, elemental and mineral composition of calculi [14,15]. Nevertheless, clinical practice did not allow the undertaking of a complete study due to the massive amount of samples and time limitation.

Nowadays, ion chromatography (IC) has been proposed as a high selective, sensitive and express method for organic and inorganic ions determination in natural and synthetic objects such as foods, biological media and water. Low detection limits and high sensitivity for the most common ions combined with a small sample volume make IC also useful for clinical practice [16-18]. However, IC has never been applied for urinary stones analysis due to the complexity of sample preparation and difficulties in quantitative calculi dissolution. Later developments of IC may lead to solving all technical issues and make it possible to analyse biominerals using IC. Metals also can be analyzed using the IC [19], nevertheless, an atomic emission spectroscopy (AES) is the most efficient and accurate method for cation analysis [20-22].

Up to now, PXRD is used as an express, reproducible and relatively simple tool for analysis of urinary and kidney stones and can be recommended for routine analysis [23,24]. Nevertheless, PXRD technique is not able to evaluate a high CO_3^{2-}/PO_4^{3-} rate of calcium phosphate which indicates a past or current urinary tract infection [25] and should be coupled with other analysis techniques. The time needed for the sample preparation, data collection and processing is approximately one hour for each individual probe. The minimal sample weight is usually 5-10 mg depending on the measurement technique. Data collection can be automated using automatic sample changer. The sample preparation is a critical step for collection of correct and representative data. The main sources of an error are: i) poor calibration of the PXRD equipment which should be avoided before measurement using external or internal standards, ii) small amount of the sample, which can be partially minimized using long measurement time and special sample holders, iii) not representative sample, iv) preferential orientation of crystallites in the sample. Due to the anisotropic and concretion-like structure of calculi, last two issues should be minimized using careful grinding of the sample. All issues can be also minimized using transmission measurement in a glass capillary, which needs less amount of sample (usually 2-3 mg) and gives reproducible results without visible preferential orientation of crystallites [26].

Both, data processing and analysis need a special qualification of the operator and cannot be issued routinely for complex samples with two or more phases and cannot be automated. After the data collection there are three main steps of PXRD-based analysis which should be mentioned: i) data transformation into the suitable format and normalization, ii) qualitative phase analysis using manual comparison with potential crystal

phases or automatic phase identification using the corresponding database-connected algorithms which is usually included in the diffractometer software, and iii) quantitative phase analysis using individual or integral intensities as well as whole-profile techniques such as Rietveld or two-stage methods. The last step is the most crucial and needs careful analysis of possible sources of experimental and calculating errors, nevertheless, in the literature there is no final protocol for correct data processing and quantitative phase analysis of calculi in terms of obtaining the complete data with a high accuracy. Standard protocols published 30-40 years ago are still actual but should be modified with inclusion of modern techniques for data collection and processing [23,24].

The quantitative phase analysis yields the numerical data (relative amount of crystalline phases presented) which can be further used for the classification of urinary stones samples as well as for the statistical analysis and clustering. Statistical analysis enables massive amount of information to be processed and to find characteristic parameters significant for samples classification and prediction behaviour of the disease in case of the lack of experimental information. Cluster analysis has been recently proposed for classification of calculi using numerical data obtained from elemental, chemical and ion composition [27]. Oxalate, phosphate and calcium concentration can be used as variables for stable classification, nevertheless, introduction of more variables such as magnesium, carbon and nitrogen concentration can improve analytical scheme. In principle, the same classification can be performed based on the data obtained by PXRD, which provides the information about concentration of oxalate, phosphate, calcium and magnesium containing phases.

Due to the massive amount of urinary and kidney stones samples in ordinary practice, there is a need for a rapid method of semi-quantitative phase analysis based on PXRD. One possibility to improve and automate the PXRD analysis is the recently developed multivariate high-throughput clustering method based on the whole-profile PXRD data [28-30]. The method may result in the classification of PXRD patterns based on the whole-profiles for multiple samples as an input without an application of any structural models, preliminary qualitative and quantitative phase analysis. Previously constructed database for typical, representative for known classes, samples can also be used for classification of new samples as software automatically detects the most representative pattern. Numerical (correlation coefficients) and graphical (dendrogram and principle component analysis) plots can be obtained as an output of the analysis. Similarities of the diffraction patterns of the different urinary stones samples and standards result in an appearance of samples in the same cluster group and allow to classify PXRD patterns (and calculi samples as well) in semi-automatic regime. Up to now, the whole-profile multivariate clustering technique has been successfully applied for a high-throughput phase analysis in pharmaceutical research for new polymorph screening, mineralogy for mapping minerals samples as well as for screening newly synthesised inorganic compounds [31,32].

Fourier transform infra-red spectroscopy (FTIR) also has been proposed as a powerful tool for calculi analysis [33,34]. Nevertheless, the difficulties in the quantitative data analysis should be mentioned as the main limitation of the technique. The presence of free, adsorbed and constituted water molecules in calculi samples makes it impossible for the correct interpretation of the spectra in the high wavenumbers region especially for qualification and quantification of oxalates with different numbers of crystal water.

As mentioned above, massive sets of analytical data appear in clinical practice should be statistically analysed using numerical and graphical methods. Nevertheless, minimising the interposition of the operator in the data mining and analysis is still actual and challenging area. The development of automatic methods of data analysis especially for PXRD and IR data performed during the last decades may lead to analyse data without detailed phase and component analysis in semi-automatic regime which reduces time and cost of the clinical a single analysis. Herein, we report a comparative analytical study of 20 non-infection urinary stones taken randomly by PXRD, IC, AES and IR-spectroscopy to compare techniques for quantification of the calculi composition. Methodological aspects of PXRD, AES, IC and FTIR methods were in the focus of the paper presented. The main goal of the investigation was an approbation of different data collection and evaluation techniques to probe easy and reproducible protocols for calculi analysis. Hierarchical clustering (with numerical and multivariate input) was chosen for classification of samples due to its potential for a high-throughput analysis of massive analytical data. The numerical and graphical data obtained for FTIR are similar to PXRD patterns and a multivariate approach for a high-throughput and automatic clustering can also be applied for FTIR spectra. Multivariate clustering has been applied for the first time for calculi analysis.

2. Experimental procedure

2.1. Samples

Symptomatic non-infection kidney stones were retrieved by surgical operation of 20 patients aged between 20 and 70 years admitted to the Department of Urology of the Krasnoyarsk Regional Hospital, Russia, during January-December. All patients were from Krasnoyarsk region. The kidney stones were washed with distilled water and dried at room temperature. Color of calculi ranged from white to dark-brown with a solid needle rough surface. The stone size ranged from 0.1 to 2 cm in length.

2.2. Reagents

All solutions were prepared from AR grade or IC grade chemicals in 18 MΩ water obtained from an aquaMAXTM Ultra 370 Series – Ultra Water Purification System (Young Lin Instrument Co, Korea). Anion stock solutions (1000 mg L^{-1}) were prepared from analytical reagent grade sodium salts (Sigma-Aldrich, USA). Stock standard solutions were stored at 4 °C and working standards were prepared fresh daily. Sodium carbonate and sodium bicarbonate (Sigma-Aldrich, USA) were used for preparation of carbonate eluent. Phthalic acid (Sigma-Aldrich, USA) was used for preparation of phallic eluent. KBr (SpectrosoL, UK) was used to make KBr pellets for FTIR-spectroscopy.

2.3. Samples treatment for IC analysis

A 5 mg sample was powdered, treated with HCl (12.39 M, 25 µL) 5 ml distilled water and 0.1 mL cation-exchange resin KU2-8 in the acid form, and the mixtures were stirred in a water bath for 20 min. The solutions were then filtered through 0.2-rtm membrane filters (Whatman, USA) and filtrate was poured into a 25 mL flask and used for IC analysis.

2.4. Samples treatment for ICP AES analysis

A 5 mg sample was powdered, treated with 5 mL HCl (12.39 M, 25 µL) and HNO_3 (98.6%, 20 µL) solution in 5 mL distilled water the mixtures were stirred in a water bath for 20 min. The solutions were then filtered through 0.2-rtm membrane filters (Whatman, USA) and filtrate was poured into a 25 mL flask and used for ICP AES analysis.

2.5. Equipment and analytical conditions

2.5.1. Ion-chromatography (IC)

Two chromatography systems were used for the analysis. A Shimadzu (Japan) LC-20A chromatograph comprising an LP-20ADsp liquid-delivery pump, a CDD-10Avp conductivity detector, SPD-M20A photodiode array detector, a CTO-20AC column oven, and an SCL-10Avp system controller was applied for oxalate, phosphate, sulphate, and nitrate analysis. The column and detection cells were placed inside the column oven to control the temperature. System control, data acquisition, and data analysis were performing using a Shimadzu LC solution Ver 1.1 workstation. A 250×4.0 mm I.D. IC SI-90 4E analytical column (Shodex, Japan) and 4.6 mm I.D.×10 mm IC SI-90G guard column (Shodex, Japan) were applied with a direct conductivity and photodiode array (for urate anion, λ=284 nm) detection. The optimized mobile phase used for rapid separation contained 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$. The flow rate was 1.0 mL min^{-1}, column temperature was 33°C, and injection volume was 20 µL.

A Shimadzu personal ion analyser PIA-1000 equipped with a conductivity detector has been used for fluoride, acetate and formate analysis. A 100×4.6 mm I.D. IC-A1S analytical column (Shim-pack, Japan) and 4.6 mm I.D.×10 mm IC GA-1S guard column (Shim-pack, Japan) were used with direct conductivity detection. The optimized mobile phase used for rapid separation contained 2.0 mM phthalic acid and 1.2 mM sodium hydroxide. The flow rate was 0.7 mL min^{-1}, column temperature was 33°C, and injection volume was 10 µL. Details of sample preparation and IC analysis of urinary stones samples were previously discussed in details [35].

2.5.2. Atomic emission spectroscopy (AES)

Metals in calculi samples were analysed with inductively coupled plasma AES on a iCAP 6500 ICP spectrometer (Thermo Scientific, USA) equipped with high performance state solid CID86 detector chip, spectral band pass was 7 pm at 200 nm. A continuous choice of wavelengths was in the range 166 to 847 nm. Mass flow of plasma Ar gas was 10 L min^{-1}, mass flow of auxiliary gas was 2 L min^{-1}, mass flow of nebulizer gas was 1.5 L min^{-1}. The temperature of thermostat was 38°C. Volume of sample was 10 mL. System control, data acquisition, and data analysis were performed on Enhanced-featured iTEVA software suite.

2.5.3. Quantitative Powder X-ray diffraction (PXRD)

The PXRD analysis of samples was carried out using a Shimadzu XRD-7000 diffractometer with standard experimental parameters: Cu-K_α-radiation, Ni-filter, position sensitive detector, Bragg–Brentano geometry, 2Θ = 5–60°, $\Delta 2\Theta$ = 0.03°, 1 s $step^{-1}$. For samples 13, 27, 31 and 35 measurement times were increased to 5 s $step^{-1}$ due to small amount of samples. All PXRD measurements were performed at room temperature.

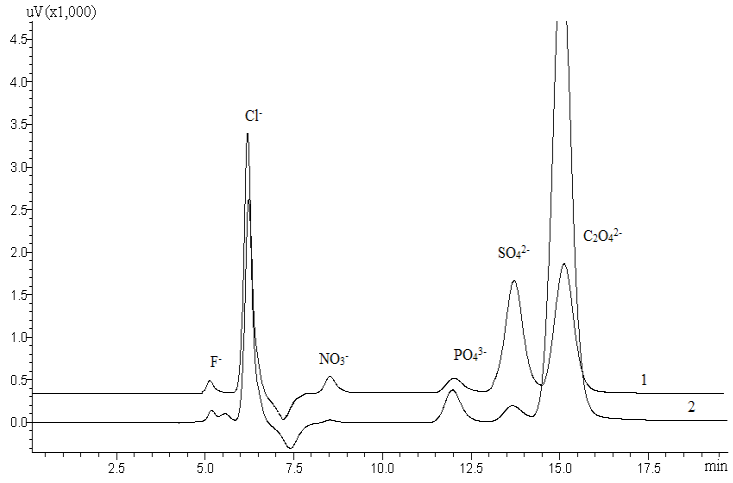

Figure 1. Chromatograms of: (1) model anions mixture F^-, 0.1 mg L^{-1}; Cl^-, 4.0 mg L^{-1}; NO_3^-, 3.0 mg L^{-1}; PO_4^{3-}, 1.5 mg L^{-1}; SO_4^{2-}, 5.0 mg L^{-1}; $C_2O_4^{2-}$, 5.0 mg L^{-1}; (2) urinary stone probe.

Silicon powder was taken as an external standard (a = 5.43075(5) Å, FWHM 2Θ = 0.12°) for the calibration of the equipment before measurements. All samples were ground with heptane in agate mortar and the suspension obtained was placed onto the quartz sample holder according to the procedure suggested in the literature [36]. A flat layer with 100 μm thickness was obtained after the evaporation of the solvent. Indexing of the diffraction patterns was carried out using the data for compounds reported in the PDF database [Powder Diffraction File, International Centre for Diffraction Data, USA (2009)]. Quantitative phase analysis was performed using the following two methods. i) Individual intensities method using maximum intensities of individual diffraction lines after the background subtraction, corundum numbers for individual phases were taken into account; and ii) Rietveld-based full-profile technique within the whole diffraction range using the POWDER CELL 2.4 software [37]. For each sample, background and peak-profile functions as well as cell parameters, zero shift, sample displacement and relative amounts of presented phases were simultaneously calculated. Preferential orientation was taken into account using March-Dollase function with a variation of o_1 parameter. Both techniques were used according to common protocols published in the literature [38,39]. Diffractograms overview can be found in the supplement information online (Supplementary Fig. 2).

2.5.4. Fourier transform Infra-red spectroscopy (FTIR)

A Thermo Scientific Nicolet 380 FT-IR Spectrometer was used for a collection of the FTIR spectra in the range of 400–4000 cm^{-1} with a 4 cm^{-1} resolution. To obtain a high signal to noise ratio 32 scans were accumulated for each sample. A 4–8 mg of ground stone sample were used to make 160-320 mg KBr pellets by a hand-press. Zero-background from the pure KBr pellet has been collected before each measurement and subtracted automatically.

2.5.5. Hierarchical cluster analysis

Cluster analysis for numerous data has been performed using Cluster 3.0 software freely available in the WEB (http://www.falw.vu/~huik/). As an input, matrixes (N×M) were used, where N is a number of samples in the series and M is a number of variations. The whole-profile clustering has been performed using PANalytical X'Pert HighScore Plus software. All PXRD (5-40° 2Θ range) and FTIR (400-2200 cm^{-1} range) patterns in the two-column data-files were normalized before processing and used as a multivariate input for the analysis.

3. Results

Before the chemical analysis, all urinary stones were completely dissolved in HCl solution. For the IC analysis, the working solutions were purified from cations (mainly Ca^{2+} and Mg^{2+}, see AES data) using cation exchanger. The data obtained for 20 urinary stones are collected in the Table 1. The numbers were calculated as mg of the corresponding ion per g of the dissolved sample. The typical chromatogram of the calculi sample is shown in Fig. 1. All peaks can be completely resolved without significant overlapping which allows the quantification of all anions present in the sample. Oxalate, phosphate, sulphate, and nitrate anions were found as the major anionic components whereas acetate, formate and

Table 1. Quantitative composition of urinary stones by IC method (ND: non detectable).

Sample	Anion concentration, mg g^{-1}							
	CH_3COO^-	$HCOO^-$	F^-	NO_3^-	PO_4^{3-}	SO_4^{2}	$C_2O_4^{2-}$	$C_5H_3N_4O^{3-}$
10	2.61±0.05	ND	0.342±0.007	0.52±0.02	24±1	2.12±0.08	47±2	ND
13	ND	ND	0.0253±0.0005	0.16±0.05	ND	0.48±0.01	24±1	ND
14	1.14±0.03	ND	0.0411±0.0008	0.34±0.01	ND	0.53±0.02	21.0±0.8	ND
20	ND	ND	0.0160±0.0003	0.095±0.003	1.72±0.05	0.36±0.01	18.9±0.8	ND
21	0.52±0.01	ND	ND	0.54±0.02	0.51±0.02	1.17±0.04	0.54±0.02	131±4
22	ND	1.72±0.03	0.64±0.01	0.055±0.002	36±1	0.86±0.03	33±1	ND
23	4.42±0.09	ND	ND	0.045±0.001	0.80±0.02	0.231±0.007	17.1±0.3	ND
27	1.51±0.03	ND	0.0330±0.0007	0.156±0.005	ND	1.08±0.03	29±1	ND
28	ND	ND	ND	0.141±0.004	0.163±0.005	0.23±0.03	7.5±0.3	ND
29	ND	ND	0.117±0.002	0.143±0.004	6.6±0.2	0.68±0.02	37±1	ND
30	ND	1.09±0.02	ND	0.87±0.03	ND	0.46±0.01	0.83±0.02	27.2±0.8
31	ND	0.271±0.005	0.161±0.003	0.82±0.02	1.19±0.04	0.148±0.004	33±1	13.4±0.4
33	ND	0.160±0.003	0.111±0.002	0.083±0.002	17±1	0.068±0.002	23±1	ND
34	ND	ND	0.122±0.002	0.108±0.003	3.6±0.1	0.185±0.005	12.9±0.5	ND
35	ND	0.264±0.005	0.0424±0.0008	0.165±0.005	1.62±0.05	0.18±0.05	44±2	ND
36	ND	ND	0.60±0.01	0,67±0.02	9.4±0.3	0.98±0.03	134±5	ND
37	6.2±0.2	0.252±0.005	0.084±0.002	0.066±0.002	16±1	0.233±0.007	12.0±0.5	ND
38	ND	ND	0.151±0.003	0.0263±0.0008	37±1	0.194±0.006	1.35±0.05	ND
39	ND	ND	0.0453±0.0003	0.102±0.003	ND	0.242±0.007	88±3	ND
41	ND	0.201±0.004	4.02±0.08	0.32±0.01	48±2	0.127±0.004	43±2	ND

fluoride were found as minor components. Oxalate, nitrate and sulphate were detected in all calculi samples, as well as acetate and format were detected in two samples (6 and 7). Urate anion was found only in three samples (21, 30 and 31) unlike oxalate which was present in all samples. At the same time, Uricite phase has been detected in samples 30 and 31 using PXRD. Concentrations of sulphate, nitrate, acetate, formate, and fluoride are relatively small and cannot be detected by other techniques especially PXRD which has a detection limit approximately 5 mass% if the phases are crystalline. The formation of amorphous inclusions and isomorphous substitution with major crystalline phases is also possible which makes difficult the detection of minor components by PXRD in comparison with IC and AES.

Ten calculi samples were also analysed using AES. As, Ca, Cu, Fe, K, Mg, Mn, Na, Pb and Zn were found in all samples analysed. The data are summarized in Table 2. Al was found in two samples (33 – 0.20±0.01 and 34 – 0.156±0.008), Co – also in two samples (30 – 0.00053±0.00003, 38 – 0.00040±0.00002) and Ni was found in a single sample (33 – 0.0032±0.0002 mg g^{-1}). The data for Al, Co and Ni are not included into the Table 1 however they were used for statistical analysis.

Common crystalline phases usually present in urinary stones are mineral Whewellite (**COM**, $CaC_2O_4 \cdot H_2O$, PDF 20-231), Weddellite (**COD**, $CaC_2O_4 \cdot 2.38H_2O$, PDF 75-1314), Hydroxylapatite (**CaP**, $Ca_{10}(PO_4)_6(OH)_2$, PDF 72-1243), and Brushite ($CaHPO_4 \cdot 2H_2O$, PDF 72-1240) as well as organic Uricite (**UC**, $C_4(NH)_2O_2C(NH)_2O$, PDF 28-2016) and Uric acid ($C_5H_4N_4O_3$, PDF 31-1982, and $C_5H_4N_4O_3 \cdot 2H_2O$, PDF 19-1996). Struvite ($MgNH_4PO_4(H_2O)_6$, PDF 77-2303), ammonium urate ($C_5H_7N_5O_3$, PDF 04-0496) and carbonate apatite ($Ca_{10}(PO_4)_6CO_3$, PDF 03-0180) known to be more common for infection urinary stones and rare for non-infection calculi [40]. The structural information deposited in the PDF-database can be used for the indexing as well as for the Rietveld-based quantitative PXRD analysis. Sixteen urinary stones were analysed using PXRD. COM, COD, CaP and UC were found with a domination of oxalate-based species (COM and/or COD). The results of quantitative analysis are presented in the Table 3 with experimental error estimated as

Table 2. Chemical analysis data obtained by AES (ND – non detectable).

Sample	Metal concentration, mg g^{-1}									
	As	Ca	Cu	Fe	K	Mg	Mn	Na	Pb	Zn
20	0,0059±0,0003	182±9	0,0124±0,0006	0,100±0,005	4,9±0,2	4,9±0,2	0,043±0,002	3,9±0,2	0,0096±0,0005	0,023±0,001
22	ND	281±14	0,0119±0,0006	0,20±0,01	6,2±0,3	5,6±0,3	0,044±0,002	4,9±0,2	0,021±0,001	0,52±0,03
28	0,00071±0,000004	270±14	0,031±0,002	0,23±0,01	6,4±0,3	6,9±0,3	0,062±0,003	5,1±0,3	ND	0,054±0,003
30	ND	30±1	0,0162±0,0008	0,24±0,01	4,4±0,2	4,8±0,2	0,056±0,003	3,3±0,2	0,0146±0,0007	0,038±0,002
31	0.00064±0,00003	103±5	0.021±0.001	0.1602±0.008	5.4±0.3	4.9±0.2	0.047±0,002	4.1±0.2	ND	0.041±0.002
33	0,0029±0,0001	188±9	0,0148±0,0005	1,01±0,05	3,7±0,2	5,0±0,3	0,053±0,003	3,1±0,2	0,029±0,001	0,63±0,03
34	0,0026±0,0001	125±6	0,025±0,001	7,5±0,4	5,1±0,3	4,2±0,2	0,072±0,004	3,2±0,2	0,028±0,001	0,127±0,006
37	0,0031±0,0002	64±0,3	0,0093±0,0005	ND	3,0±0,2	1,05±0,05	0,0045±0,0002	2,3±0,1	ND	0,0160±0,0008
38	0,0028±0,0001	7,0±0,4	0,0067±0,0003	ND	3,1±0,2	1,26±0,06	0,0077±0,0004	2,5±0,1	ND	0,019±0,001
41	0,0147±0,0007	253±13	0,0129±0,0006	0,27±0,01	3,3±0,2	4,9±0,2	0,034±0,002	3,2±0,2	0,0154±0,0008	0,54±0,03

Table 3. Results of quantitative PXRD analysis of the urinary stones (mass%) (ND: non detectable).

Sample	Whewellite $CaC_2O_4*H_2O$	Weddellit $CaC_2O_4*2.375(H_2O)$	Hydroxylapatite $Ca_{10}(PO_4)_6(OH)_2$	Uricite $C_4(NH)_2O_2C(NH)_2O$	Calculation method
10	13	61	22	ND	B
	16	69	15	ND	C
13	100	ND	ND	ND	D
14	95	5	ND	ND	B
	87	13	ND	ND	E
	81	19	ND	ND	C
20	100	ND	ND	ND	-
22	93	3	4	ND	B
23	100	ND	ND	ND	-
27	100	ND	ND	ND	-
28	100	ND	ND	ND	-
30	ND	ND	ND	100	-
31	90	ND	ND	10	B
	85	ND	ND	15	C
33	57	37	6	ND	B
	54	41	5	ND	C
34	95	ND	5	ND	B
	91	ND	9	ND	C
35	100	ND	ND	ND	D
37	86	ND	14	ND	B
	81	ND	19	ND	C
38	33	ND	67	ND	B
	22	ND	78[A]	ND	C
41	40	46	14	ND	B

A) anisotropic broadening of (002) diffraction line;
B) individual intensities;
C) whole-profile fitting with preferential orientation;
D) strong amorphous background;
E) whole-profile fitting without preferential orientation.

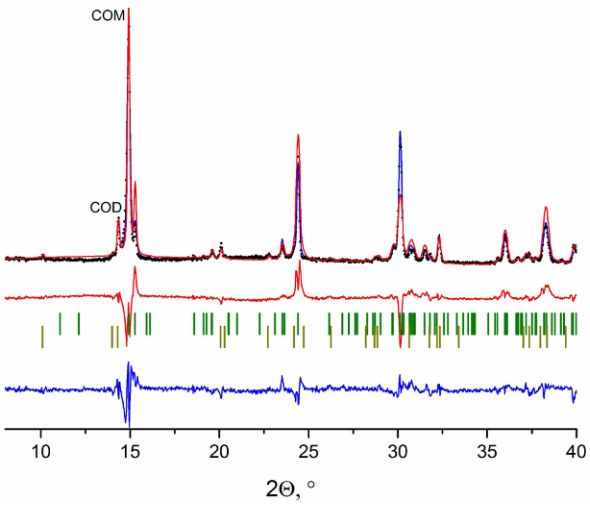

Figure 2. PXRD profile fitting of two-phase (black circles – experimental PXRD profile, Whewellite calculated diffraction lines positions – green marks and Weddellite – brown marks) sample 14 without applying a preferential orientation (red line) and with a preferential orientation applied for Whewellite phase in [1 0 0] crystallographic direction (blue line) with the corresponding differential curves (red and blue lines on the bottom). COM (1 0 0) at 14.93° diffraction line for Whewellite phase and COD is (2 0 0) at 14.36° 2Θ diffraction line for Weddelite were used for single-line quantitative analysis.

5 mass%. As found, 6 kidney stones were composed on the pure COM as well as 1 stone was composed on the pure UC. All other samples were composed on two (4) or three phases (5). Samples 13 and 35 showed a relatively strong amorphous background in the range 15-25° 2Θ.

A combination of quantitative analysis of urinary stones with IC, AES and PXRD methods gives possibility to compare three analytical techniques and to detect the limitations of all approaches in the application to such a complex object. We analyzed 10 samples using three techniques. In all samples, except the sample 30, calcium oxalate was detected as a major phase by PXRD. These findings were confirmed by IC analysis. In addition to calcium oxalates, CaP phase was detected in 44% of samples by PXRD. Based on IC analysis, phosphates were detected in 75% of samples. So, CaP was not detected in samples 20, 23, 28, 31 by PXRD, on the contrary with IC. Not only oxalate and phosphate were detected by IC but also sulfate and nitrate ions. The analytical information about minor anions may allow the classification of calculi using more variables and also to understand their formation mechanism and behavior during the medical treatment as well as the reformation after chirurgic treatment.

Quantitative phase compositions for 16 samples obtained using individual PXRD intensities and the whole-profile technique were comparable but slightly different. The major phases presented in calculi had the strongest diffraction intensities in the range of 5-45° 2Θ. Strong overlapping between diffraction lines makes the indicating of individual intensities characteristic for each phase difficult especially for samples composed of 3 and more crystal phases. For the analysis of individual intensities, the following diagnostic lines were chosen: (1 0 0) at 14.93° for COM, (2 0 0) at 14.36° for COD, (1 2 1) at 13.74° for CaP, and (0 2 1) at 28.84° 2Θ for UC.

The careful grinding and preparation on the quartz low-background sample holder were used to minimize the effect of the preferential orientation. Nevertheless, whole-profile analysis of phase pure COM samples showed the strong influence of preferential orientation on the relative intensities in PXRD profiles. The average orientation coefficient o_1 for (1 0 0) diffraction line was 0.75-0.80, which is relatively high. Preferential orientation in the [0 0 1] crystallographic direction was applied for COD and [0 0 1] for CaP. All attempts to completely avoid an influence of the preferential orientation of crystallites in the samples were unsuccessful. The differences in the calculated relative intensities with and without application of preferential orientation in the whole-profile fitting were sufficient and show its importance for the correct data processing. (1 0 0) at 14.93° 2Θ for COM phase and (2 0 0) at 14.36° 2Θ for COD diffraction lines can be strictly recommended for single-line quantitative analysis due to the relatively low dependence of their intensities on the preferential orientation (see Fig. 2). Diffraction lines at higher angles cannot be recommended for single-line qualitative analysis due to the dependence of their relative intensities on the preferential orientations of crystallites.

FTIR spectra have been collected for 10 samples. All spectra were analysed qualitatively to detect possible compounds having pronounced FTIR spectral signatures. All peaks with their attribution are collected in the Supplementary Table 1. Peaks characteristic for oxalates, phosphates and Uric acid were found. Differences between COD- and COM-rich samples cannot be detected using FTIR spectroscopy. Nevertheless, differences between oxalate-pure and mixed samples are pronounced.

4. Discussion

There are several possibilities for a statistical analysis and classification of the experimental data. The input data can include all possible variables (phase, anion and cation composition) and/or can be based only on major/minor components of each sample. The criterion

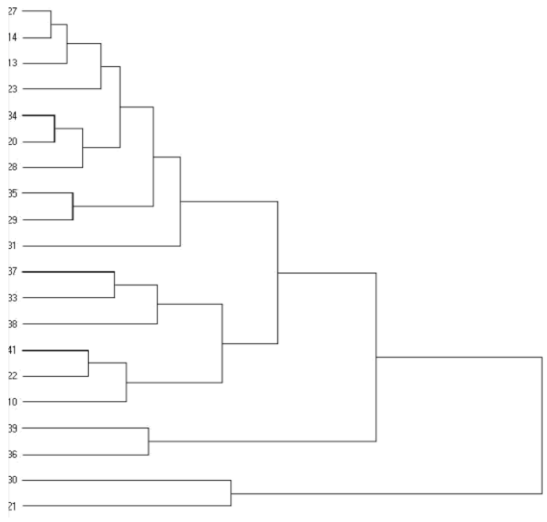

Figure 3. Hierarchical cluster analysis dendrogram obtained on the basis of the anionic composition data (7 variables, 20 samples, fursthest neghbour strategy with chord distance).

for inclusion of any variables is state of the art and it is always difficult to find the correct minimal set of parameters and not excessed for complete grouping the data [41,42]. The sets of the data of different scales could be analysed directly, or with an application of Z-score for the data of different dimensions and logarithm-scale for the numerical data with high absolute differences. Such corrections give more clear comparability for major and minor components as well as for the data obtained by different techniques (*e.g.* chemical, ion and phase analysis) [43]. Here, no special data correction was applied due to the comparable scales of the data obtained.

Hierarchical analysis is a powerful tool with numerical and graphical output for grouping the data and obtaining the main classification criteria in massive sets of input data. Clustering of the data was performed to find significant parameters for samples grouping and classification as well as to compare two parallel classifications based on two different sets of experimental data: phase and ion composition. Ion components obtained using IC analysis result in the [20×8] matrix, where 20 – number of calculi samples, 8 – the number of anions. Both cations and anions were analysed in 10 samples and resulted in the [10×20] matrix, where 20 is the total number of cations (13) and anions (8). Hierarchical analysis of the anion content data allowed to classify the data onto 5 groups (Fig. 3): samples with major oxalate content (13, 14, 27, 23, 34, 20, 28, 35, 31, 29) as well as two oxalate samples with extremely high oxalate content (36, 39); mixed samples with high oxalate and phosphate content (in two sub-groups: 37, 33, 38 with $C_2O_4^{2-}$>PO_4^{3-} and 10, 22, 41, with $C_2O_4^{2-}$<PO_4^{3-}) as well as two samples (21 and 30) are anion-pure. Sample 30 shows pure UC phase in PXRD which is in agreement with the analytical data.

The classification obtained is mainly based on the oxalate/phosphate composition and other anionic content did not have much influence on the clustering output. To understand the influence of minor components on the calculi classification, oxalate and phosphate concentrations should be further excluded or Z-scale correction could be performed before the data analysis. Combined analytical data obtained by IC and AES can be also used for the classification. The main cationic content is presented by Ca^{2+}, Mg^{2+} K^{+} and Na^{+}. The main cation was Ca^{2+} which is in concordance with PXRD data, where no any Mg^{2+}-containing phases were found. All other metals also present as minor components. Only 10 calculi were analysed by AES method which is not enough for a comparison of hierarchical clustering with 7 and 20 variables.

The first data set was found by the qualitative phase analysis using individual intensities or Rietveld technique. A [16×4] numerical matrix (Table 3) was used as an input for the cluster analysis. The second strategy was a multivariate clustering where normalised whole profiles are used as an input. Both approaches provided the comparable results. The first strategy requires preliminary quantitative and qualitative analysis of each diffractogram which is a time-consuming procedure as well as a high qualification of the analyst is required for the correct data interpretation. The method, as shown above, is sensitive to the data collection as well as to the data quality and sample crystallinity. The whole-profile multivariate clustering does not require any preliminary data processing and can be performed in a semi-automatical regime.

The numerical clustering using the first strategy yielded four clusters (Fig. 4):

COM samples (13, 20, 23, 27, 28, 35) and mixed samples with domination of COM phase (22, 14, 31, 37, 34) were placed into the separate cluster; mixed samples with a prevalence of non COM phases (41, 33, 10) were separated as well as sample 30 (pure UC) and sample 38 (domination of CaP) have been placed into two separate clusters. Such classification seems to be reasonable nevertheless mixed samples with different minor components cannot be separated into the different clusters.

The whole-profile multivariate clustering also results in four clusters which can be analysed as classical dendrograms or as principal analysis three dimensional diagrams (Fig. 4):

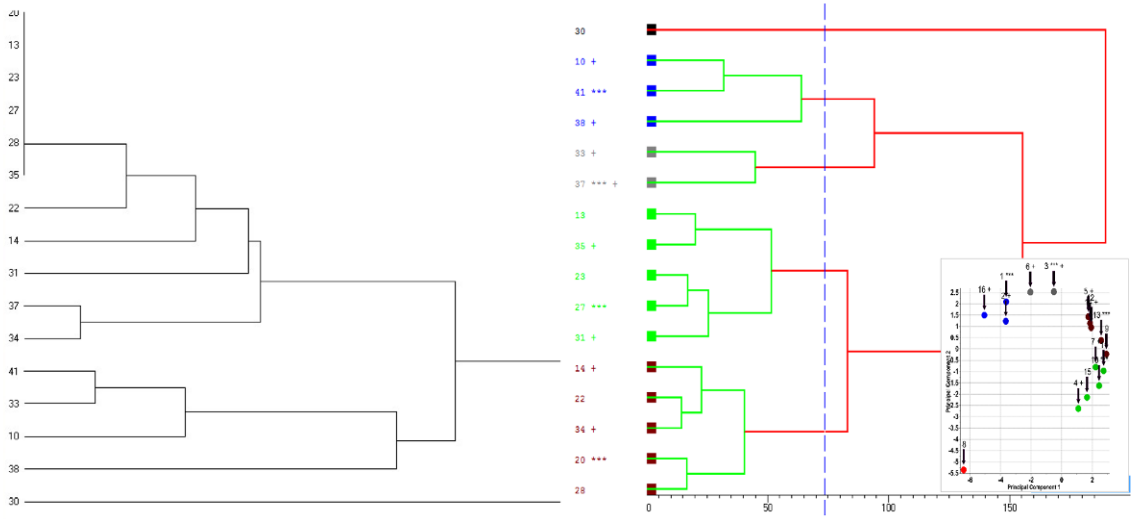

Figure 4. Left: hierarchical cluster analysis dendrogram obtained on the basis of the quantitative phase composition data calculated using PXRD whole-profile approach (4 variables, 16 samples, fursthest neghbour strategy with chord distance). Right: Hierarchical cluster analysis dendrogram obtained on the basis of the whole-profile method (multiple whole-profile variables, 16 samples, and principal component analysis plot (insert)).

Table 4. Comparison of calculi grouping based on chemical and PXRD analysis.

Method		Anionic content	Numerical PXRD	Multivariate PXRD	Quantitative FTIR	Multivariate FTIR
	Uricit/anion-pure	21, 30	30	30	30, 31	30
	Whewellite/Oxalate:					
cluster	Pure	36, 39 + 13, 14, 23, 27	13, 20, 23, 27, 28, 35	23, 27, 31	20, 28, 33	20, 28, 31
	Amorpous background			13, 35		
	Mixed	20, 27, 28, 29, 31, 34, 35	14, 22, 31, 34, 37	14, 20, 22, 28, 34	22, 34	22, 33, 34
	Mixed	33, 37, 38 + 10, 22, 41	10, 33, 38, 41	10, 33, 37, 38, 41	37, 38, 41	41, 37+38

COM samples (23, 27, 31, 13, 35) with pure COM or predominant COM phase are placed into the main cluster. Samples 13 and 35 are placed in a separate sub-group with a high amorphous background. Mixed samples (41, 10, 38) and (37, 33) form two clusters of three- and two-phase calculi. Again, UC calculus has been placed into a separate cluster.

The normalized FTIR spectra can be clustered in the same manner. Spectroscopic region between 4000 and 2200 cm^{-1} cannot be used due to the high intensity and broadness of spectroscopic lines characteristic for water frequencies. ATR measurements should be performed to reduce the relative intensities of water specific bands. Ten samples were clustered into five groups (Fig. 5): oxalate pure samples, mixed oxalate-rich samples, mixed oxalate-poor samples (two sub-groups), and uricite sample.

All classifications are comparable for samples with clear predominant component. Nevertheless, intermediate samples with mixed behaviour can be clustered in different groups depending on the classification method and type of input data (Table 4). The same variability for appearance of mixed samples in different groups depending on the criteria has also been found for manual grouping according to the known empirical criteria. Otnes [44] and later Abdel-Halim [45] have formulated the corresponding empirical criteria for grouping non-infection calculi into three groups: URATE (>20% of urate), OXALATE (>40% of oxalate) and PHOAPHATE (>10% of phosphate). According to the empirical classification, the sample 30 should be placed into the URATE group, samples 37, 38 and 41 should be separated into the PHOSPHATE group and all other samples should be found in the OXALATE group.

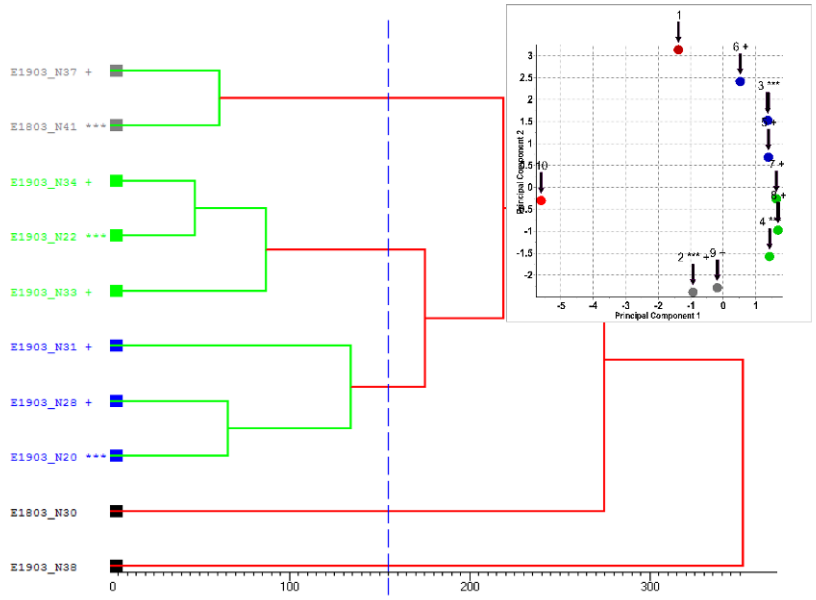

Figure 5. Hierarchical cluster analysis dendrogram obtained on the basis of the IR whole-profile method (multipl whole-profile variables, 10 samples, and principal component analysis plot (insert)).

Such classification is poor and not clear, especially for mixed samples. The authors implemented intermediate mixed groups but limits for such groups were taken arbitrary. Having the "natural" criteria obtained from the clustering analysis of big sets of clinical samples diagnosticians can find precise limits of pure and mixed groups.

5. Conclusions

Ion chromatography analysis can be applied for simultaneous quantification of numerous inorganic and organic anions. Eight anions have been detected in the presented set of samples at the same time. The coupling of IC and AES may allow to achieve a reproducible quantification of all chemical components in calculi samples. Nevertheless, PXRD and FTIR methods can be automatized in terms of data collection and analysis, especially the grouping of the large data sets can be performed using high-throughput approaches. The data presented show the same trends for the samples grouping using all available techniques.

Hierarchical clusters obtained using numerical (ion and phase composition) and multivariate (PXRD profiles) inputs are similar which demonstrate that multivariate strategy can be recommended for a high-throughput analysis of the PXRD data obtained in clinical analysis of bio-mineral samples (Table 4). Good correlation between independent quantitative methods shows the correctness of both techniques and a possibility from one hand to complement both analyses, on other hand, to displace missing data in the case of the lack of detailed experimental information. A relatively new multivariate whole-profile approach can be used as a tool for a high-throughput time reducing technique for clinical practice, when a quick and stable classification of samples is required. All three sets of the data can be automatically separated onto three clusters: oxalate-reach, oxalate-pure and non-oxalate samples. A more detailed classification of oxalate-pure samples should be performed with a more representative selection of calculi samples. PXRD and IR techniques allow the separation of COM and COD samples based on the specific signatures of the phases. IC analysis is not able to recognize COM and COD reach samples and should be coupled with other techniques.

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