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## Assessment of Lipid Extraction from Human Bone on Subsequent Demineralization

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**ASSESSMENT OF LIPID EXTRACTION  
FROM HUMAN BONE ON SUBSEQUENT DEMINERALIZATION**

by

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University in Partial Fulfillment of the  
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December, 1990

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## **ABSTRACT**

### **ASSESSMENT OF LIPID EXTRACTION FROM HUMAN BONE ON SUBSEQUENT DEMINERALIZATION**

**JaeEun Lee**  
**OLD DOMINION UNIVERSITY, 1990.**  
**Director: Dr. Lloyd Wolfinbarger, Jr.**

The purpose of this study was to assess the effectiveness of various solvents in removal of lipids from human bone, and to determine the effects of lipid extraction on subsequent acid solubilization of the mineral components of bone. Specifically, the following processing steps were studied; 1) ground human bone was defatted in various ethanol / water mixtures, and / or chloroform / methanol ( 2:1, v:v ), 2) ground human bone which had lipids extracted ( in absolute ethanol ) and bone which had not had lipids extracted were demineralized using various concentrations of hydrochloric acid to assess the value of lipid extraction on subsequent demineralization. For most of the studies, ground bone was placed into columns and the various combinations of solvent systems allowed to flow through the bone matrix while lipid and calcium concentrations were monitored in the eluent solutions. The data demonstrate that chloroform / methanol ( 2:1, v:v ) removes bulk lipid more effectively than various combinations of ethanol / water mixtures. Lipid removal prior to demineralization with HCl improves subsequent demineralization of bone and the rate and extent of demineralization were

significantly affected by the concentration of HCl used in the demineralization process. The process of demineralization was monitored using a calcium-specific electrode or by measuring the pH of the eluent solutions. All data were statistically evaluated.

The results indicate that the capacities of solvents for lipid extraction from human bone matrix did not differ significantly among the various solvents tested. However, lipid extraction using column extraction methods and chloroform / methanol mixtures required less volume than absolute ethanol. Generally, lipid extraction of bone prior to demineralization made the calcium more extractable than from non lipid extracted bone. Higher concentrations of HCl solution usually required less time for calcium extraction from bone matrix, although 0.3 N HCl extracted more calcium than 0.5 N HCl. The present data indicate that pH values of eluent acidic solutions remain between pH 3.0 and 4.0 while the demineralization process occurs. This value declines to below or near pH 1.0 when demineralization is completed.

## **DEDICATION**

This work is dedicated to my loving mother and sister whose moral, spiritual and financial support have made this work possible.

I express my great appreciation to Mary and James Merritt who took me into their home over the past year just like family.

## ACKNOWLEDGEMENTS

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To Dr. Mark S. Elliott, for his advice and much needed encouragement and to Dr. Deborah A. Waller for her unselfish support of this project and advice, I owe a very strong debt of gratitude.

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## I. INTRODUCTION

The use of ground demineralized bone ( GDB ) as an inducer of new bone formation has received the attention of many workers in the field of bone and cartilage research.

Bone is one of the most frequently transplanted tissues in humans and is routinely used for the repair of skeletal defects caused by trauma, neoplasia, and infection. For centuries physicians have used skeletal remains from cadavers to fill voids in living bone caused by trauma, cancer or other diseases. Unlike organs, transplanted bone, tendon and other tissue is not normally rejected by the body's immune system.

Marshall R. Urist ( 1965 ) discovered that acid demineralized bone fragments possess the ability to elicit new bone formation when implanted intramuscularly. Subsequent research has substantiated this early work ( Syftestad and Urist, 1982; Tenenbaum, 1981; Urist, 1976; Urist et al., 1967; Urist and Strates, 1970 ) and ground-demineralized bone is used clinically in a variety of applications. For instance, ground bone is routinely used in repair of extraosseous periodontic operations and defects. Experimenters have suggested that there are noncollagenous proteins that have the ability to induce bone formation ( Syftestad, 1982; Urist et al., 1967; Urist and Strates, 1970; Urist and Strates, 1971; Urist et al., 1983 ).

A soluble protein component of bone, bone morphogenetic protein ( BMP ), and decalcified bone matrix have been shown to induce the formation of bone in extraosseous tissue. The formation of bone begins with chemotaxis of progenitor cells and their attachment to the demineralized matrix ( Reddi, 1983; Urist et al., 1983 ). This process is followed by proliferation and differentiation of attached cells into chondrocytes. Bone is formed when the cartilaginous matrix undergoes calcification and replacement by osteoid, which then mineralizes. Weiss and Reddi ( 1980, 1981 ) have demonstrated that the appearance of fibronectin on the surface of the demineralized bone matrix is essential to the induction of bone, and that antibodies to fibronectin prevent the normal process of endochondral ossification around the demineralized bone matrix. Fibronectin is a large, adhesive glycoprotein with many biological properties. Initial mineral formation occurs within small cytoplasmic structures, the alleged matrix vesicles. These structures are membrane-bound and enriched in alkaline phosphatase ( AP ) activity ( Matsuzawa et al., 1971 ). AP is presumably involved in the initiation of calcification processes by raising the local concentration of phosphate ions ( Beertsen and Van den Bos, 1989 ).

Tenenbaum and coworkers ( Tenenbaum, 1981; Tenenbaum and Heersche, 1982; Tenenbaum and Palangio, 1987 ) have recently studied the possible role of organic phosphates and alkaline phosphatase in the initiation of calcification during the formation of bone. They discovered that  $\beta$ -glycerophosphate ( GP )-treated cultures would produce mineralized bone *in vitro* ( Tenenbaum, 1981; Tenenbaum and Heersche, 1981; Tenenbaum and Palangio, 1987 ) and that GP-induced

calcification of newly formed bone was blocked by the drug levamisole, an inhibitor of alkaline phosphatase activity ( Tenenbaum, 1987 ).

Although it is well known that acid demineralized bone induces new bone formation, it has not been clearly elucidated what affects the processing steps used in the demineralization of human bone have on remineralization. It is known that bone matrix vesicles contain lipids and accumulate calcium. These vesicles also have a relatively high alkaline phosphatase activity ( Anderson, 1976 ). Lipids are soluble in organic solvents, eg., ethanol, methanol, chloroform, and acetone. Inorganic salts, i.e.,  $\text{Ca}^{++}$ , are soluble in acidic solutions.

The objective of this research was to examine and validate the role of solvent extraction of lipids on subsequent solubilization of the mineral components of bone by acid solutions.

## II. LITERATURE REVIEW

### Bone as a Tissue

**Bone** is a highly specialized form of connective tissue. It is distinguished from other forms of connective tissue by the fact that it is extremely hard, owing to the deposition within a relatively soft organic matrix of a complex mineral substance, largely composed of calcium, phosphate, and carbonate. By far the greater number of cells of connective tissue are fibroblasts which are a spindle-shaped cells present in loose and dense connective tissue, with the capacity to form the fibers of these tissues.

Bone is described as developing by two different methods: intramembranous ( in membrane ), and endochondral ( in cartilage ). The fundamental process in both methods is similar. Bone becomes calcified with the deposition of amorphous and crystalline apatite. The bones of the calvarium of the skull are formed by intramembranous ossification, whereas the basal bones of the skull and the majority of bones of the skeleton are formed by endochondral ossification. In early fetal life a condensation of mesenchyme cells occurs in the case of both membrane and endochondral bone formation. A *mesenchymal cell* is an embryonic connective tissue cell, with an outstanding capacity for proliferation, capable of further differentiation into reticular cells or osteoblasts. In the former a group of cells differentiate into *osteoblasts*, so forming centers of ossification ( Ham, 1974 ). These cells secrete osteoid and in so doing some of them become surrounded and become *osteocytes*

lying in their lacunae. Other cells continue to form osteoid and surround ingrowing capillaries which will bring in the haemopoietic cells of the future marrow ( Ham, 1974 ). In an area of mesenchymal condensation, in the case of endochondral ossification, cells with oval or round nuclei appear packed together forming a model of the future bone. These cells surround themselves with an extracellular matrix or ground substance which is largely composed of glycoproteins peculiar to cartilage. The cells at the periphery of the original condensation become orientated to form the osteoblasts. These differentiated cells lay down a layer of osteoid, i.e., the matrix characteristic of bone. This layer immediately calcifies, becoming a collar of periosteal bone directly in contact with the cartilaginous model. The cartilage cells in the centers of the model have in the meantime undergone degenerative changes which are associated with some calcification ( Ham, 1974 & Cormack, 1979 ).

Bone is much stronger than cartilage because normal cartilage persisting in the body is not calcified and hence cartilaginous structures of any great dimension would bend if called upon to bear weight. The matrix of bone however, is calcified and hence stone-like, so it resists bending and can bear much weight. Bone consists of matrix and cells. The matrix includes collagen and proteoglycans as the principle organic component and phosphates, calcium carbonate, calcium fluoride and magnesium fluoride as the principle inorganic component. The bones also serve as a reservoir of calcium and phosphate, available for the other needs of the body, as well as for supplying minerals for deposition when needed in other parts of the skeleton. Collagen fibers in the bone matrix contribute to the strength and resilience of bone, whereas the inorganic salts, composed chiefly of calcium, phosphate, and



carbonate, are responsible for the hardness and rigidity.

Cells present in the matrix are classified into three types: *osteoblasts*, *osteocytes*, and *osteoclasts*. The cellular components of bone are associated with specific functions: *osteoblasts* with the formation of bone; *osteocytes* with the maintenance of bone as a living tissue; and *osteoclasts* with the destruction or resorption of bone. *Osteoclasts* are large multinuclear cells engaged in the absorption and removal of the bone substance. *Osteoblasts* produce the organic bone matrix and some enzymes needed for the process of calcification. *Osteocytes*, the principle cells of the bone, are *osteoblasts* which have been enclosed in the developing matrix of the bone. Thus, *osteocytes* and *osteoblasts* have much the same morphological features, though the granular endoplasmic reticulum is less developed in the *osteocyte* ( Tatsuo and Shigern, 1972 ). These cells, having common ancestors, are closely interrelated.

Matrix vesicles are minute, more or less rounded structures that range from 30 nm to 1  $\mu\text{m}$ , in size and have been seen in the matrix of cartilage and osteoid tissue and at other sites undergoing calcification. These vesicles which are surrounded with a membrane identical with the cell membrane. They are known to contain lipid and to accumulate calcium. They also exhibit certain enzyme activities. Of particular interest is the observation that they have a relatively high alkaline phosphatase activity ( Anderson, 1976 ). Organic phosphates ( alkaline phosphatase substrates ) such as  $\beta$ -glycerophosphate ( GP ) and phosphoethanolamine could serve as sources of phosphate during mineralization of bone formed *in vitro* ( Sampathe and Reddi, 1985 ). That organic phosphates may "induce" mineralization is not

clear, but the rationale for their use is based on the supposition that the organic phosphates would be metabolized by the enzyme alkaline phosphatase ( AP ) ( Anderson, 1976 & Sampathe and Reddi, 1985 ). The extracellular matrices of the mineralized connective tissue ( bone, dentin, calcified cartilage, cementum, etc. ) consist of hydroxyapatite ( mineral ), collagen, noncollagenous proteins, lipids and water. The mineral has mechanical and homeostatic roles. The collagen, interacting with the mineral, plays a structural role. The functions of the noncollagenous matrix proteins have not all been established. It is apparent that they must be involved in secretion, assembly, maturation, mineralization and / or maintenance of the extracellular matrix ( Kinne and Fisher, 1987 ).

### **Demineralized Bone and Calcification**

Transplants of bone are not only of interest in experimental osteogenesis but are also of major importance in the surgical treatment of fractures and of other skeletal disabilities. This role is attested to by the establishment of bone banks and the employment of preserved bone, as well as by the widespread use of fresh autogenous bone grafts. The term transplant implies that at least some of the cells of the donor survive the injury caused by relocating the tissue. The term tissue transfer is more suitable for bone, since it states that the donor cells are transported from one location to another, without reference to the end-result. The word graft as used in the term bone graft means ordinarily a tissue transfer, since the new growth does not necessarily depend upon survival of any of the cells of the donor

tissue. In this sense a bone graft differs from a skin graft, in which the success of the latter procedure depends entirely upon survival of the donor cells. Bone is one of the most frequently transplanted tissues in humans, and is routinely used for the repair of skeletal defects caused by trauma, neoplasia, and infection.

Two mechanisms have been postulated to account for the deposition of bone after bone-grafting: first, autogenous grafts retain viable *osteoblasts* that participate in the formation of bone ( Ham and Harris, 1971 ); and second, both autografts and allografts provide the lattice for the deposition of bone by the process of creeping substitution ( Ham and Harris, 1971 ). Recent investigations of the formation of bone in response to demineralized bone grafts has demonstrated that a third mechanism, induced *osteogenesis*, also contributes to the formation of bone. The development of bone is described as being due to osteogenesis. Another term for the process by which bone develops is ossification. Since the primary function of the *osteoblast* is to form bone, osteogenic capacity appears to be the most reliable parameter for characterizing a cell type as osteoblastic, and several reports have demonstrated the ability of cultured *osteoblasts* to produce a mineralized matrix ( Bellows et al., 1986 & Tenenbaum and Heersche, 1982 ).

Matrix decalcified with ethylenediamine tetraacetic acid ( EDTA ), mixed formic and citric acids or acetic acid produced osteogenesis in the same way as matrix decalcified with HCl, but EDTA produced a slightly lower percentage of positive results ( Urist, 1965 ). Lactic acid failed to remove all the mineral, and diffuse deposit which remained seemed to increase inflammation and prevent osteogenesis ( Urist, 1965 ). The chemical composition of HCl-decalcified bone

matrix, in millimoles per liter, per kilogram, was total Ca,  $4.4 \pm 2.6$ ; total P,  $17.5 \pm 3.0$ ; Na,  $163.3 \pm 1.8$ ; hexosamine  $41.4 \pm 11.5$  ( mean and standard deviation ); in percentage dry weight, total N was  $44 \pm 0.5$  ( Urist, 1965 ).

Intensive laboratory investigations by Urist et al. ( 1982 ), and Reddi (1983) have demonstrated the effect of demineralized bone matrix and bone morphogenetic protein on the induction of bone formation. A soluble protein component of bone, bone morphogenetic protein ( BMP ), and demineralized bone matrix have been shown to induce the formation of bone in extraosseous tissue ( Urist and Strates, 1971 ). It has been reported by Hosny and Sharaway ( 1985 ), Vandersteenhoven and Spector ( 1983 ), and Linden ( 1975 ) that the demineralized bone may mineralize following implantation. Hosny and Sharaway ( 1985 ) believed that the mineralization of DB may inhibit bone formation by interfering with the release of BMP. Firschein and Urist ( 1971 ) suggested that remineralization of the old matrix is brought about by osteoblasts, and that it is not a causal factor in bone induction, but a consequence of it. Matrix vesicles appear to promote mineralization by concentrating calcium and possibly phosphate in their membrane and interior spaces. Most of this uptake, especially that of  $\text{Ca}^{++}$ , occurs after the vesicles are released into the extracellular matrix space ( Wuthier, 1976 ). However, the rate, pattern, and ultimate degree of repair of defects in bone that has been grafted with demineralized bone matrix remain unknown. This lack of knowledge has restricted the successful application of the principle of the induction of bone formation to clinical situations. One possibility is that  $\text{Ca}^{++}$  may be transported across the vesicle membrane by energy-dependent enzymatic pumps ( Ali, 1976 ). Urist ( 1981 ) and

Urist et al. ( 1982 ) have suggested that the active components of demineralized bone matrix reside in the low-molecular weight proteins, or bone morphogenetic protein.

Anastassiades et al. ( 1978 ) have further demonstrated that these low-molecular weight proteins can be extracted from demineralized bone with guanidinium hydrochloride. Sampathe and Reddi ( 1985 ) have shown that although, after separation neither the extracted protein nor the residue is capable of the induction of bone formation by itself, they can be recombined to reconstitute a bioactive bone-inducing matrix. In addition to proteins of bone matrix, fibronectin ( a cell-surface protein that is also present in plasma, i.e. plasma fibronectin ) has been demonstrated on the surface of particles of demineralized bone matrix in subcutaneous implants of demineralized bone matrix. The localization of fibronectin on the surface of the particles has been shown to be necessary for ossification induced by demineralized bone matrix. Weiss and Reddi ( 1981 ) have ascribed a critical function to fibronectin, which is a circulating protein with collagen binding, adhesive, and chemotactic properties, in bone formation mediating the attachment of cells to the demineralized bone matrix ( Urist and Strates, 1970 & Urist et al., 1983 ).

Most of the attention to the enzyme systems in cartilage and in bone has been directed to their possible role in calcification. Many of the enzymes in these tissues, however, as in the soft tissues, have primary functions with relation to the metabolic activities of the cells. There is no evidence that any enzyme system is required for calcification of the matrix of bone, as distinguished from cartilage matrix. The

abundance of phosphatase in *osteoblasts* has suggested, that the presence of this enzyme is involved in the formation and deposition of the bone mineral.

### **Lipid Content of Bone**

Decalcified dentin and bone consists primarily of collagen, a protein widely distributed throughout the mammalian body. Some lipids are so tightly bound to the organic matrix of calcified tissues that removal is difficult. Therefore following extraction of lipids in ethanol certain lipid classes are not removed from bone and teeth unless acidified solvents are also employed ( Shapiro, 1970a & 1970b ). Such lipid extracts contain a very high percentage of acidic phospholipids ( phosphatidylserine, phosphatidylinositol ) known to complex with calcium ( Cotmore et al., 1971 ). Localization of lipid components in developing femurs of normal and tetracycline-treated chick embryos has been described by Rolle ( 1965 ) and alcohol-extractable lipids ( probably neutral fat ) were found in the cytoplasm of osteoid cells ( Rolle, 1965 ). Phospholipids have also been detected in the cytoplasm of chondrocytes ( Rolle, 1965 ).

Following extraction of bone with chloroform / methanol, free and esterified cholesterol and phospholipids have been found in the chloroform / methanol ( lower ) phase. The cholesterol content of compact bone has been assayed by Pikular ( Pikular, 1955 ) after demineralization of the sample material with hydrochloric acid. Cholesterol was found to range from 0.0028 to 0.0040 gms / 100 gms dry ash-free protein. Leach ( 1958 ) determined the lipid content of compact

ox leg bone which he rinsed with acetone during the cleaning procedures: no demineralization step was used. Lipid extracts were found to contain triacylglycerols, cholesterol, cholesterol esters, phospholipids, and possibly a beta-carotene.

Quantitative analysis of human bone, obtained following autopsy or after surgery, was demonstrated by paper chromatography ( Dirksen and Marinetti, 1970 ). The major portion of total lipids was found to be extracted in chloroform-methanol before demineralization and consisted primarily of triacylglycerols ( 85% ). Phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, and cardiolipin were all extracted before demineralization. The major portion of acidic phospholipids obtained before demineralization was believed to have originated from cell membranes and other vascular elements rather than from the matrix itself. Shapiro ( 1970a ) observed similar results with mature bovine cortical bone except that phosphatidylserine, phosphatidylinositol, and phosphatidic acid were not extractable until after demineralization. Shapiro ( 1971 ) obtained a total of 1120 mg neutral lipid / 100 g calcified tissue. Some 777.3 mg of this was triacylglycerol, 88% of which was extracted before EDTA demineralization.

### III. MATERIALS AND METHODS

#### SEM of Fresh and Demineralized Bone

Fresh and demineralized ground bone particles were observed in a Cambridge Stereoscan Scanning Electron Microscope according to the following steps.

The fresh and demineralized ground bone particles were mounted on aluminum stubs using double sided tape. The stubs were sputter coated with 130 Å of Au / Pd using a Palaron E5200 sputter coater for 4 minutes. The bone particles were observed in a Cambridge Stereoscan 100 scanning electron microscope at 10 Kv. They were photographed using polaroid type 55 film.

#### Preparation of Ground-Human Bone

The bone to be used in this study was provided by the *LifeNet* ( *LN*, Virginia Tissue Bank, 5809 Ward Court, Virginia Beach, VA 23455 ) and was equivalent to that bone material currently utilized in the production of the ground-demineralized bone product provided for clinical usage. Bone was procured by *LN* staff from cadavers shortly ( less than 24 hrs ) after death ( range of donor age; 16 to 65 ). It was stored frozen at -84°C until processed. For processing, bone was thawed at room temperature, mechanically debrided, ground using a Telsa Analytical Bone Mill, sized ( 2-3 mm diameter ), and refrozen. Ground bone was stored frozen until used in experiments. Just prior to use, sufficient bone was thawed at room



temperature and used without further processing.

### **General Lipid Extraction / Column Extraction from Ground-Human Bone**

Two independent studies were conducted. The first study was to determine the weight percentage of lipids extracted from bone by each solvent system. The second study was performed to determine the volume of solvent necessary to extract the "extractable lipids" ( for absolute ethanol and chloroform / methanol, 2:1, v:v, solvent systems ) from 10 grams of bone.

#### **A. General Extraction of Lipids**

Lipids were extracted from 10 gms of ground-bone by stirring in 100 ml of various combinations of ethanol and water mixtures, i.e., absolute ethanol, 95% ethanol, 70% ethanol, and chloroform / methanol ( 2:1, v:v ) for 1 and half hours at room temperature. Each mixture was filtered through Whatman 3M filter paper using a Büchner funnel. The supernatant was evaporated using Rotavapor-M ( Buchi HB-140 ) until volume of residuals no longer changed. This residue was dissolved in chloroform and transferred into a tared measuring flask. Extracted lipids were weighed after the chloroform was evaporated. Delipidized bone from each solvent system was freeze dried after washing with water. This material was weighed ( Figure 1 ).

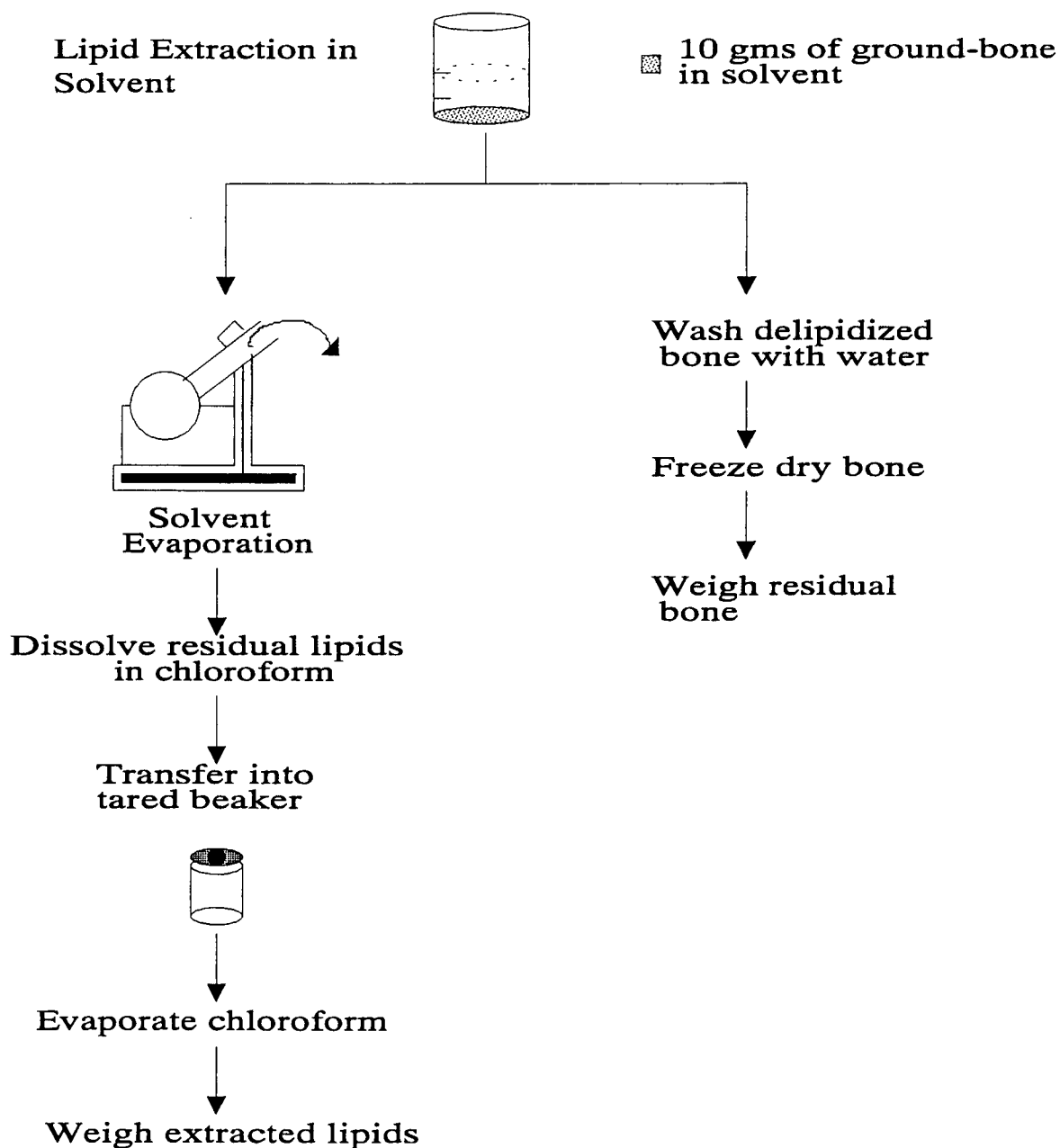


Fig. 1. Diagram of process used in bulk lipid extraction. Lipids were extracted from 10 gms of bone by stirring in 100 ml of various solvents for 1.5 hours at room temperature.

## **B. Column Extraction of Lipids**

In this study, two solvent systems, absolute ethanol and chloroform / methanol ( 2:1, v:v ), were studied. Based on results from the preceding experiment ( A. General Extraction of Lipids ) these two solvent systems were most effective in lipid extraction. Absolute ethanol is in common usage in bone processing for lipid removal and is generally considered as the preferred solvent system for tissue processing because of its limited toxicity, low volatility; it minimizes problems with its use in the processing facility, and it is relatively inexpensive. Chloroform / methanol is the preferred solvent system for lipid extractions; it finds common usage where the greatest numbers and quantities of lipids are to be extracted, but it is rarely used where clinical applications are intended because of toxicity to tissues and animals. It was chosen for use in this study primarily as a "control" solvent by which lipid extraction by ethanol could be compared.

For the "column extraction of lipids", 10 gms of thawed ground bone were packed into a glass column ( length x diameter, 30 cm x 1.5 cm ) and a "plug" of glass wool added to the top of the matrix such that the column plunger did not rest directly on top of the bone. Each solvent system tested was pumped through the bone matrix using a Pharmacia P-3 pump at a flow rate of 1 ml / minute. Effluent liquid was collected in 300 drop ( volume for ethanol was 5.2 ml per tube, chloroform / methanol ( 2:1, v:v ) was 3.2 ml per tube since each solution had different drop sizes ) fractions using an ISCO fraction collector. All column fittings were teflon. This procedure was designed to determine the optimal volume of each solvent system necessary for extraction of lipids from 10 gms of ground-bone ( Figure 2 ).

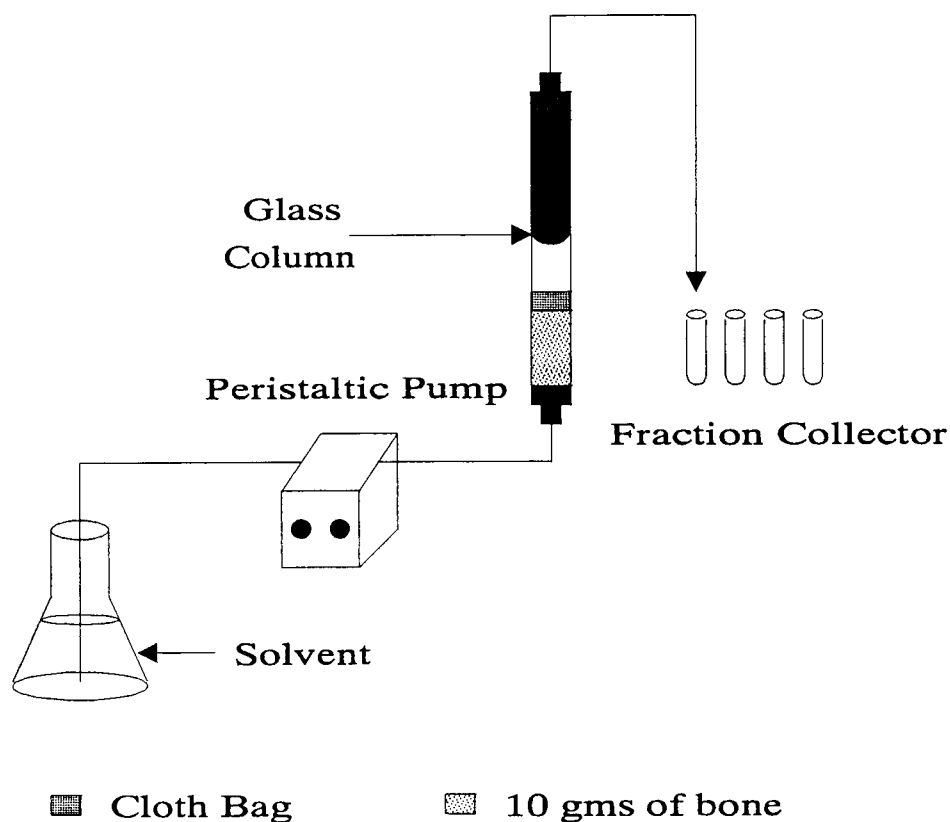


Fig. 2. Diagram of process used in column mediated extraction of lipids and minerals from ground bone. Ground bone was placed into the column and the various combination of solvent systems allowed to flow through the bone matrix using a peristaltic pump (flow rate of 1 ml / minute) with monitoring lipid and / or calcium concentrations in the eluent solutions.

### Analysis of Lipid Content in Lipid Extracts

Cholesterol standards were prepared for use in calculation of lipids extracted from bone. Although the solvent systems employed extracted a variety of lipids, i.e, cholesterol, phospholipids, etc., cholesterol was chosen to provide a relative measure of lipids extracted from bone. Cholesterol standards at 0.025 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1.0 mM, 5.0 mM, 10.0 mM, and 50.0 mM were prepared in absolute ethanol and in cholesterol / methanol ( 2:1, v:v ). Aliquots of each solution were transferred to a quartz cuvette for measurement of absorbance. Standard solutions were also examined on the scanning spectrophotometer ( Beckman, Model 26 ) in the UV range ( 200 nm to 360 nm ). A chart speed of 5 inches / min was used at room temperature. Each solvent system, absolute ethanol and chloroform / methanol ( 2:1, v:v ), was used as a "blank". Optimal wavelengths for absorbance by cholesterol in each solvent system could be determined using the scanning spectrophotometer ( Figures 5 & 6 ). After determining the optimal wavelength for absorbance by cholesterol in each solvent system, absorbance by each concentration of cholesterol was determined and a standard curve generated ( Figures 7 & 9 ). Absorbance values of lipids in each fraction of eluent obtained from the column extraction of bone by each solvent were determined, at the appropriate wavelength, and the concentration of "lipid" in each fraction calculated by use of the appropriate standard curve.

### **Demineralization of Ground-Human Bone**

All demineralization steps were performed on ground-bone; absolute ethanol extracted or non ethanol extracted. The ground-bone ( 10 gms ) was packed into a small glass column ( length x diameter, 30 cm x 1.5 cm ). Various concentrations of hydrochloric acid ( 0.1 N, 0.3 N, 0.5 N, 0.8 N, and 1 N ) were pumped through the packed bone matrix using a peristaltic pump ( 1 ml / min ) and eluent ( 300 drops per fraction ) was collected using a fraction collector ( Figure 2 ). This process was performed at room temperature.

### **Calcium Assays**

#### **A. Standard Calibration Curve for $[Ca^{++}]$ using an Orion 407A Specific Ion Meter and Calcium Specific Electrode**

Calcium ion standards at 0.010 M, 0.025 M, 0.050 M, 0.075 M, 0.10 M, 0.25 M, 0.50 M, 0.75 M, and 1.0 M ( as  $CaCl_2$  ) were prepared in various concentrations of hydrochloric acid ( 0.1 N, 0.3 N, 0.5 N, 0.8 N, and 1.0 N ). Aliquots ( 100 ml ) of each solution were transferred to a beaker containing 2 ml of ISA ( ionic strength adjuster, 4 M KCl ) reagent. The different concentrations of HCl used with the calcium ion standards were necessary because the "unknown" calcium ion solutions were obtained using differed concentrations of HCl in the demineralization process. Correction of voltage shown on the meter with calcium ion concentration was obtained by placing the calcium specific electrode in 100 ml of a 0.10 M standard.

The function switch was turned to "X<sup>+</sup>". Solutions were stirred continuously and when a stable reading was obtained, the meter needle was adjusted to "1" on the "red" logarithmic scale using the calibration control knob. The electrode was then rinsed with distilled water, blotted dry and placed in a 1.0 M standard. When reading was stable, the temperature adjust knob was turned until the meter read "10" ( full scale right ) on the red logarithmic scale. The mV / red value for each standard solution was obtained using constant stirring. Millivolt / red values were obtained for each eluent fraction and calcium concentrations calculated using the appropriate standard curve.

#### **B. Determination of pH in Eluent**

The pH value of each fraction was read at room temperature using a pH meter. The pH electrode was first calibrated using pH standards and the pH of eluent fractions obtained during column demineralization of bone determined.

#### **Statistical Analysis**

Each experimental group consisted of 2 to 3 replicate assays. Millivolt readings using the Orion 407A Ion Analyzer were performed three times on each sample and the mean  $\pm$  range calculated. Calcium ion concentrations in each acidic solution were also determined in replicates of three and results presented as the mean  $\pm$  range.

All statistical tests employed in this study were from the ANOVA program ( SPSS ) at Old Dominion University, Norfolk, VA. Experiments were analyzed by comparing means and standard deviations from single assays. Bon's tests for critical differences were used to determine if mean values of bone demineralization were different. Significance was assigned at the  $p < 0.05$  level. All values in tables and figures represent the mean  $\pm$  the standard error of the means.



## **IV. RESULTS**

### **1. Description of Bone Product**

#### **Scanning Electron Microscopy of Fresh and Demineralized Ground Human Bone**

Scanning electron microscopy was used to visualize the ground demineralized bone utilized in this study. Figures 3.a and b show representative electron micrographs of fresh ground dried bone fragments, showing a dense network, and indication of granular fine structure. As may be seen in figures 4.a and b, granular fine structure is less dense in demineralized than in fresh bone fragments and bone matrices are difficult to see.

### **2. Lipid Removal from Ground-Human Bone**

Two methods were used in this study. Each method was designed to evaluate different aspects of lipid extraction.

#### **A) Lipid Extraction from Ground-Bone Using Column Extraction**

This study used two solvent systems, absolute ethanol and chloroform / methanol ( 2:1, v:v ) to assess the quantity of solvent necessary for extraction of lipids from 10 gms of ground human bone. Because absolute ethanol and chloroform / methanol can be expected to extract different lipids from the bone, it was necessary to choose some component of extractable lipid that could be easily monitored and which would be extracted by both solvent systems.

Fig. 3. Electron micrograph of a section of an undecalcified human ground bone. to illustrate pattern of arrangement of crystals of bone mineral in relation to collagen fibers. The underlying collagen is barely visible, but the crystals are easily seen in the regularly arranged bands corresponding to the main collagen striation. The longitudinal direction of the fibers is horizontal.

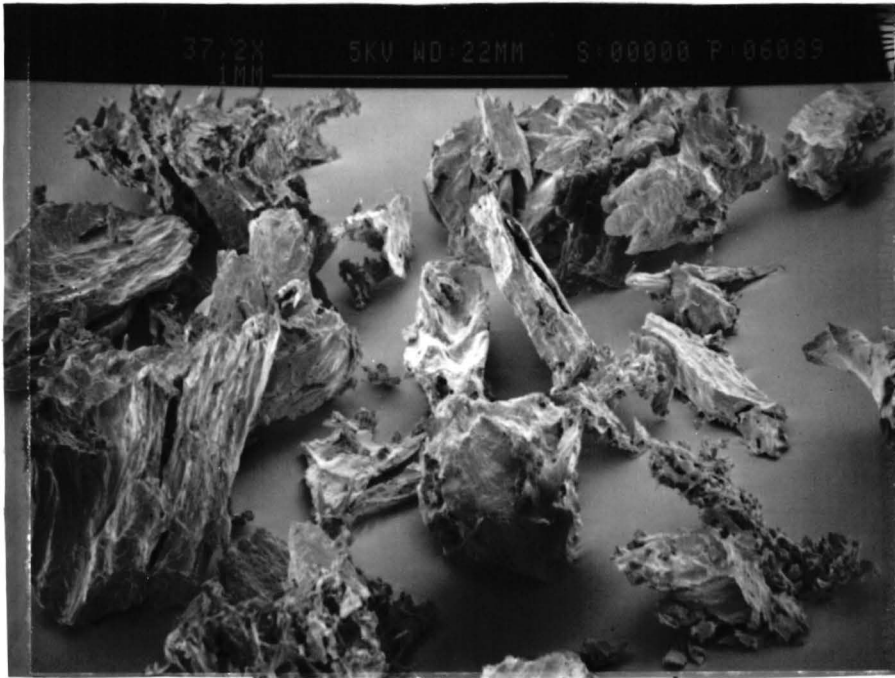


a

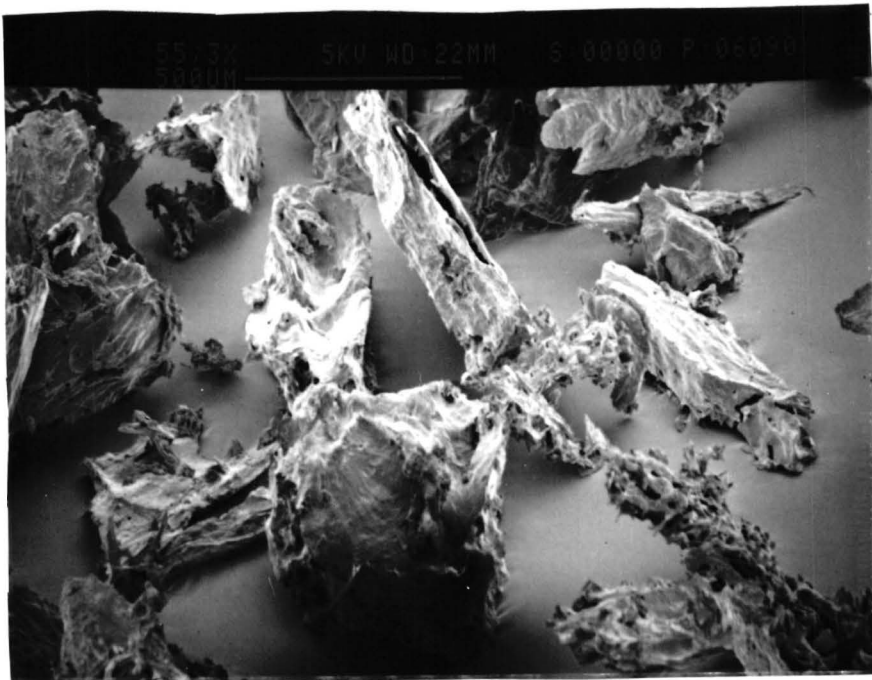


b

Fig. 4. Electron micrograph illustrating the appearance of human bone in decalcified sections, illustrating less dense structure than undemineralized bone.



a



b

Cholesterol represents a common component of mammalian lipids, it exhibits a characteristic absorbance spectra permitting monitoring of a column eluent, and it is soluble in both absolute ethanol and chloroform / methanol. Because the absorption characteristic of cholesterol is a function of the solvent in which it is dissolved, cholesterol was dissolved in known quantities in both absolute ethanol and chloroform / methanol. Using each solvent as a reference solution, the absorbance spectra of cholesterol, between 200 and 360 nm, were obtained ( Figures 5 & 6 ). As may be seen in figure 5, cholesterol exhibits an absorbance peak in absolute ethanol at 210 nm. In chloroform / methanol, an equivalent quantity of cholesterol exhibits an absorbance peak at 252 nm ( Figure 6 ). Based on these data, lipids eluting from ground bone during absolute ethanol extraction were routinely monitored at 210 nm whereas lipids eluting from ground bone during chloroform / methanol extraction were monitored at 252 nm. It must be understood that the total absorbance value at either of these wavelengths for a particular elution volume is due to the sum total of lipids within that elution volume and thus can not be directly correlated with cholesterol concentration in that volume. The total lipid composition of each elution volume would be expected to be different with each solvent and within a particular chromatographic elution of bone by a particular solvent. These particular experiments were designed to determine how much solvent needed to be passed through the bone matrix to effect a "complete", i.e., extraction of those lipids soluble in that particular solvent, removal of solvent extractable lipids. As may be seen in figure 8, absolute ethanol has effectively removed all 210 nm absorbing material from 10 grams of ground human bone by fraction number 18 for a total

ethanol elution volume of 90 ml. In a similar procedure, chloroform / methanol extracts all 252 nm absorbing material by fraction number 12 for a total chloroform / methanol elution volume of 38.4 ml ( Figure 10 & Table 2 ). Because absorbance for both elutions occurs at different wavelengths, a comparison of total absorbance values is not useful in determining the total amount of lipid extracted by each solvent system and an alternative assay was utilized to quantitate total lipid extracted by absolute ethanol and chloroform / methanol.

## **B) Bulk Lipid Extraction**

Lipids were extracted from 10 grams of ground bone in each solvent under equivalent conditions of time and volume ( extraction time: 1.5 hours, volume: 100 ml of each solvent ). Ethanol / water solutions, i.e., 70% ethanol, 95% ethanol, and absolute ethanol, and chloroform / methanol ( 2:1, v:v ) were used for extraction of lipids. Water extraction of ground bone was used as a control. After lipid extraction, the ground-bone was weighed. The results of these experiments are shown in Table 1. It may be seen that the chloroform / methanol solvent was the most effective solvent system used, removing 180 mgs of lipid from 10 grams of bone matrix. Absolute ethanol was the second most effective solvent, removing 108 mg of lipid. Although chloroform / methanol appeared to be the most effective solvent system in this study, analysis of the data using the multiple comparison test reveals that extractions with chloroform / methanol and absolute ethanol were not significantly different in lipid extraction. Lipid extraction by 95% ethanol and 70% ethanol treatments were also not significantly different from each other, however

Table 1. Total Lipids Extracted from 10 gms of Ground-Bone Using Various Solvents.

Solvent	a (mg)	b (%)	c (gm)	d (gm)
Water	0.0	0.00	9.22±0.16	0.78
70% EtOH	26.0±8.52	0.26	9.17±0.21	0.80
95% EtOH	83.7±2.87	0.83	9.07±0.20	0.85
100% EtOH	108.0±13.49	1.08	8.67±0.32	1.32
Chloroform/ MeOH	179.7±46.45	1.80	8.24±0.36	1.58

Values are means  $\pm$  SD , n=3, p < 0.05, Extraction time was for 1.5 hours and same volume of each solvent system was utilized.

- a. Extracted lipid in mg per 10 gms of bone. Distilled water was used as a control, i.e., no lipid extraction.
- b. Total weight percent ( mg % ) indicates the weight of lipids extracted divided by the weight of bone extracted multiplied by 100.
- c. Indicates the weight of solvent extracted bone.
- d. "Extra weight" indicates the difference in the sums of mg extracted lipid plus lipid extracted bone subtracted from the 10 gms of bone used. This value probably represents water.



Table 2. Total Volume Used for Lipid Extraction with Absolute EtOH and Chloroform / Methanol ( 2:1, v:v ) Using Column Extraction.

Solvent	Volume (ml / 10 gms of bone)	a (gms)	b (gms)
Ethanol	90.0±5.49	8.70±0.85	1.3
Chloroform/ MeOH	38.4±3.87	8.50±0.87	1.5

Values are means  $\pm$  SD, n = 3, p < 0.05, Optimal volume for lipid extraction was determined when absorbance readings declined to a base-line value.

Volume: Used volume for lipid extraction

- a. Bone weight: weight of bone after lipid extraction
- b. "Extra weight": Indicates the difference in weight of extracted bone plus weight of extracted lipid subtracted from 10 gms. This "extra weight" probably represents water.

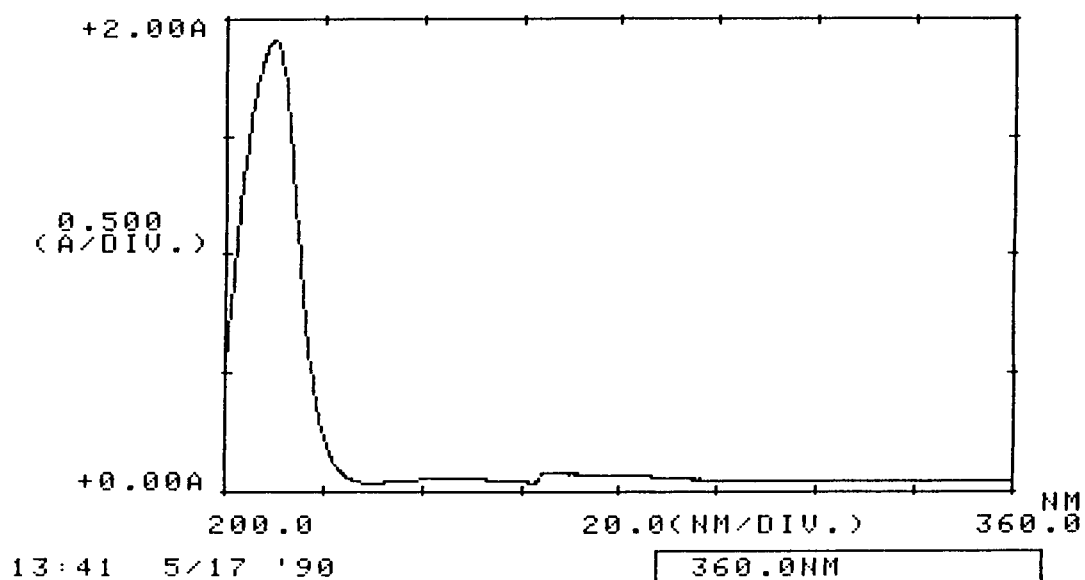


Fig. 5. Absorbance spectra of cholesterol in absolute ethanol between 200 and 360 nm. Cholesterol exhibits an absorbance peak in absolute ethanol at 210 nm (Absorbance of 1 mM of cholesterol is 1.912 at 210 nm).

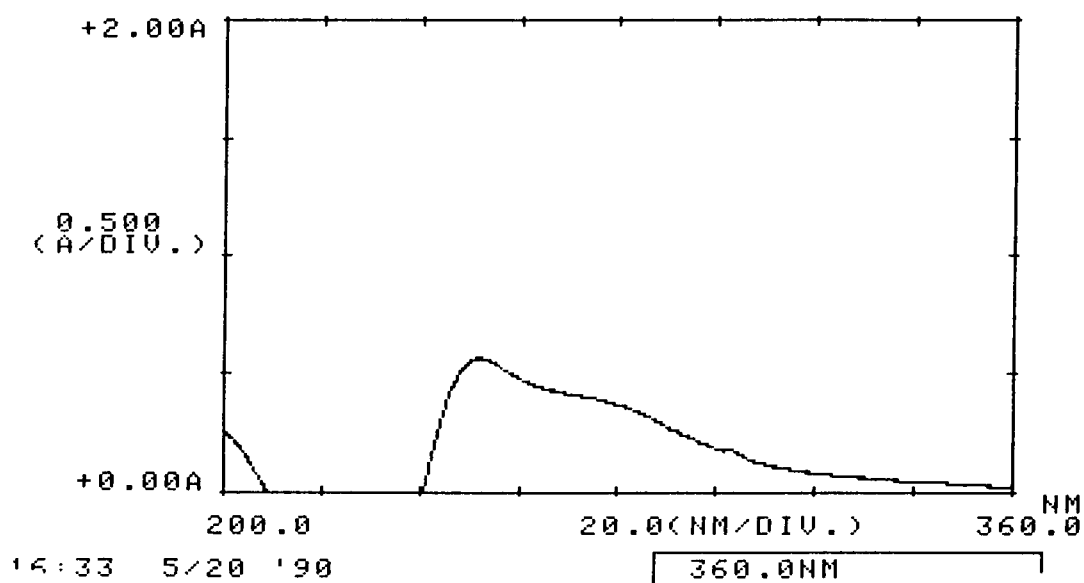
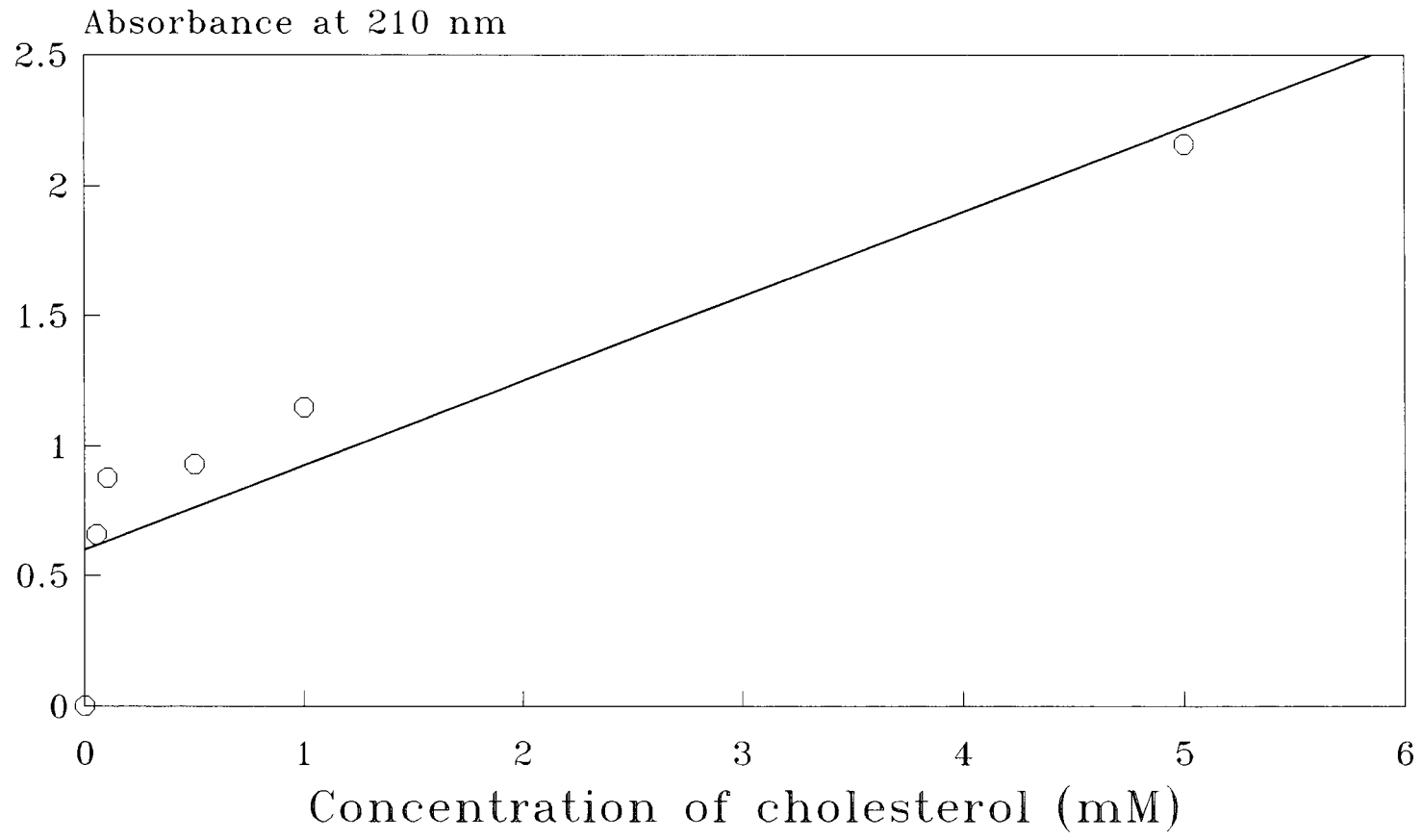


Fig. 6. Absorbance spectra of cholesterol in chloroform / methanol (2:1, v:v) between 200 and 360 nm. In chloroform / methanol, an equivalent quantity of cholesterol exhibits an absorbance peak at 252 nm (Absorbance is 0.567 at 252 nm).

Fig. 7. Standard curve of absorbance at 210 nm versus cholesterol concentration in absolute ethanol.



$$Y = 0.3244X + 0.6018$$

Fig. 8. Analysis of lipid extraction by absolute ethanol from ground bone matrix (10 grams) utilizing absorbance at 210 nm. Ethanol was pumped through the bone matrix at a rate of 1 ml / minute 5.0 ml were collected in each fraction. The absorbance values represent the mean  $\pm$  range of two replicate assays.

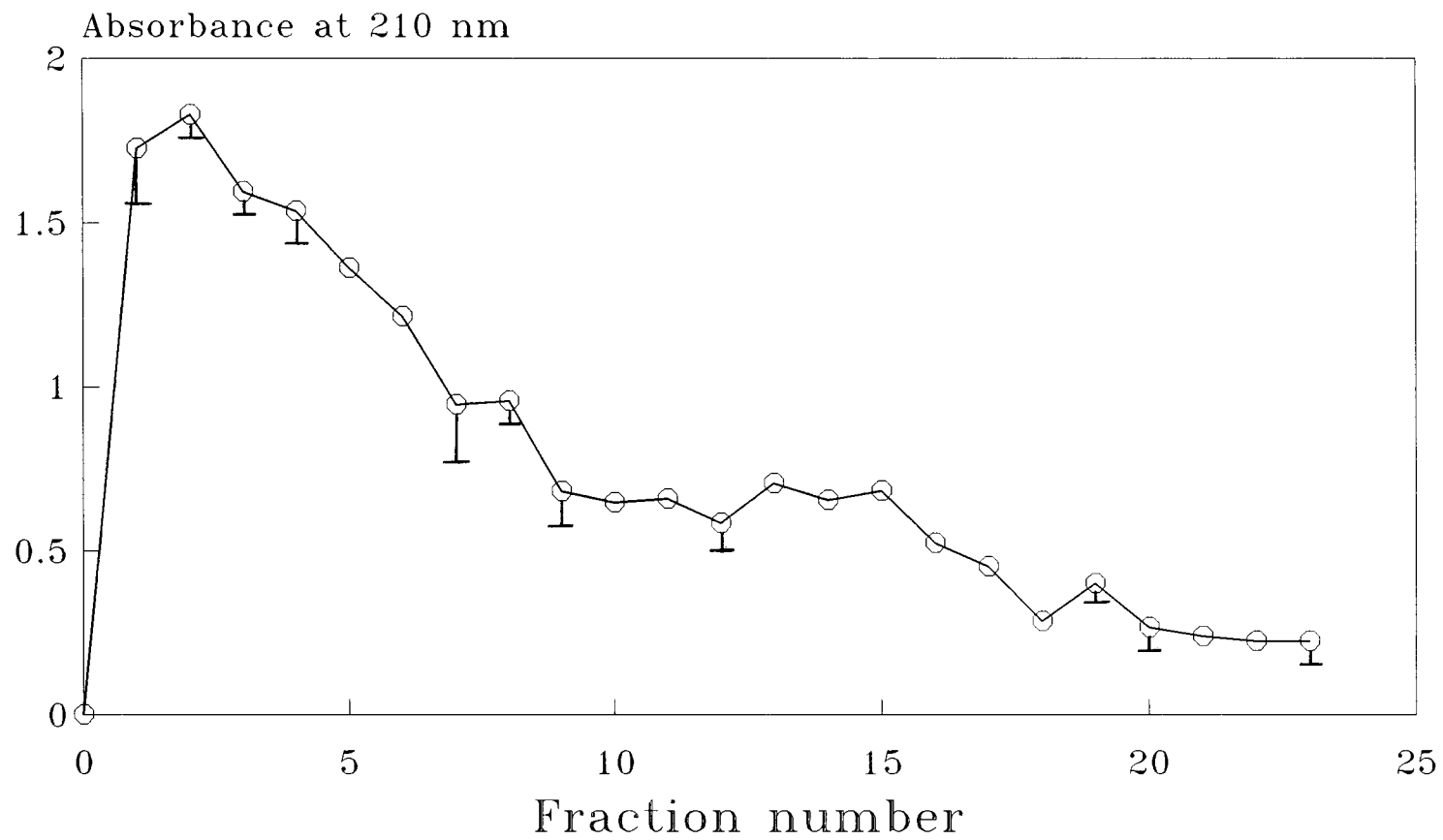
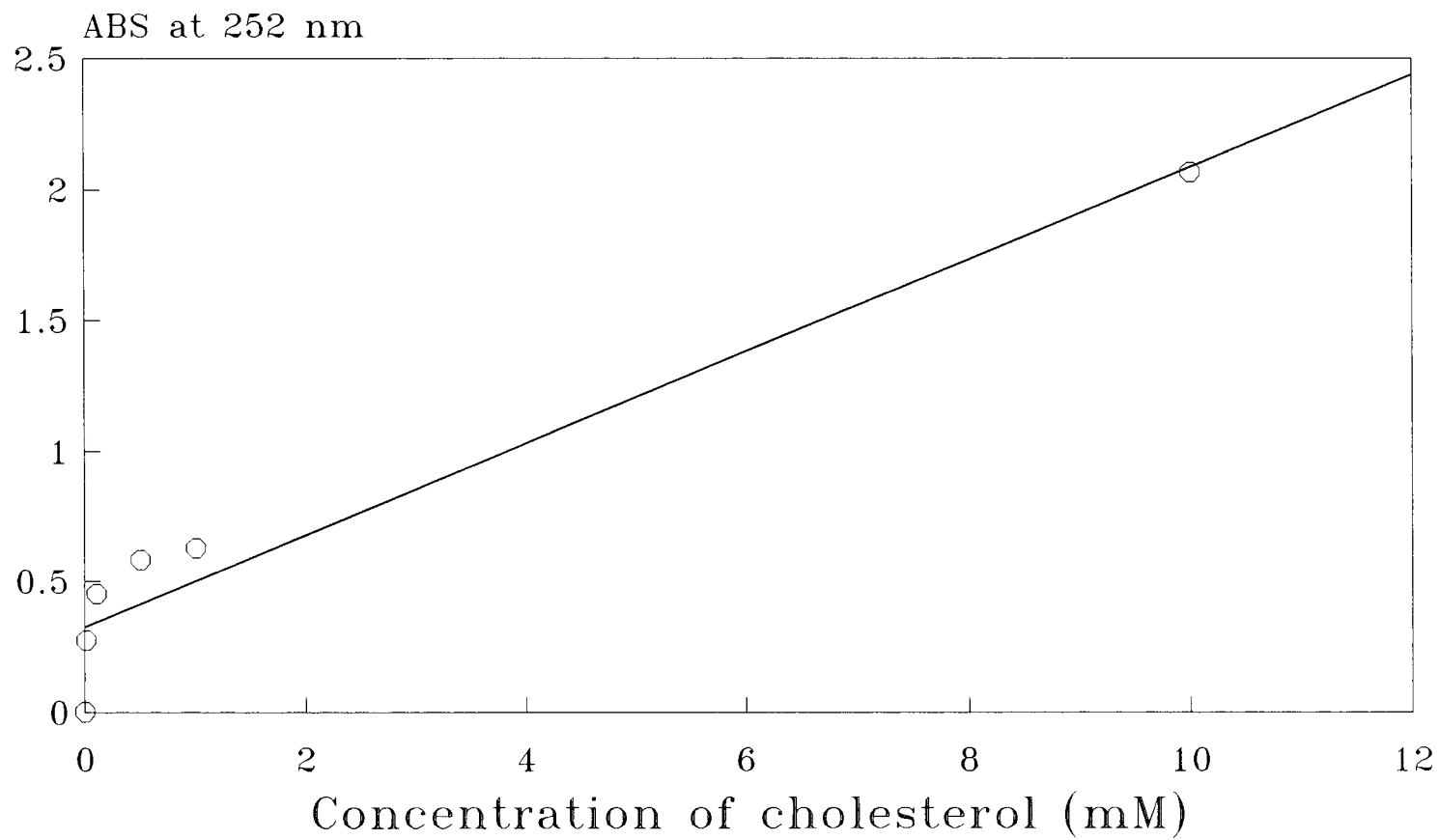


Fig. 9. Standard curve of absorbance at 252 nm versus cholesterol concentration in chloroform / methanol (2:1, v:v).

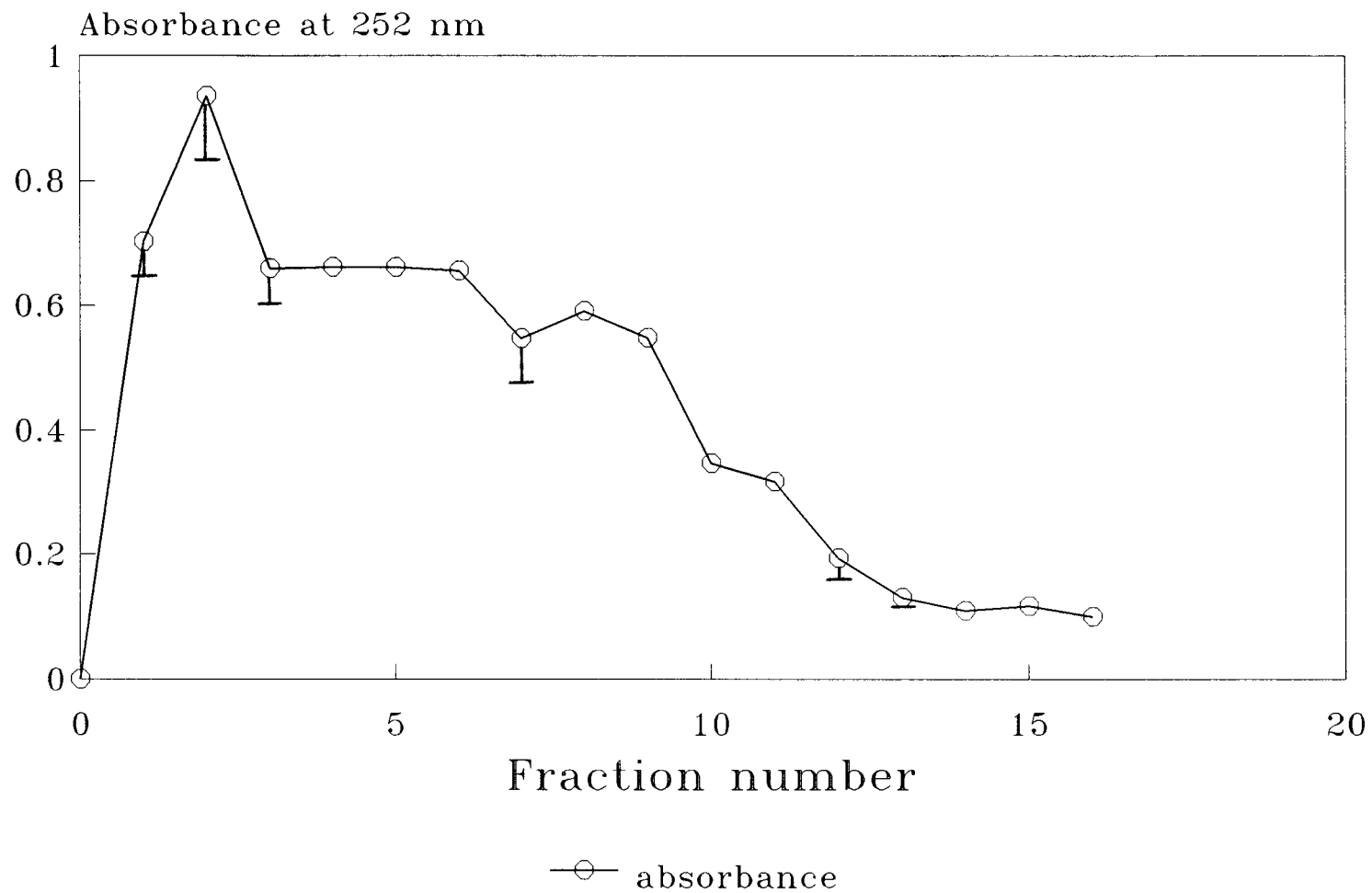




—○— Absorbance

$$Y = 0.1761X + 0.3255$$

Fig. 10. Analysis of lipid extraction by chloroform / methanol from ground bone matrix (10 grams) utilizing absorbance at 252 nm. Chloroform / methanol was pumped through the bone matrix at rate of 1 ml / minute and 3.2 ml were collected in each fraction. The absorbance values represent the mean  $\pm$  range of two replicate assays.



there were significant differences between two groups ( absolute ethanol, chloroform / methanol and 95%, 70% ethanol ). It is therefore, suggested that absolute ethanol and chloroform / methanol are the most effective solvents for use in lipid extraction from bone.

### **3. Demineralization**

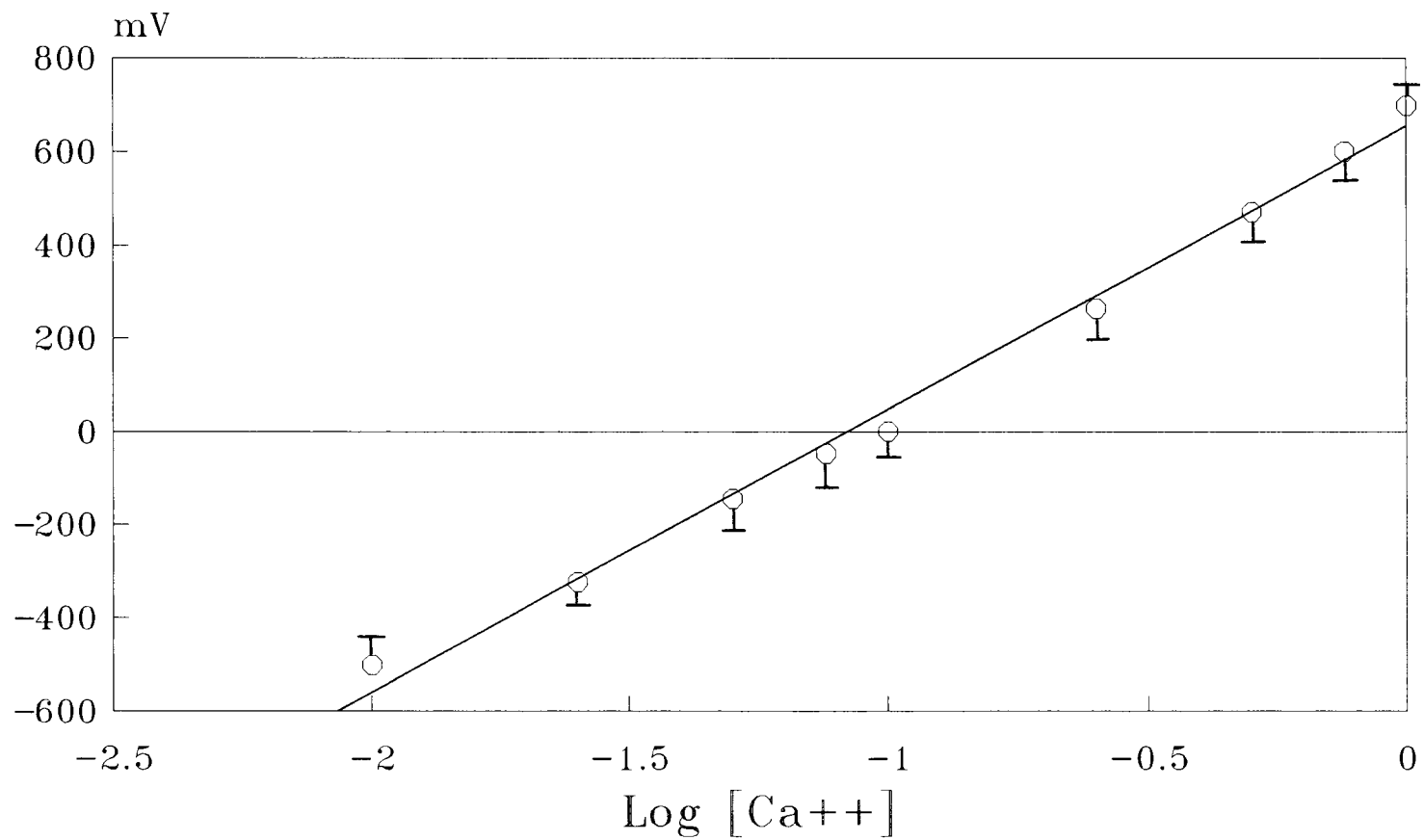
#### **A) Standard Calibration Curve in Each Concentration of HCl**

Standard calcium concentrations in each different concentration of acid solution ( 0.1 N, 0.3 N, 0.5 N, 0.8 N and 1.0 N HCl ) were read as millivolt ( mV ) using the calcium ion specific electrode. Standard curves for calcium ion in each acid concentration are shown in figures 11, 12, 13, 14, & 15. Each calibration curve was repeated a minimum of two times. It may be seen from the data, that mV readings versus calcium ion concentration are quite linear in the different HCl concentrations and that the minimum detection limits for calcium ion was slightly less than 10 mM.

#### **B) Demineralization**

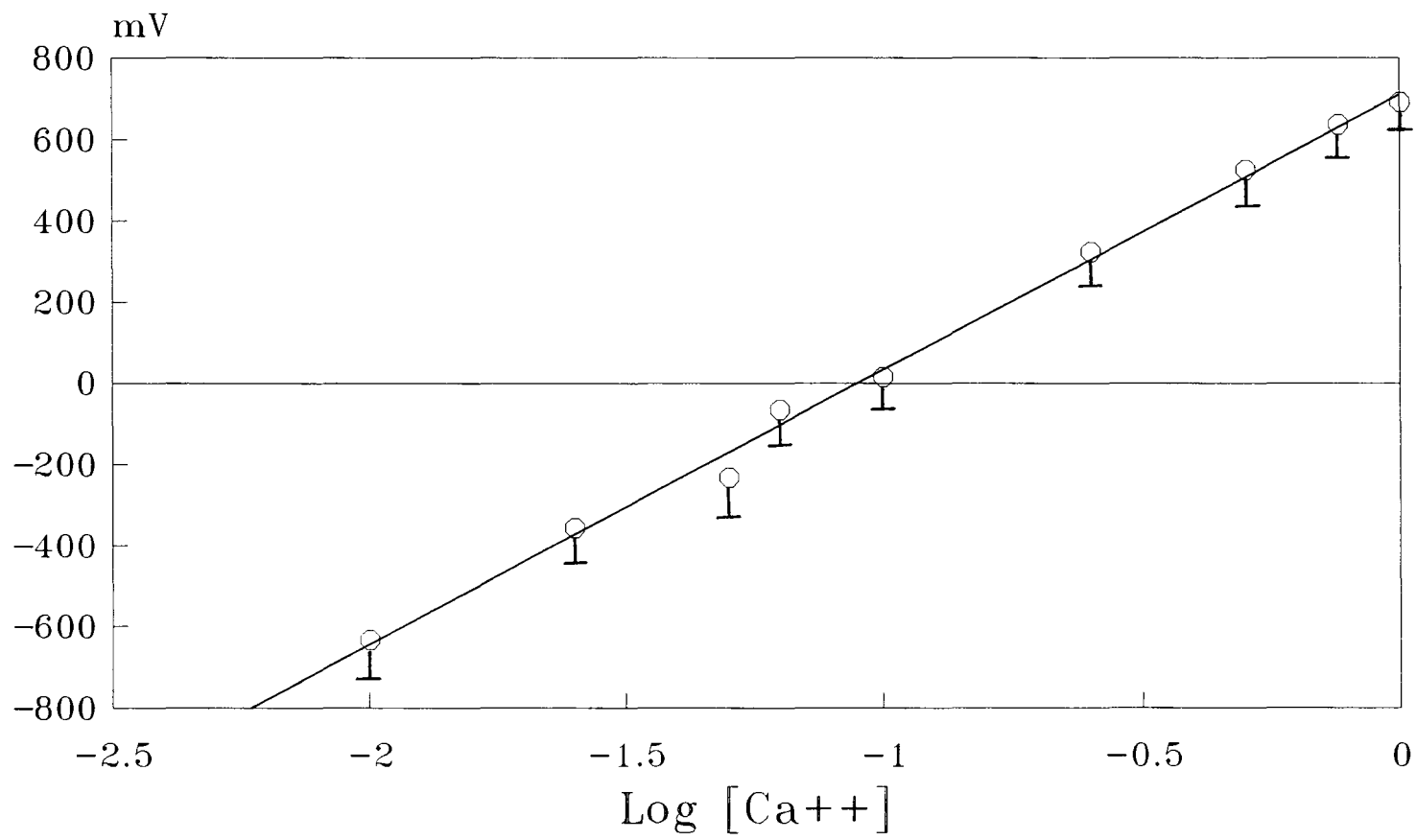
Demineralization of bone matrix was performed using five different concentrations of hydrochloric acid ( 0.1 N, 0.3 N, 0.5 N, 0.8 N, and 1.0 N ). The process of demineralization was monitored using the calcium-specific electrode and by measuring the pH of the eluent acidic solutions. Calcium concentrations in eluent fractions were calculated using the appropriate standard curve.

Fig. 11. Standard calibration curve for calcium ion in 0.1 N hydrochloric acid.  
Values represent the mean  $\pm$  range of two replicate assays.



$$Y = 607.5X + 654.9, [\text{Ca}^{++}] = M$$

Fig. 12. Standard calibration curve for calcium ion in 0.3 N hydrochloric acid.  
Values represent the mean  $\pm$  range of two replicate assays.

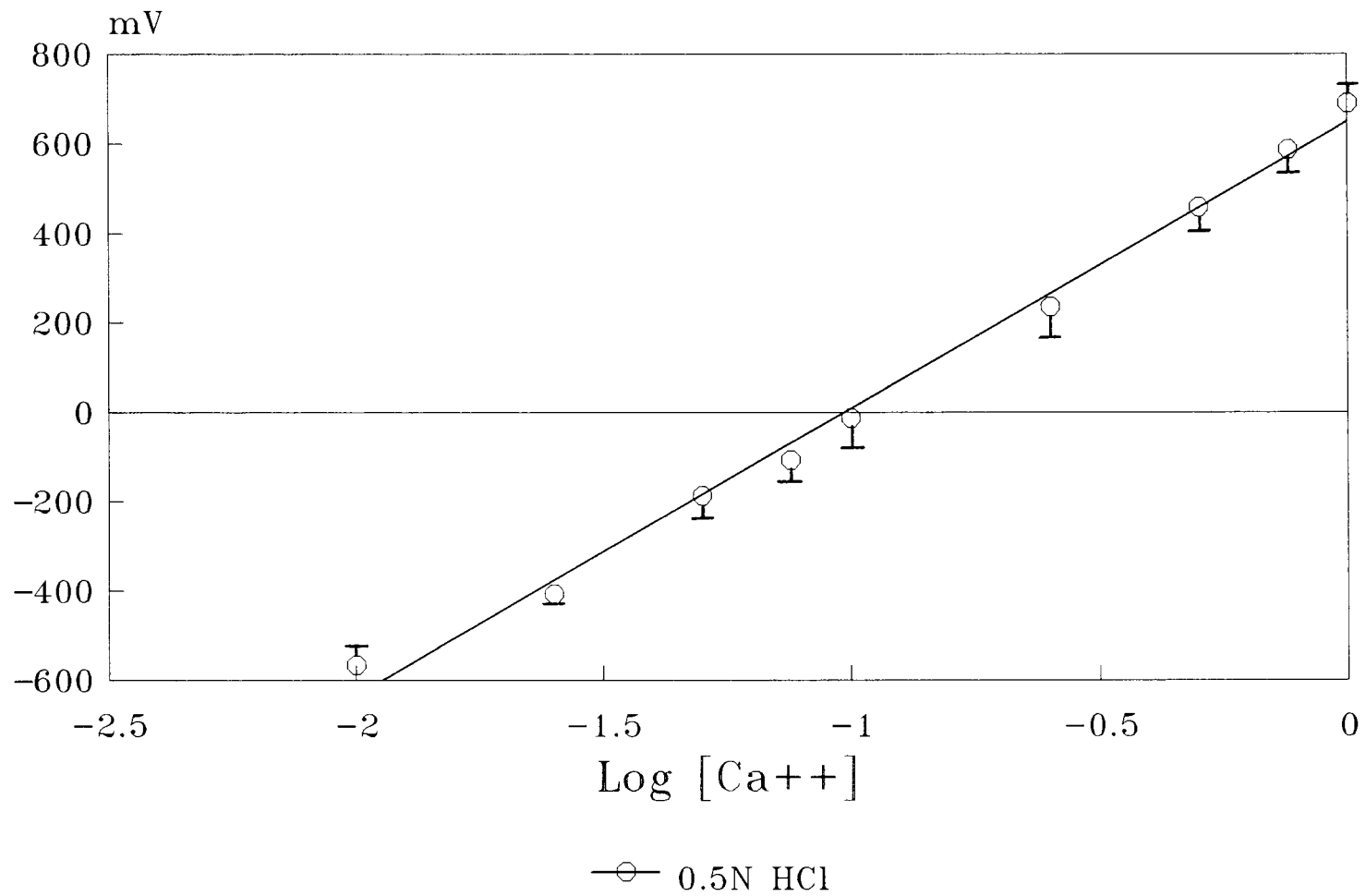


—○— 0.3 N

$Y = 68.0X + 708.1, [\text{Ca}^{++}] = \text{M}$

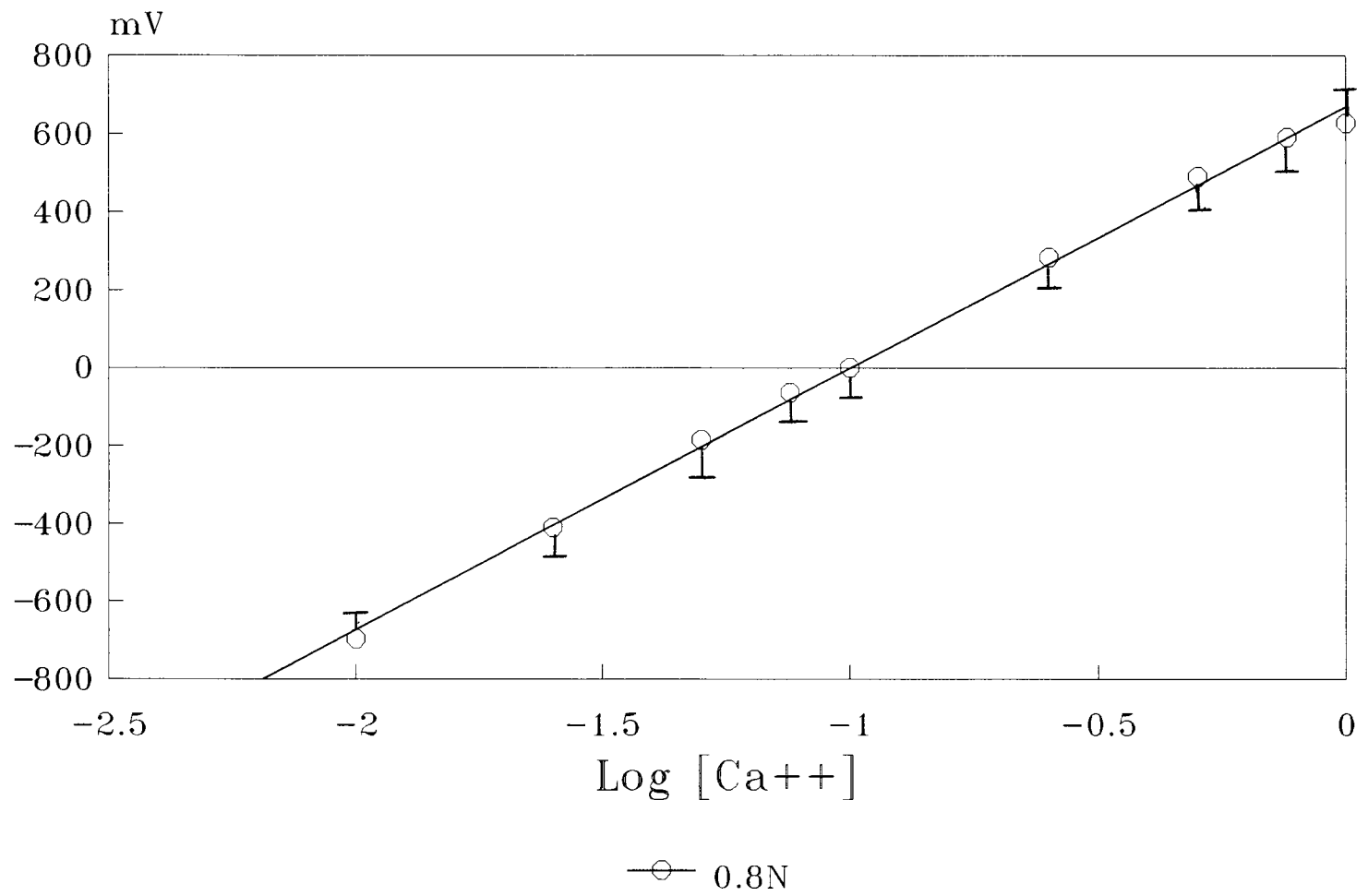


Fig. 13. Standard calibration curve for calcium ion in 0.5 N hydrochloric acid. Values represent the mean  $\pm$  SD of three replicate assays.



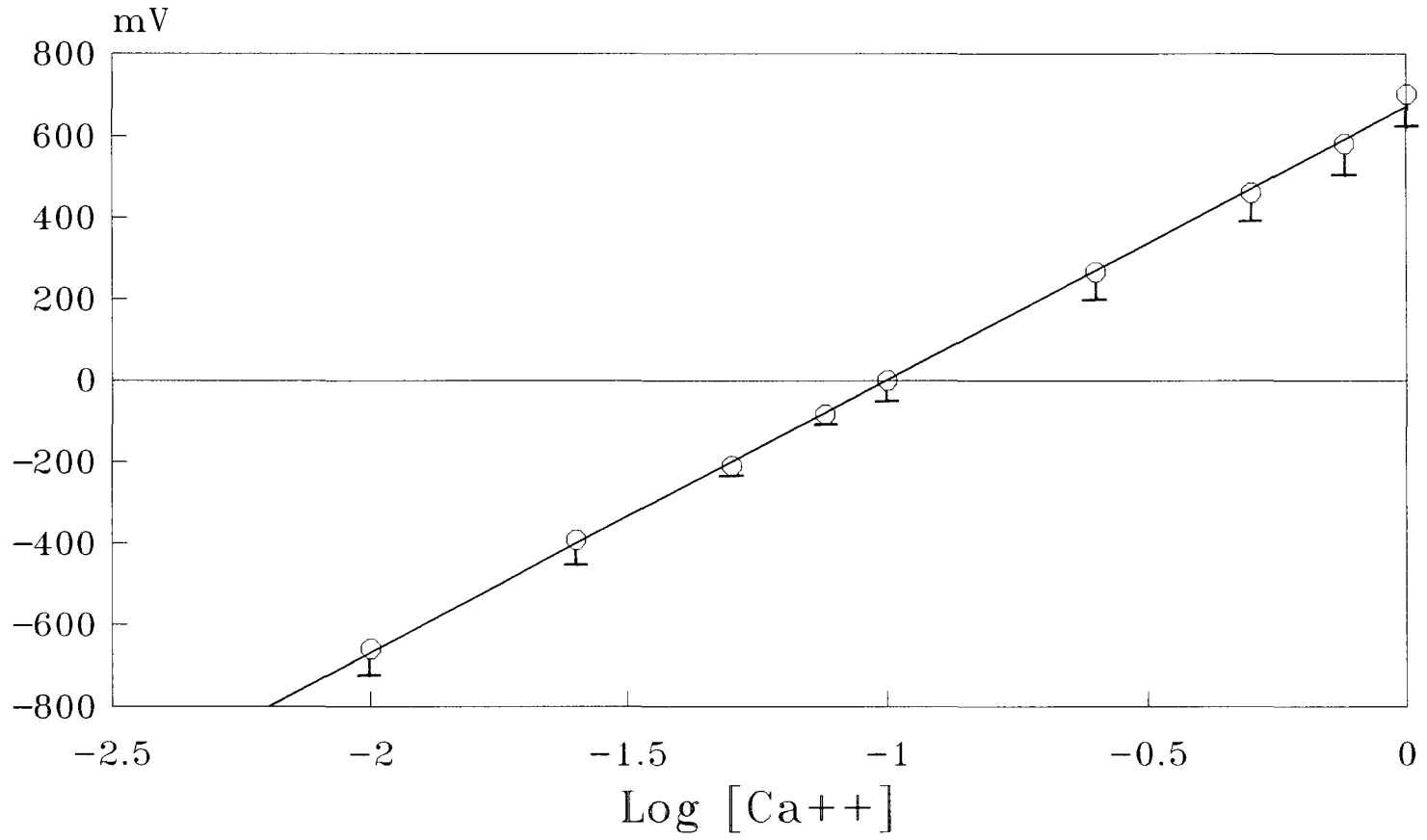
$$Y = 641.9X + 649.4, [\text{Ca}^{++}] = \text{M}$$

Fig. 14. Standard calibration curve for calcium ion in 0.8 N hydrochloric acid.  
Values represent the mean  $\pm$  SD of three replicate assays.



$$Y = 671.9X + 670, [\text{Ca}^{++}] = M$$

Fig. 15. Standard calibration curve for calcium ion in 1.0 N hydrochloric acid.  
Values represent the mean  $\pm$  range of two replicate assays.



—○— 1.0N

$Y = 605.2X + 577.5, [\text{Ca}^{++}] = \text{M}$

The data to be presented in this portion of the results section will focus on several objectives. These objectives were: 1.) to determine whether or not lipid extraction affected subsequent acid mediated demineralization, 2.) to determine which acid concentration might be most appropriate for demineralization ( monitoring total calcium ion extracted and the volume of acid used in the extraction ), and 3.) to determine if monitoring of pH of eluent fractions correlated with calcium ion, i.e., mineral, content of each fraction ( which the ultimate objective of being able to use pH monitoring as an easier method to determine completion of the demineralization process ). Consequently, the data are presented in the form of figures to demonstrate graphically the calcium ion concentrations and pH of eluent fractions in each extraction process. Data from these figures are further manipulated mathematically to express the total mg's of calcium ( expressed as equivalents of  $\text{CaCl}_2$  ) extracted from 10 grams of bone matrix and mg's of calcium per unit volume of extracting solution. It was not always obvious when extraction of calcium ion was completed, i.e., where the calcium ion concentration in eluent fractions ceased to decrease, and thus completion of demineralization was routinely calculated as occurring: 1.) when the calcium ion concentration in eluent fractions dropped below the minimum detection limits of the assay, or 2.) when two or more successive data plots ( at some minimum values ) were not different from each other. It should thus be emphasized that the calculated volume of acid used in completion of demineralization is based on a more subjective estimate than total calcium extracted.

### a. Demineralization Without Lipid Extraction

Figures 16 through 20 demonstrate demineralization of ground human bone which has not been subjected to lipid extraction. Demineralization using 0.1 N HCl was unsatisfactory in that although calcium ions were present in eluent fractions, the extent of measurable concentrations of calcium extraction was so minimal that effective demineralization, i.e., complete removal of calcium from the bone, did not occur in what would be considered as an "economically usable" time interval.

As may be seen in figure 16, the concentration of calcium ion in eluent fractions of 0.1 N HCl rapidly increase to a value approximating 0.06 to 0.07 M and remain at this "value" through the time ( and acid volume ) allowed. There was a transient increase in pH of the early eluent fractions, but pH of subsequent fractions declined to approximately pH 4.2 and remained at or near this value.

Demineralization of non lipid extracted bone matrix using 0.3 N HCl was clearly more effective than with 0.1 N HCl ( Figure 17 ). Here, the calcium ion concentration rapidly increased to a value approximating 0.20 M  $\text{CaCl}_2$  within the first four fractions, and remained at or near this value until fraction 20. By fraction 26 the calcium ion concentration in the eluent had rapidly declined to a value approximating 0.03 M  $\text{CaCl}_2$  ( nearing the minimum detection limits of the assay ). Using fraction number 25 as "completion of demineralization" it was possible to calculate the total grams ( 7.78 grams, Table 3 ) of calcium ( as calcium chloride ) extracted from the 10 grams of bone matrix. The total volume of 0.3 N HCl used in this extraction was calculated to be 325 ml ( Table 3 ). The pH of each eluent fraction was also monitored. As with 0.1 N HCl, a transient increase in pH of the



early fractions occurred until the concentration of calcium ion in the eluent fractions occurred. At this eluent volume, the pH of each fraction declined to a value near pH 3.5 and remained at this value until fraction 17 or 18. It appeared as though the pH profile of eluent fractions paralleled, but preceded, the calcium ion profile of eluent fractions. By fraction 25 the pH of eluent had declined to a value near pH 1.0, remaining at or near this value to termination of the experiment.

Demineralization of non lipid extracted bone matrix with 0.5 N HCl is shown in figure 18. The calcium ion concentration rapidly increased to a value approximating 0.30 M to 0.33 M  $\text{CaCl}_2$  and remained near this value until fraction 10. By fraction 21 the calcium ion concentration in the eluent had rapidly declined to a value approximating 0.03 M  $\text{CaCl}_2$ , which was near the minimum detection limits of the assay. There was a transient increase in pH of the early eluent fractions, but pH of subsequent fractions varied from approximately pH 3.2 to pH 1.4 until fraction 13. By fraction 14 the pH of eluent had declined to a value near pH 1.0, remaining at or near this value to termination of the experiment. Using fraction number 15 as "completion of demineralization" a calculated total of 6.29 grams of calcium ( as calcium chloride, Table 3 ) was extracted from the 10 grams of bone matrix. The total volume of 0.5 N HCl used in this extraction was calculated to be 166.5 ml ( Table 3 ). Demineralization of non lipid extracted bone matrix with 0.5 N HCl extracted less calcium ion than with 0.3 N HCl, but less volume of acid was used for demineralization ( demineralization with 0.3 N HCl took more time than with 0.5 N HCl ).

Figure 19 shows demineralization of non lipid extracted bone matrix with 0.8 N HCl. The calcium ion concentration of the early eluent fraction rapidly increased to a value approximating 0.59 M as  $\text{CaCl}_2$  and concentrations of subsequent fractions rapidly declined from 0.57 M to 0.02 M by fraction 18. From fraction 18 to fraction 25 the calcium ion concentration of eluent fractions remained at or near 0.02 M to termination of the experiment. The pH value of each eluent fraction rapidly increased to a value approximating pH 3.0 to pH 3.18 within the first 4 fractions. Subsequent pH values of eluent fractions rapidly declined to a value approximating pH 1.2 by fraction number 10, and remaining at or near this value for the remaining fractions. A total of 6.15 grams of calcium ion ( as calcium chloride ) was extracted from 10 grams of bone matrix using fraction number 11 as "completion of demineralization" ( Table 3 ). The total volume of 0.8 N HCl used in this extraction was calculated to be 137.5 ml ( Table 3 ). As with other demineralizations, as may be seen in figure 19, the pH profile of eluent fractions paralleled the concentration of calcium ion in eluent fractions of 0.8 N HCl.

Demineralization of non lipid extracted bone matrix with 1.0 N HCl was clearly faster than with lesser concentrations of HCl ( Figure 20 ). The calcium ion concentrations in eluent fractions rapidly increased to a value approximating 1.4 M to 1.5 M  $\text{CaCl}_2$  within the first 4 fractions. The calcium ion concentration rapidly decreased after fraction number 4. After fraction 11, the calcium ion profile of eluent fractions remained at or near 0.05 M. There was a transient increase in pH of the early eluent fractions, but pH of subsequent fractions rapidly declined to a value approximating pH 0.8 and remained at or near this value to termination of the

Fig. 16. Demineralization of non lipid extracted bone matrix by 0.1 N hydrochloric acid. The volume of each fraction was 15 ml and the flow rate of HCl was 1.0 ml / minute. The values represent mean  $\pm$  range, n = 2.

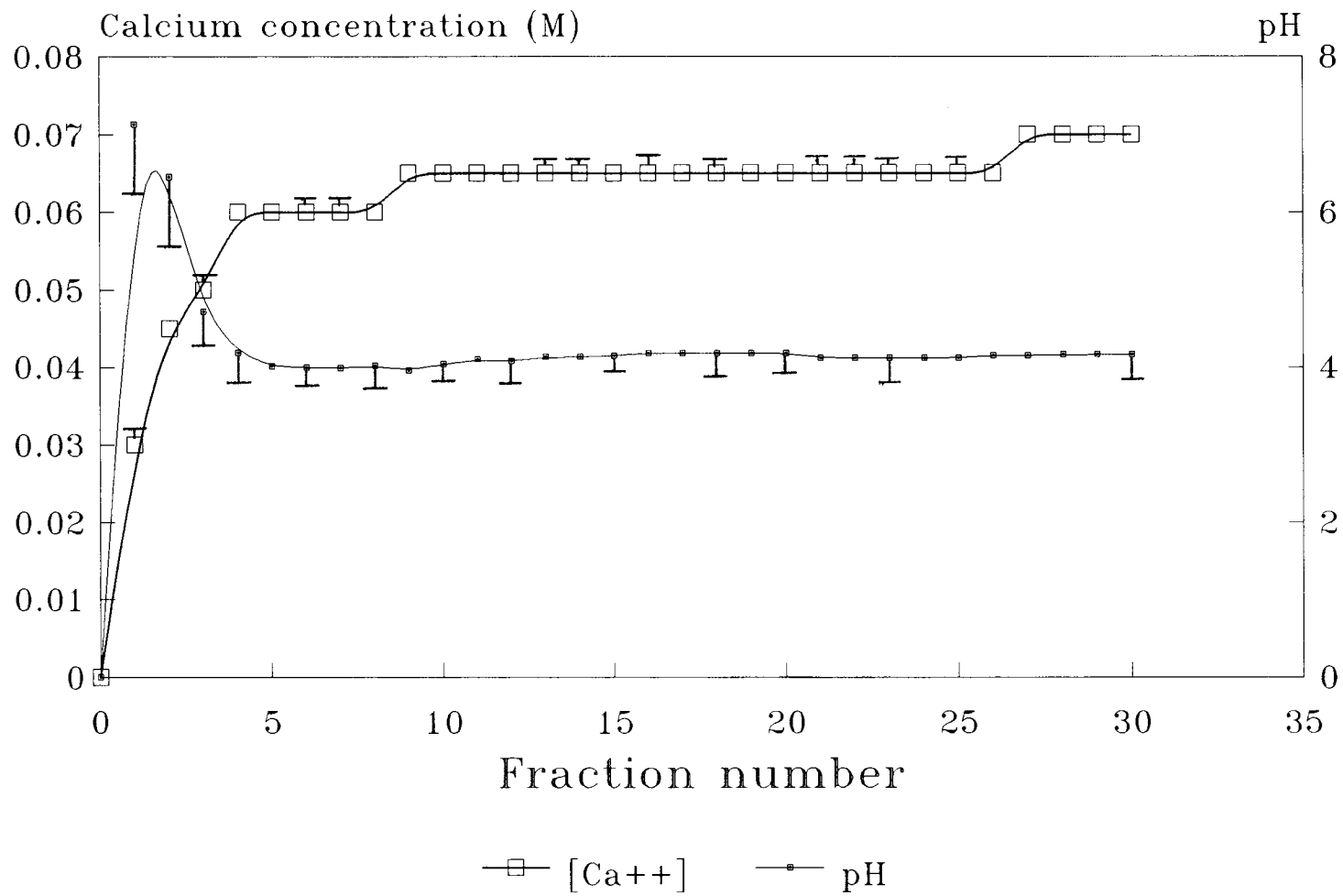


Fig. 17. Demineralization of non lipid extracted bone matrix by 0.3 N hydrochloric acid. The volume of each fraction was 13.0 ml and the flow rate followed as described in figure 16. The values are mean  $\pm$  range, n = 2.

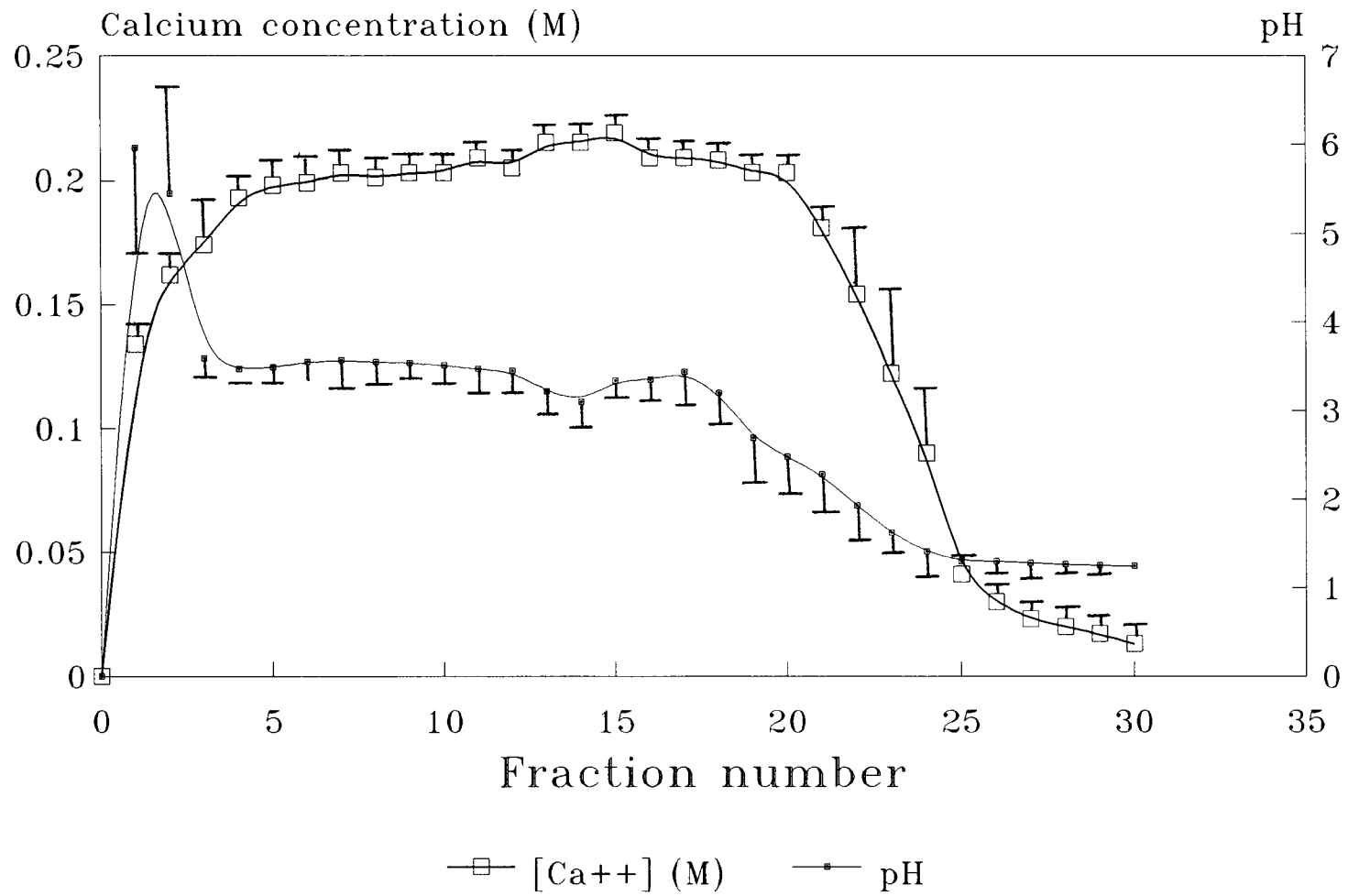


Fig. 18. Demineralization of non lipid extracted bone matrix by 0.5 N hydrochloric acid. The volume of each fraction was 11.1 ml and the flow rate was as described in figure 16. Values represent mean  $\pm$ SD, n = 3.

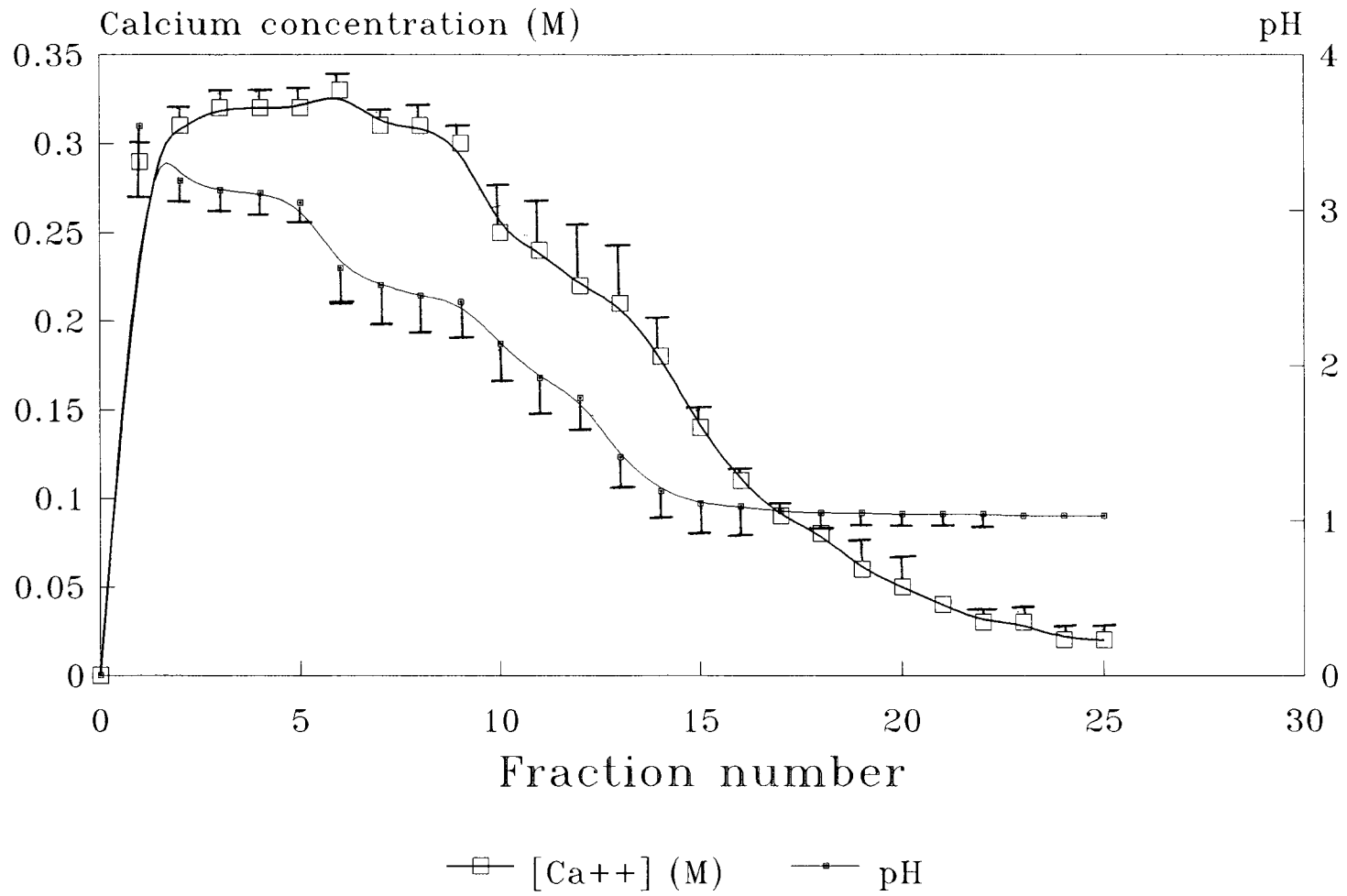




Fig. 19. Demineralization of non lipid extracted bone matrix by 0.8 N hydrochloric acid. The volume of each fraction was 12.5 ml and the flow rate was as described in figure 16. Values are mean  $\pm$  SD, n = 3.

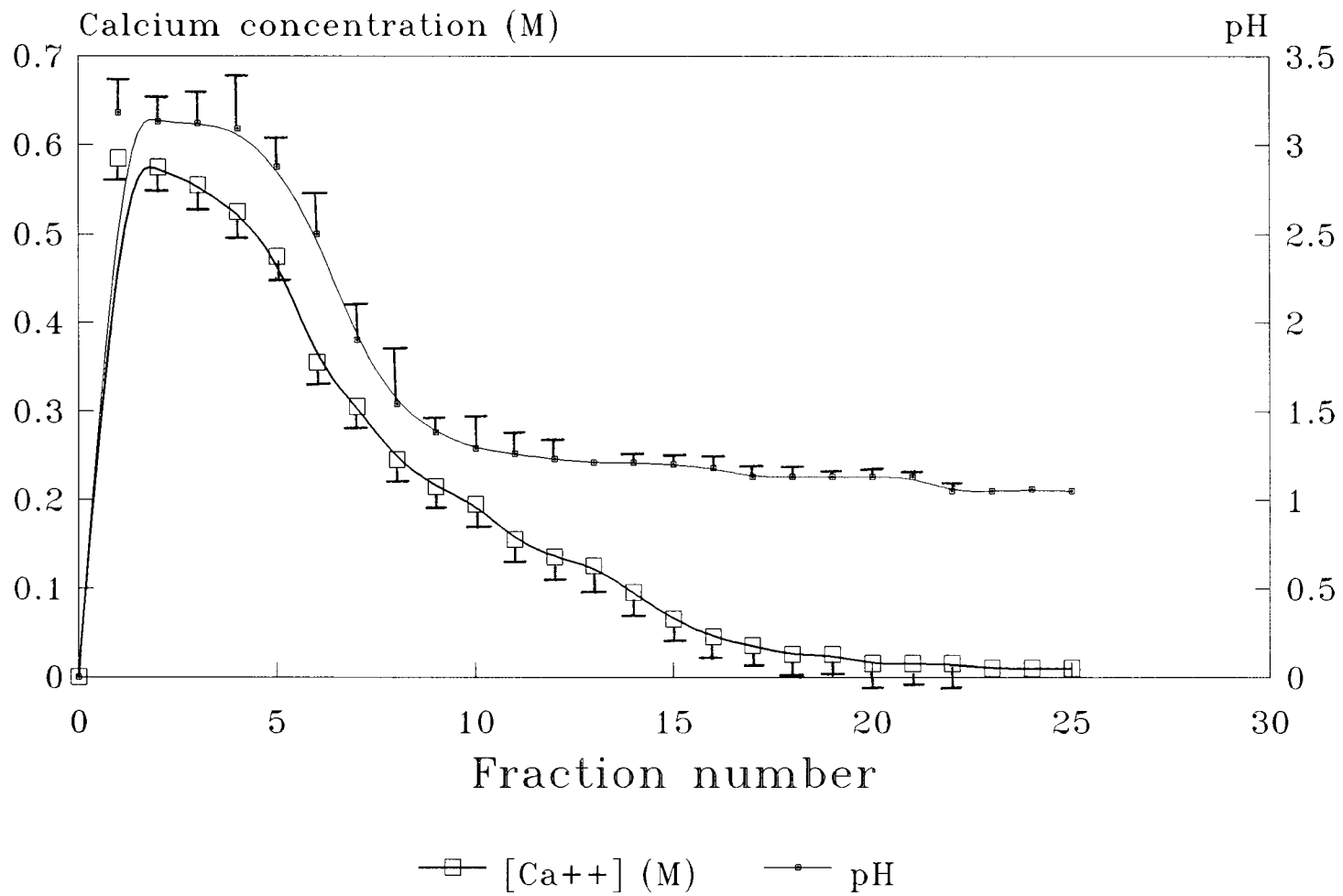
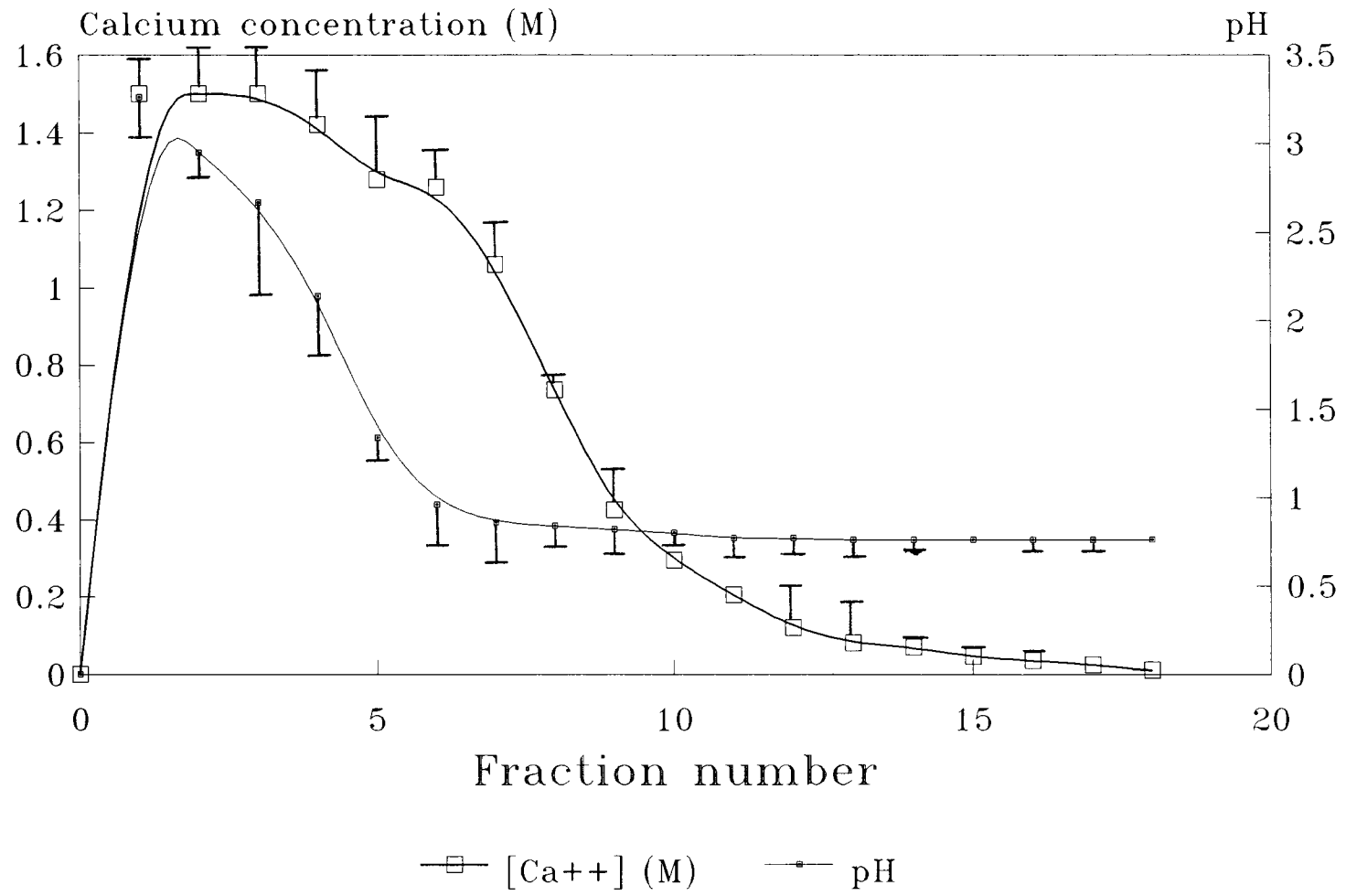


Fig. 20. Demineralization of non lipid extracted bone matrix by 1.0 N hydrochloric acid. The volume of each fraction was 11.0 ml. Values represent mean  $\pm$  SD of three replicate assays.



experiment. A total of 18.09 grams of calcium ion as  $\text{CaCl}_2$ , was extracted from 10 grams of bone matrix using fraction number 11 as "completion of demineralization". In this extraction the total volume of 1.0 N HCl used approximated 121.0 ml ( Table 3 ).

#### **b. Demineralization With Lipid Extraction**

Figures 21 through 25 show demineralization of ground human bone which has been subjected to lipid extraction using absolute ethanol. Lipid extraction from ground bone was based on the results from preceding experiment ( II. Lipid removal from human bone, a, part ). Like demineralization of non lipid extracted bone matrix using 0.1 N HCl, demineralization of lipid extracted bone was not satisfactory. The extent of measurable concentrations of calcium extraction was so minimal that effective demineralization, i.e., complete removal of calcium from the bone did not occur in what would be considered as an "economically usable" time interval.

As shown in figure 21, the concentration of calcium ion in eluent fractions of 0.1 N HCl rapidly increase to a value approximating 0.06 M to 0.07 M and remain at this value through the time ( and acid volume ) allowed for the experiment. There was a transient increase in pH of the early eluent fractions, but pH of subsequent fractions declined to approximately pH 4.3 and remained at or near this value.

Demineralization of lipid extracted bone matrix using 0.3 N HCl was clearly more effective than with 0.1 HCl ( Figure 22 ). The calcium ion concentration rapidly increased to a value approximating 0.20 M  $\text{CaCl}_2$ , within the first four

fractions, and remained at or near this value until fraction 19. By fraction 30 the calcium ion concentration in the eluent had rapidly declined to a value approximating 0.03 M  $\text{CaCl}_2$  ( nearing the minimum detection limits of the assay ). The pH of each eluent fraction was also monitored. As with 0.1 N HCl, a transient increase in pH of the early fractions occurred until the concentration of calcium ion in the eluent fractions stabilized. At these eluent volumes, the pH of each fraction declined to a value near pH 3.6 and remained at or near this value until fraction number 17. It appeared as though the pH profile of eluent fractions paralleled, but preceded, the calcium ion profile of eluent fractions. By fraction 25 the pH of eluent had declined to a value near pH 1.0, remaining at or near this value to termination of the experiment. Therefore using fraction number 25 as "completion of demineralization" it was possible to calculate the total grams ( 8.79 grams, Table 3 ) of calcium ( as calcium chloride ) extracted from the 10 grams of bone matrix. The total volume of 0.3 N HCl used in this extraction was calculated to be 311 ml ( Table 3 ).

Figure 23 shows demineralization of lipid extracted bone matrix with 0.5 N HCl, the calcium ion concentration rapidly increased to a value approximating 0.32 M to 0.35 M calcium ( as calcium chloride ) and remained near this value until fraction 7. The calcium ion concentration in the eluent had rapidly declined to a value approximating 0.05 M  $\text{CaCl}_2$  by fraction number 14 ( which was near the minimum detection limits of the assay ). Using fraction number 14 as "completion of demineralization", a total of 6.78 grams ( Table 3 ) of calcium was extracted from the 10 grams of bone matrix. The total volume of 0.5 N HCl used in this extraction

was calculated to be 159.8 ml ( Table 3 ). The pH of the early eluent fractions increased to a value approximating pH 3.5 to pH 3.3 and remained at these values for the first 5 fractions and then rapidly declined to a value approximating pH 1.0 by fraction 13. The changing of the eluent pH values paralleled the calcium ion concentrations of the eluents. Like demineralization of non lipid extracted bone matrix with 0.5 N HCl, extraction of lipid extracted bone with 0.5 N HCl also resulted in less calcium extracted than with 0.3 N HCl.

Demineralization of lipid extracted bone matrix with 0.8 N HCl resulted in more calcium ion extraction than with 0.5 N HCl ( Figure 24, Table 3 ). As may be seen in figure 24 the concentration of calcium ion in eluent fractions of 0.8 N HCl rapidly increased to a value approximating 0.8 M to 0.7 M  $\text{CaCl}_2$  within the first 5 fractions, and rapidly declined to a value approximating 0.06 M calcium ion by fraction 14, remaining at or near this value in subsequent fractions. The pH value of each eluent fraction was also monitored. There was a transient increase in pH of the early eluent fractions within the first 3 fractions, which then rapidly decreased until fraction 12. By fraction 12 the pH value in the eluent had rapidly declined to approximately pH 1.1, remaining at or near this value. Using fraction number 11 as "completion of demineralization", 11.05 grams of calcium ion were extracted from the 10 grams of bone matrix. The total volume of 0.8 N HCl used in this demineralization was calculated to be 110.0 ml ( Table 3 ). The results showed that the pH values of eluent fraction paralleled, but preceded, the calcium ion values of eluent fractions.

Figure 25 shows demineralization of lipid extracted bone matrix with 1.0 N HCl. Like preceding demineralizations, the pH profile of eluent fractions paralleled, but preceded, the calcium ion profile of eluent fractions. The concentrations of calcium ion in eluent fractions of 1.0 N HCl rapidly declined to a value approximating 0.16 M by fraction number 9. The values of calcium ion concentrations remained at or near this value after fraction 9. There was a transient increase in pH of the early eluent fractions, but pH of subsequent fractions rapidly declined to approximately pH 0.99 until fraction 5, remaining at or near this value to termination of the experiment. Using fraction number 8 as "completion of demineralization", it was possible to calculate the total grams ( 15.83 grams, Table 3 ) of calcium ion extracted from the 10 grams of bone matrix.



Table 3. Total Calcium Ion Extracted from 10 gms of Ground Bone Matrix in Various Concentrations of HCl.

HCl (N)	aI (gms)	aII (gms)	a <sup>@</sup> I (gms/ml)	a <sup>@</sup> II (gms/ml)	bI (ml of HCl)	bII (ml of HCl)
0.1	3.34±0.30	3.52±0.20	0.009	0.009	375.2±3.41	375.0±2.97
0.3	7.78±0.20	8.79±0.10	0.024	0.028	325.2±5.46	311.0±3.24
0.5	*6.29±0.90	*6.78±0.60	0.038	0.042	166.5±4.67	159.8±2.78
0.8	*6.15±0.50	*11.05±0.20	0.045	0.100	137.5±3.40	110.0±3.20
1.0	*18.09±1.10	*15.83±0.30	0.150	0.171	121.0±2.40	92.8±2.95

Values are means ± range, N = 2 or 3, \* marked ones were replicated 3 times.

aI : Total extracted calcium from 10 gms of non lipid extracted bone in various concentrations of HCl.

aII: Total extracted calcium from 10 gms of lipid extracted bone in various concentrations of HCl.

a<sup>@</sup>I: Indicates grams of calcium extracted per unit volume of extraction solution in non lipid extracted bone, i.e., for 0.3 N HCl, a I ÷ b I,  $7.78 \div 325.2 = 0.024$ .

a<sup>@</sup>II: Indicates grams of calcium extracted per unit volume of extraction solution in lipid extracted bone, i.e., for 0.3 N HCl, a II ÷ b II,  $8.79 \div 311 = 0.028$ .

bI : Indicates total acid volume used for demineralization of 10 gms of non lipid extracted bone.

bII: Indicates total acid volume used for demineralization of 10 gms of lipid extracted bone.

Fig. 21. Demineralization of lipid extracted bone using 0.1 N hydrochloric acid. The volume of eluent collected in each fraction was 15.0 ml and the solvent flow rate was 1.0 ml / minute. Values are mean  $\pm$  range, n = 2.

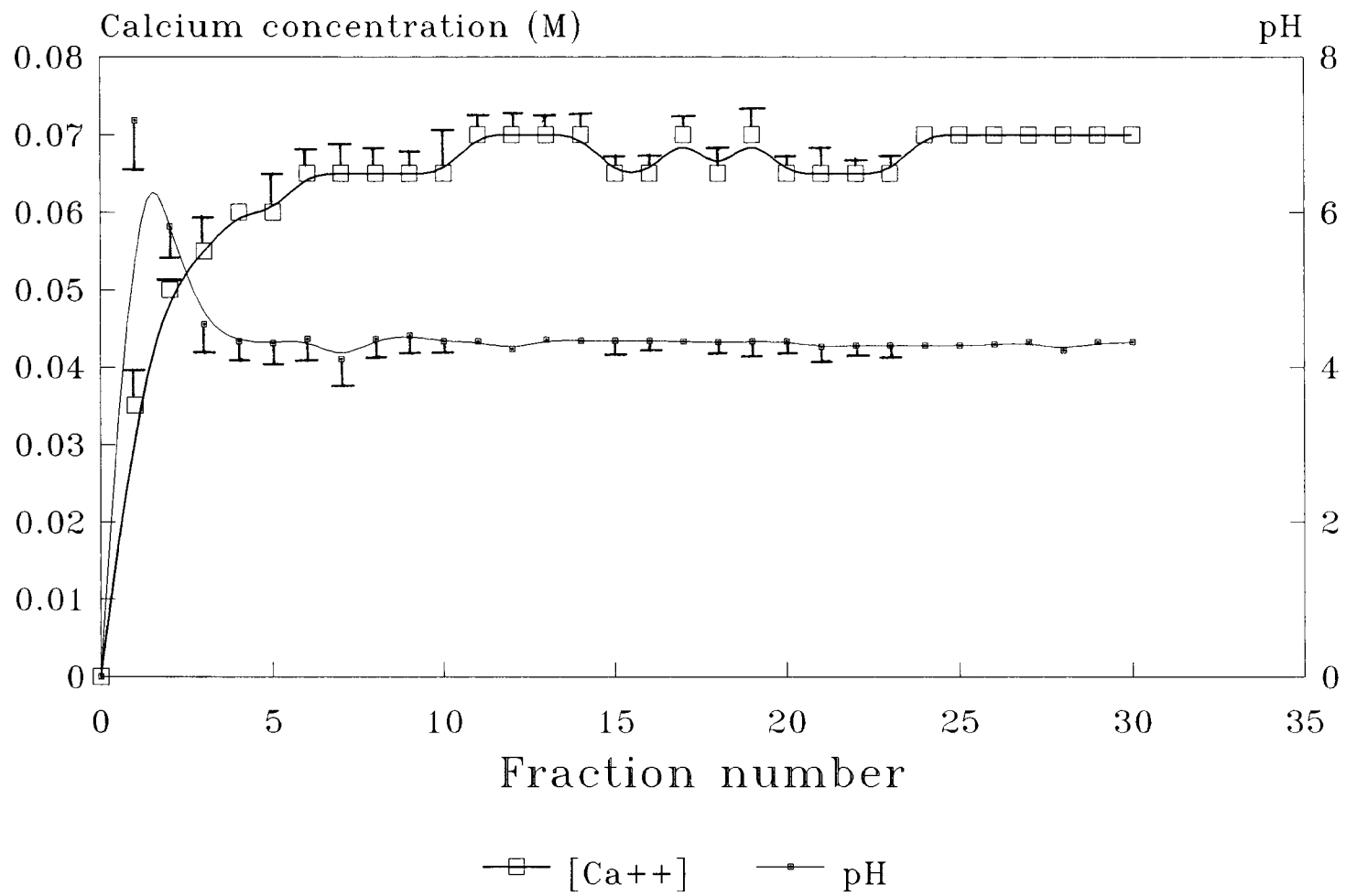


Fig. 22. Demineralization of lipid extracted bone using 0.3 N hydrochloric acid. The volume of eluent collected in each fraction was 12.44 ml and the solvent flow rate was as described in figure 21. Values are mean  $\pm$  range, n = 2.

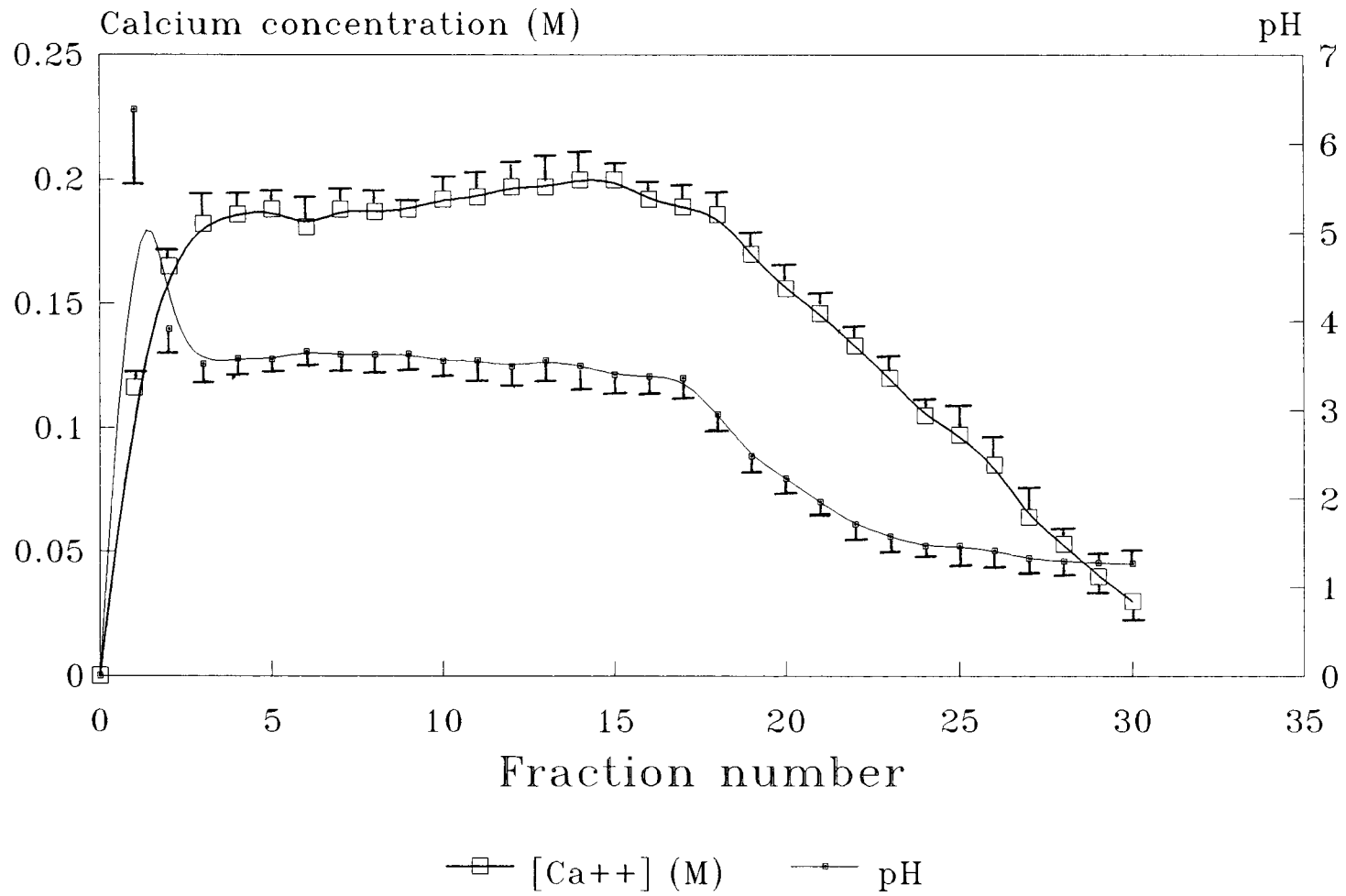


Fig. 23. Demineralization of lipid extracted bone using 0.5 N hydrochloric acid. The volume of eluent collected in each fraction was 11.4 ml and the solvent flow rate was as described in figure 21. Values are mean  $\pm$  SD, n = 3. Bars represent standard deviations.

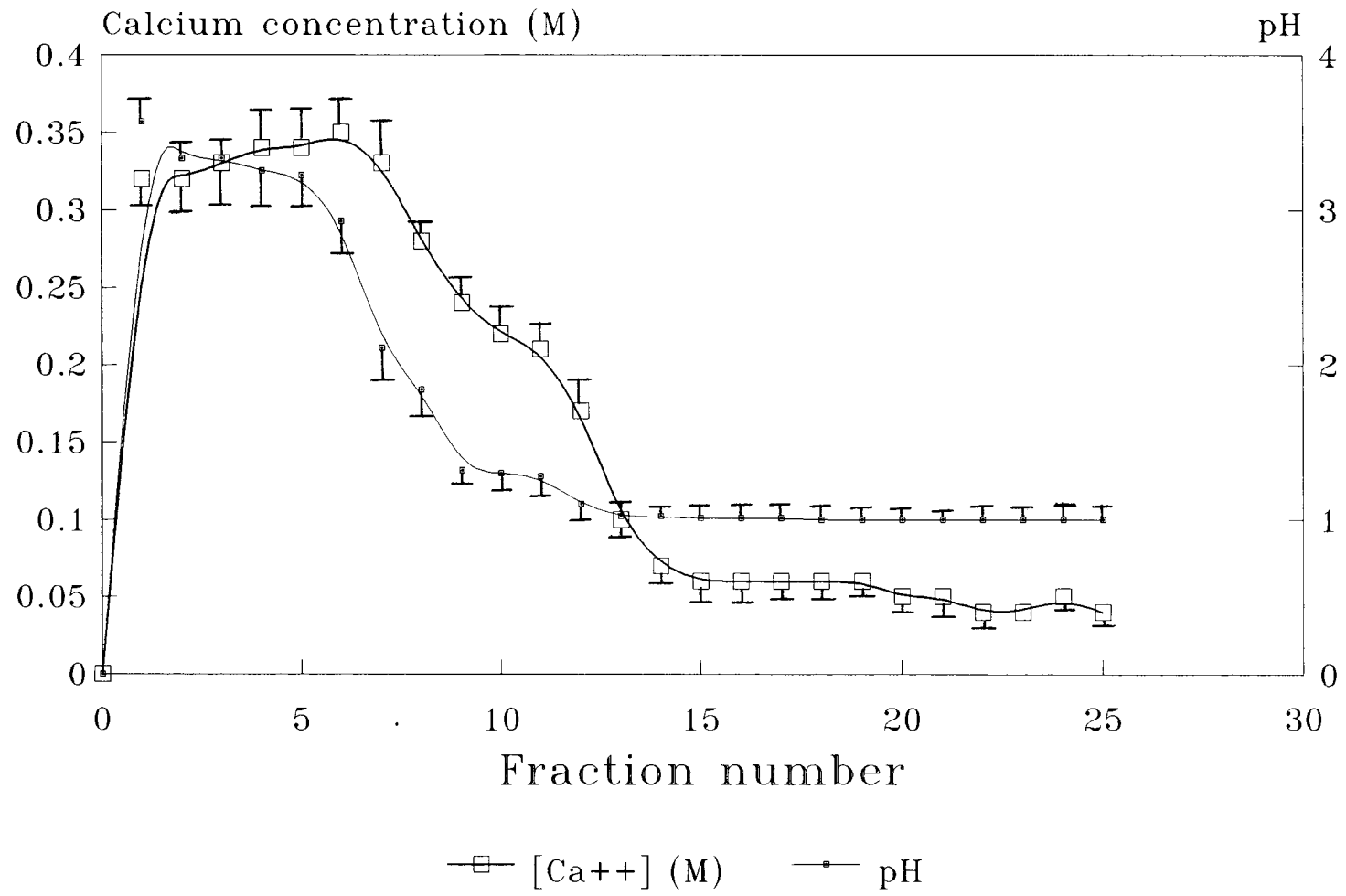


Fig. 24. Demineralization of lipid extracted bone using 0.8 N hydrochloric acid. The volume of eluent collected in each fraction was 10.0 ml and the flow rate was as described in figure 21. Values are mean  $\pm$  SD, n = 3.



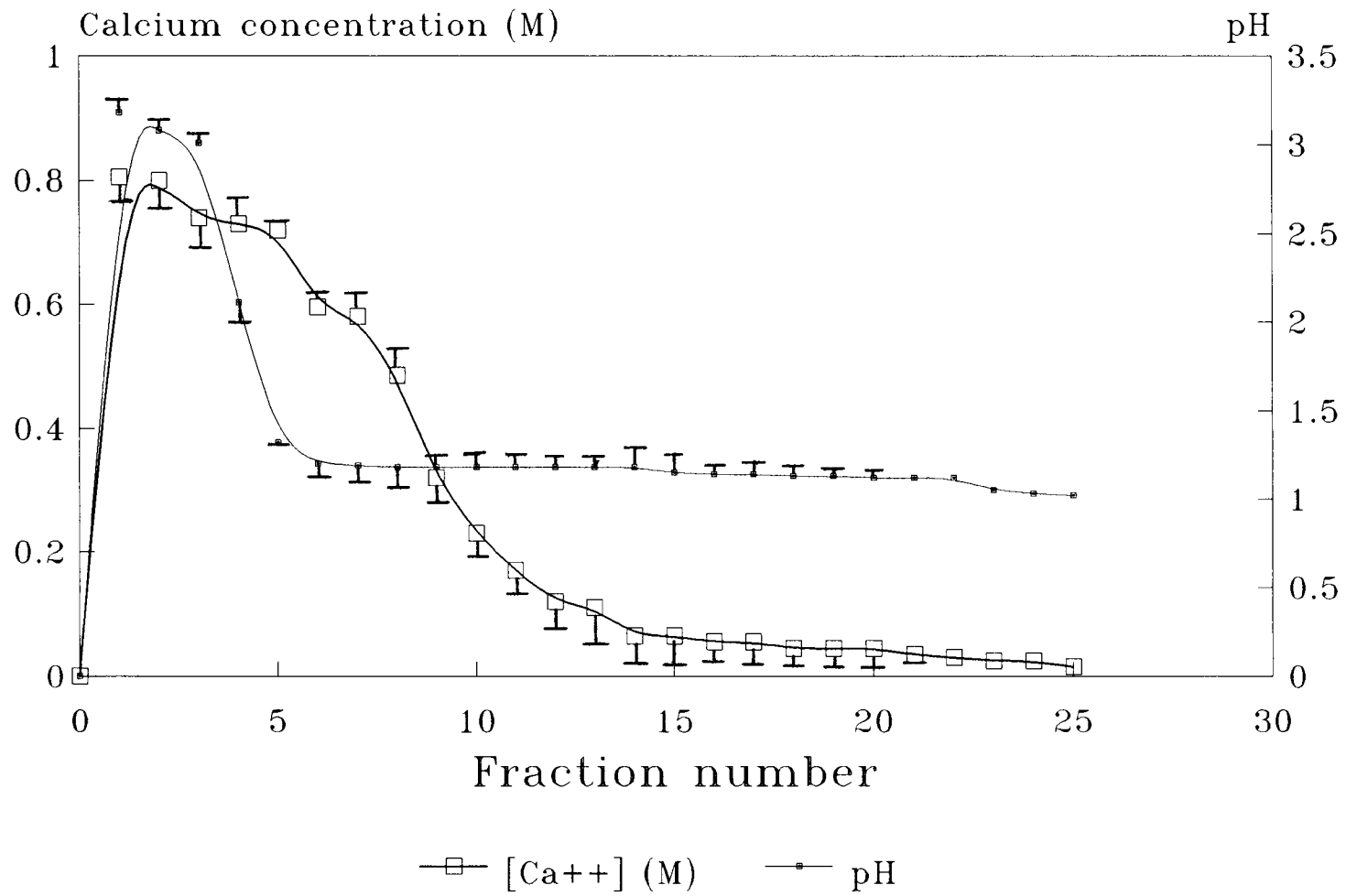


Fig. 25. Demineralization of lipid extracted bone using 1.0 N hydrochloric acid. The volume of eluent collected in each fraction was 11.6 ml and the flow rate was as described in figure 21. Values are mean  $\pm$  SD, n = 3.

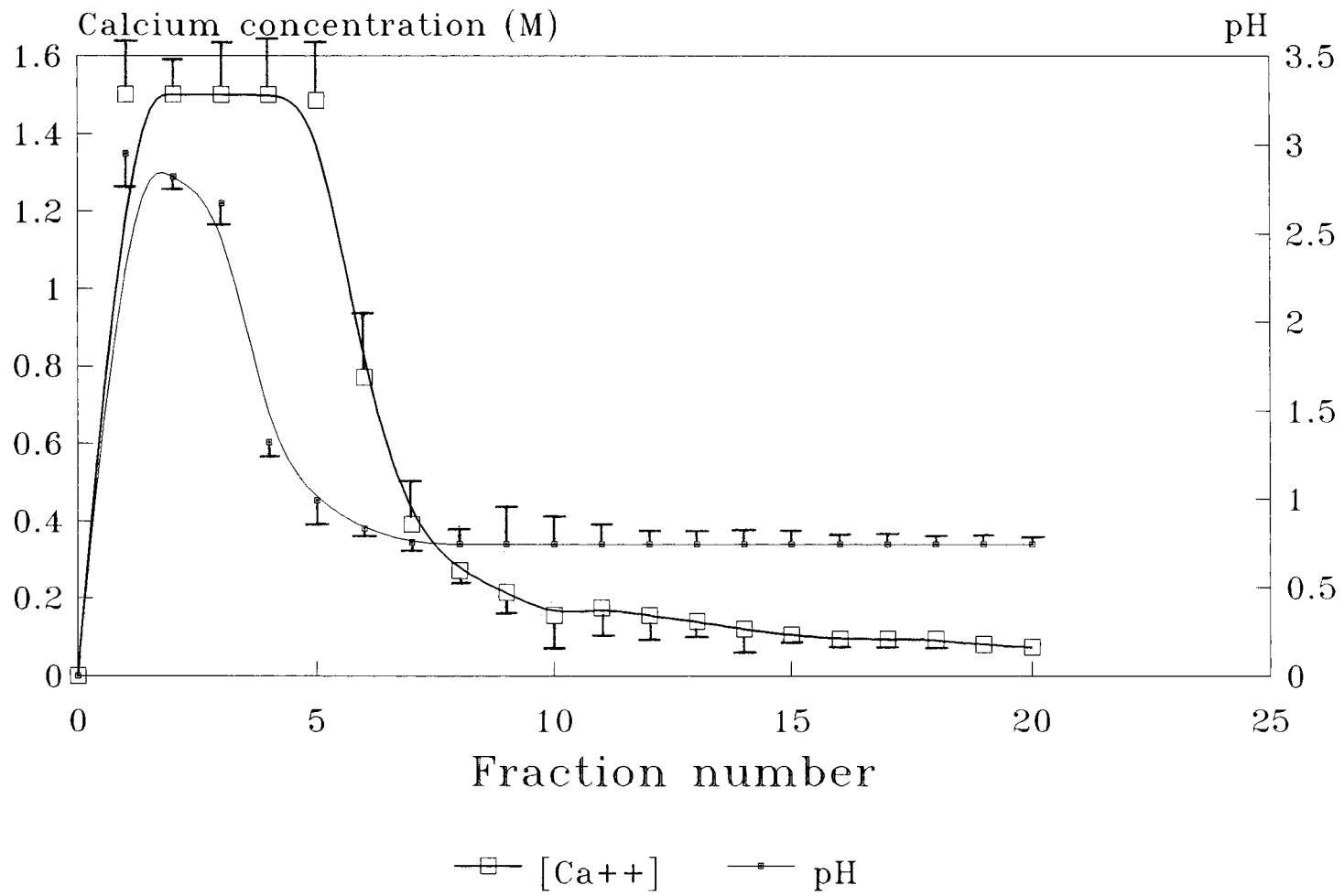
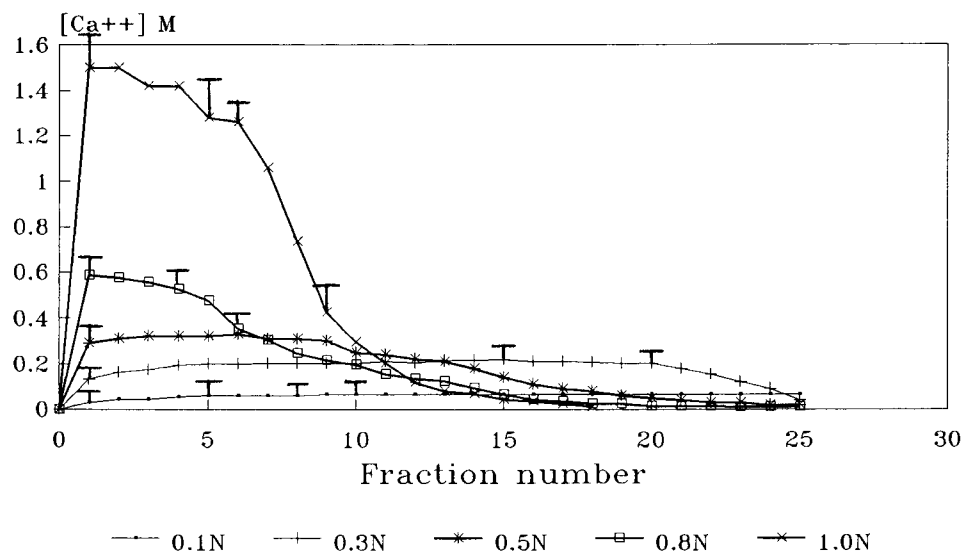


Fig. 26. Calcium concentrations in eluent fractions at various concentrations of acid used for demineralization.

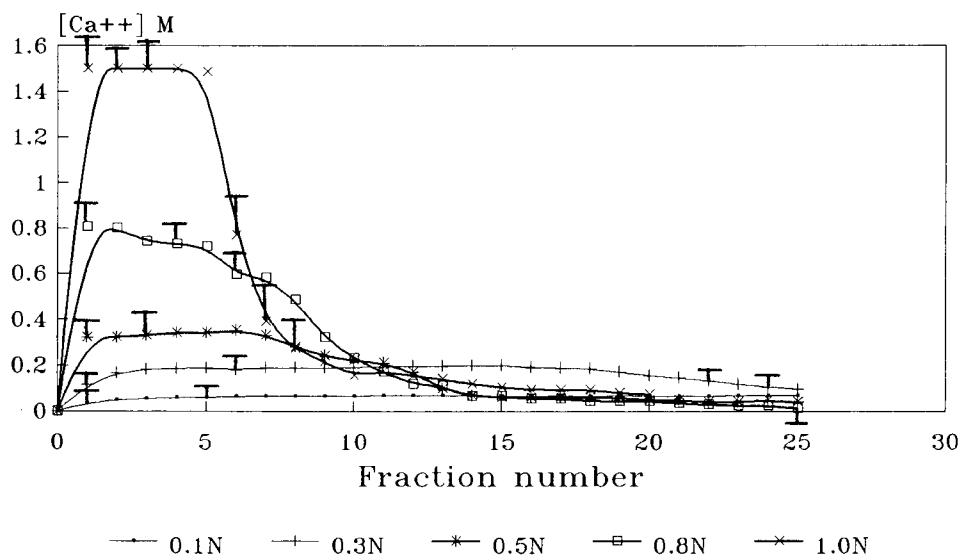
a.) without lipid extraction

b.) with lipid extraction

a



b



## V. DISCUSSION

Bone tissue is the most widely utilized transplantable tissue. Approximately 250 tissue banks in the United States procure human bone and process it into some form of clinically usable material. These materials, or products, include femur heads for use in "hip replacement" therapy, mandibles, ribs, as well as parts of specific bones such as iliac crest wedges ( used in spinal fusion operations ), cloward dowels, and ground demineralized bone matrix. The ground demineralized bone matrix particles are used to repair gross bone defects such as may occur with osteosarcomas or damage occurring due to accidental mutilation of bone. Smaller particle bone matrix, generally referred to as "dental dust" is used by periodontal surgeons for alveolar ridge augmentation and to restore damage to alveolar ridge occurring during periodontal surgery.

Ground demineralized bone matrix has been used extensively and considerable work has been performed to substantiate its role in eliciting the formation of new bone. Less work has been performed to assess the effects of the various processing effects utilized in production of ground demineralized bone matrix on subsequent osteoinductive activity of the product. Minimal work has also been performed to assess ( validate ) the efficacy of the methods utilized in production of ground demineralized bone. The majority of tissue processing groups, tissue banks, utilize what is commonly referred to as "bulk processing". The proximal and distal ends of long bones, i.e., femur, tibia, are removed and the shaft portions of the bones mechanically debrided to remove periosteum, bone marrow, and other

loosely associated cellular materials. The shaft portions are then cut into chunks, and the chunks further ground into smaller and smaller particles, i.e., 250 to 700  $\mu\text{m}$  size range for "dental dust". These ground particles are sized using sieves, and particles of defined size are added to extraction flasks. Absolute ethanol is added and this bone suspension is extracted at 4°C, with shaking, for timed intervals. The bone particles are eventually separated from the ethanol and added to 0.5 N hydrochloric acid where additional extraction occurs at 4°C, with shaking. Eventually the bone matrix is washed with water and buffered solutions until the bone matrix is restored to physiological pH after which the bone matrix is freeze dried, packaged, and distributed as ground demineralized bone. Although it is unknown whether or not "complete" demineralization is necessary for the bone matrix to possess osteoinductive activity, most demineralized bone matrix available from tissue banking organizations typically contains less than 5 %, by weight, residual calcium. The ethanolic extraction is used to extract lipids. This extraction procedure is normally conceded to improve subsequent demineralization with acid, but is perhaps most commonly utilized to inactivate potential viral contaminants and / or to improve the matrix properties of the bone product. Various groups have suggested that bone particles demineralized without lipid extraction fail to hydrate as well as demineralized bone particles also subjected to lipid extraction, and thus do not "work as well" ( Wolfinbarger, personal communication ). Ethanolic extraction is also preferred for use in bone processing because of what is perceived as a reduced potential for toxicity of residual extraction solvent. It is well established that chloroform / methanol, and other organic solvents, is superior in extraction of lipids

from tissues, but its potential toxicity combined with its flammability and other undesirable properties restrict its use on tissues to be used clinically.

The research presented in the context of this study was sponsored by *LifeNet*, the Virginia Tissue Bank. Freshly ground bone matrix was provided as a frozen, sterile, matrix. This study was performed to investigate the effects of different solvent systems in removal of lipids from the bone fragments and the effect of this lipid removal on subsequent demineralization. The study was divided essentially into two phases. The first phase of the study was designed to assess the efficacy of different combinations of ethanol / water mixtures in extraction of lipids. Chloroform / methanol was used primarily for comparative purposes. The first phase included experiments designed to determine the total quantity of lipid extracted by different solvent systems. Differential extraction of various lipid types by the different solvent systems was not considered within the confines of this study since no data are available to suggest that different compositions of lipids within bone particles may affect subsequent demineralization and / or osteoinductive potential of the bone matrix. Additional experiments were included to determine the optimal volume of extraction solvent to be used in lipid extraction. The "column extraction" method employed was chosen because *LifeNet* is attempting to change from its current "bulk extraction" process to a "column-based extraction" process and was interested in methods to validate when lipid extraction was "completed".

The second phase of the study focused on demineralization of the bone matrix using hydrochloric acid. This phase was designed to examine the effects of lipid extraction on subsequent demineralization, the optimal concentration of hydrochloric



acid to use in the demineralization process, and was undertaken using column extraction procedures for the same reasons as for lipid extraction using the column mediated process. With acid demineralization of bone matrix using the column mediated process, it was important to develop an assay which could be used to effectively monitor the demineralization process. Although the calcium specific probe was chosen for this study, the pH of eluent was also monitored with the expectation that pH changes in column eluent could be used to assess the extent ( completion ) of demineralization.

As was shown using scanning electron microscopic examination of the ground bone matrix, the bone particles utilized in this study consisted of a rather uniform particle consisting of both cancellous and cortical bone matrix. This mixture of bone matrix is consistent with the grinding of the shank portions of leg bones. Although not well visualized by the SEM photographs, the demineralized bone matrix obtained using lipid extraction exhibits different handling characteristics when compared to similar bone matrix without lipid extraction. The bone matrix produced without lipid extraction appears "lighter and fluffier" than bone matrix produced with lipid extraction. No quantitative means were used to assess this difference and thus these described differences must be presented as a subjective appraisal of the two bone matrices. It is unknown what effect this difference will have on subsequent clinical usage and this aspect of ground demineralized bone will require additional, future, studies.

The lipid extraction portion of the study was quite informative. From the data obtained, it may be suggested that absolute ethanol represents the best solvent

system, of those tried, for lipid extraction of human bone matrix. The quantities of lipid extracted by 70 % and 95 % ethanol were not significantly different from each other and the quantities of lipid extracted by absolute ethanol and chloroform / methanol were not significantly different from each other. However, absolute ethanol extracted significantly (  $p < 0.05$  ) more lipid than extracted by water mixtures of ethanol. Utilizing the column extraction process, it was determined that approximately 9 ml of absolute ethanol are required for each gram ( wet weight ) of bone placed in the column. Whether or not this value may differ with different column geometries, i.e., length versus diameter of the "column bed", is unknown at present. The volume of absolute ethanol required for "lipid" extraction was significantly greater than the volume of chloroform / methanol, i.e., 90 ml versus 38 ml, respectively. It should also be noted that the rate of flow of the extraction solvents through the bone matrix may affect lipid extraction and value noted above applies to a solvent flow rate of 1 ml / minute. The use of absorbance of eluent solutions to monitor lipid extraction was based on the premise that cholesterol could be used to monitor total solvent extractable lipid. Cholesterol is readily soluble in both solvent systems used in the column mediated process and absorbance was monitored at the wavelength demonstrated to be "optimal" for cholesterol in the different solvent systems. Considering that a UV / visible spectrophotometer may not be available to tissue bank staff, an alternative monitoring procedure for lipid extraction was tested. The alternative assay consisted on allowing drops of the eluent to drop into glass distilled water. When lipids are present in the eluent, as verified by absorbance at appropriate wavelengths, a cloudy residue appears when

the eluent fails into the water. This event presumably results from the limited solubility of lipids in water and as the ethanol, or chloroform / methanol eluents, used in bone extraction, "disperse" in the water, the lipids form micellar structures which appear as a cloudy material. This alternative monitoring procedure offers minimal opportunity to quantitate the process and is not useful when lipid content of the extraction eluent is low, but it is suggested that it may be used for the "routine" monitoring of lipid extraction from bone matrix.

The second phase, acid demineralization aspect, of the study provided the most usable data. As mentioned earlier, the majority of tissue banking operations employ 0.5 N HCl in the demineralization of bone matrix. They also utilize bone matrix which has had lipids extracted using absolute ethanol. The data suggest that the greater the acid concentration, the greater the quantity of calcium extracted from the bone matrix. Hydrochloric acid at 0.1 N is not effective in extraction of appreciable quantities of calcium from the bone matrix over the time and acid volume utilized in this study. It is thus suggested that this concentration of acid is perhaps not an "economically" usable solution for use by tissue banking organizations. An HCl concentration of 0.3 N appears to extract significantly more calcium than 0.5 N HCl, i.e.,  $7.78 \pm 0.20$  and  $6.29 \pm 0.90$ , respectively. At this concentration of HCl, it is also suggested that lipid extraction with absolute ethanol significantly improves calcium extraction, i.e., 0.3 N HCl extracted only  $7.78 \pm 0.20$  grams of calcium from non lipid extracted bone whereas it extracts  $8.79 \pm 0.10$  grams of calcium from lipid extracted bone. Although the volume of acid used in these extractions is more difficult to quantitate, it would appear that approximately

325 ml of 0.3 N HCl were necessary to extract calcium from non lipid extracted bone whereas only 311 ml of 0.3 N HCl were needed for lipid extracted bone.

Hydrochloric acid concentrations of 1.0 N were capable of extracting the greatest quantities of calcium from both lipid and non lipid extracted bone matrix. This acid concentration also required the least volume of acid to effect the extraction of calcium. In short, the data presented in this study suggest that, although individual values are not always statistically significantly different, the quantities of calcium extracted increase with increasing HCl concentration and the quantity of calcium extracted with each acid concentration was always consistently greater when lipids were extracted prior to demineralization. In addition, as the concentration of acid used in the extraction process increased, the volume of acid necessary to effect calcium extraction decreased. Again, although not always significantly different, less volumes of acid were consistently required for lipid extracted bone matrix suggesting a trend in that lipid extraction improves subsequent demineralization.

The use of pH of the eluent solution as a means of monitoring the demineralization process appears to be effective. It is suggested that as the demineralization process occurs, the extracted salts act to buffer the actual hydrogen ion concentration and pH of the eluent solution remains near pH 3.0 to 4.0. As the demineralization process proceeds, the pH of the eluent consistently declines before a decline in calcium ion concentration. This observation suggests that the salt content, and hence its buffering capacity, declines as the demineralization process nears completion and the pH begins to decline. Once the pH declines to less than 1.0, demineralization is essentially completed.

Since the study described here was designed to investigate the effects of lipid extracted and non lipid extracted ground bone on subsequent demineralization of ground bone, the potential requirements for remineralization can be answered only by future studies using *in vitro* and / or *in vivo* systems.

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