UNIVERSITI SAINS MALAYSIA

A STUDY INTO THE PREVALENCE OF DIATOMS IN THE SOFT DRINKS IN MALAYSIA

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science in Forensic Science

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CERTIFICATE

This is to certificate that the dissertation entitled

"A Study into the Prevalence of Diatoms in the Soft Drinks in Malaysia"

is the bonafide record of research work done by

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ABSTRACT

Drowning is defined as death due to immersion or partial immersion in a liquid and it is one of the most common causes of unnatural death. A large volume of water is absorbed into the circulation because the low salt (sodium chloride) in the freshwater. Together with the water inhaled, diatoms are also inhaled reaching throughout circulation. Pathologists are often faced with the problem, while examining the dead body taken from the water, when asked whether the case was one of drowning or was the victim dead before entering the water? In a situation in which the dead body is highly decomposed or only the skeletal remains are recovered, direct diagnosis of death by drowning is indeed difficult. In highly decomposed dead body, when there is nothing left except those skeletal remains, and in which there is no tissue remains, histopathological examination can not be done, and the only method left is considered to be diatom test. However, many researchers have demonstrated diatoms in tissue samples from non- drowned individuals. The channel through which the diatoms reach the internal organs seems primarily through the lungs. Respired air normally contains a fairly large number of diatoms cells and valves. Also through the alimentary canal when there is a possibility of presence of diatoms in the food. Raw fruit and vegetables which have been in contact with soil diatom and shellfish which feed on diatoms contains diatoms. In this research, only juicy drink sold in restaurants are considered for analysis. The result shows the absence of diatoms in all the drink samples examined here. It is possible that

increasing the volume of the drink may indicate diatom. Due to practical constraints, the volume analyzed could not be increased.

INTRODUCTION

Diatoms are aquatic unicellular algae that belong to *Bacillariophyta* representing about 35 % of the source of oxygen producers in the biosphere (Pollanen, 1996). There are more than 200 genera of living diatoms, and it is estimated that there are approximately 10,000 extant species (Round & Crowford, 1990). Diatoms are widespread and can be found in the ocean, in freshwater, in soil and on damp surfaces. Most live pelagically in open water, although some live as surface films at the water- sediment interface, or even under damp atmospheric condition. They are especially important in oceans where they are estimated to contribute up to 45% of total oceanic primary production (Mann, 1999)

Diatom cells are contained within a unique silicate cell wall comprised of two separate valves (or shells). The biogenic silica that the cell wall composed of is synthesized intracellularly by the polymerization of silica acid monomers. This material is then extruded to the cell exterior and added to the wall. Diatom cell walls are also called frustules; these unique frustules are crystalline in nature characterized by unique pattern of symmetry and microstructure (Round et al., 1990; Pollanen, 1998). On this basis, vast structural diversity of the frustules leads to a remarkable number of morphologically distinctive varieties of diatoms (Round et al., 1990; Peabody, 1997).

In forensic pathology, test for diatoms have been used in cases of death by drowning (Knight, 1996). It is also a common practice, particularly in European Medicolegal departments to consider the finding of diatoms in the organs of the major circulation as pathognomonic evidence for the diagnosis of death by drowning (Mueller, 1949).

However, the use of diatoms as indicator of drowning is a controversial subject opposed by many authorities. This is because it has been demonstrated that the diatoms were found in the non- drowned victims' organs too (Spitz and Schneider, 1964; Peabody, 1980; Foged, 1983; Polson et al., 1985; Pachar, 1993). Inhalation of atmospheric air containing diatoms or drinking water containing diatoms, can lead to the presence of diatom in the bone marrow (Hendy, 1980; Foged, 1983; Shapiro et al., 1988; Pachar and Cameron, 1993; Dimiao et al., 2001; Kristic et al., 2002). Also, it has been suggested that the diatoms can enter the human body through the food that contains diatoms (Spitz and Fisher, 1982; Pollanen, 1988; Shapiro et al., 1988; Hurlimann et al., 2000). The diatoms that enter along the food may also be absorbed from the gastrointestinal tract and reach to the bloodstream (Spitz and Schneider, 1964; Foged, 1983; Shapiro et al., 1988). As the blood circulates through out the body, it will deposit the diatoms to such organs and tissues including the bone marrow in the same way the diatoms that enter the respiratory tract during the drowning are deposited (Foged, 1983; Pollanen, 1998; Knight, 1996). Deposition of diatoms inside the body by ingestion can lead to misinterpretation of ante mortem

drowning (Spitz and Schneider, 1964; Hendy, 1980; Foged, 1983; Shapiro et al., 1988).

The possibility of diatoms entering the blood stream along with ingested material that is absorbed can not be excluded since no studies show that diatoms can not enter the circulation through the alimentary tract (Shapiro et al., 1988). Here the possibility of diatoms deposition through the juicy drinks is studied among the local people of Kota Bharu, Kelantan, Malaysia. The interference from this study can be useful to indicate the cumulative ingestion of diatoms by the Malaysian, which may influence the body level diatoms.

REVIEW OF LITERATURE

Diatoms are microscopic unicellular algae of the class *Bacillariophycea* which is division or phylum *Bacillariophyta* (Peabody, 1977). Diatoms are unicellular plants that represent the most abundant single source of oxygen producers in the biosphere (Pollanen, 1996). Diatoms are photosynthesizing algae that contain chloroplasts with special pigment content. A very few diatom species can also live heterotrophically in the dark if they are supplied enough suitable organic carbon (Round et al., 1990). The sizes of diatoms range from less than 5µ to more than 500µ (Peabody, 1977). These diatoms are widespread which can be found in the ocean, in freshwater, in soils and damp surface (Peabody, 1977). They are estimated to contribute up to 45% of total oceanic primary production (Mann, 1999). They also can be found anywhere where there is light, moisture, and nutrients (Peabody, 1977). In the atmospheric air, diatoms also can be found (Shamsudin, 1991).

The only entity that is truly specific to diatom is the cell wall, which is the extracellular coat or frustules that is composed of silica. This frustule is characterized by unique pattern of symmetry and microstructure which is crystalline in nature (Round et al., 1990; Pollanen, 1998). The diatom cell is divided into two halves, the epitheca and the hypotheca (halves= theca). The face of each half is called a valve, and joining the two thecae together is a series of girdle bands, known collectively as the cincture (Round et al., 1990). The cincture is an element of the siliceous endoskeleton that beside form enclosing

and protecting the cell is also capable of accommodating the increase in cell volume during the cell cycle (Round et al., 1990). This silica-based extracellular coat or frustule is resistant to enzymatic and acid digestion (Pollanen, 1998; Ludes et al., 1999). This silica skeleton resists decomposition, so in drowning cases demonstration of diatom in decomposed bodies are valuable sign (Matsubara et al., 1980). Moreover, they can be recognized even after digestion by strong acid (Matsubara et al., 1980).

Diatom blooms follow a pattern of seasonal periodicity (Damann, 1951; Beeton, 1963; Peterson, 1986). A massive diatom bloom occurs in freshwater in the early spring (April). This is followed by a decline in the live diatom population but a persistence of high level of dead diatoms in the water in the summer (Pollanen, 1997). In the autumn, another diatom bloom occurs and there is a progressive decline in the diatom population in the winter months until the next spring bloom. Therefore, diatom population dynamics suggest that the monthly incidence of positive test outcome should correlate with the cyclic variation in the diatom population (Pollanen, 1997). In addition to seasonal alterations in diatom concentration, it has been found that different genera of diatoms predominated at geographically distinct sites which likely relates to intrinsic features in the local water in those area (Ludes et al., 1996)

Drowning is death due to aspiration of fluid into air passage (Giertsen, 1977). In both seawater and freshwater drowning, death may be due to hypoxia (Modell, 1978). Death by aspiration of water led to the use of diatoms as indicators of death by drowning since in this way diatoms are introduced into human body from the drowning medium (Peabody, 1980). The pathway of diatoms reaching the bone marrow in freshwater drowning is well established (Pollanen, 1998). In marine water drowning, due to hypertonicity of the seawater, diatom seldom is present in the systemic circulation (Knight, 1996). Apart from drowning, diatoms are also introduced to human body through food intake as well as due to drinking water in daily life (Foged, 1983; Krstic et al., 2001). Raw fruits and vegetables may have been in contact with soil diatoms (Tedeschi et al., 1977). Shellfish that feed diatoms have immense quantities of diatom that may enter the circulation and reach the tissues as we ingest them (Knight, 1996). Besides that, diatom are also encountered in living person through the inspired air (Shapiro et al., 1988; Pachar and Cameron, 1993; Dimaio et al., 2001; Krstic et al., 2002)

Establishing a positive diagnosis in case of death by drowning has been indicated to be difficult (Ludes et al., 1999). The diagnosis of the death will become much more difficult than straightforward when the body is recovered from the water weeks or months after death (Peabody, 1980). In this stage, the

body may be decomposed, making histopathological examination impossible (Peabody, 1980). In addition to the decomposition of the dead body, such severe fragmentation or dismembering by the action of ships' propellors, lock gates or aquatic organisms during its passage through the water also will cause further difficulties during the process of diagnosis. The skeleton only remains of a dead body in some extreme cases (Ferris and Stockdale, 1972), or the body gets reduced to a few bones loosely clad with the adipocere (Peabody, 1977).

Under the conditions described above, the reliable method for determining death by drowning using the histopathological treatment become unsuitable and led to investigation of the chemical changes involved in drowning (Spitz et al., 1969; Schwar, 1972) and into the use of diatom as indicators of death by drowning (Peabody, 1980). Yet, in freshwater drowning in the absence of other evidence, detection of diatoms in the body tissues is the most reliable method left (Shapiro et al., 1988). Furthermore, these diatoms will be deposited among other internal organs beside of lungs (Knight, 1996).

However, in practice, diatom test had become quite controversial as indicator of drowning. It has been observed that diatom test can not led to conclusive opinion on ante- mortem drowning (Foged, 1983; Pollanen, 1998). Even the use of diatom test for the diagnosis of death by drowning based on the findings of diatoms in the tissues of subjects thought to have drowned was

doubtful (Spitz and Schneider, 1964). This is supported by the finding of diatoms in the tissue samples from non- drowned individuals (Peabody, 1980).Yet, findings of planktons in the internal organs and tissues were not particularly convincing proof (Eidlin, 1968). Beside that, in the lung of routine human autopsies, diatoms were consistently found in cases other than drowning cases (Spitz and Schneider, 1964; Foged, 1983; Polson et al., 1985; Pachar, 1993). In addition, in a case of a death because of poisoning, numerous of diatom cells also had been found in all available organs (Krstic et al., 2002). This shows that demonstration of diatoms from a subject's tissue cannot be construed as definitive evidence. Thus, this (diatom) technique acts as a supportive evidence rather than proof evidence (Rushton, 1961).

OBJECTIVE AND HYPOTHESIS

OBJECTIVE

Review of the literature indicates that the study on the prevalence of diatoms and on its quantity in the juicy fruit drinks had not been done so far. Drinking the juicy drinks can be one of the sources for the diatoms to get into the human body and, possibly, for being deposited in the bone marrow. The objective of this study is to find out the presence or absence of diatoms in the juicy fruit drinks in Kota Bharu, Malaysia taking into consideration the drink preferences of the local population.

HYPOTHESIS

Water is the source for the plants or trees to grow. Diatoms are rich in the water and wet soil. Most plants belonging to root vegetables category such as carrot may contain diatoms. All fruit juices are consumed by human beings and it is hypothesized that the juices impending consumption would reveal the presence of diatom. We can estimate the average consumption of the juicy drinks and the relative amount of the diatom which will be entering the human body. These findings can be useful for interpreting the result relating to the detection of diatoms in bone marrow in drowning cases.

MATERIALS AND METHODS

MATERIALS

The samples for this study included the juicy fruit drinks prevalent in Kota Bharu, Kelantan, Malaysia. These juicy drinks are those that are mixed or prepared on the spot and processed for human consumption in local restaurants and food stalls. To assess the preference of drinks among the local people, a survey has been done obtaining responses to a questionnaire (Appendix 1). Based on the responses, the most frequent juicy drinks were identified, purchased and had been studied during this research. Table 1 is the list of juicy drinks in the order of preference by the local people.

Table 1: Juices purchased

No.	Juice drinks
1.	Soy drink
2.	Carrot
3.	Milked carrot
4.	Coconut
5.	Sugar cane
6.	Apple
7.	Mango
8.	Guava
9.	Honey Dew
10.	Watermelon

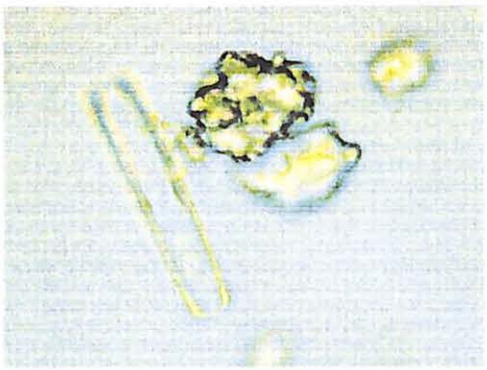
METHODS

Pre- test: Examination of freshwater diatom under microscope

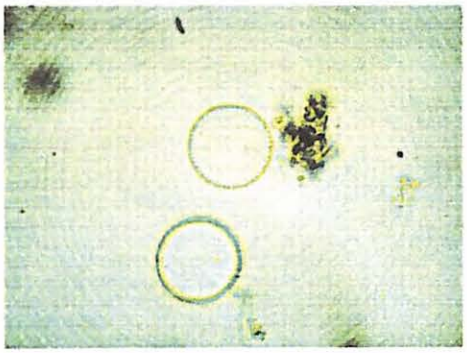
Freshwater sample was collected from the pond located in the USM campus and it was used as control. The water sample was examined for the presence of diatoms in the laboratory. The freshwater was collected in 500 ml plastic bottles. In the laboratory, 200 ml of the freshwater was transferred into a 500ml beaker. Then, equal amount of 65% concentrated nitric (HNO_3) acid was added onto the same beaker. The acid –water mixer was then boiled using the Bunsen burner and the HNO_3 was added during the boiling process until completion of digestion. The boiled material was then centrifuged and the pellet was examined using a light microscope. This pre-trial indicated that (a) the frustules of the diatoms could be identified based on their distinctive bilateral symmetry; and (b) the morphology-based identification indicated that the navicular (boat-shaped) diatoms predominated the fresh water sample that was examined.

Figure 1: Photomicroscopy of variation forms of diatoms recovered from fresh water samples (400X)

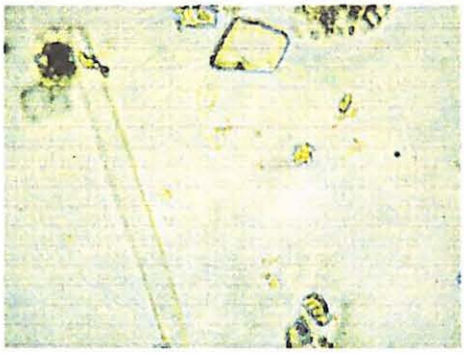
1. Naviculoid diatom



2. Pennate diatom



3. Centric diatom



4. Filamentous diatom

METHODS

Each drink sample was processed through the following stages:

1. Preparation of the samples
2. Extraction of frustules from sample- strong acid digestion
3. Isolation of diatoms from the acid extract
4. Microscopic examination

PREPARATION OF THE SAMPLES

Apparatus

1. Beaker (500 ml)
2. Gloves
3. Labeling sticker
4. Pen

Procedures

Before the sample was poured into the beaker, the beaker itself was cleaned by washing it with the tap water followed by rinsing with the distilled water and left to dry in the autoclave. The sample material was then poured into the beaker. The amount of each sample which was poured into the beaker was 200ml. Then each beaker was labeled.

EXTRACTION OF FRUSTULES FROM THE SAMPLE

Apparatus

1. Beaker 100ml
2. Bunsen Burner
3. Gloves
4. Stand for Bunsen Burner

Reagent

65% concentrated nitric acid (HNO_3)

Precaution:

1. The extraction process was carried out within the fume hood. This is because the irritant nitric acid oxide vapor generated from the boiling process will contaminate the laboratory ambience if done in open space.
2. Avoid the acid- sample mixture from drying by adding the concentrated nitric acid throughout the boiling process.
3. When dealing with the concentrated acid always wearing the glove for avoiding contact with the acid which is strong corrosive nature.

Procedures

Equal amount of concentrated nitric acid was added to each beaker that contained the sample drink. Then the mixture of acid-sample was boiled using direct flame. The boiling process was continued until the volume of acid-sample mixture was one- fourth (100 ml) of the initial volume (400ml). During the boiling

process, the concentrated nitric acid was repeatedly added while continuing boiling until obtaining a clear content. Here, clear mean there was no suspended material inside the content. After the volume became one- fourth of previous volume, the boiling process was stopped and the content was left to cool in room temperature. Thus, the final volume of acid digested material was 100 ml for each sample.

ISOLATION OF DIATOMS FROM THE ACID EXTRACT

Apparatus

1. Centrifuge tube (2.0 ml)
2. Glass tube (10 ml)
3. Disposable tips
4. Micropipette (1000 µl)
5. Macro centrifuge Hettich, Universal 32R
6. Micro centrifuge Spectrofuge 16M
7. Vortex mixer EVM-6000
8. Labeling material : sticker and pen

Reagent

Distilled water (dH_2O)

Procedures

After the content of the beaker was cooled, 10 ml of the content was transferred to the 10 ml centrifuge glass tube using the micropipette. All the tubes were labeled accordingly and then centrifuged at 4500 rpm for 15 minutes using macro centrifuge. After the first centrifugation, the supernatant of the sample was discarded using the Pasteur pipette while leaving the discernible sediment in the tube. The sediment was stirred well with distilled water and made up to 10 ml. This washing step was carried out twice. After that, the sediment was transferred to 2.0 ml centrifuge tube from each tube and labeled accordingly. Again the sediment was stirred well with distilled water and now made up to 1.0 ml. Then the tubes were centrifuged at 14,000 rpm for 15 minutes using the micro centrifuge. After the first centrifugation, the supernatant of the sample was discarded using Pasteur pipette while leaving the discernible pellet in the tube. Again, the sediment left was stirred well with the distilled water and made up to 1.0ml. This washing step for micro centrifugation also carried out twice.

MICROSCOPIC EXAMINATION OF THE SLIDES

Apparatus

1. Coverslips
2. Marker pen
3. Light microscope
4. Pasteur pipette
5. slides

Reagents

DPX mounting medium

Procedures

After the final centrifugation using micro centrifuge, the supernatant was discarded leaving only 0.1 ml of supernatant and the pellet in the graduated tube. Then the content left was agitated using the vortex so that the pellet will dislodge. By using the Pasteur pipette the content in the centrifuge tube was aspirated and placed onto ten of glass slides. The glass slides were then covered with the coverslip and sealed with DPX along the edges and labeled accordingly. This seal prevents air from outside from drying the slide and so act as preservation of the slide for further microscopic examination. Then the slides prepared for each sample was examined under light microscope starting with low magnification of 100 X followed by 400X.

Qualitative Assessment

Starting with low magnification, the slides were examined thoroughly for the determination of diatoms based on their morphological structure, shaped, and bilateral symmetry of frustules. After initial identification of diatoms in the microscopic field using low magnification, further observation of every diatom was done by using high magnification (40 X magnification). This revealed more details regarding the morphological structures, and the image of diatom observed was captured by using the camera attached to the microscope.

Quantitative Assessment

This step dealt with counting the number of the diatoms found per slide. In every slide the number of diatom was counted by using the strip technique commencing from top left end of the area of the slide under the coverslip. Here, 10 slides were prepared for each sample and this accounted for the number of diatoms that were present in 10 ml of acid digested material.

The estimated number of diatoms in 10 ml was multiplied by 100 for obtaining the estimated number of diatoms in the 100 ml of acid digested material, which was obtained by digesting 200 ml of juice.