# The effect of enzyme digestion time on the detection of diatom species

**Zhengliang Yu[1], *Chao Liu[2], Huijun Wang[1], Ling Chen[1], Sunlin Hu[2], Jian Zhao[2], Huipin Wang[1], Yu Zhou[2] and Weibing Xie[1]**

[1]Faculty of Forensic Medicine, Southern Medical University, Guangzhou, China
[2]Guangzhou Institute of Criminal Science and Technology, Key Laboratory of Forensic Pathology, Ministry of Public Security, Guangzhou, China

**Abstract**: This study is aimed at detecting diatom in lung, liver and kidney tissues using PCR - DHPLC technology after different periods of enzyme digestion to assess the effect of enzyme digestion on the detection of diatom species. Twenty Randomly selected experimental rabbits were drowned at the same place. Their liver, kidney, and lung tissues were removed for sampling. After the extraction of DNA from the samples, amplification was conducted with specific primers of the SSU gene of diatom. Then, an analysis was performed with agarose gel electrophoresis and DHPLC. Within 2 h-8 h, the amount of the diatom species found in the lung gradually increased over time and was statistically significant ($P<0.05$). After 8 h, with enzyme digestion, the amount of the diatom species found in lung showed no significant increase ($P>0.05$). However, as for the liver and kidney, within 2h-6h, the amount of the diatom species gradually increased over time and was statistically significant ($P<0.05$). After 6h, the fig. did not present significant growth ($P>0.05$). The amount of the diatom species found in the organs after different periods of digestion time had significant differences, which provides a reference for the detection of diatoms and also, has a good application prospect in the forensic identification of drowning.

**Keywords**: Forensic medicine, drowning; enzyme digestion, detection of diatom, SSU gene, PCR-DHPLC.

## INTRODUCTION

There are many dead animal bodies floating along the river in the cities, and many of them have corrupted. Diatom test is commonly used for analyzing the cause of death. The mainstream of traditional diatom test is an acid digestion method. The diatom detection rate of this method is low, and it poses risk related to operation, etc. Therefore, this method was controversial in academia. In 2003, Guanying He applied an enzyme digestion method to detect diatoms in organs (Guanying *et al*. 2003). He concluded that with regard to the diatom detection rate, the enzyme digestion method was superior to the acid digestion method. Additionally, he pointed out that compared to other enzymes, the digestion ability of trypsin was better and its diatom detection rate was higher. In 2006, Shuling Ma detected diatoms with the enzyme digestion method in the organs from 59 cases of human bodies (Shuling *et al*., 2006). He considered that the enzyme digestion method was simple and of high detection rate and the results were reliable. However, previous studies on the enzyme digestion method simply focused on the digestive ability of different enzymes, but not on the amount of diatom species detected after different periods of digestion time. And previously, the method used for diatom detection after enzyme digestion was smear microscopy, which was inferior to the molecular biology method. In 2008, Fang gang He used primers designed by belU *et al.* (1997) to amplify the water samples from three lakes and the lung tissues of drowned animals from the two of the three lakes (Fanggang *et al.*, 2008). The results successfully verified that the amplified products in the lung tissues of the drowned animals were similar to those of the corresponding drowning site, but were significantly different from those of the non-drowning places. These methods have no longer depended on the morphological, physical and chemical characteristics of planktons. But on the molecular level, PCR is of high sensitivity and specificity for amplifying the specific sequences of planktons, and can effectively eliminate the interference of spoiled organism. It has been a new method for the identification of drowned animals. The primers designed by LAURA *et al.*, (2011) were chosen for this research, with the application of PCR-DHPLC technology to detect the amount of diatom species after different periods of digestion time.

## MATERIALS AND METHODS

The 20 rabbits with either gender were provided by laboratory animal center of Guangdong province, weighing around 2.4~3.4kg. The rabbits were taken into a cage and carried to the Nansha Humen Bridge in winter. The cage was suspended into the sea with 0.5m under the surface for 5 seconds and then, taken out. After 10 seconds, the cage was suspended again into the sea with the same depth. We repeated the steps above until the rabbits died. Afterwards, the cage was suspended into the sea with the same depth for 24 h. Finally, the lung, liver and kidney tissues were removed from the drowned rabbits.

**Corresponding author:* e-mail: liuchaogaj@21cn.com

***Diatom separation***

After removal, each kind of tissues was made into 20 samples. The enzyme (trypsin) at the concentration of 20 mg/ml was added into the lung tissue of 1 g. Respectively, the suspension was incubated at 50°C for 2h, 4h, 6h, 8h and 10h. the dispose of the liver and kidney based on the lung. After digestion, centrifugation was performed at 3000 rpm for 30 minutes and the supernatant was discarded.

***Diatom DNA extraction***

The Power Soil™ DNA Isolation Kit was used to extract DNA from the sediment. The DNA extracted with this Kit could be directly used for the downstream experiment without further purification.

***PCR amplification***

The primers reported by LAURA were used to amplify the SSU gene of the diatom (table.1. for the sequences of the primers) (LAURA *et al*., 2011). The primers were combined by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

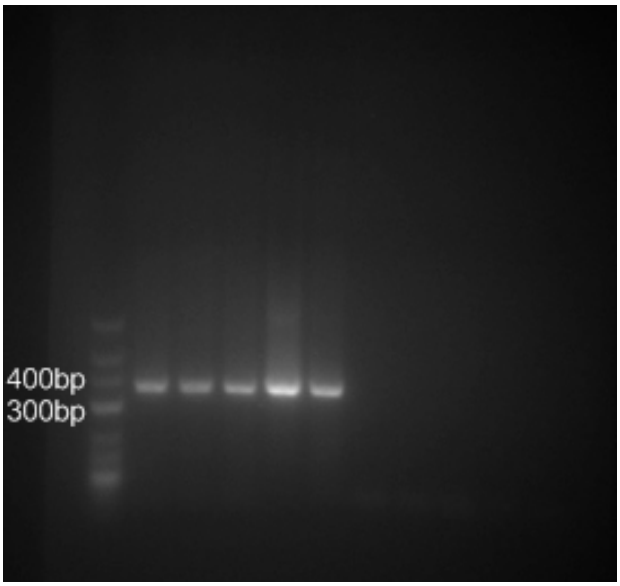


**Fig. 1**: Amplification products generated from *Cyclotella meneghiniana*, *Melosira varians*, *Nitzschia sp*, *Synedra sp*, *Navicula sp*, human genome, rabbit genome, *Scrippsiella trochoidea*, *Platymonas elliptica*, *prorocentrales* (from left to right).

***DHPLC technology***

The PCR products of the five most common diatoms (*Cyclotella meneghinianas*, *Melosira varians*, *Nitzschia sp*, *Synedra sp*, *Navicula* sp) along Guangzhou section of the Pearl River (Sunlin 2009) were detected with DHPLC technology. We set different column temperature and flow velocity until every single species showed single peak. Under the same column temperature and flow velocity, the other samples of the PCR products were tested. Because for each species, the time point that the peak appears is the same, different PCR products can present different peaks and then, the diatom species can be differentiated (Xuewei Zhang 2007 and Ulrike Scharrer *et al*., 2010).

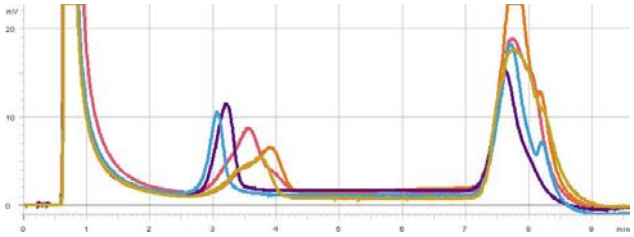

**Fig. 2**: shows the DHPLC results of the five most common diatoms in the Guangzhou section of the Pearl River (The peak time from left to right in turn shows *Melosira varians*, *Synedra sp*, *Cyclotella meneghiniana*, *Nitzschia sp*, *Navicula sp*).

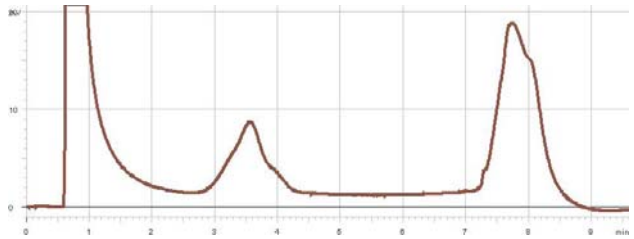

**Fig. 3**: DHPLC result of the diatom in rabbit kidney (*Cyclotella meneghiniana*)

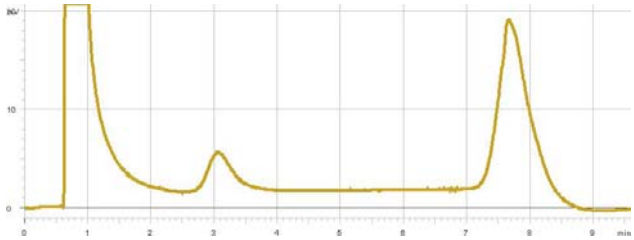

**Fig. 4**: DHPLC result of the diatom in rabbit kidney (Melosira varians)

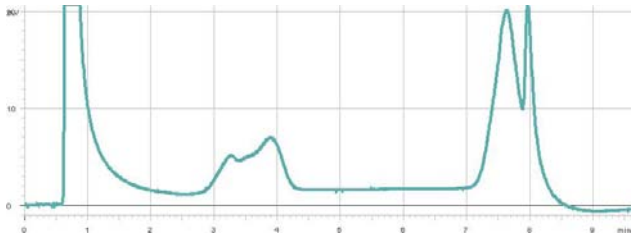

**Fig. 5**: DHPLC results of the diatoms in rabbit liver (*Synedra sp* and *Navicula sp*)

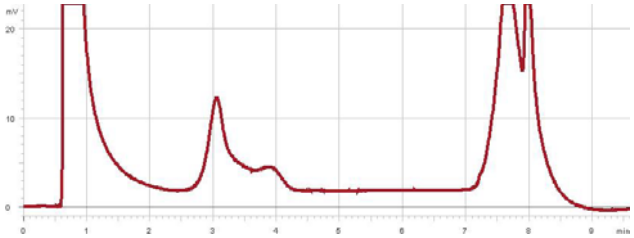

**Fig. 6**: DHPLC results of the diatoms in rabbit liver (*Melosira varians* and *Navicula sp*)

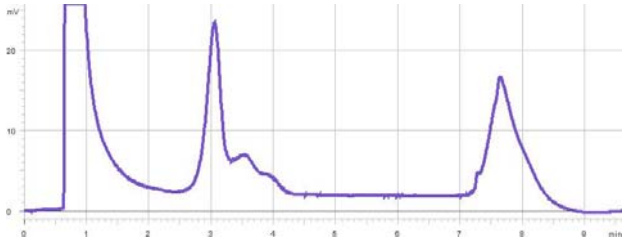

**Fig. 7**: DHPLC results of the diatoms in rabbit lung (*Melosira varians, Cyclotella meneghiniana* and *Navicula sp*)

**Table 1**: The sequences of the primers for the SSU gene of diatoms

| Primer | Sequences |
|---|---|
| Dia-516R | CTCATTCCAATTGCCAGACC |
| A145F | CCGTAGTAATTCTAGAGCTAATA |

**Table 2**: The amounts of diatom species detected in lung

| | Digestion time | | | | |
|---|---|---|---|---|---|
| | 2h | 4h | 6h | 8h | 10h |
| Rabbit 1 | 1 | 1 | 2 | 3 | 2 |
| Rabbit 2 | 1 | 2 | 2 | 2 | 3 |
| Rabbit 3 | 0 | 1 | 1 | 2 | 1 |
| Rabbit 4 | 1 | 1 | 2 | 1 | 2 |
| Rabbit 5 | 1 | 2 | 1 | 3 | 2 |
| Rabbit 6 | 2 | 1 | 2 | 1 | 1 |
| Rabbit 7 | 1 | 2 | 3 | 2 | 3 |
| Rabbit 8 | 0 | 1 | 3 | 2 | 2 |
| Rabbit 9 | 1 | 2 | 1 | 1 | 2 |
| Rabbit 10 | 0 | 1 | 2 | 2 | 1 |
| Rabbit 11 | 0 | 1 | 2 | 1 | 3 |
| Rabbit 12 | 0 | 0 | 1 | 2 | 2 |
| Rabbit 13 | 1 | 2 | 1 | 2 | 3 |
| Rabbit 14 | 0 | 1 | 2 | 3 | 2 |
| Rabbit 15 | 1 | 0 | 2 | 3 | 2 |
| Rabbit 16 | 2 | 1 | 2 | 2 | 3 |
| Rabbit 17 | 2 | 1 | 1 | 2 | 3 |
| Rabbit 18 | 1 | 1 | 2 | 3 | 3 |
| Rabbit 19 | 0 | 1 | 0 | 2 | 1 |
| Rabbit 20 | 1 | 1 | 2 | 3 | 2 |

The results were analyzed with the SPSS13.0 software, and the paired rank sum test results were as follows, R1=32, R2=32, = 37.5 R3, R4=37.5 n=20, $R_{0.05}$ =52, R1, R2, R3 < $R_{0.05}$, R4 > $R_{0.05}$. From 2 h to 8 h, the amount of diatom species detected in lung increased gradually, and it appeared to be statistically significant ($P<0.05$). From 8 h to 10 h, the fig. did not present significant increase ($P>0.05$).

## RESULTS

The amplification product size of the SSU gene is 340 bp. The specificity test results of the primers were shown in fig. 1.

The five most common diatoms in Guangzhou section of the Pearl river (*Cyclotella meneghinianas, Melosira varians, Nitzschia sp, Synedra sp, Navicula sp*) were provided by The Institute of Hydrobiology of the Chinese Academy of Sciences and the other three kinds of common algae (*Scrippsiella trochoidea, Platymonas elliptica, Prorocentrales*) were provided by Red Tide and Marine Biology Research Center, Jinan University.

**Table 3**: The amount of diatom species detected in liver

| | Digestion time | | | | |
|---|---|---|---|---|---|
| | 2h | 4h | 6h | 8h | 10h |
| Rabbit 1 | 0 | 1 | 1 | 2 | 1 |
| Rabbit 2 | 1 | 0 | 0 | 1 | 2 |
| Rabbit 3 | 0 | 0 | 1 | 2 | 1 |
| Rabbit 4 | 0 | 1 | 2 | 1 | 1 |
| Rabbit 5 | 1 | 0 | 1 | 2 | 1 |
| Rabbit 6 | 1 | 1 | 2 | 1 | 2 |
| Rabbit 7 | 1 | 2 | 1 | 1 | 2 |
| Rabbit 8 | 0 | 1 | 2 | 2 | 3 |
| Rabbit 9 | 1 | 2 | 1 | 3 | 2 |
| Rabbit 10 | 0 | 1 | 1 | 2 | 3 |
| Rabbit 11 | 0 | 0 | 1 | 0 | 1 |
| Rabbit 12 | 0 | 1 | 1 | 2 | 2 |
| Rabbit 13 | 1 | 2 | 1 | 3 | 1 |
| Rabbit 14 | 0 | 1 | 2 | 1 | 2 |
| Rabbit 15 | 0 | 0 | 1 | 0 | 0 |
| Rabbit 16 | 1 | 2 | 2 | 1 | 2 |
| Rabbit 17 | 1 | 0 | 1 | 2 | 3 |
| Rabbit 18 | 1 | 2 | 1 | 2 | 1 |
| Rabbit 19 | 0 | 0 | 2 | 1 | 3 |
| Rabbit 20 | 1 | 1 | 1 | 2 | 2 |

The results were analyzed with the SPSS13.0 software, and the paired rank sum test results were as follows, R1 =22.5,R2 =28, R3=59.5, R4=53. n=20, $R_{0.05}$ =52, R1, R2 <$R_{0.05}$, R3, R4 > $R_{0.05}$. From 2 h to 6 h, the amount of diatom species detected in liver increased gradually, and it had statistical significance ($P < 0.05$). From 6 h to 10 h, the fig. did not show significant increase ($P>0.05$).

The diatom species involved in the tables 2, 3 and 4 were the five most common diatoms in Guangzhou section of the pearl river as the entry standard.

## DISCUSSION

At present, there have been a number of reports about the diatom detection. And in practice, the acid digestion method is the most widely used and remains the mainstream (Zhao *et al*., 2013). However, the diatom detection rate of this method is comparatively low. Moreover, as the operating environment is open, corruption is involved with the whole process and it is difficult to eliminate pollution factors. Therefore, the acid digestion method is full of controversy in practice (Deqing *et al*., 2004 and Fanggang *et al*., 2006). In this study, the PCR - DHPLC method was used to detect diatom in lung, liver and kidney after enzyme digestion for different periods and assess the effect of enzyme digestion time on the detection of diatom species. Based on molecular biology method, this study is of high sensitivity and specificity for the detection of diatoms. In

this study, with the diatom genomic DNA as the template, the other three kinds of common algae (scrippsiella trochoidea and platymonas elliptica; prorocentrales) genomic DNA, human genome DNA, rabbit genomic DNA underwent PCR under the same PCR condition. The products were tested by electrophoresis. The results turned out that the diatom genomic DNA was the only one to present the positive band and others did not, which proves the primers are specific to the diatoms. Within 2 h-8 h, the amount of the diatom species detected in the lung tissue gradually rose over time and it had statistical significance ($P<0.05$). After 8 h, the amount of the diatom species identified in lung tissue showed no significant increase ($P>0.05$);.Within 2 h-6 h, the amount of the diatom species found in the liver and kidney tissues gradually increased and it had statistical significance ($P<0.05$). However, after 6 h, the amount of the diatom species found in liver and kidney tissues did not present significant growth ($P>0.05$). For the next step, we are intended to investigate more diatom species and increase the diversity of diatoms, which facilitates the replenishness the diatom species library. Furthermore, large animal experiments should be conducted in the near future and also, we would increase the material types and their quantity. Hopefully, we could simulate drowned human bodies, which would improve its application value of the investigation of drowning in forensic medicine.

**Table 4**: The amount of diatom species detected in kidney

| Digestion time | | | | | |
|---|---|---|---|---|---|
| | 2h | 4h | 6h | 8h | 10h |
| Rabbit1 | 0 | 1 | 1 | 2 | 1 |
| Rabbit2 | 0 | 0 | 1 | 1 | 2 |
| Rabbit3 | 1 | 0 | 1 | 2 | 1 |
| Rabbit4 | 1 | 1 | 2 | 1 | 2 |
| Rabbit5 | 0 | 1 | 0 | 1 | 2 |
| Rabbit6 | 1 | 0 | 2 | 1 | 3 |
| Rabbit7 | 1 | 1 | 2 | 1 | 1 |
| Rabbit8 | 0 | 0 | 1 | 2 | 3 |
| Rabbit9 | 1 | 1 | 1 | 2 | 1 |
| Rabbit10 | 0 | 1 | 2 | 1 | 2 |
| rabbit11 | 0 | 0 | 0 | 0 | 0 |
| rabbit12 | 1 | 2 | 1 | 2 | 1 |
| rabbit13 | 1 | 1 | 2 | 1 | 1 |
| rabbit14 | 0 | 1 | 1 | 1 | 2 |
| rabbit15 | 1 | 0 | 1 | 2 | 1 |
| rabbit16 | 0 | 0 | 1 | 2 | 2 |
| rabbit17 | 1 | 1 | 2 | 1 | 2 |
| rabbit18 | 1 | 0 | 1 | 2 | 1 |
| rabbit19 | 0 | 1 | 2 | 1 | 1 |
| Rabbit20 | 0 | 1 | 1 | 2 | 1 |

## CONCLUSION

PCR-DHPLC method is a new method for the diagnosis of drowning, this method has the advantages of high sensitivity and specificity; PCR-DHPLC method can separate and identify different diatom species, it can compare the diatom species in drowning organs to drowning waters to determine whether the diatom species are the same and thus it provides a better way to infer the drowning locations.

## REFERENCES


belU N and F Garcia-Pichel G (1997). Muyzer PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.,* **63**: 3327-3332.

Deqing Wu, Xiaohu Xu and Xiaojun Yu (2004). The present situation of the diatom inspection. *Chinese Journal of Forensic Medicine,* **19**(supplement): 13-15.

EPP LS, Stoof-Leichsenring KR, MH Trauth and Tiedemann R (2011). Molecular profiling of diatom assemblages in tropical lake sediments using taxon-specific PCR and denaturing high-performance liquid chromatography (PCR-DHPLC). *Mol. Ecol. Res.*, **5**(11): 842-853.

Fanggang He, Daixin Huang and Liang Liu, etc (2008). The PCR - DGGE method to detect 16 s rDNA of the plankton in the identification of drowning. *Chinese Journal of Forensic Medicine,* **23**(4): 234-237.

Fanggang He, Liang Liu and Daixin Huang, etc (2006). The application value of the detection of Plankton 16 s rDNA in the identification of drowned rats. *Chinese Journal of Forensic Medicine,* **21**(6): 331- 333.

Guanying He, Weidong Zhang and Fei Li, etc (2003). The Enzyme digestion method to inspect diatom in organs. *Chinese Journal of Forensic Medicine,* **18**(3): 171-173.

Laura S. Epp; Kathleen R. Stoof-Leichsenring;Martin H. Trauth and Ralph Tiedemann (2011). Molecular profiling of diatom assemblages in tropical lake sediments using taxon-specific PCR and Denaturing High-Performance Liquid Chromatography (PCR-DHPLC) [J], *Mol. Ecol. Resour.*,**5**(11): 842-853.

Shuling Ma, Fan Li, Haojie Qin and Yaonan Mo (2006). The analysis of enzymatic detection of diatom in 59 cases. *Journal of Henan University of Science and Technology*, **24** (1): 60-61.

Sunlin Hu (2009). Study on the applications of two micro-beam analysis techniques in the diagnosis of drowning. Sun yat-sen university, Ph.D. Thesis, p.6.

Ulrike Scharrer, Marzena Skrzypczak-Zielinska, Weronika Wituszynska, Marek Mierzejewski, Kristin Krause, Cezary Cybulski and Ursula G Froster (2010). A simple method of investigating mutations in CHEK2 by DHPLC: A study of the German populations of Saxony, Saxony-Anhalt and Thuringia. *Cancer Genetics and Cytogenetics*, **199**(1): 48-52.

Xuewei Zhang (2007). The inspection of the PINK1 gene mutations with DHPLC technology. Central south university master's degree thesis.

Zhao J, Liu C, Hu S, He S and Lu S (2013). Microwave digestion-vacuum filtration-automated scanning electron microscopy as a sensitive method for forensic diatom test. *Int. J. Legal Med.,* **127**(2): 459-463.