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# A pilot study of the histo-morphological and loss of crystallites from human mineralised tissues due to daily consumption of everyday local Malaysian condiments. A radiological-variable pressure scanning electron microscope investigation.


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**Abstract**
Local condiments consumption is a key constituent of diets throughout the east coast of Peninsular Malaysia. Little evidence-based knowledge's is known of the morphological changes that may impinge on oral health due to consumption of these local condiments. VPSEM, a new highly developed model of scanning electron microscope with simplified preparation of the tested material before use (Billy, 2000), has never been utilized before to study the effects of these locally available Malaysian condiments. This study is an attempt to pioneer the utility of high-resolution microscopy especially with VPSEM in elucidating a need to qualitate these condiments by scientific investigation thus, hoping to improve this commercially viable products present quality. At present their quality attributes is based mainly on flavor and smell.

## Introduction

Fermented foods are essential components of the diet in a number of developing countries, and are consumed either as main dishes or as condiments (Steinkraus, 1996). Fermented condiments are product of fermentation evolved for the development of taste or aroma; it often resulted in enhanced nutrition, stabilization of the original raw materials, and detoxification of anti-nutrient factors. Several fermented condiments relied on the introduction of various organisms such as from the Bacillus species, including Bacillus natto and B. subtilis. With the selected ingredient and additives, the finished products are of a very local character and usually will exhibit sensory properties resulting from unique selected flora and ritual processing technologies applied in small scale, home-based fermentations (Beaumont M, 2002). Due to the lack of scientific and technological know-how, these commercially viable locally produced fermented condiments are generally evaluated on the basis of subjective qualitative attributes such as odour and flavour.

Kelantan is a state in the east coast of Peninsular Malaysia. Among its traditional foods that remain to be part of the regular diet of a large segment of the population, is a type of condiment known locally as 'budu'. The lavious enjoyment of local condiments consumption remains a key constituent of diets throughout many parts of the east coast Malaysia. Most of these condiments were prepared by incubating the fish or shrimp in high concentrations of salt and under high humidity at ambient temperature over several months. Generally, the fish sauces were only made from selected fish species such as anchovy and sardine (Jung-Nim Park, *et al,* 2001). Fish and other types of marine-derived food are good sources of long-chain polyunsaturated fatty acids. Fatty acids are essential elements for neural development in the infant *in utero* and during the first few years after birth (Uauy, Mena, & Rojas, 2000). The particular fatty acid

incorporated in the brain and retina of the developing infant is docosahexaenoic acid or DHA. Since the human body lacks the enzymes to manufacture fatty acids, DHA must be supplied by the diet, or produced *in vivo* from diet-derived fatty acid precursors such as linolenic acid.

Other condiments consumed are the traditionally fermented soybean condiments, such as soybean paste and soy sauce, that are commonly consumed by other people in South East Asian countries (Mongkolwai, Assavanig, Amnajsongsiri, Flegel, & Bhumiratana, 1997). Soy sauce has been known to contain strong anti-oxidative and free radical scavenging activity (Yamaguchi, N. and Fujimaki, M. 1974). Although the nitrogen compounds were emphasized as one of the important factors on antioxidative activity of soy sauce, the main components of brown color products in soy sauce were recognized as melanoidin, the end products of Maillard reaction process, which is also strongly suggested as a compound holding antioxidative activity (Gapsoon Moona, *et al.*, 2002)

It is well known that mineralised human tissues such as its bones and teeth react to various physical and chemical stimuli. These reactions are often observed quite differently to that of other tissues and organs of the body. The demineralisation of bone matrix and of fully calcified bone have been studied by Boothroyd (1964), and Thorogood and Gray, (1975), whereas the mineralisation research was conducted on tooth tissue by Watson (1960) and Decker (1973). However studies have yet to be carried out to observe the relationship between the consumption of local sauces with the natural process of demineralisation and loss of crystallites from the mineralised tissues that might be induced and attributed by regular consumption of these local delights. Thus little is known of the histo morphological changes that might occurs and that might give rise to high incidences of oral health issues such as white spots and fissures caries tooth formations that may be associated with the consumption of the condiments.

**Design and setting:**

- This current study was designed to investigate the predisposing role of selected local condiments on white spots formation on healthy mineralised teeth structure.
- The study was conducted in the Health campus of Universiti Sains Malaysia that is situated in the east coast of Peninsular Malaysia. The primary objective was to assess the ultra-morphology changes that may occur and correlate the finding with the adherence properties of the selected condiments.
- To register a national morphological database on effects of local consumption of these sauces especially to the mineralised tissues of the human body.
- It is hope that insights gained from this study will further update knowledge of regular consumptions of the condiments. The data obtained will also be

useful in nutritional evaluation of segments of the population consuming these food products.

**Experimental methodology**

Fish anchovies, tomato sauce ketchup and soya sauce were randomly selected from the wide range of commercially available product list for the pilot study. Only fresh varieties of the identified local condiments based on expiry dates will be used. Each portion of the identified condiments was placed into various labelled 100 ml bottle containers. These specimen containers will then be fixed to a variable low torque speed vial rotator at room temperature; (this will help to improve the penetration of the condiments ingredients into the mineralised tissue). Ph and the expiry dates of each condiment will be regularly noted. The mineralised tissue will be initially disinfected with Tymol gargle, then directly immersed into each labelled condiment containers. Regular daily changes of fresh portions of the various condiments were subjected to the mineralised tissues. Each day the various morphological changes were examined macroscopically using a stereomicroscope; by when every each morphological change observed was clerked and recorded. Under normal circumstance a complete demineralisation of a tooth structure will hypothetically be achieved within the late fourth to the fifth week, hence the morphological changes will be studied by late fifth week. On the fifth week, each mineralised tissues was chemically fixed for a full duration of one day with full strength Karnovsky's solution (pH 7.0) following which they were rinsed gently in Ringers solutions. (If needed, the chemically fixed specimens were then x-rayed to check for radiolucency and radio-opacity). The specimen's were prepared as ground section where they were sectioned as unstained sections using a diamond saw microtome at selected 20□m specimen thickness increments. Each ground sections were then grinded and polished for optical microscope observations. The ground sections were observed under the LEICA stereomicroscope and variable pressure scanning electron microscope. The ground section was initially washed with phosphate buffers solution. No osmification was carried out. The preparation was examined under the LEO-VPSEM using its peltier cooling sub-stage at 35 to 48 Pascal pressure at an accelerating voltage of 15 kV and around 9mm to 15mm working distance. The tooth ground sections they was examined initially from their occlusal surface, then to the pulpal border and clerked. Photomicrographs images of the sections were taken and then transferred to Leica imaging workstation to enable accurate analysis of the demineralised areas. The resultant image was measured and colour-coded in accordance to various histogram classes.

The immersion experiments were based on various time intervals recorded at 24-Hours, 72 hours to 4 days and finally 4 weeks and after. Visual stereo and VPSEM charting of various morphological changes will be recorded.

# Flow chart of the study

**SELECTION OF CONDIMENTS**
**SATISFY INCLUSION AND EXCLUSION CRITERIA**

**COLLECTION OF MINERALISED TISSUES**
(TOOTH AND CADEVERIC BONE TISSUES)

↓

**DISINFECTION WITH TYMOL GARGLE**
(2-4hours)

↓

**IMMERSION AND DEMINERALISATION EXPERIMENTS**
(Immersion timetable: 2days, 5days, 1 week, 10days, 2 weeks, 28 days, 30 days. 4weeks, 40days, -5weeks)

↓

**STEREOMICROSCOPE OBSERVATIONS**
(Morphological changes recording with respect to the above immersion timetable)

↓

**CHEMICAL FIXATION**
(Full strength Karnovsky's solution)

↓

**RADIOLOGICAL INVESTIGATION (RADIOLUCENCY OR RADIO-OPACITY)**

↓

**DIAMOND SAW MICROTOME SECTIONING**
(Ground section)

↓

(Rinse in sodium phosphate buffers)

↓

**SECTIONS GRIND AND POLISHED**

↓

**RINSE RINGER'S SOLUTION**

(3X)

↓

**EXAMINATION UNDER VPSEM**

**(Peltier cooling sub-stage at 35 Pascal pressure at an accelerating voltage of 15 kV)**

↓

DATA COLLECTION
ELECTRON PHOTOMICROGRAPHS IMAGES

↓

IMAGING WORKSTATION, RESULTS AND DATA ANALYSIS

↓

REPORT WRITING + CONFERENCE PRESENTATION

## Results

### 1. PH and immersion in condiments experiments

The results from this study indicate that the pH of the condiments used were consistent through the period of immersion experiments. The mean Ph are as follows: Tomato sauce mean Ph = 4.3. Anchovies sauce mean Ph = 5.97. Soya bean sauce mean Ph = 5.1.

### 24-Hours, immersion

(i). Stereomicroscope observations.
No changes or deformation was observed on the surface occlusal morphology of the entire tooth crown immersed in the various condiments selected.

(ii). Variable pressure SEM observations.
The thread like structure observed spiraling at some regions of the mid dentine in the fish anchovies condiments immersion experiment preparations.

### 72 hours to 4 days immersion

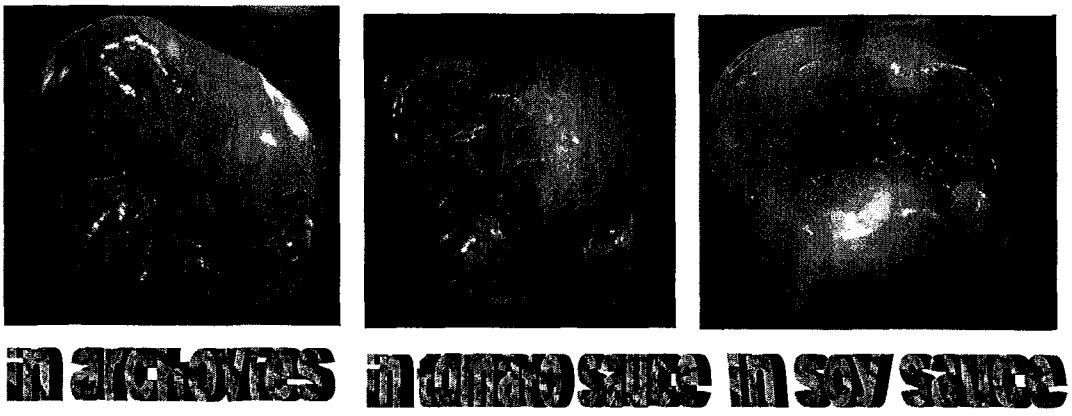
(ii). Variable pressure SEM observations.
In fish anchovies condiments immersion experiments, the finding observed was consistent with the 24 hours finding observation. The thread like structure suggestive of putative odontoblast process was still observed in mid-dentine region.

In the tomato sauce condiments immersion experiments, at the coronal end of the pulp chamber the pulpal tissue seems to be totally dissolved and absent of any soft tissues remnants. The mid dentine tubules was well defined and also clean of any putative structures. At 2000X magnifications, the dentinal tubules was observed as shallow furrows with blunt borders and edges, no sharp demarcation edges was observed. Such an observation gives this region of the dentine an appearance, dentinal tubules of a wider diameter width. At the enamel dentine junction (EDJ), literatures have reported that the dentinal tubules here are filled with peritubular dentine with its main mineral component of calcium phosphate. Although considered amorphous, this peritubular dentine was reported to be crystalline octacalsium phosphate (Berkovitz, Holland and Moxham, 1992). In this experiments when observation under the VPSEM at the

enamel dentine junction was conducted, the sample inserted in tomato sauce condiment shows no crystalline peritubular dentine present. What was observed at the EDJ was the folding waves/contour of the gaps or the intertubular dentine between the tubules. It is hypothetically suggestive that this folding wave is an indication that demineralization process is occurring at that site.

In the soy sauce condiments immersion experiments the pulp tissue was observed to be present and well intact as a whole connective tissue bundle yet it is a bit displaced form its actual adherent location i.e., to the coronal part of the pulp chamber. It is suggestive the pulpal tissue here is undergoing a slow process of disorganization and displacement. No folding waves were observed in the intertubular dentine. Soy sauce was also present in the tubules. The soy sauce is the only condiment that was observed present at this time interval in the dentinal tubules although all preparations from the immersion experiments was gently washed before placement on to the stud of the peltier stage camber for VPSEM observations.

**Figure I**
Stereomicroscope photomicrograph of tooth enamels post-immersed in the selected condiments



**4 weeks immersion**

(i). Stereomicroscope observations.
Surface occlusal morphology of the tooth crown was observed using the stereomicroscope at 16X. Tomato sauce specimens showed signs of demineralisation as early as week 4 in immersion. They were soft in texture and flabby like in nature, this phenomenon was not observed in the other condiment immersion samples.

(ii). Variable pressure SEM observations.
The use of the saw microtome leads to the formation of smear layer impregnated on the cut surface of the tooth. The smear layers were thick and visible seems especially in the tomato sauce specimens. Observation of its surface topography was almost impossible, although attempt was made to remove the smear layer with the use of 35 phosphoric acid for 1 minute. The

specimens were then gently fractured to ovoid the cut surface. The fracturing technique was conducted gently with precision hammering with the use of a mallet. Observation was made using the VPSEM at 9mm working distance at 47 Pascal pressure with the (back scattering electron) BSED mode. The results can be summarised as below;

a. there was no smear layer observed around the fractured surface in all specimens. Smear layer was only observed on the cut surface and of the three groups of specimens-condiments samples; the least coated was the surface of specimens immersed with Soya bean ketchup.

The tomato sauce specimens were covered with a well-defined coating believed to be the adherence of the sauce and smear layer on the surface area of the specimens (figure A & B below). This was interesting to be observed and noted as the phenomenon of coating was not observed to occur in other specimens with other condiments although all of them have been thoroughly washed under running tap water and the naked eye do not show any present of a coating or acquired pellicle like surface of what ever sort of colour to be present.

**Figure A:**

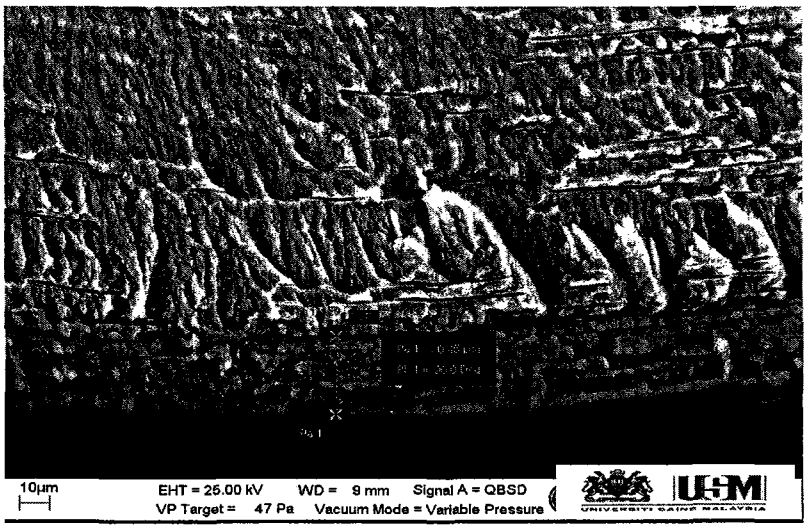
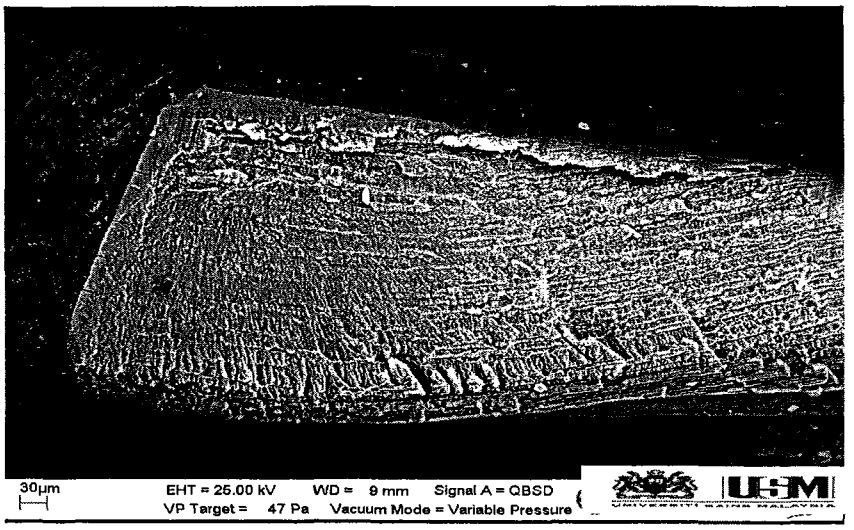



**Figure B:**



## Discussions

The mean average Ph of our selected fish anchovies condiment budu, is 5.97. At this average Ph, it seems that of the three selected condiments used in this study, the fish anchovies showed the least and slow characteristic changes observed macro and micro-morphologically. From past studies the mean average Ph value of fish sauces produced in the South East Asia region was cited within 5.4 to 5.8 Ph level, Myanmar fish sauce is at an average mean Ph value of 6.23, Laotian 4.90 and Chinese fish sauces at 6.15 (Ren, *et al,* 1993, Fujii, *et al.,* 1992). The Ph value of fish sauce was also noted to be of a higher reading then soya sauce where they ranged from 4 to 5 Ph. It will be interesting to study if the various morphological changes observed here will be similarly observed when immersed in the above condiments. The fish sauce was also reported to be of high salt concentration (15 to 25%w/w), hence not recommended to be consumed in high quantities (Aryanta, *et al.,* 1991).

The tooth preparations used in this experiment, are from freshly surgically removed wisdom teeth gained from consented patients at the government dental clinic Jalan Mahmood Kelantan. The tooth preparation comprises of enamel-dentine and coronal pulp tissues. This tooth model was selected as the mineralised preparation for not only of the anatomical features but due to the fact that this structures of the tooth are in direct instance contact especially linked to adherence principals with the selected condiment once consumed. The various stereo and VPSEM morphological changes reported should be database beneficial to the oral care worker. This information can increase awareness and scientific-based oral heath promotion towards better lifestyle eating habits especially from the consumptions of local pride condiments from locally available home based industries.

The early demineralization stage observed under the stereomicroscope was identified as well defined whitish chalky areas or island patch that are usually visible on the occlusal margin and ridges of the tooth crown preparations. This whitish chalky island is a sign of an early caries formation identified as white spots and this is related to demineralization of the tooth hardest tissue, the enamel. We believed that this process of enamel demineralization is maximized by the fourth week especially in tomato condiments immersion experiments. However, if the soft tissue such as the pulpal tissue is allowed to be exposed and thus allowing the condiments to adhere to it, then as early as within 24 hours there will be total disintegration of the soft tissue leading to a non-vital stage of a former vital tissue structure.

The variable pressure SEM technique for microstructural observation requires no chemical fixation process, and thus reduces the preparation time and possible artifacts, as compared with the usage of the conventional scanning electron microscopy. The local condiments selected here is a series of widely available commercial home based products that were prepared by ritual and anecdotal.

So far, with no scientific based microscopy efforts have ever been recorded to enhance and hence to regulate further its quality. To the knowledge of the present researcher the closest quality control evaluation is limited to subjective qualitative attributes such as flavor and odour. Hence, high-resolution microscopy monitoring and evaluation investigations especially with the usage of VPSEM is highly recommended and as shown here have proven necessary for improving quality and for advocacy.

Small-scale home industries based food fermentation technologies in Malaysia and its neighboring countries is by ritual have evolved through years of experience (or village-art methodologies), rather than through novel scientific breakthroughs. Many of these small-scale manufacturers are therefore, illiterate thus reluctant to accept changes and modify their fermentation processes. Upgrading the quality and safety of fermented foods, while reducing their production cost and maintaining their authenticity and uniqueness, is of utmost importance and high-resolution microscopy can do its part here.

**Conclusion**

Consumption of local condiments is a delight that is commercially viable and demographic specific that colors one's culture. Although Ph and mineral content is believed to have played hypothetically a significant role in the changes observed, it was most likely that the adherence, viscosity and permeability capabilities of each selected condiments to the dentine pulp complex preparations that have lead to the morphological changes observed.

**References**


Beaumont, M. (2002). Flavoring composition prepared by fermentation with Bacillus spp. *Int J Food Microbiol.* May 25; 75(3): 189-96.

Berkovitz, B. K. B., Holland, G. R., Moxham, B. J. (1992). Secondary dentine and post eruptive features. In: A colour atlas and textbook of oral anatomy, histology and embryology. 2nd Eds, Wolfe Publishing Ltd. 142-143.

Billy, (2000), Secondary imaging in variable pressure mode-short communication. *Microsom* vol 3, no: 2, August. P18.

Boothroyd, B. (1964), The problem of demineralization in thin sections of fully calcified bone. *J. Cell Biol.*, 20: 165-173.

Decker, J.D. (1973), Fixation effects on the fine structure of enamel crystal-matrix relationships. *J. Ultrastruc. Res.*, 44: 58-74.

Fujii, T. Nikkuni, S. and Iida, H. (1992). Chemical composition and putrescible potential of commercial "Shottsuru", Japanese fish sauce. *Nippon Shokuhin Kogyo Gakkaishi.* 39, pp 2013; 706.

Gapsoon, Moona, Minja Leeb, Youngsun Lee and Gassinee Trakoontivakorn (2002). Main component of soy sauce representing antioxidative activity. *International Congress Series.* Volume 1245, November, Pages 509-510.

Jung-Nim Park, Yuki Fukumoto, Eriko Fujita, Tadayoshi Tanaka, Takuya Washio, Soichiro Otsuka, Tetsuji Shimizu, Katsuko Watanabe and Hiroki Abe. (2001).

Chemical Composition of Fish Sauces Produced in Southeast and East Asian Countries. *Journal of Food Composition and Analysis*. Volume 14, Issue 2, April, Pages 113-125.
Leach, S.A. &. (1983) Demineralisation and Remineralization of the Teeth. Oxford University Press.
Mongkolwai, T., Assavanig, A. Amnajsongsiri, C., Flegel, T.W and Bhumiratana, A. (1997). Technology transfer for small and medium soy sauce fermentation factories in Thailand: a consortium approach. *Food Research International,* 30 (1997), pp 2013; 563.
Ren, H. Hayashi, T. Endo, H. and Watanabe, E. (1993). Characteristics of Chinese and Korean soy and fish sauces on the basis of their free amino acid composition. *Bull. Jpn. Soc. Sci. Fish*. 59, pp 2013; 1935.
Silwood, C. J. Prinz, J. Grootveld, M and. Lynch, E. (2000). Protective Effects of Hexaflurotitinate against demineralisation of the hard dental tissues. *Proceeding of IADR meeting* Washington.
Steinkraus, K.H. (1996). In: Handbook of Indigenous Fermented Foods, Marcel Dekker, New York, pp 2013; 274.
Thorogood, P. V., J.C. Gray, (1975), Demineralisation of bone matrix: Observations from electron microscope and electron probe analysis. *Calcif. Tissue Res.,* 19: 17-26.
Thylstrup, A. Leach, S. A. Qvist, V. (1987). Dentine and Dentine Reactions in the Oral Cavity. Oxford University Press.
Uauy, R., Mena, P. and Rojas, C., (2000). Essential fatty acids in early life: structural and functional role. *Proceedings of the Nutrition Society* 59, pp.2013; 15.
Watson, M. L. (1960), The extracellular nature of enamel in rat. *J. Biophys.* BioChem. Cytol., 7: 487-492.
Yamaguchi, N. and Fujimaki, M. (1974). Studies on browning reaction products from reducing sugars and amino acids: Part 14. Antioxidative activities of purified melanoidin and their comparison with those of legal antioxidants. *Journal of Food Science and Technology* (Japan) 21. p. 6.

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Below are the VPSEM photomicrographs illustrations of the dentine slads preparations that were immersed in various condiments selected.

**Figure II.**

***Comparison between 3 controlled VPSEM micrographs dentinal surfaces with peritubular dentine present***

| Plot | Area | Count | Area Fract | Area % | Mean Frame |
|---|---|---|---|---|---|
| 1 | 377.72 | 220 | 0.0525 | 5.25 | 7188.96 |
| 2 | 454.98 | 237 | 0.0615 | 6.15 | 7395.83 |
| 3 | 140.27 | 95 | 0.0195 | 1.95 | 7188.96 |

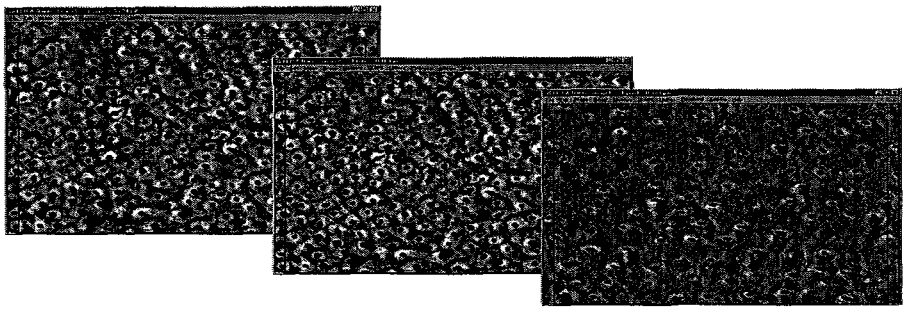

**Figure III.** The 3 VPSEM micrographs of fish anchovies, soy sauce and tomato sauce dentine pulp tissue morphological changes observed in clockwise manner post immersion.