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Atomic Absorption Spectrometric Determination of Mineral Elements in Mammalian Bones

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The phosphorus content of the major bones of male and female selected mammals was determined using the yellow vanadomolybdate colorimetric method. For each animal, the bone with the highest phosphorus content was used as pilot sample. Varying concentrations of strontium were added to solutions of the ashed pilot samples to minimize phosphorus interference in the determination of calcium and magnesium using flame atomic absorption spectrophotometry operated on the air-acetylene mode. At least 6,000 ppm (0.6%) of strontium was required to give optimum results for calcium. The amount of magnesium obtained from the analysis was not affected by the addition of strontium. With the incorporation of strontium in the sample solution, all elements of interest can be determined in the same sample solution. Based on this, a procedure is proposed for the determination of calcium and other elements in bones. Average recoveries of spiked calcium and magnesium were 97.85% and 98.16%, respectively at the 95% confidence level. The coefficients of variation obtained for replicate determinations using one of the samples were 0.00% for calcium, lead and sodium, 2.93% for magnesium, 3.27% for iron and 3.92% for zinc at the concentration levels found in that sample. Results from the proposed procedure compared well with those from classical chemical methods at the 95% confidence level. It is evident that calcium, phosphorus, magnesium and sodium which are the most abundant elements in the bones are distributed in varying amounts both in the different types of bones and different animal species, although the general trend is Ca > P > Na > Mg for each bone considered. The calcium – phosphorus ratio is generally 3:1. The work set out to propose an atomic absorption spectrometric method for the multi-element analysis of mammalian bones with a single sample preparation and to study the distribution pattern of these elements in the bones.

Key words: Phosphorus interference, bone minerals, distribution, calcium-phosphorus ratio

Bones constitute the principal component of all adult vertebrate skeletal structures and may exist in either dense or spongy form known as compact and cancellous bones, respectively. The bone is known to be hard and elastic. The hardness is due to the inorganic salts, which constitute about 70% of bone chemical mixture. The elastic nature of the bone is a result of the organic substances which make up the remaining 30% of the chemical mixture. Although the outer surface of the bone is extremely hard, it is actually a living tissue, which derives its nourishment from blood vessels and nerve tissues.

There are various methods available for the examination of the mineral content of bones. These include polarography (Ladanyi & Stalder 1983), stripping voltammetry (Yixiang 1985), inductively coupled atomic emission spectroscopy (Lee 1983, Barnett 1987), atomic absorption spectroscopy (Lindh et al. 1980, Drash 1982, Simon & Liese 1983, Drash et al. 1987, Drash & Ott 1988,

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Kowal et al. 1989, Samuel et al. 1989, Keinonem 1992, Baranowska et al. 1995) and x-ray fluorescence (Lind 1983, Armstrong et al. 1992, Price et al. 1992). Most of the reports are concerned with the determination of toxic elements especially lead and cadmium in bones. One disadvantage of some of these reports is that the elements analyzed for are in most cases not determined in the same sample solution thereby making such analysis laborious. Although the atomic absorption spectrophotometer is very common, no method has been described yet for the determination of calcium in bones using this instrument. Since bones contain phosphorus, the delay or lack of interest in the development of such a procedure is perhaps due to the interference of phosphorus in the determination of calcium in biological materials by this technique. Phosphorus usually combines with calcium ions in the sample solution to form $Ca_3(PO_4)_2$ which in the flame is converted to $Ca_2P_2O_7$, a very thermally stable compound and which reduces substantially the concentration of free calcium ions in the flame. This effect of phosphorus is usually overcome by the addition of strontium or lanthanum solution to the sample solution before analysis. The strontium or lanthanum preferentially reacts with PO_4^{3-} ions to form $Sr_3(PO_4)_2$ or $La_2(PO_4)_2$ which are more stable than $Ca_3(PO_4)_2$ thereby preventing the interaction of PO_4^{3-} ions with Ca^{2+} ions.

Various concentrations of strontium or lantanum in the sample solution have been recommended when analyzing different classes of materials: 1.0% for calcium and magnesium in water (APHA/AWWA 1975) and plant materials (Ranjhan & Krishna 1980), and calcium in urine (Briscoe & Rogan 1965); 0.3% for calcium in unused petrol engine lubricating oils (Udoh 1995); 0.1% for calcium in plant materials (AOAC 1984); 0.08% for calcium and magnesium in soil extract (Allen et al. 1974) and 0.04% for calcium and magnesium in plant digest (Allen et al. 1984). If the interference due to phosphorus is eliminated for the determination of calcium, the same bone sample solution can be used for the determination of all other elements. This work was designed to study the distribution pattern of mineral elements in bones and to show that the addition of the correct amount of strontium not only eliminates the interference of phosphorus in the analysis of calcium in bones using air-acetylene flame atomic absorption spectroscopy but also permits multi-element analysis with a single sample preparation.

Experimental

Apparatus

(i) A Unicam ultraviolet/visible spectrophotometer (Pye Unicam, England) equipped with a print-out recorder and a computer).

(ii) A Unicam 919 atomic absorption spectrophotometer (Pye Unicam, England) which was operated on the air-acetylene mode at wavelength settings as detailed in the instrument's data book for each element: calcium at 422.7, magnesium at 285.2, iron at 248.3, lead at 217.0, zinc at 213.9, copper at 324.8, cadmium at 228.8, manganese at 279.5, nickel at 232.0, cobalt at 240.7 and chromium at 357.9 nm.

(iii) A flame photometer (Model PFP7, Jenway, England)

(iv) A Gallenkamp muffle furnace (Gallenkamp, England) with a temperature range 0-1000°C was used.

Reagents, solutions and calibration curves

All reagents were of analytical reagent grade. Deionized distilled water was used in all preparations and dilutions. Stock strontium solution (50,000 ppm) was prepared by dissolving 152.156 g $SrCl_2.6H_2O$ in water and diluting to IL in a volumetric flask. Stock standard solutions of the elements (1000 ppm in each case) were prepared as detailed in the AAS data book. All stock solutions were standardized before use. Calibration solutions were prepared by serial dilutions of the stock solutions. The concentrations of the elements in the samples were determined by measuring the absorbance and referring to the respective calibration curve. New calibration curves were prepared on each subsequent analysis.

Sample collection

Nine live animals (see names in Table 1) were bought from sellers, slaughtered and the bones removed. The bones were soaked in water for 48 h to ease removal of the adhering flesh. The bones were then dried in an oven at 50°C to constant weight and blended into powder with a Belltone Minshun electric blender. All the powdered samples were stored in plastic containers.

Determination of phosphorus by uv-vis method

One gram of each sample powder was weighed out in triplicate into crucibles. They were then put in a muffle furnace and ashed at 600°C. The ash was treated with 20.0 mL of 1 + 1 ($HCl:H_2O$) solution and the resulting solution concentrated on a steam bath to about 5.0 mL. The solution was transferred quantitatively to a 50- mL volumetric flask and diluted to the mark with water. The resulting solutions were used for the determination of phosphorus by the yellow vanadomolybdate method (AOAC 1984) using the uv-vis spectrophotometer. In this method, an aliquot of the sample solution is pipetted into a 100- mL volumetric flask and 20.0 mL of vanadomdybdate

reagent is added to it and the solution diluted to the mark with water. (The vanadomdybdate reagent is prepared thus: 20.0 g ammonium molybdate is dissolved in 200.0 mL hot water and then cooled, then 1.0g ammonium metavanadate is also dissolved separately in 120.0 mL hot water, cooled and then 140.0 mL concentrated nitric acid is added to it in a IL volumetric flask. The molybdate solution is gradually added to the vanadate solution and the resulting solution diluted to the IL mark with water). For the calibration curve, 0 - 5 ppm phosphorus standard solutions are prepared in 100 – mL volumetric flasks each containing 20.0 mL vandomolybdate reagent. The absorbance of the resulting yellow solutions of increasing intensity are read at 400 nm.

Effect of the varying concentrations of strontium on the determination of calcium and magnesium by atomic absorption spectroscopy

A fixed mass (0.1 g) of each pilot sample (identified by bold face numbers in Table 1) was weighed in duplicates, ashed, dissolved in 20.0 mL of 1+1 (HCl : H_2O) solution and concentrated on a steam bath to about 5.0 mL. The solutions were transferred quantitatively to 50- mL volumetric flasks. Varying volumes of stock strontium solution corresponding to strontium concentrations given in Table 2 were added. The absorbance of calcium and magnesium in each was determined by atomic absorption spectroscopy. The corresponding concentrations were then read from the calibration curves.

Proposed procedure

Each sample powder (0.1 g) is weighed into a crucible and put in a muffle furnace set at a temperature of 200°C first to char and then 600°C and heated until the ash is free from all visible traces of carbon. The crucible is removed from the furnace and allowed to cool. The ash is treated with 20.0 mL 1+1 (HCl : H_2O) solution and the resulting solution concentrated on a steam bath to about 5.0 mL. The solution is transferred quantitatively to a 50- mL volumetric flask. 6.0 mL of 50,000 ppm strontium solution is added and the resulting solution diluted to the mark with water. The resulting solution is analyzed for the elements of interest using an atomic absorption spectrophotometer and a flame photometer for sodium. A blank solution is also prepared and analyzed.

Reproducibility studies

Triplicate weighings of 0.1 g of the femur of female *B. indicus* was ashed and solubilized as described under proposed procedure. In each case, 6.0 mL of stock strontium solution was added and the solution transferred quantitatively to a 50- mL volumetric flask and made to volume with water. A blank solution was also prepared. All the solutions were analyzed for the elements of interest by atomic absorption spectrophotometry and flame photometry in the case of sodium.

Recovery studies

Known amounts of calcium and magnesium (50 mg each) as oxides were added to 0.1 g portion of the femur of female *B. indicus* in triplicate. The samples were then ashed and analyzed as described under proposed procedure.

Comparison of results from proposed procedure with those from classical chemical methods

Duplicate weighings of 0.1 g of each sample were ashed and the solutions made as described under proposed procedure but without the addition of strontium solution. A blank solution was also prepared for these. The solutions were analyzed by both AAS and classical chemical methods – calcium by permanganate titration after precipitation as oxalate, magnesium by precipitation as the quinaldinate, iron by titration with permanganate and zinc by titration with EDTA (Vogel 1961). The results using the femur of female *B. indicus* were compared with those from reproducibility studies involving the same sample.

Application of procedure

Duplicate weighings of 0.1 g of each of the other samples not used in the above preliminary works were ashed and the solutions prepared as described in the proposed procedure. A blank solution was also prepared. The solutions were analyzed for the different elements as in the proposed procedure.

Results and Discussion

Determination of phosphorus

Table 1 shows the phosphorus content of the samples. It is evident from the results that the bone accumulating the highest amount of phosphorus varies from one organism to another, irrespective of sex. Generally, tibia and fibula has the highest phosphorus content for most of the animals. The degree of interference of phosphorus on the absorption behaviour of calcium is supposed to be maximum in the bone sample with the highest concentration of phosphorus for each animal. Bones with the highest phosphorus content were therefore selected as pilot samples for the respective animals (see bold face in

Table 1. Analysis of phosphorus content of the bones by spectrometri c method (AOAC 1984).

Bone	Animal/phosphorus content (µg/g x 10^3 bone powder)								
	Bos indicus (dwarf cow)		*Capra reversa* (goat)		*Ovis aries* (sheep)		*Sus scrofa* (pig)	*Canis familiaries* (dog)	*Oryctolagus cuniculus* (rabbit)
	Male	Female	Male	Female	Male	Female	Female	Female	Male
Femur	**125.26***	125.81	90.20	93.59	98.42	86.50	113.45	93.81	**111.22***
	±0.01	±0.20	±0.05	±0.10	±0.09	±0.09	±0.02	±0.09	**±0.07**
Humerus	115.69	114.75	92.34	91.02	95.63	104.41	**136.00***	94.80	84.74
	±0.12	±0.05	±0.09	±0.02	±0.09	±0.11	**±0.10**	±0.03	±0.09
Radius/ulna	114.84	95.30	99.16	100.46	**122.50***	104.61	92.72	98.65	85.59
	±0.04	±0.17	±0.01	±0.04	**±0.10**	±0.01	±0.07	±0.05	±0.04
Ribs	108.16	106.73	94.69	80.45	96.82	104.40	101.70	77.92	74.78
	±0.04	±0.02	±0.10	±0.13	±0.07	±0.10	±0.02	±0.02	±0.07
Vertebral	107.19	107.86	90.09	93.57	67.70	82.52	91.61	78.61	103.52
column	±0.01	±0.03	±0.01	±0.08	±0.17	±0.04	±0.03	±0.03	±0.03
Tibria/fibula	107.18	**135.64***	**102.96***	**106.69***	99.21	**113.50***	90.40	**98.96***	102.79
	±0.16	**±0.05**	**±0.09**	**±0.19**	±0.08	**±0.09**	±0.01	**±0.04**	±0.09

*Bold face identifies animal and bone with the highest phosphorus content.

Table 1). If the interference due to phosphorus is eliminated in ash solutions of those bones, then that quantity of strontium needed to achieve this will also be sufficient when handling bones with lower phosphorus content than the pilot bones.

Effect of varying the concentration of strontium on the determination of calcium and magnesium

Table 2 shows the amount of calcium obtained with varying concentration of strontium. It is evident that the amount of calcium increased, with increasing concentration of strontium until a peak value was obtained generally at strontium concentration of 6,000 ppm. Higher concentrations of strontium did not produce any better result. Table 3 shows that increase in the concentration of strontium did not give any corresponding significant increase in the amount of magnesium obtained whether strontium was added or not. A similar result was obtained in the determination of additive elements in unused petrol engine lubricating oils (Udoh 1995), metallic elements in vegetables (Udoh 1999) and animal protein sources (Udoh 2000).

Reproducibility and recovery studies

Table 4 shows that the proposed procedure gives highly reproducible results. The coefficients of variation calculated were 0.00% for calcium, lead and sodium, 2.93% for magnesium, 3.27% for iron and 3.92% for zinc at the concentration levels found in that sample. Copper, cadmium, chromium, manganese, nickel and cobalt were not detected in that sample.

For the recovery studies (Table 5), analysis of the ash solutions showed recovery of calcium to be 97.85± 0.35% while that of magnesium was 98.16± 0.31%. It follows that the procedure is quantitative and accurate in the determination of the ultimate element content of the bones. Application of the student t – test at 95% confidence level to replicate recovery values showed no significant differences between the amount of element added and the amount recovered. Observable variations in results could be attributed to current fluctuations and variations in instrument response.

Comparison of results

Table 4 shows that the results obtained from the proposed procedure are more enhanced for calcium than those obtained from solutions without strontium. It is also observed from the table that the proposed procedure gives comparatively good results for all other elements as those obtained from solutions without strontium. Although solutions with and without strontium give comparatively good results except for calcium, it would be uneconomic to determine other elements without the addition of strontium and only calcium with strontium. Hence to determine all metallic elements including calcium in bones by atomic absorption spectroscopy using air – acetylene flame, the correct amount of strontium (not less than 6,000 ppm) must be added to the bone stock solution prior to analysis. Although only the femur of female *B. indicus* is used to illustrate this, the same trend was observed for all other bone samples. Table 4 also shows that the results obtained from the proposed procedure compare well with those from classical chemical methods but the latter methods are laborious and more time-consuming.

Distribution of the elements

The concentration levels of the elements in the bones are illustrated in Figs. 1 to 6. The concentration of calcium in the bones ranged from 207.04 µg/g in

Table 2. Effect of the increasing concentration of strontium on the amount of calcium obtained.

Concentration of strontium, ppm	Pilot bone/amount of magnesium (μg/g X 10^3)								
	B. indicus		*C. reversa*		*O. aries*		*S. scrofa*	*C. familiaris*	*O. cuniculus*
	Male (Femur)	Female (Tibia and fibula)	Male (Tibia and fibula)	Female (Tibia and fibula)	Male Radius and ulna	Female (Tibia and fibula)	Female (Humerus)	Female (Tibia and fibula)	Male (Femur)
0	67.50	57.78	55.66	87.50	70.38	61.11	64.61	66.67	58.25
1,000	200.00	138.90	111.10	250.00	168.92	221.78	223.70	183.33	174.76
3,000	249.00	333.07	166.37	281.25	281.25	227.92	230.42	221.94	319.23
5,000	349.00	345.65	200.37	311.25	308.56	332.19	251.68	276.64	319.23
6,000	349.00	357.99	332.24	405.00	336.71	332.19	364.03	304.44	348.35
9,000	349.00	359.99	332.24	405.00	420.17	332.19	364.03	304.44	348.30
12,000	348.50	359.43	332.24	404.38	420.61	331.63	363.46	303.89	347.77
15,000	348.50	359.66	331.68	403.75	420.04	331.08	362.90	303.33	347.19

Table 3. Effect of the increasing concentration of strontium on the amount of magnesium obtained.

Concentration of strontium, ppm	Pilot bone/amount of magnesium (μg/g X 10^3)								
	B. indicus		*C. reversa*		*O. aries*		*S. scrofa*	*C. familiaris*	*O. cuniculus*
	Male (Femur)	Female (Tibia and fibula)	Male (Tibia and fibula)	Female (Tibia and fibula)	Male Radius and ulna	Female (Tibia and fibula)	Female (Humerus)	Female (Tibia and fibula)	Male (Femur)
0	7.13	6.41	11.43	10.99	5.12	6.84	4.34	11.46	4.11
1,000	7.12	6.78	11.43	11.05	5.18	6.33	4.97	10.98	5.40
3,000	6.76	6.77	11.02	10.33	7.46	6.12	4.97	10.71	5.03
5,000	7.10	6.71	10.63	10.32	7.85	7.11	4.96	10.96	4.30
6,000	7.09	6.41	11.41	10.30	7.43	7.09	4.24	10.71	5.00
9,000	7.44	6.39	10.99	12.08	7.78	7.09	4.59	11.07	5.36
12,000	7.07	6.74	10.64	12.07	7.06	7.08	4.93	11.42	5.71
15,000	7.06	6.73	11.36	12.05	7.76	6.36	4.56	11.42	5.34

Table 4. Reproducibility of proposed procedure and comparison of results using the femur of femal *B. indicus*.

Element	Concentration, μg/g X 10^{-1}		
	With Sr	Without Sr	Other Methods
Calcium	332.52 ± 0.00	100.35 ± 5.65	340.20 ± 0.50
Magnesium	653 ± 0.19	6.51 ± 0.10	6.71 ± 0.25
Sodium	155.00 ± 0.00	a	a
Iron	0.14 ± 0.05	0.14 ± 0.07	0.15 ± 0.01
Lead	0.12 ± 0.00	a	a
Zinc	0.10 ± 0.04	0.09 ± 0.02	0.10 ± 0.03
Copper	N.D.		
Cadmium	N.D.		
Manganese	N.D.		
Nickel	N.D.		
Cobalt	N.D.		
Chromium	N.D.		

a: Element not analyzed for

Table 5. Recovery studies data using the femur of female *B. indicus*.

Element	Concentration, mg		$\bar{X}$	S.D.	% recovery
	Added	Recovered			
Ca	50	49.11			
	50	48.92			
	50	48.77	48.93	0.17	97.85 ± 0.35
Mg	50	49.23			
	50	48.93			
	50	49.09	49.08	0.15	98.16 ± 0.31

the ribs of male *O. Cuniculus* to 420.11 μg/g in the radius and ulna of male *O. aries*. The concentration of phosphorus ranged from 67.70 μg/g in the vertebral column of *O. aries* to 136.00 μg/g in the femur of *S. scrofa* (see Table 1). A close examination of the results reveals that the bone sample containing the highest amount of phosphorus need not contain the highest amount of calcium. Generally, the tibia and fibula of the different animals (see Table 1) contain more phosphorus than other bones. The concentration of magnesium ranged from 3.70 μg/g in the humerus of female *C. reversa* to 10.81 μg/g in the tibia and fibula of male *C. reversa*. The concentration of sodium ranged from 10.00 μg/g in the femur of female *C. familiaris* to 27.50 μg/g in the ribs of male *C. reversa*. The concentration of iron was generally low but ranged from 0.07 μg/g in the radius and ulna of male *O. cuniculus* to 0.27 μg/g in the femur of female *O. aries* and the vertebral column of male *O. aries*. The concentration of zinc was also generally low and

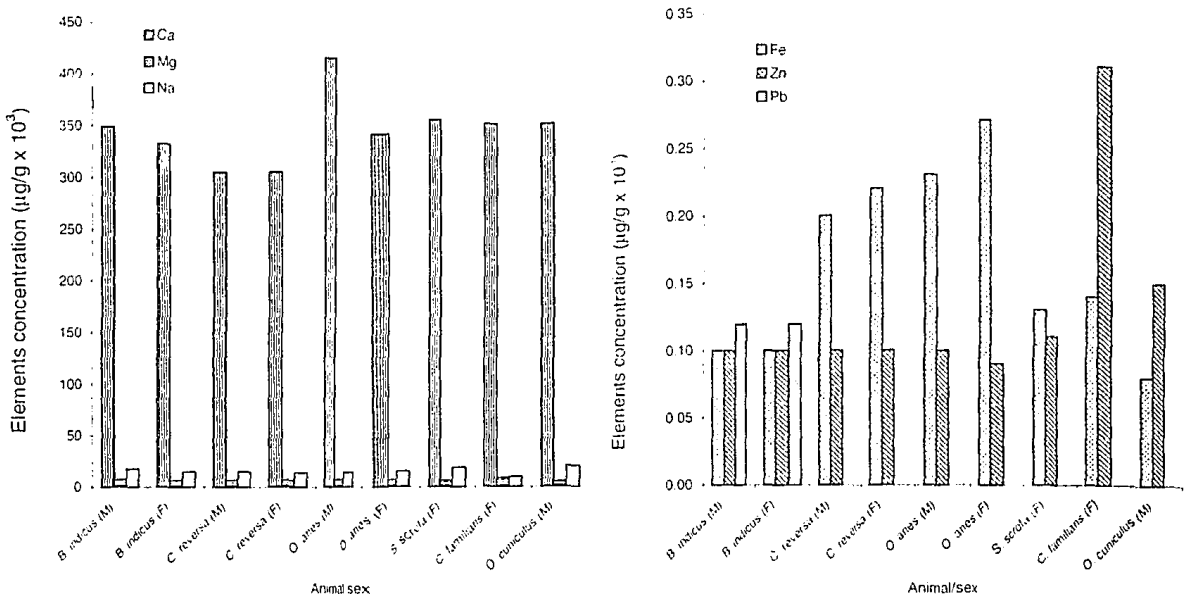

Figure 1. Distribution of elements (µg/g x 10^3 bone powder) in the femur.

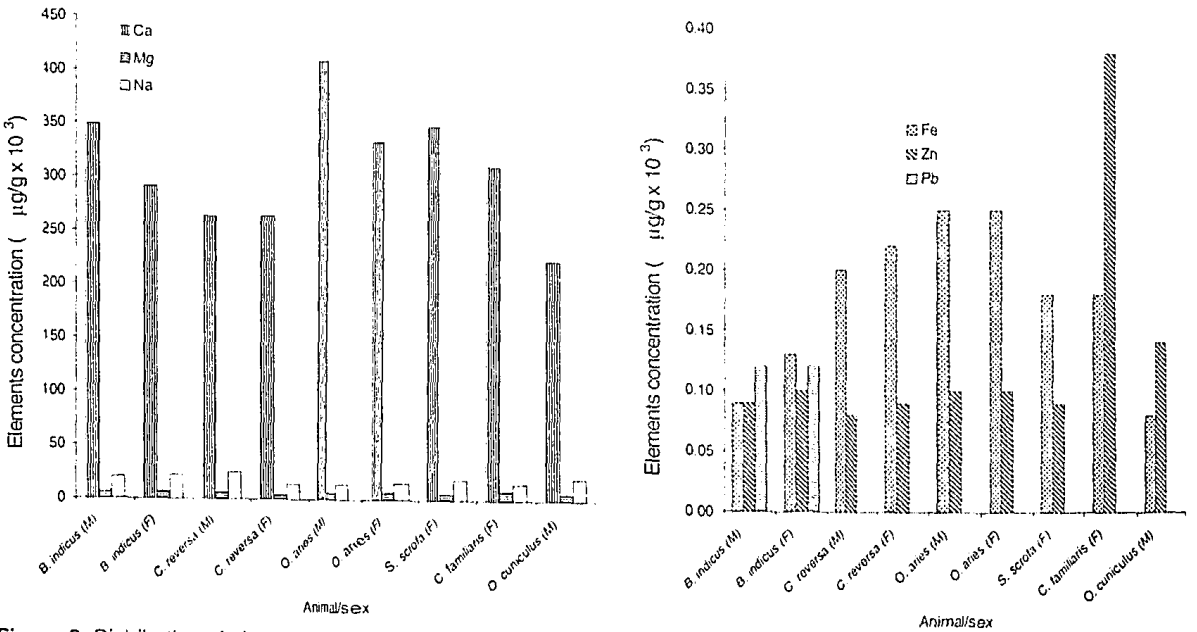

Figure 2. Distribution of elements (µg/g x 10^3 bone powder) in the humerus.

ranged from 0.08 µg/g in the ribs of male *C. reversa* to 0.38 µg/g in the humerus of female *C. familiaris* and radius and ulna of female *C. familiaris*. Lead was only detected in the bones of male and female *B. indicus*. The results reveal that the abundancy of the elements is in the order Ca > P > Na >Mg > Fe > Zn > Pb. The concentrations of zinc are in most cases less than those reported for human bones of the upper Silesian industrial district (Baranowska et al. 1995).

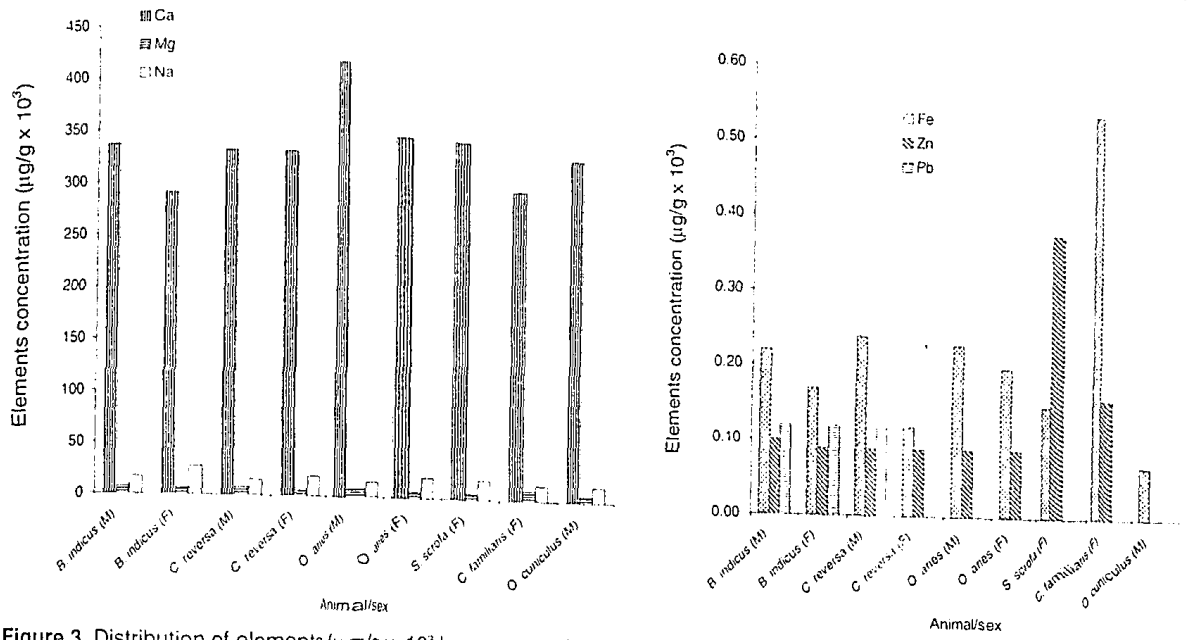

Figure 3. Distribution of elements (µg/g x 10^3 bone powder) in the radius and ulna.

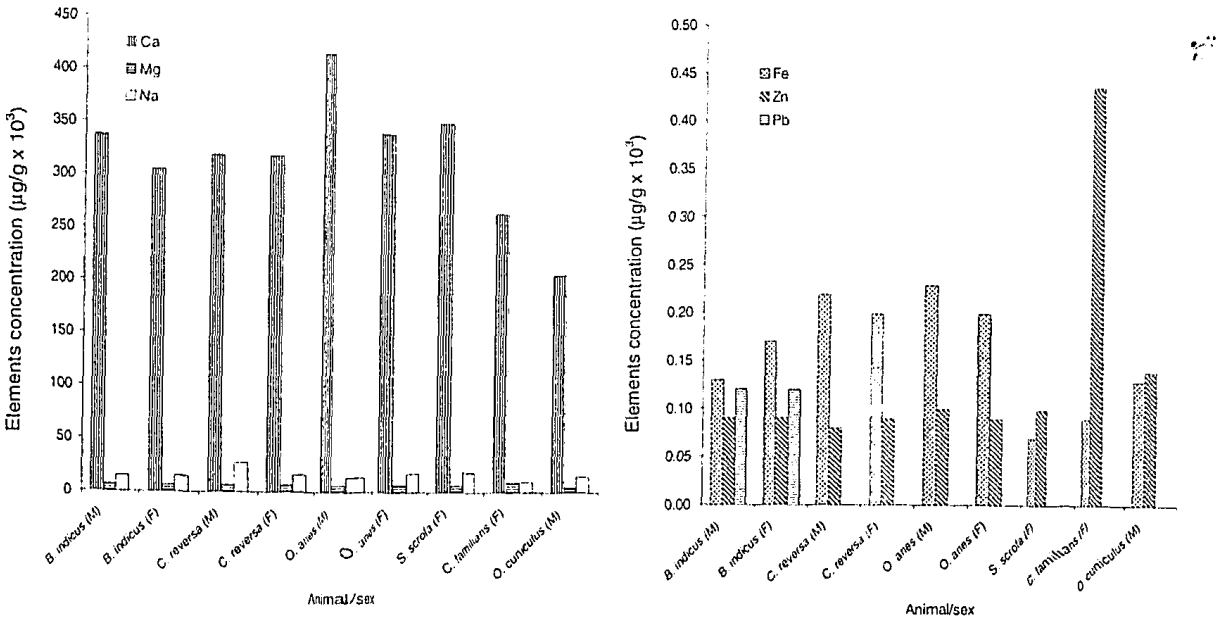

Figure 4. Distribution of elements (µg/g x 10^3 bone powder) in the ribs.

Conclusion

For a total recovery of calcium in bone ash solutions in an analysis by AAS, at least 6,000ppm (0.6%) of strontium should be present in the sample stock solution. The method is fast, simple, allows for the determination of all elements in a single sample preparation and may be extended to the analysis of other bones not covered in this work. Although the use of strontium or lanthanum to eliminate interference

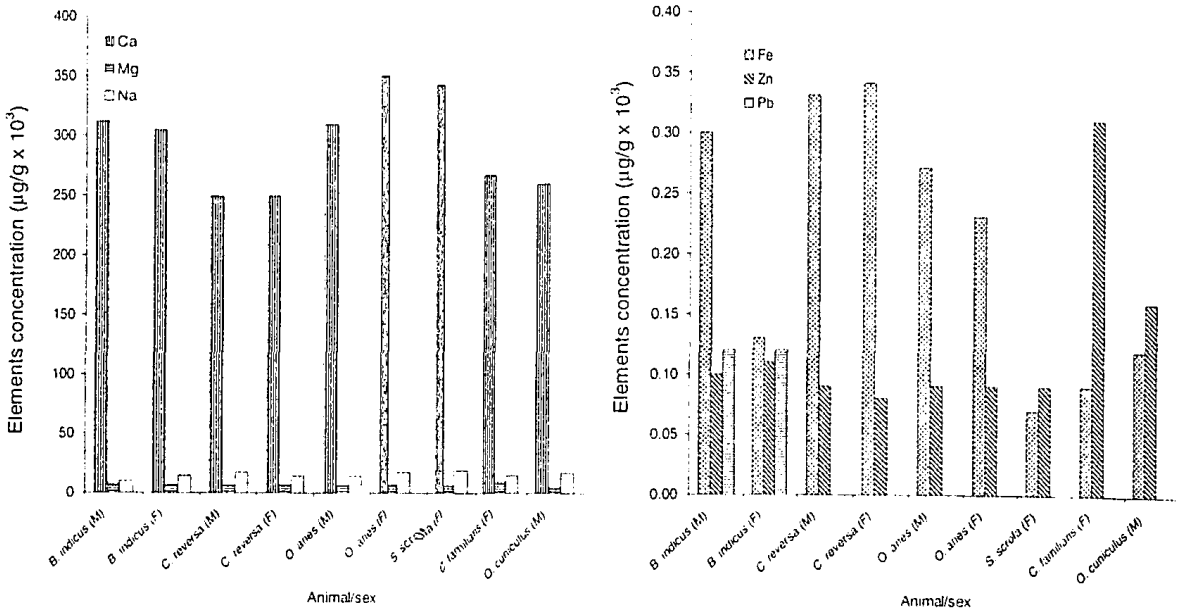

Figure 5. Distribution of elements (µg/g x 10^{-3} bone powder) in the vertebral column.

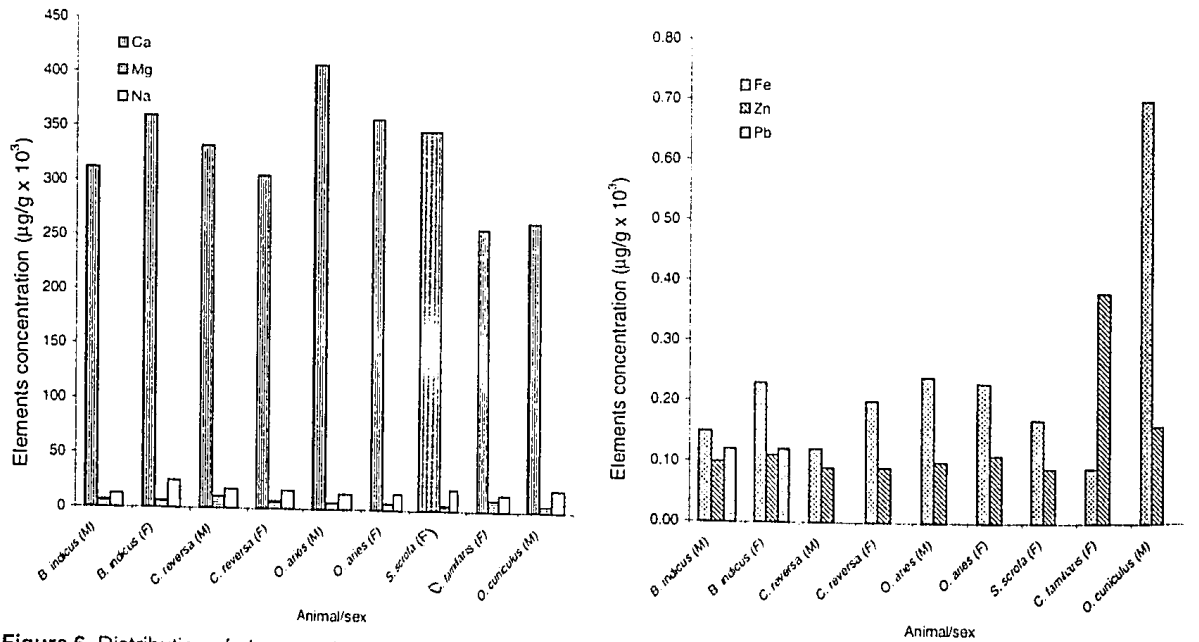

Figure 6. Distribution of elements (µg/g x 10^{-3} bone powder) in the tibia and fibula.

by phosphate has long been known, the exact amount which brings about the desired effect in different matrices will continue to be a subject of research.

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