

Physical and chemical properties of fish and chicken bones as calcium source for mineral supplements

Pianpan Phiraphinyo¹, Sutachai Taepakpurenat¹,
Paungkaew Lakkanatinaporn², Worapot Suntornsuk³,
and Leena Suntornsuk⁴

Abstract

Phiraphinyo, P., Taepakpurenat, S., Lakkanatinaporn, P.,
Suntornsuk, W. and Suntornsuk, L.

**Physical and chemical properties of fish and chicken bones as calcium source
for mineral supplements**

Songklanakarin J. Sci. Technol., 2006, 28(2) : 327-335

Physical and chemical properties of two bones of two species of fish, hoki (*Macruronus novaezelandiae*) and giant seaperch (*Lates calcarifer Bloch.*), were compared with chicken bone to evaluate their composition for use as natural calcium supplement. The information could be useful for waste utilization in the food and pharmaceutical industries. Physical testing and chemical analyses were performed according to the USP 24 and BP 1998 standards under calcium carbonate monograph. Loss on drying found in hoki, giant seaperch and chicken bones was 12.4, 11.3 and 5.9 % w/w, calculated on dried basis, respectively. Total calcium

¹B.S.(Pharmacy), ²M.S.(Pharmaceutical Chemistry), Asst. Prof., Department of Pharmaceutical Chemistry, ³Ph.D.(Pharmaceutical Chemistry), Assoc. Prof., Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok 10400 Thailand. ⁴Ph.D.(Food Science), Assoc. Prof., Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140 Thailand.

Corresponding e-mail: pylll@mahidol.ac.th, worapot.sun@kmutt.ac.th

Received, 10 April 2005 Accepted, 7 October 2005

determined by complexometric titration was 31.8, 28.1 and 32.2% w/w in hoki, giant seaperch and chicken bones, respectively. All samples contained carbonate and phosphate anion residues but gluconate, acetate and citrate were absent. The presence of calcium carbonate was confirmed by thermogravimetry. Results from all bones showed that limit tests for heavy metals, arsenic and iron complied with the USP standard, whereas barium, chloride and sulfate conformed to the BP standard. The magnesium and alkali metals in giant seaperch bone were within the BP limit (1.5%), but those of hoki and chicken bone exceeded the limit.

Key words : calcium, fish bone, chicken bone, food supplement

บทคัดย่อ

เพ็ชรพันธุ์¹ พิระภิญโญ¹ สุธาชัย เตภักภูรีนาถ¹ พวงแก้ว ลัคนทินพร¹ วรพจน์ สุนทรสุข²
และ ลีณา สุนทรสุข¹

สมบัติทางกายภาพและเคมีของก้างปลาและกระดูกไก่เพื่อเป็นแหล่งอาหารเสริมแคลเซียม

ว. สงขลานครินทร์ วทท. 2549 28(2) : 327-335

งานวิจัยนี้ศึกษาสมบัติทางกายภาพและเคมีของก้างปลา 2 ชนิดคือ ก้างปลาโฮกิ (*Macrurus novaezelandiae*) และก้างปลากะพงขาว (*Lates calcarifer* Bloch.) เปรียบเทียบกับกระดูกไก่ และประเมินองค์ประกอบ ทางเคมีเพื่อนำมาใช้เป็นแหล่งอาหารเสริมแคลเซียมจากธรรมชาติ ข้อมูลที่ได้จะเป็นประโยชน์ในการนำวัสดุเหลือทิ้ง มาเพิ่มมูลค่า ในอุตสาหกรรมอาหารและยา การทดสอบทางกายภาพและวิเคราะห์ทางเคมีเชิงคุณภาพและเชิงปริมาณใช้อ้างอิงตามข้อกำหนดของแคลเซียมคาร์บอเนตในตำรายา USP 24 และ BP 1998 พบปริมาณความชื้น (Loss on drying) ซึ่งคำนวณจากน้ำหนักแห้งเท่ากับ 12.4% 11.3% และ 5.9% โดยน้ำหนัก ในก้างปลาโฮกิ ก้างปลากะพงขาว และกระดูกไก่ ตามลำดับ พบปริมาณแคลเซียมรวม (Total calcium) ในก้างปลาโฮกิ 31.8% ในก้างปลากะพงขาว 28.1% และในกระดูกไก่ 32.2% โดยน้ำหนัก จากการวิเคราะห์ทางเคมีตรวจพบอนุมูลคาร์บอเนตและฟอสเฟต แต่ไม่พบกลูโคเนต อะซิเตต และซิเตรต จากนั้นได้ทำการศึกษาเพิ่มเติมโดยเทคนิค Thermogravimetry พบแคลเซียมในรูปแบบแคลเซียมคาร์บอเนต ผลการทดสอบ Limit Test พบว่าตัวอย่างทั้งสามชนิดมีปริมาณโลหะหนัก เหล็ก และสารหนู ไม่เกินมาตรฐานที่กำหนดใน USP และมีปริมาณแบเรียม คลอไรด์ และซัลเฟตไม่เกินมาตรฐานที่กำหนดใน BP ส่วนแมกนีเซียมและโลหะอัลคาไลน์ที่ตรวจพบในปลากะพงขาวมีปริมาณไม่เกินมาตรฐานที่กำหนดใน BP แต่ที่ตรวจพบในก้างปลาโฮกิและกระดูกไก่มิมีปริมาณเกินมาตรฐานที่กำหนดใน BP

¹ภาควิชาเภสัชเคมี คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล พญาไท กรุงเทพฯ 10400 ²ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี บางมด กรุงเทพฯ 10140

Fish and chicken are major sources of protein for human consumption. Additionally, their bones are high in calcium, which is an essential mineral for normal body function (e.g. bone growth, blood clotting and neurotransmission) (Nordin *et al.*, 1998). Calcium is distributed throughout the body, 99% in the bone and 1% in the blood circulation with the plasma level of 8.5-10.5 mg/dL. Lack or an insufficiency of calcium can cause osteoporosis, heart disease and hemorrhage.

Therefore, fish and chicken bones can be used as a food supplement to enhance the calcium content in various kinds of foods (Pearson and Dutson, 1992; Subasinghe, 1996; Kim, Choi and Koo, 1998; Steinmetz, 1999).

The fishery, especially hoki and giant seaperch, and poultry processing industries in Thailand are enormous and they provide significant national income annually. Each year, a considerable amount of by-products (e.g. skin, bone, head

and internal organs) is generated by these industries. These by-products can be used as feed meal. In addition, protein residue from these wastes could be recovered by enzymatic hydrolysis (Linder, Fanni, Parmentier, and Sergent, 1995). On the other hand, bones from the wastes are valuable as a calcium source for humans. However, these bones have not been well characterized. The objective of this study was to investigate the physical (i.e. appearance and loss on drying) and chemical (i.e. limit test, total calcium content and other residues) properties of the bones of two fish species, hoki (*Macruronus novaezelandiae*) and giant seaperch (*Lates calcarifer*), and chicken bone according to the British Pharmacopoeia (BP) and the United States Pharmacopoeia (USP) monographs. Both pharmacopoeias are widely used and well accepted as standard specification for pharmaceutical raw materials and dosage forms. Additionally, other anion residues (e.g. gluconate, acetate, citrate and phosphate) were qualitatively analyzed in order to investigate whether the calcium in the bones is present in these respective anion forms (e.g. calcium gluconate, calcium acetate, calcium citrate and calcium phosphate). The basic information on properties of the bones could be useful for the pharmaceutical and food industries. It could lead to the utilization of these bones as a natural source of calcium in pharmaceutical dosage forms and in functional foods.

Materials and Methods

Samples

Hoki, giant seaperch and chicken bones were supplied by local fish and poultry processing factories. The bones were deproteinized to remove all protein residues in the bone by enzymatic hydrolysis using the modified procedure described by Linder and co-workers (Linder, Fanni, Parmentier, and Sergent, 1995). The bones were cut uniformly to the lengths of 2-4 cm and mixed with Allzyme FPD (Beacon Research, UK) in a ratio of 2.7 to 1 (w/w). The mixture suspended in citrate-phosphate buffer (pH 5.0) was maintained at 50°C and shaken at the rate of 150 rpm for

3 days. After 3 days, the solution was filtered through Whatman paper no. 3 and the residue was washed twice with distilled water. The bone was dried at 60°C for 24 h, and ground to a fine powder. The powder was passed through a sieve with a mesh no. 60 prior to analysis.

Physical testing

Physical appearance (i.e. texture, odor and color) of the bone before and after drying in an oven was compared. Loss on drying (LOD) was performed by drying 200 mg of the bone at 200°C for 4 h, according to the USP limit under calcium carbonate monograph, the LOD should not be more than 2% of its weight (USP 24).

Chemical analyses

Identification of calcium and carbonate

Identification of calcium and carbonate was performed according to the monographs described in USP 24. Positive identification of calcium was indicated by the opaque white precipitate after addition of ammonium oxalate to the sample solution. The precipitate was insoluble in 6 N acetic acid, but soluble in hydrochloric acid. The presence of carbonate was confirmed by the effervescence after addition of acetic acid to the sample solution. The resulting gas was passed through calcium hydroxide test solution, which gave a white precipitate of calcium carbonate immediately. Additionally, the sample solution gave red color with phenolphthalein test solution, which confirmed the presence of carbonate instead of bicarbonate.

The presence of calcium carbonate in the bone sample was confirmed by thermogravimetry. About 10 mg of bone sample was placed into the thermogravimetry analyzer. A heating rate of 25°C/min was used to heat the sample from ambient to 850°C. Thermogravimetric spectrum of the bone sample was compared with that obtained from standard calcium carbonate.

Assay of total calcium

Assay of total calcium content in fish and chicken bone was modified from the assay of

calcium under calcium carbonate monograph described in USP 24. Bone sample ca 200 mg was mixed with water and hydrochloric acid, 2 mL each, adjusted to 50 mL with water and placed in an ultrasonic bath for 10 min. Prior titration, 18 mL of 0.05 N edetate disodium was added into the mixture to prevent the precipitation of calcium hydroxide, then neutralized by adding 25 mL of 1 N sodium hydroxide and the final volume adjusted to 100 mL. The solution was titrated with 0.05 N edetate disodium using hydroxy naphthol blue as an indicator. Each mL of 0.05 N edetate disodium is equivalent to 20.04 mg of Ca.

Limit tests

Limit tests for the fish and chicken bones were carried out by using the method described in BP 1998 or USP 24 depending upon the availability of the instruments and chemicals. Heavy metals, arsenic and iron tests were performed by USP methods, whereas magnesium and alkali metal, barium, chloride and sulfate were tested by BP methods.

Heavy metal

Stock standard lead solution was prepared by dissolving 8 mg of lead nitrate with 5 mL water, adding 1 mL nitric acid and adjusting to 50 mL with water. Standard lead solution (10 µg/mL) was prepared by diluting 10 mL of the stock standard lead solution to 100 mL with water. Test preparations were prepared according to Method II of the heavy metal limit tests described in USP 24. A bone sample (1 g each) was put in a crucible, moistened with sulfuric acid and ignited at 100°C until charred. 2 mL nitric acid and 4 drops sulfuric acid was added to the residue, which was then ignited at 600°C until no white fumes were evolved and the residue was allowed to cool down. 4 mL of 6 N hydrochloric acid were added to the ash, which was then covered and digested on a steam bath for 15 min, uncovered, and slowly evaporated on a steam bath to dryness. The crucible lid was closed and allowed to stand for 25 min. The residue was again evaporated to dryness on a water bath, moistened with 1 drop of hydrochloric acid and

10 mL of hot water and further digested for 2 min. 6 N ammonium hydroxide was added to the solution dropwise, until the solution was just alkaline to litmus paper and the volume was adjusted to 25 mL with water. The mixture was adjusted to pH 3-4 with 1 N acetic acid. The standard lead solution and test preparations were transferred into a Nessler's tube, 10 mL of freshly prepared hydrogen sulfide added, mixed and the preparations allowed to stand for 5 min. The color produced from the sample solution should not be more intense than that of the standard solution (0.002%).

Arsenic

Arsenic trioxide stock solution was prepared by dissolving 132 mg arsenic trioxide, previously dried at 105°C for 1 h, in 5 mL of 1 in 5 sodium hydroxide. The solution was neutralized with 2 N sulfuric acid, and a further 10 mL of 2 N sulfuric acid added. The solution was adjusted to 1,000 mL with boiled and cooled water and mixed. Standard arsenic solution was prepared by diluting 10 mL arsenic trioxide stock solution in 10 mL of 2 N sulfuric acid and adjusting the volume to 1,000 mL with boiled and cooled water. Sample solution was prepared by dissolving 1 g of the bone in 15 mL hydrochloric acid and diluting with water to 55 mL in a generator flask. Testing was performed by consecutively adding 20 mL of 7 N sulfuric acid, 2 mL of potassium iodide TS, 0.5 mL of stronger acid stannous chloride TS and 1 mL isopropyl alcohol into the generator flask, mixing and allowing to stand for 30 min. Lead acetate cotton was inserted into the lower tube and 3 mL of silver diethyldithiocarbamate was added into the absorber tube of the generator flask. Three grams of granular zinc mesh no. 20 were added into the flask and allowed to stand for 45 min with 10 min mixing interval. The color produced by the sample solution should not be more intense than that obtained by treating a 3 mL of standard arsenic solution in the same manner (3 mg/kg As).

Magnesium and alkali metals

A 1 g sample of bone was dissolved in 12

mL of diluted hydrochloric acid, boiled for 2 min and diluted with 20 mL water. To sample was added 1 g of ammonium chloride, 0.1 mL of methyl red solution, and dilute ammonia until the color of the indicator changed and then 2 mL in excess. The mixture was heated to boiling, 50 mL of hot ammonium oxalate solution added, allowed to stand for 4 h, and diluted to 100 mL with water. The mixture was filtered and 50 mL of the filtrate was aliquoted, 0.25 mL sulfuric acid added and evaporated to dryness on a water-bath and ignited to a constant mass at 600°C. The residual weight should not be more than 7.5 mg (1.5%).

Iron

Iron stock solution was prepared by dissolving 863.4 mg ferric ammonium sulfate with water and adding 10 mL of 2 N sulfuric acid and diluting with water to 100 mL. 10 mL of the iron stock solution was pipetted into a 1000 mL volumetric flask, 10 mL of 2 N sulfuric acid added, diluted to volume with water and mixed. This solution contained the equivalent of 0.01 mg (10 µg) iron per mL. One mL of this stock solution was transferred into a Nessler's tube, 2 mL hydrochloric acid was added and diluted with water to 50 mL. Sample solution was prepared by dissolving 50 mg of the bone with 5 mL hydrochloric acid and diluting to 10 mL with water. Sample solution was transferred to a Nessler's tube, 2 mL of 20% w/v citric acid and 0.1 mL mercaptoacetic acid added, basified with 10 M ammonia and adjusted to 20 mL with water and allowed to stand for 5 minutes. The color produced from the sample solution should not be more intense than that of the standard solution (200 mg/kg).

Preparation of Solution S for testing of barium, chloride and sulfate

Solution S was prepared by dissolving 5 g of the bone in 80 mL acetic acid. The solution was boiled for 2 min and allowed to cool at room temperature. The solution was adjusted to 100 mL with dilute acetic acid, filtered and used for testing of barium, chloride and sulfate.

Barium

Solution S 10 mL was aliquoted, 10 mL of calcium sulfate solution added and allowed to stand for 15 min. The opalescence of the solution should not be more intense than the mixture of 10 mL solution S and 10 mL of distilled water.

Chloride

Standard chloride solution was prepared by diluting 10 mL standard chloride solution (5 mg/kg) with 5 mL water. For sample solution, 3 mL of solution S was diluted to 15 mL with water. To the standard chloride and sample solutions, 1 mL of 2 N nitric acid was added, the mixture was poured into 1 mL of 0.1 M silver nitrate and allowed to stand for 5 min protected from light. The opalescence of the sample solution should not be more intense than that of the standard solution (330 mg/kg).

Sulfate

Solution S 1.2 mL was added with 1 mL of 25 %w/v barium chloride solution and diluted to 15 mL with distilled water. To the sample solution, 0.5 mL of 5 M acetic acid was added and allowed to stand for 5 min. The opalescence of the sample solution should not be more intense than that obtained by treating a 15 mL of standard barium chloride solution (10 mg/kg SO₄) in the same manner (0.25%).

Other anion residues

Gluconate

Standard and sample solutions were prepared separately by dissolving 20 mg of calcium gluconate and the bone sample, respectively, with 1 mL of water. About 5 µL of each solution was spotted on a silica gel 60 F₂₅₄ plate (20 x 20 cm) and developed in a thin layer chromatography tank containing a mixture of alcohol: water: ammonia: ethylacetate (50:30:10:10 v/v) as a mobile phase. After developing, the plate was dried at 100°C for 20 min, cooled and sprayed with potassium dichromate solution (50 g/L in 40% sulfuric acid). Gluconate residue in the sample solution was indicated by the spot with the same R_f value of that obtained from standard solution.

Acetate

Identification of acetate residue in the bone samples was performed by the method described for identification test for acetate. Firstly, sulfuric acid and ethyl alcohol was added to a small amount of bone sample and the preparation then poured into cool water. Acetate residue was identified by the specific odor of ethyl acetate. Additionally, positive identification of acetate was confirmed by the deep red color after adding ferric chloride solution into basidified bone sample solution, which was diminished by mineral acid.

Citrate

About 2-3 mg of the bone sample was dissolved in 1 mL water and 15 mL pyridine. Citrate residue was spotted by the reddish color after addition of 5 mL acetic anhydride into the sample solution.

Phosphate

Any yellow precipitate of ammonium phosphomolybdate, produced after mixing the slightly acidic bone sample with ammonium molybdate solution, which could be dissolved in 6 N ammonium hydroxide, confirmed the phosphate residue in the bone sample.

Table 1. Physical appearance of the investigated bones

Sample	Physical appearance	
	Before drying	After drying
Standard CaCO ₃	white odorless powder	yellowish odorless powder
Hoki bone	white strong odorous powder	brownish odorless powder
Giant seaperch bone	yellow odorous powder	brownish odorless powder
Chicken bone	yellow odorless powder	dark yellow odorless powder

Table 2. Loss on drying (LOD) and total calcium content in the investigated bone (n = 3)

	% LOD	% Calcium content (%RSD)
Limit for CaCO₃	< 2	98.0-100.5 (CaCO₃)
Sample		
Hoki bone	12.4	31.8 (0.7)
Giant seaperch bone	11.3	28.1 (0.1)
Chicken bone	5.9	32.2 (0.7)

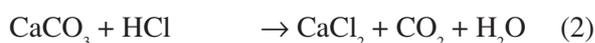
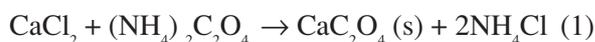
Table 3 Limit tests of the investigated bone (n = 2)

	Ba ²⁺	Cl ⁻ (mg/kg)	SO ₄ ²⁻ (%)	Iron (mg/kg)	Heavy metals (%)	As (mg/kg)	Mg and alkali metals (%)
Limit for CaCO₃	NP	< 330	< 0.25	< 200	< 0.002	< 3	< 1.5
Sample							
Hoki bone	NP	< 330	< 0.25	< 200	< 0.002	<3	1.6
Giant seaperch bone	NP	< 330	< 0.25	< 200	< 0.002	<3	1.0
Chicken bone	NP	< 330	< 0.25	< 200	< 0.002	<3	4.0

NP = no opalescence

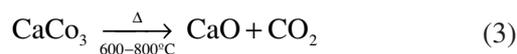
Results and Discussion

The physical appearances of bones of hoki, giant seaperch and chicken are compared in Table 1. Except for color, all bones generally had similar physical appearances. Chemical analyses of the bones are shown in Tables 2 and 3. The moisture content, as indicated by LOD, in the fish bones (e.g. hoki and giant seaperch) varied from 11 to 12%, whereas that of the chicken bone was about 6% (Table 2). All bone gave positive identification for calcium (reaction 1) and carbonate (reaction 2).



The total calcium content in the fish and chicken bone was determined by complexometric titration using edetate disodium as a titrant. During titration, calcium hydroxide could precipitate and interfere with the visual end-point detection. Auxiliary complexing agent such as ammonia ammonium acetate buffer (pH 10) was not sufficient to prevent the precipitation of calcium hydroxide. In addition, the complexing agent interfered with the end-point detection. To prevent this phenomenon, the sample solution should be added with the titrant and neutralized prior the actual titration. The optimal amount of edetate disodium required was 18 mL. The total calcium content in all bones, determined by complexometric titration, varied from 28-32 % w/w with the % RSDs of 0.1-0.7% (n = 3) (Table 2). These results are similar to those of the previous study, which showed the calcium content in various fishes (e.g. cod, Alaska pollack, yellowfin sole, hoki, conger eel and mackerel) are in a range of 37.1-38.6 % w/w (Kim, Choi and Koo 1998). For the bone of other animals such as bovine and other mammals, the calcium content is in a range of 37 % w/w and 35.6-36.3 % w/w, respectively (Field, 2000). However, the calcium content in each bone may vary depending on age, species and feed nutrition. All samples contained calcium in carbonate form,

which was confirmed by a positive peak around 600-800°C from thermogravimetry (Figure 1). The mass loss at this region was the loss of the carbonate composition (reaction 3). Interestingly, the magnitude of these peaks in giant seaperch and chicken bones were lower than that obtained from hoki bone, despite the calcium content in all bones were similar (28-32 % w/w). We reasoned that this peak only indicated the loss of carbonate decomposition from the bones. Since all bones gave positive identification for carbonate and phosphate, the calcium in giant seaperch and chicken bones may present as calcium phosphate more than as calcium carbonate compared to hoki bones. The peak around 100°C was the loss of water; however the peak about 300-500°C remains unknown. Identification of this peak is under investigation.



Limit tests were performed in order to check for chemical impurities associated with the bone samples. Limit tests for heavy metals, arsenic, barium, chloride, sulfate and iron in the investigated fish and chicken bones complied with the requirements under calcium carbonate monograph in the pharmacopeias except for Mg and alkali metal in hoki and chicken bones (Table 3).

Heavy metal and arsenic contents in hoki, giant seaperch and chicken bone were within the USP 24 limits, which are less than 0.002% and 3 mg/kg, respectively.

The magnesium and alkali metal contents of giant seaperch bone were within the BP limit (1.5%), while those of hoki and chicken bones exceeded the limit (Table 3). Iron content in all bones complied with USP 24 standard (200 mg/kg). Limit test for barium, chloride (330 mg/kg) and sulphate (0.25%) in all bones complied with the BP 1998 standard. Additionally, all bones showed negative results for gluconate, acetate and citrate but positive results for carbonate and phosphate. The absence of these anion residues indicates that calcium in the investigated bones was not in the forms of calcium gluconate, calcium

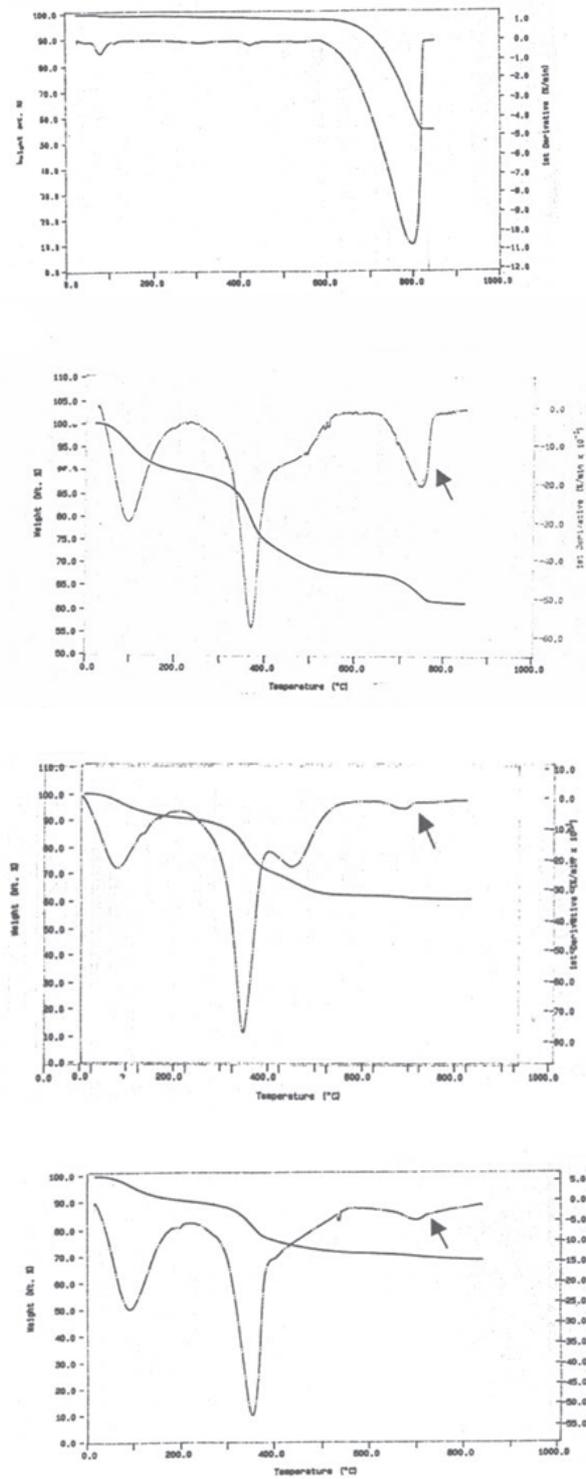


Figure 1. Thermogravimetry curve of a) calcium carbonate b) Hoki bone c) Giant seaperch bone and d) chicken bone. An arrow represents the loss of CO₂ from CaCO₃.

acetate or calcium citrate but was present in calcium phosphate form as well as calcium carbonate. These data demonstrated that fish bones show higher quality than chicken bones in terms of chemical impurities (i.e. magnesium and alkali metals). The results indicated that fish bones have potential to be developed as calcium preparations (e.g. tablets or capsules), supplements or health food for human consumption. For the pharmaceutical aspect, the dissolution test and bioavailability of calcium from fish bone should be determined prior to manufacturing process.

Conclusions

The present study demonstrated that the investigated fish and chicken bones had similar characteristics in terms of the calcium content and limit tests (except for the magnesium and alkali metal content) based on the calcium carbonate monograph in the USP 24 and BP 1998 pharmacopoeias. The information obtained from this study can be valuable for future development of the bones as calcium supplements for human consumption.

Acknowledgement

The authors wish to thank the Faculty of Pharmacy, Mahidol University for providing financial support and facilities for this work and Faculty of Science, King Mongkut's University of Technology Thonburi, for providing the facilities for enzyme hydrolysis. Assoc. Prof. Satit Putipipathkhachorn, Ms Surin Uyoung and Ms. Praditha Ratanawichit are thanked for their technical assistance.

References

- Alexander, K.S., Dollimore, D., Dunn, J.G., Gao, X. and Patel, D. 1993. The determination of cholesterol, calcium carbonate and calcium oxalate in gallstones by thermogravimetry. *Thermochemica Acta*, 215: 171-181.
- British Pharmacopoeia (BP) Vol. I and II. 1998. The Stationery Office, London, United Kingdom.
- Field, R.A., 2000. Ash and calcium as measures of bone in meat and bone mixture. *Meat Sci.*, 55: 225-264.
- Kim, J. S., Choi, J.D. and Koo, J.G. 1998. Component characteristics of fish bone as a food source. *Agric. Chem. Biotechnol.*, 41(1): 67-72.
- Linder, M., Fanni, J., Parmentier, M., Sergent, M. and Phan-Tan-Luu, R. 1995. Protein recovery from veal bones by enzymatic hydrolysis. *J. Food Sci.*, 60(5): 949-952.
- Nordin, B.E.C., Gurr, M.I., McIntosh, G.H., Schaafsma, G., Miller, G.D., Groziak, S.M., Sieber, R. and Goulding, A. 1997. Dietary calcium in health. *Bull. Int. Dairy Fed.*, 322: 36-40.
- Pearson, A.M. and Dutson, T.R. 1992. Inedible meat by-products. *Adv. Meat Res.*, 8: 416-432.
- Steinmetz, T. 1998. Functional food in focus. *Food Mar. Technol.*, 12(4): 11-15.
- Subasinghe, S. 1996. Innovative and value-added tuna products and markets. *Infofish. Int.*, 1: 43-45, 48-50.
- The United States Pharmacopoeia (USP 24), the National Formulary (NF 19) 2000. United State Pharmacopoeial Convention Inc., Rockville.