A Simple Method for Determination of the Unsaturated Fatty Acids Concentration in the Fish and Shellfish by Hemoglobin Denaturation

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Summary

To develop a simple and rapid method for the determination of unsaturated fatty acid (USFA) in lipophilic fraction of marine organisms such as the fish and shellfish, a method measuring the change of oxyhemoglobin into methemoglobin by USFA, when added to blood, was established. The method measuring the formation of methemoglobin at 630nm, as an indicator of EPA concentration, was better in linearity of dose- and time-dependency than that measuring the decrease of oxyhemoglobin at 540nm or 578nm. As a result of the determination of EPA and LNA concentrations using dual wavelength spectrophotometry between 700nm as λ_1 and 630nm as λ_2, significant correlations were observed between concentration of USFA and synthesis ratio of methemoglobin up to the concentration at 5.0 mM. The correlation between the synthesis ratio of methemoglobin as an indicator of USFA concentrations and the toxicity obtained by the mouse bioassay was significant ($P<0.05$) and the coefficient of correlation was 0.876.

From those results, it is considered that this method, using the change of hemoglobin spectrum by the result of a reaction taken place by USFA but not by the diarrhetic shellfish toxin derived from planktons, is simple and rapid for determination of USFA abundantly contained in lipophilic fraction of marine organisms.

Introduction

The toxin in the lipophilic fraction from bivalves, is called diarrhetic shellfish toxin by the manifesting clinical symptoms of intoxicated humans. The toxin is measured by a bioassay to observe the killing effect on mice given by intraperitoneal injection. The presence of toxin has been monitored based on the standard method[1] for detection of the toxin from bivalves in several areas with occasional occurrence of toxic planktons in Japan. The method[1] has been used mainly for the detection of toxins such as okadaic acid (OA) and dinophysis toxin (DTX) which was isolated and purified from the toxic plankton by Yasumoto *et al.*[2]

It has been reported[3] that toxic samples which killed mice were occasionally found even at areas in absence of causative toxic planktons while monitoring bivalves using this method. Thus, so far as the assay of diarrhetic shellfish toxin was performed according to the bioassay with lethal toxicty to mice, any lipophilic toxins other than well-known toxins such as OA or DTXs should be considered.

We also observed the toxicity according to bioassay with mice in lipophilic fraction of bivalves[4] independently from the appearance of toxic planktons and in that of some kinds of fishes[5]. In regard to unknown substances, we[4] suggested that acidic substance other than OA and DTXs in marine organisms was thought to be one of the toxins. It was clarified in the previous paper[6] that unsaturated fatty acid (USFA) affected blood hemoglobin in converting oxyhemoglobin (O_2Hb) into methemoglobin (MetHb). The same hemoglobin change was observed in lipophilic fraction of toxic

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bivalves and several fishes[5]. Recently, Takagi *et al*[7] suggested that USFA in marine organisms was one of marine toxins from their experiments, in which major toxic component in digestive gland of poisonous scallop was found to be the fraction of polyenoic free fatty acids and highly unsaturated fatty acids such as eicosapentaenoic acid since they showed higher toxicity to mice.

Therefore, evaluation of USFA as a lethal toxin to mice becomes necessary, independently from the studies on toxins derived from planktons. For determination of USFA in marine organisms, development of a rapid and simple method was attempted in the present study. For measuring the total USFA in the lipophilic fraction of marine organisms, a method using hemoglobin spectrum changed by USFA was investigated using a dual wavelength spectrophotometer but without help of sophisticated instruments, such as a gas chromatography (GC) or a high performance liquid chromatography (HPLC).

Materials and Methods

Fresh bivalves with 0.4MU／g toxicity and fresh and dry fish viscera with various toxicity were used. Bivalves were collected along the Pacific coast in Chiba Prefecture as previously reported[4]. Fishes were purchased from markets.

Eicosapentaenoic acid (EPA ; $C_{20:5}$) and linolenic acid (LNA ; $C_{18:3}$) were purchased from Sigma Co.Ltd.

Red blood cells (RBC ; from kohjin Co.Ltd) were used after rinsing twice or three times with 0.01M phosphate buffered saline at pH7.4 (PBS). Then, they were suspended in the same buffer at 2％ (v／v).

Dual wavelength spectrophotometry (DWLS) was performed by Hitachi model 557 to determine the concentrations of oxyhemoglobin (O_2Hb) and methemoglobin (MetHb) from the spectrum.

Results

1) Changes of the spectrum of hemoglobin by lipophilic fraction of the fish and shellfish

Ether fractions of bivalves with 0.4MU／g and fish with various toxicities (1.0, 0.25 and <0.25 MU／g) were prepared according to the standard method[1] for diarrhetic shellfish toxin, and dissolved in PBS containing 1％ Tween 60 to make equivalents of 20 g／ml for bivalves and 4 g／ml for fish, respectively. After 0.1ml of them was added individually to 2 ml of 2％ RBC suspension, the mixture was stood at room temperature. Aliquots of RBC suspension were taken out 1 h after addition of the fraction, and they were hemolysed in seven fold volume of distilled water. A photometric spectrum of hemoglobin was immediately drawn at a range from 700nm to 450nm by scanning with DWLS.

Hemoglobin spectra were drawn 1 h after addition of ether extract from the fish and shellfish with various toxicities to 2％ RBC suspension (Fig. 1). The decrease of O_2Hb peaks at 540nm and 578nm was more remarkably observed than increase of MetHb peaks at 500 and 630nm. Though a sample of 0.25MU／g toxicity had a small peak around 675nm, it gave little influence on reading the optical density (OD) at 700nm.

2) Spectrum change of hemoglobin after addition of EPA

One half mg of EPA dissolved in 0.2ml of methanol was added to 2 ml of 2％ RBC suspension and the mixture was stood at room temperature. One half ml of the suspension was taken out 0.5, 1, 2, and 3 h after addition of EPA, and hemolysed by mixing in 3 ml distilled water. The hem-

olysate was immediately scanned by DWLS (model 557) in the same manner as mentioned above. Two ml of the RBC suspension added with 0.2ml of methanol was prepared as a control.

The change of spectra of hemoglobin along the time course after addition of 0.5mg of EPA to blood suspension is shown in Fig. 2. Along the time courese after adding EPA, two peaks of O_2Hb at 540nm and 578nm were decreasing and two peaks of MetHb at 500nm and 630nm were increasing.

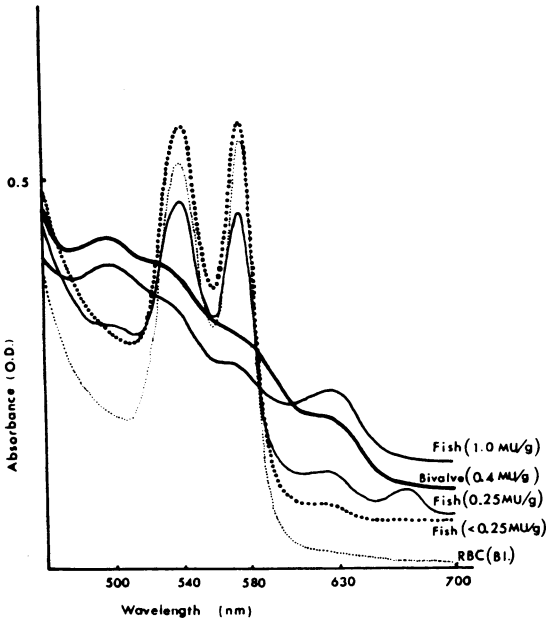

Fig. 1 Changes of hemoglobin spectra of RBC 1 h after addition of ether extracts from three fish viscera having 1.0, 0.25 and <0.25MU/g and bivalves having 0.4MU/g toxicities.

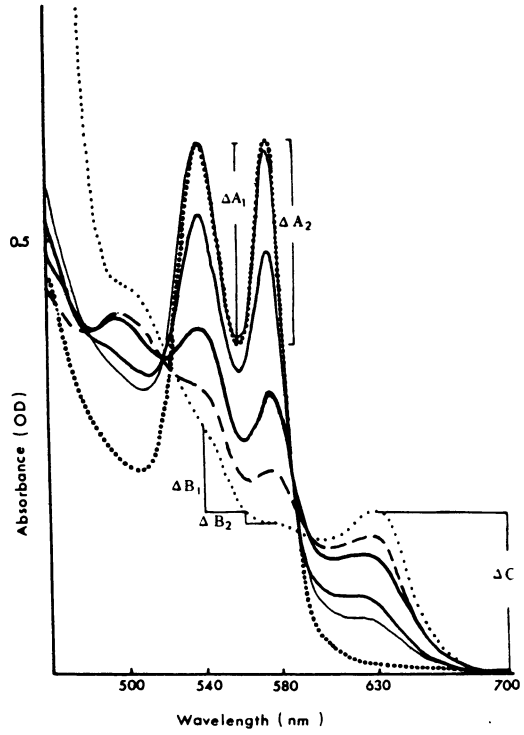

Fig. 2 Changes of hemoglobin spectra of RBC 0.5h(——), 1 h (——), 2 h (——) and 3 h (– –) after additi on of 0.5mg EPA in 2 ml of 2% RBC. Broken lines represent the O_2Hb spectrum of blank RBC (•••) and the MetHb spectrum after the addition of potassium ferricyanide to RBC (· · ·).

3) Method for determination of USFA concentration by the change of hemoglobin spectrum

To select the best photometric method for determination of EPA concentration, three methods to measure O_2Hb or MetHb were examined, namely, A) Measuring the decrease of O_2Hb at 540nm, B) Measuring the decrease of O_2Hb at 578nm, and C) Measuring the increase of MetHb at 630nm. Absorbance of each peak of the above 3 was calculated by the dual wavelength spectrophotometry[8] by the difference of absorbance between 540nm and 560nm for the peak at 540nm, by that between 578nm and 560nm for the peak at 578nm, and by that between 630nm and 700nm for the peak at 630nm, respectively. In order to change 100% O_2Hb of hemolysate to MetHb, 20mg of potassium ferricyanide, a famous agent converting O_2Hb to MetHb[9], was added to control RBC hemolysate. To examine the reliability of the method to measure the amount of USFA, the linearity of LNA concentration determined by this method was also examined besides EPA.

In regular method to measure O_2Hb concentration by absorbance at 540nm, the difference of abs-

orbance between 540nm and 560nm is utilized for DWLS according to the spectrum in Fig. 2. However, in the spectrum of MetHb in the same figure, the curve is too steep at this area and a big difference of ΔB_1 can not be disregarded. Therefore, correction was made to ΔA_1 by subtracting ΔB_1. The ratio of remaining O_2Hb after addition of EPA is calculated by the formula, (sample ΔA_1 - MetHbΔB_1)／($O_2Hb\Delta A_1$ - MetHb ΔB_1), where $O_2Hb\Delta A_1$ and MetHbΔB_1 are constants under this experimental condition.

Likewise, absorbance of O_2Hb at 578nm was measured by the difference of absorbance between 578nm and 560nm by DWLS. The remaining ratio of O_2Hb is calculated by the same manner, (sampleΔA_2 - MetHbΔB_2)／($O_2Hb\Delta A_2$ - MetHbΔB_2), where O_2Hb ΔA_2 and MetHb ΔB_2 are constants.

On the other hand, for measuring the amount of MetHb, the difference of absorbance between 630nm and 700nm was used in the same way (Fig. 2). The synthesis ratio of MetHb was calculated simply by the formula, sample ΔC／MetHbΔC. Various concentrations of EPA were added to RBC suspension, and synthesis ratio of MetHb and remaining ratio of O_2Hb were measured along the time course by the 3 methods mentioned above. Results are illustrated in Fig. 3. The method to determine EPA by using the increase of MetHb was considered to be best among 3 from the view points of linearity of dose-dependency between 0.5h and 2h after addition of EPA and up to the maximal concentration at 1.4mM EPA. On the contrary, the determination of EPA concentration using the decrease of O_2Hb was of little advantage by absence of linear dose and time dependency.

The relationships between synthesis ratios of MetHb (Y) by the reaction for 1h and concentrations (X) of EPA or LNA are shown in Fig. 4. The regression equations and coefficients of correlation were $Y=0.42+0.45\ \log_{10}X$; $r=0.946$ for EPA, and $Y=0.43+0.6\ \log_{10}X$; $r=0.995$ for LNA, respectively, and the significant correlations were observed.

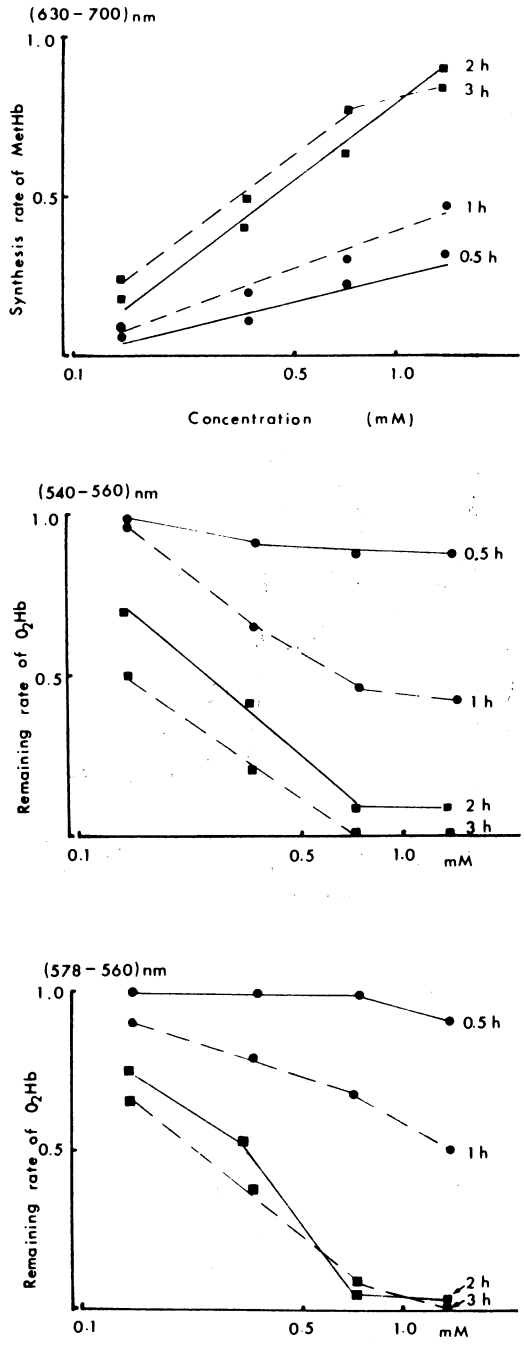

Fig. 3　Changes of hemoglobin after addition of various concentrations of EPA to RBC at different reaction times. Figures represent the change of synthesis ratio of MetHb measured at 630 and 700nm (upper) and the change of remaining ratios of O_2Hb measured at 540 and 560nm (middle) and at 578 and 560nm (lower) by DWLS.

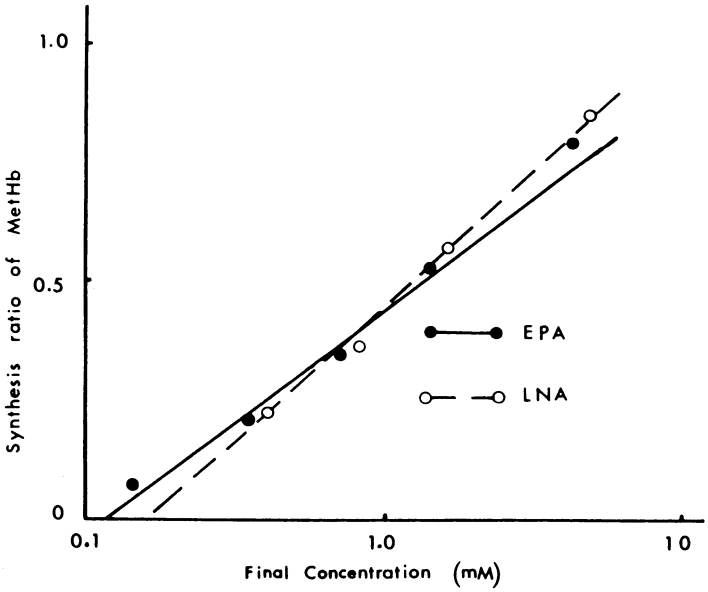

Fig. 4 Correlation between added EPA or LNA concentration (mM) and synthesis ratio of MetHb 1 h after addition of USFA.
Relationships are as follows ;
EPA (MetHb) $=0.45 \log_{10}$EPA (mM) $+0.42$, $r=0.946$
LNA (MetHb) $=0.60 \log_{10}$LNA (mM) $+0.43$, $r=0.995$

4) Effect of methanol as an emulsifier of USFA on the determination of USFA concentrations

In the present study, methanol was used to improve the solubility of USFA in RBC suspension. In order to select the optimum concentration of methanol, methanol was added to 2 ml RBC suspension containing 0.5, 1 and 3 mg of LNA to make the final methanol concentrations at 5, 9, 20 and 33%. RBC was adjusted to 1.8% as the final concentration in each reaction mixture. One half ml of the mixture was taken out at different intervals (0.5, 1, 2, 3 h) and hemolyzed by adding six fold volume of distilled water. Effect of methanol on hemoglobin change was measured by synthesis ratio of MetHb in the reaction mixture against each blank with the same dose of PBS (5, 9, 20 and 33%) added in place of methanol.

As shown in Fig. 5, a significant suppressive effects on MetHb synthesis were induced in 0.5 and 1.0mg LNA when methanol was added at 33%. At 0.5mg of LNA, even 20% of methanol showed the suppressive effect during the whole experimental period. Therefore, it was determined that 9 % was the permissible maximal concentration of methanol to be added as an emulsifier of USFA.

5) Correlation between the concentration of USFA in ether fraction of fish viscera and the degree of toxicity shown by mouse bioassay

Using 16 fresh samples of fish viscera and 2 dry samples, the correlation between the synthesis ratio of MetHb as the indicator of concentration of USFA and the toxicity shown by mouse bioassay was studied. The correlation between the synthesis ratio of MetHb after 1 h and the toxic value according to the mouse bioassay for the above 18 samples was examined. As in Fig. 6, a significant positive correlation was found with the correlation coefficient of $r=0.876$.

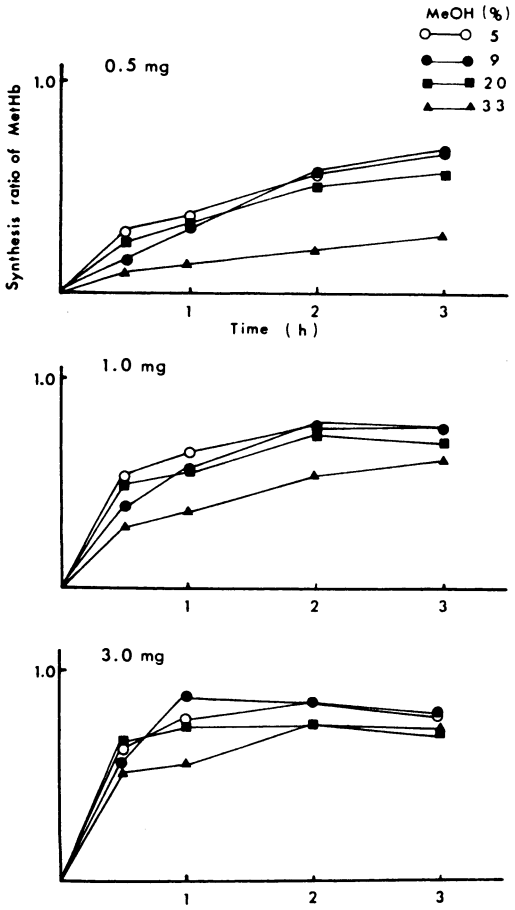

Fig. 5　Effect of methanol as emulsifier on synthesis ratio of MetHb at various concentrations of LNA (0.5, 1.0 and 3.0mg)

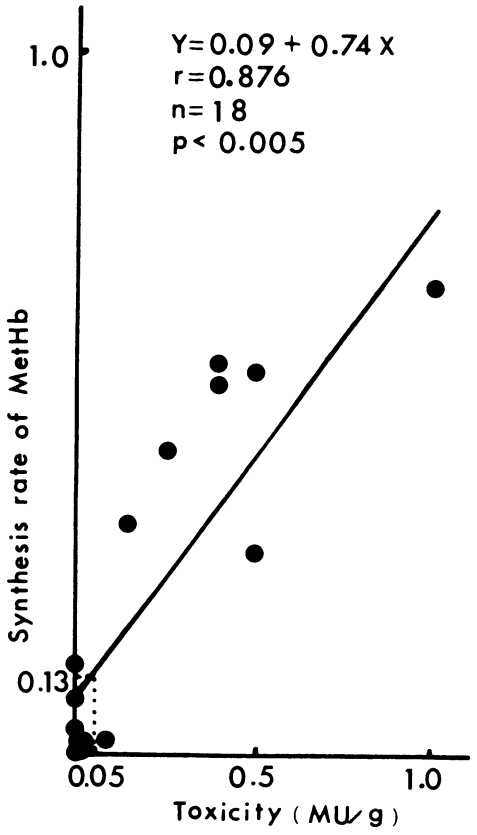

Fig. 6　Correlations between synthesis ratio of MetHb 1 h after addition of ether extracts from fish viscera and toxicity of the extracts according to the mouse bioassay.

Discussion

Since the standard method[1] adopted today for testing the toxicity of bivalves is primarily the one for testing all toxic substances in lipophilic fraction of bivales, there is a possibility of containing the toxic substances other than the known toxins derived from planktons such as DTX or OA.

It is considered that USFA which shows toxicity to mice and is contained at high percentage in marine organisms should not be ignored as one of the other toxic substances mentioned above. Therefore, in the present study, a new method was established to detect USFA by using their action to convert O_2Hb to MetHb which were measured by DWLS.

In the converting reaction of hemoglobim by USFA, selection of appropriate reaction time was considered to be important for the accurate assay of USFA. When the reaction time was more than 2h after addition of the sample, linearity of dose dependency was not sustained at the concentrations above 1.4mM of EPA because MetHb synthesis reached to the maximal equilibrium. Crude lipid concentrations in fish and shellfish used in this experiment varied from 1 to 5 % in wet weight. Generally, average EPA concentration of fish and shellfish was known about 16% of total fatty acid.[10] From those figures, the EPA concentration of fish and shellfish added to blood suspension appeared to be in a range of 0.9−4.5mM. By this range of EPA concentrations, it was concl-

uded that the determination of EPA had to be carried out within 1 h after addition of samples to RBC. In this assay system, O_2Hb should be stable during the reaction period. This was proven in our study that oxyhemoglobin did not change for 24 hrs.

Fortunately, in this method, a linear relationship was obtained between synthesis ratio of MetHb and concentrations of EPA and LNA up to the concentration of 5 mM as shown in Fig. 4. It is clear that the degree of hemoglobin change is varied more or less depending on the characteristics of USFA, especially on the number of double bond.[6] Since EPA and LNA are at average degree of unsaturation among various USFA, it seemed to be possible to expand the results with EPA or LNA to the determination of total USFA quantitatively.

As it was clarified in our previous work[3] that OA had no effect on blood hemoglobin at toxicity less than 1.0MU, this method for determination of USFA concentrations using the effect of hemoglobin change seems to be significant to measure the toxin independently of the well-known toxins such as OA and DTX.

There was a high correlation ($r=0.876$) between synthesis ratio of MetHb and toxic values of 18 fish viscera (Fig. 6). For example, when 0.05MU／g, the permissible minimal concentration of diarrhetic shellfish toxin[11], is corresponding to 0.13 in the synthesis ratio of MetHb from Fig. 6. If 0.13 is referred in the Fig. 4, it corresponds to 0.23mM of USFA or EPA equivalent. From this calculation, USFA is formed to be an important causative substance from marine organisms to give lethal effects in the mouse bioassay when synthesis ratio of MetHb was above 0.13.

In conclusion, the rapid and simple method established in the present study for determination of USFA concentrations using the change of hemoglobin spectrum will be helpful for estimating the causative substance in the toxic marine organisms in which USFA are abundantly contained.

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ヘモグロビン変性を用いた魚貝類中の不飽和脂肪酸の簡易測定法

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抄録

不飽和脂肪酸によるオキシヘモグロビンからメトヘモグロビンへの変性を用いた魚貝類脂溶性分画における不飽和脂肪酸の簡易測定法について検討した。

その結果，630nmにおけるメトヘモグロビンの生成を測定する方法が，540nm，578nmにおけるオキシヘモグロビンの減少を測定する方法に比べ，量依存性，時間依存性ともにすぐれていた。

エイコサペンタエン酸（EPA），リノレン酸（LNA）を用いた測定では，5 mMまで濃度とメトヘモグロビン生成率との間に有意な相関関係が認められた。

魚の内臓を用い，マウス毒性値とメトヘモグロビン生成率の相関関係を調べた結果，相関係数0.876と良好であった（$P<0.05$）