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# Quantitative analysis of monofluoroacetate in biological samples by high-performance liquid chromatography using fluorescence labeling with 9-chloromethylanthracene

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**Abstract**

A rapid and sensitive RP-HPLC method with fluorescence detection has been developed for the quantitative analysis of trace amounts of monofluoroacetate (MFA) in biological samples as serum, food and meat. 9-Chloromethylanthracene (9-CMA) is used as the fluorescence labeling reagent. Samples were extracted and reacted with 9-chloromethylanthracene together with tetrabutylammonium bromide as catalyst at 80 °C for 50 min to give a new fluorescent derivative as 9-methyleneanthracene monofluoroacetate (MA-MFA). The resulting MA-MFA was characterized with IR, $^{1}$H NMR, $^{13}$C NMR and MS. Chromatography separation is performed on an Agilent Hypersil ODS column with a fluorescent detector employed with the excitation and emission wavelengths as 256 nm and 412 nm, respectively. Optimal conditions for derivatization, fluorescence detection and chromatographic separation have been established. The novel method yields a good linear relationship when the MFA concentration in serum within 1 and 250 ng/mL ($r = 0.9988$). The detection limit (signal-to-noise ratio = 3 with 2 μL injected) was 0.25 ng/mL. The practical applicability of this method was demonstrated by quantitative determination of MFA-Na in a blood sample from a person who had ingested the poison.


*Keywords:* Monofluoroacetate (MFA); 9-Chloromethylanthracene (9-CMA); 9-Methyleneanthracene monofluoroacetate (MA-MFA); High-performance liquid chromatography

## 1. Introduction

Sodium monofluoroacetate (MFA-Na), commonly known as 1080, is a highly toxic compound. Monofluoroacetate (MFA) was incorporated into the citric acid cycle to form fluoro-citric acid, which in turn inhibited the citric acid metabolizing enzyme aconitase. MFA-Na was initially developed for use as a rodenticide in the 1940s. Now it is widely used around the world as a vertebrate pest control agent [1]. MFA is also a toxic component in many poisonous plants native to Brazil [2], South and West Africa and Australia [3,4]. $LD_{50}$ values for MFA-Na are extremely low, ranging from 6 μg/kg to 100 μg/kg for canine and murine animals, and to 714 μg/kg for human beings [5].

Monofluoroacetate, together with its sodium salt and its derivative fluoroacetamide were widely used as a rodenticide. Unfortunately, as they are cheap and accessible, these fluoroacetate derivatives were often used in criminal poisoning cases [6,7]. Because of the potent toxicity of MFA-Na to the non-target subjects, the possibility of secondary poisoning is high. Therefore, there is a need to develop a sensitive method for quantitative determination of MFA-Na and MFA in biological samples.

The common method for determination of MFA-Na is liquid–liquid extraction followed by derivatization and gas chromatography analysis. This method has been adopted by the US Environmental Protection Agency (EPA) [8]. Since MFA-Na is non-volatile and has no chromophore, it is often modified before analysis. Recently, a great deal of emphasis has been placed on the development of an effective, sensitive and reliable analytical method for the quantitative determination of trace levels of MFA-Na in biological samples. For example, Guan et al. [9]

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analyzed MFA-Na using capillary zone electrophoresis with a detection limit of 0.4 μg/mL. Baron et al. [10] used $^{19}$F NMR for detection and measurement of MFA-Na with a detection limit of 4 μg/g. A headspace gas chromatography method was developed for the determination of fluoroacetate in aqueous samples with a detection limit of 0.5 μg (s/n = 14) [11]. Yang et al. [12] used ion-chromatography to quantitatively analyze MFA-Na in human serum and food samples with a detection limit of 0.1 μg/mL. These methods are convenient but have poor sensitivities. Kimball and Mishalanie [13] had developed the determination of MFA-Na as free acid by GC/MS with the detection limit of 200 pg, but the highly acidic sample had special requirement of the capillary column.

Before chromatographic analysis, MFA-Na has often been converted to various derivatives with appropriate chromatographic properties. For instance, Ozawa and Tsukioka [14] successfully used an anion exchange resin (Dowex 1-X8) to isolate MFA-Na from biological samples or soils and then detected MFA-Na by GC after derivatizing with 2,4-dichloroaniline in the presence of *N*,*N*′-dicyclohexylcarbodiimide. Allender [15] derivatized MFA-Na with pentafluorobenzyl bromide to form an ester followed by GC detection. Shi et al. [16] synthesized 4-diethylamino monofluoroacetanilide from MFA-Na for GC analysis. However, the complicated chromatogram and the poor selective detector make it difficult to differentiate the peak of MFA derivative from other impurity.

High-performance liquid chromatography (HPLC), one of the most effective separation tools, has widely been used for the analysis of variety samples. Due to the low level of samples, fluorescence labeling has attracted a great deal of interest because it is a way to achieve high sensitivity. Moreover, fluorescence labeling can achieve a high degree of selectivity because specific fluorescence detection can efficiently eliminate interferences. The anthracene nucleus is stronglyluorescent. It has been modified with various reactive functional groups to form analytically useful fluorescence labeling agents, such as 9-chloromethylanthracene (9-CMA), which is a reactive nucleophilic agent with strong fluorescence properties.

The purpose of this paper is to develop a sensitive method for the quantitative determination of MFA-Na in biological samples as serum, rice and meat. 9-CMA was used as a derivatization reagent to convert MFA-Na to a new fluorescent derivative, 9-methyleneanthracene monofluoroacetate (MA-MFA), which was then separated by high-performance liquid chromatography and detected by fluorescent detector with better sensitivity and selectivity.

## 2. Experimental

### 2.1. Materials and reagents

9-Chloromethylanthracene obtained from Merck (Darmstadt, Germany) was prepared at a concentration of 10 mM in acetonitrile. Tetrabutylammonium bromide and triethanolamine were purchased from Aldrich (St. Louis, USA). 10% tetrabutylammonium bromide solution in acetonitrile and 10% triethanolamine solution in acetone were made. These solutions were stored at 4 °C in refrigerator before use. MFA-Na (content > 98%) and human serum containing MFA-Na from poisoned individuals were friendly obtained from the Public Security Bureau of Guangdong Province, China. Blank serum, rice and meat were also obtained. Water was purified with a Millipore Milli-Q system. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). All HPLC buffers were filtered through 0.45 μm Millipore filters prior to use. Acetone and all other reagents and organic solvents were of analytical grade purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China).

### 2.2. Synthesis and characterization of 9-methyleneanthracene monofluoroacetate

The fluorescent derivative of MFA-Na, 9-methyleneanthracene monofluoroacetate was prepared and confirmed as followed.

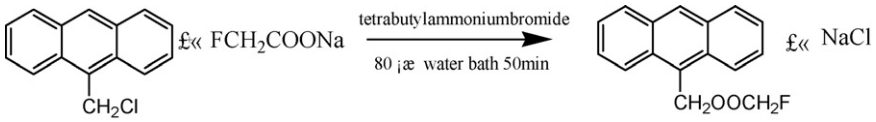
9-Chloromethylanthracene (0.224 g, $1 \times 10^{-3}$ mol) was dissolved in 25 mL acetonitrile and reacted with three molar excess of MFA-Na (0.300 g, $3 \times 10^{-3}$ mol) in the presence of phase transfer catalyst tetrabutylammonium bromide in a water bath at 80 °C for 50 min in the dark. The excessive amount of MFA-Na was removed by filtration and the solvent was evaporated under reduced pressure. The residue was washed with 20 mL water, dried, and recrystallized from hexane once before structure confirmation with IR, GC/MS, $^{13}$C NMR and $^{1}$H NMR.

Infrared spectra were recorded in KBr pellets on an EQUINOX 55 (Bruker, USA) infrared spectrophotometer. $^{1}$H NMR and $^{13}$C NMR were recorded on a nuclear magnetic resonance spectrometer (Mercury–Plus 300 MHZ, Varian) using $CDCl_3$ as solvent with TMS as internal standard. Mass spectrometry was obtained on an Agilent 6890/5973 N gas chromatograph/mass spectrometer with HP-5 (30 m × 320 μm I.D., 0.25 μm) capillary column.

UV absorbance spectra were obtained with a Shimadzu UV-3150 UV-Vis-NIR spectrophotometer (Shimadzu, Japan). Fluorescence excitation and emission spectra were obtained with a RF-5301PC spectrophotometer (Shimadzu, Japan). The fluorescence quantum yield was determined at 25 °C as described by Bella et al. [17] using quinine sulfate as a standard ($\varphi_f = 0.545$ in 0.1 M $H_2SO_4$).

### 2.3. Sample pretreatment

Biological samples as rice and meat were homogenized by a blender. To a 200 μL serum sample or 0.5 g homogenized sample, 500 μL water/acetone (2:8, v/v) was added. It was extracted by ultrasonic for 5 min and centrifuged at 4000 r/min for 5 min at 25 °C to remove proteins. The extract was collected. The extraction process was repeated three times and to the pooled extract 20 μL of 10% triethanolamine solution was added to basify the solution and was evaporated to dryness at 40 °C under nitrogen. And the sample was ready for the derivatization procedure. Blank samples were treated as described above.




Scheme 1. Derivatization reaction of MFA-Na with 9-chloromethyl anthracene.

### 2.4. Derivatization procedure

9-CMA solution (100 μL, 10 mM) and 10% tetrabutylammonium bromide solution (10 μL) with 500 μL acetonitrile were added to the sample after the sample pretreatment. The mixture was shaken thoroughly and allowed to stand for 50 min in water bath at 80 °C in the dark. The reaction was shown in Scheme 1. The derivatization solution was diluted to 1000 μL with acetonitrile and was ready for HPLC analysis. Triplicate injections of 2 μL for each sample were made. The concentration of MFA was calculated from the calibration curve.

### 2.5. Preparation of serum calibration samples

A standard solution of MFA-Na at the concentration of 1 μg/mL was prepared by dissolving 0.01 mL of 1 mg/mL MFA-Na solution in 10 mL distilled water. Human serum without MFA-Na was diluted five-fold with distilled water and was used as blank. Various amounts of MFA-Na standard solution were spiked into 200 μL blank sample with the final MFA-Na concentrations of 0, 1, 5, 25, 100, 200, and 250 ng/mL, respectively. Sample preparation were repeated the step as described in Sections 2.3 and 2.4. The linear calibration graph was plotted by peak area of MA-MFA versus the concentration of MFA-Na. The slope, intercept and correlation coefficient ($r$) were calculated using Origin software. The detection limit was defined as the amount that could be detected with a signal-to-noise ratio of three.

### 2.6. Instrument and chromatographic conditions

Chromatographic analyses were performed on an HP 1100 series (USA) HPLC system. It consisted of a vacuum degasser (Model G1322A), a quaternary pump (Model G1311A), a thermo-controlled column compartment (Model G1316A), and a fluorescence detector (FLD) (Model G1321A). An Agilent Hypersil ODS column (5 μm, 250 mm × 4.6 mm I.D.) was used. The chromatographic data were collected and processed by the HP Chemstation software. Acetonitrile and water (85:15, v/v) were employed as the mobile phase and the flow rate was 0.5 mL/min. The column temperature was set at 26 °C and the inject volume was 2 μL. The fluorescence excitation and emission wavelengths were set at $\lambda_{ex}$ 256 nm and $\lambda_{em}$ 412 nm, respectively.

## 3. Results and discussion

### 3.1. Characterization of MA-MFA

MA-MFA is a new fluorescent derivative of MFA-Na. It was in the form of bright yellow crystals with mp 137–139 °C. Gas chromatography showed that the estimated purity was more than 98%. The structure of MA-MFA was confirmed with IR, MS, $^{13}$C NMR and $^{1}$H NMR.

The molecular weight of MA-MFA is 268. Its mass spectrum contained a large characteristic molecular ion at $m/z$ 268 ($M^+$) and other fragment ions of $m/z$ 235($M^+ - FCH_2$), $m/z$ 191($M^+ - FCH_2COO$), $m/z$ 178(anthrancene ion), $m/z$ 61$(FCH_2CO)^+$ and $m/z$ 33$(FCH_2)^+$. The IR spectrum showed absorption peaks for ester carbonyl group (1 756, 1 240, 1 085 $cm^{-1}$), anthrancene ring absorption signals (3 044, 1 624, 1 525 $cm^{-1}$), and the $FCH_2$ signal (732.8 $cm^{-1}$). The $^{13}$C NMR spectrum showed a peak for carbonyl group (δ168 ppm), anthrancene ring carbons ($\delta$ 123.8, 125.1, 125.4, 127.1, 129.4, 129.9, 131.3, 131.5 ppm), the $FCH_2$ carbon ($\delta$ 76.6, 79.0 ppm). Coupling of the C atom with the F atom caused the $^{13}$C NMR signal of the $FCH_2$ carbon to form a double peak. The $^{1}$H NMR spectrum showed typical signals of anthrancene ring hydrogen at $\delta$ 7.25, 7.56, 7.59, 8.01, 8.30, 8.51 ppm, the $CH_2$ hydrogen at $\delta$ 6.30 ppm, and the $CH_2F$ hydrogen at $\delta$ 4.78, 4.93 ppm. All these data are consistent with the structure of the target compound as 9-methyleneanthracene monofluoroacetate.

### 3.2. Fluorescence characteristics of MA-MFA

9-Methyleneanthracene monofluoroacetate was dissolved in solution contained 85% acetonitrile and 15% water, and its fluorescent properties were studied. The maximum absorption wavelengths ($\lambda_{ex}$) and the maximum emission wavelengths ($\lambda_{em}$) were determined at 256 nm and 412 nm, respectively (Fig. 1). Its molar absorption coefficient ($\varepsilon$) was calculated as $2.23 \times 10^5$ L/mol cm and the fluorescence quantum yield ($\Phi$) was 0.703, referred to quinine sulfate as the standard.

### 3.3. Optimization of derivatization reaction conditions

The formation of MA-MFA indicated that 9-chloromethylanthracene could be a useful labeling agent for the determination of MFA-Na. Prior to develop a separation procedure for the analysis,factors that could affect the derivatization yields of trace amounts of MFA-Na were investigated. The derivatization of MFA-Na with 9-chloromethyl anthracene was carefully optimized with respect to reagent ratios, derivatization temperatures and reaction times.

First, the effects of 9-chloromethylanthracene concentration on the derivatization reaction were studied. To $1 \times 10^{-9}$ mol of MFA-Na, 1-, 2-, 3-, 4-, and 5-fold excess of 9-chloromethylanthracene were added. The derivatization reaction and HPLC analysis were carried out according to the methods described in the Section 2. The result (Fig. 2) showed that the yield reached maximum at the ratio of 1:3 (MFA-Na versus 9-

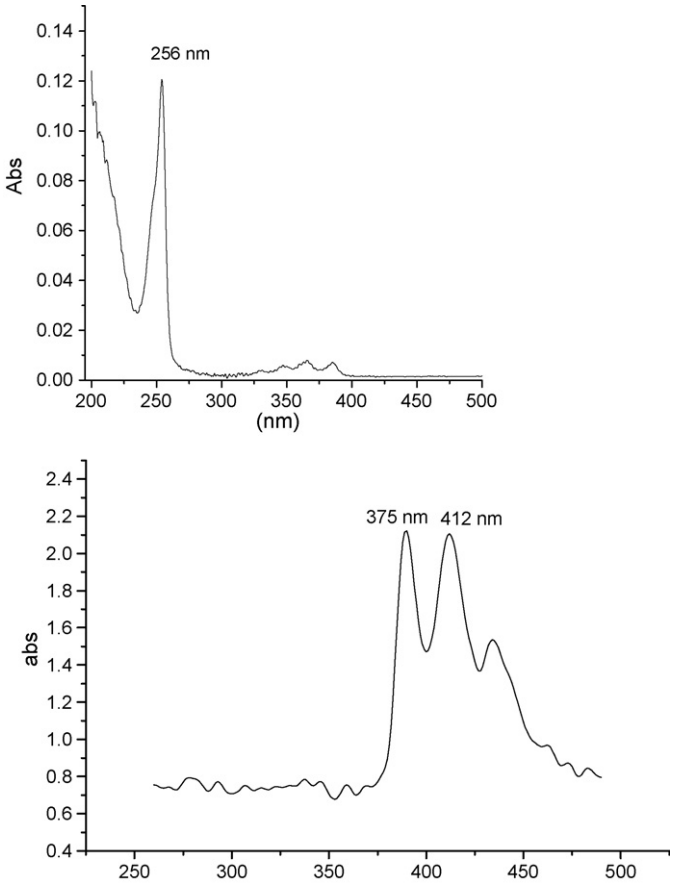



Fig. 1. Absorption and emission spectra of MA-MFA dissolved in acetonitrile.

chloromethylanthracene) and reached a plateau with increasing amounts of 9-chloromethylanthracene. Therefore, the minimum amount of 9-chloromethylanthracene required is three times the concentration of MFA-Na. However, in order to keep safe excess amounts of labeling agent, no less than five-fold excess of 9-chloromethylanthracene was used in this study.

The effects of temperature were also studied. Samples with an MFA-Na versus 9-chloromethylanthracene ratio of 1:5 were heated at 60, 70, 80, 90, 100 °C for 50 min. The HPLC peak areas of the product at different temperatures are shown in Fig. 3.

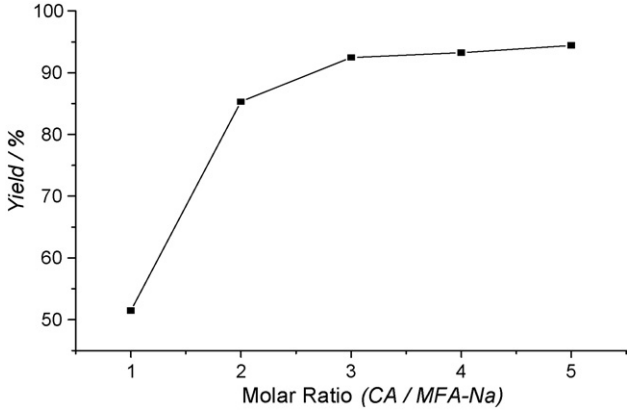



Fig. 2. Reaction yields vs. the ratios of 9-chloromethyl anthracene (CA) vs. MFA-Na.

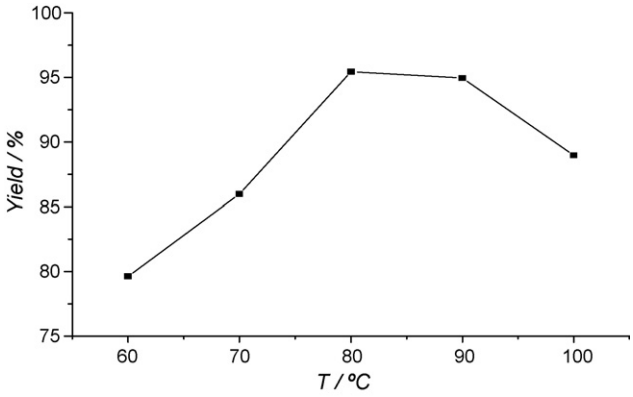



Fig. 3. Effects of reaction temperature on the yields of MA-MFA.

This result indicated that the optimal reaction temperature was 80 °C.

The reaction time was then optimized. At a reaction temperature of 80 °C and an MFA-Na versus 9-chloromethylanthracene ratio of 1:5, the maximum yield of MA-MFA was reached after 40 min. The yield remained constant from 40 min to 70 min (Fig. 4). A reaction time of 50 min was used in this study.

### 3.4. Assay validation

#### 3.4.1. Recovery

Samples including serum, rice and pork meat were spiked with MFA-Na solution to make final concentrations ranging from low (10 ng/mL or ng/g), middle (100 ng/mL or ng/g) to high (500 ng/mL or ng/g). Extracts of these quality control samples as serum, rice and meat were prepared and then reacted with 9-chloromethylanthracene and analyzed by HPLC. The peak areas of MA-MFA were recorded and the recovery and RSD% were calculated. The results are shown in Table 1. Different types of samples including serum, rice and pork meat had similar satisfactory recovery of more than 82%.

#### 3.4.2. Resolution

Biological samples are complex, other fatty acids such as formic acid, acetic acid, propionic acid, etc. can also react with 9-chloromethylanthracene. The influence of the existing fatty

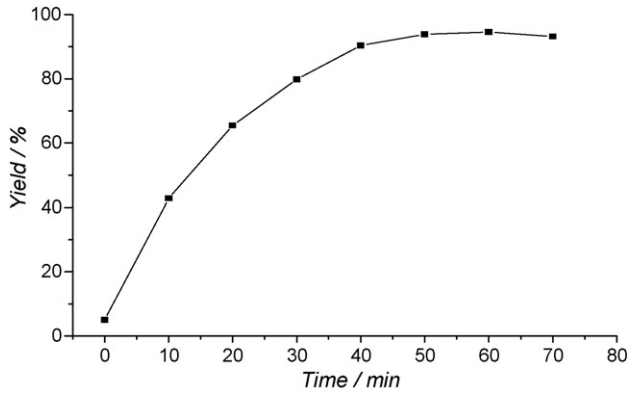



Fig. 4. Effects of reaction times on the yields of MA-MFA.

Table 1
Recoveries and RSD% of extraction of different QC samples

| Samples | Low level (10 ng/mL or 10 ng/g) | | Medium level (100 ng/mL or 100 ng/g) | | High level (500 ng/mL or 500 ng/g) | |
|---|---|---|---|---|---|---|
| | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| Serum (0.2 mL) | 83.1 | ±4.0 | 85.4 | ±3.5 | 92.4 | ±2.9 |
| Rice (0.5 g) | 84.2 | ±3.9 | 85.9 | ±2.9 | 90.0 | ±1.8 |
| Meat (0.5 g) | 82.7 | ±4.1 | 84.7 | ±3.3 | 88.2 | ±2.5 |

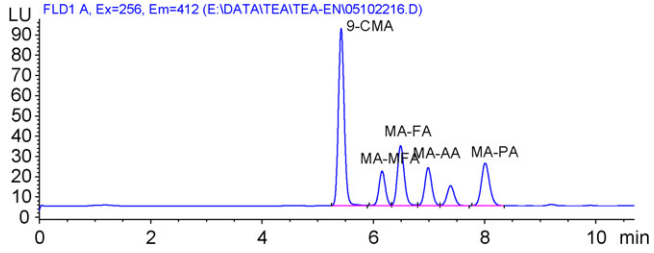



Fig. 5. HPLC Chromatogram of sample containing MFA-Na and other fatty acids after derivatization with 9-chloromethyl anthracene. The retention times of the derivatives of MFA, formic acid, acetic acid, and propionic acid were 6.157, 6.490, 6.985, and 8.010 min, respectively.

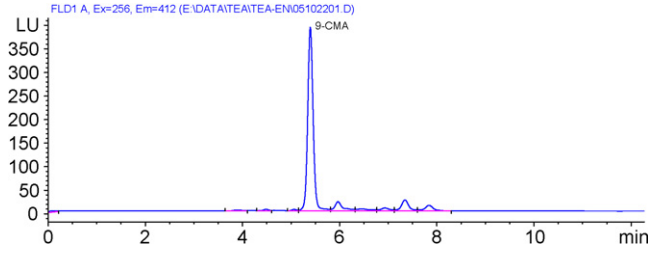



Fig. 6. HPLC Chromatogram of the MFA free serum sample.

acids with their 9-CMA esters was investigated. Under the optimized HPLC conditions, the retention time ($t_R$) of MA-MFA was 6.157 min, $t_R$ of 9-chloromethylanthracene was 5.422 min, $t_R$ of formic acid was 6.490 min, acetic acid 6.985 min, and propionic acid 8.010 min (Fig. 5). The result indicated the existing of fatty acids had minor influence on chromatographic separation.

### 3.4.3. Selectivity

Samples of rice and pork meat were homogenized, together with serum from adult healthy volunteers, extracted, modified with 9-chloromethylanthracene and analyzed by HPLC. As shown in Fig. 6, there is no peak at the retention time of MA-MFA at 6.16 min in the serum sample, suggesting that this method is highly selective for MFA-Na. Similarly, no MA-MFA peak was found in the rice and meat samples (data not shown). The use of the anthracene fluorescence probe likely contributed to the selectivity of this method.

### 3.4.4. Linear range and lower limit of quantitation of serum samples

The linearity of the serum calibration plot was investigated. The standard curve range was 1–250 ng/mL for the final concentration of MA-MFA. Calibration samples were extracted, derivatized, dissolved in acetonitrile and injected for HPLC analysis. A good linear relationship was obtained with the equation of $y = 14.56x - 6.51$, when $y$ was the peak area of MA-MFA and $x$ was MFA (MFA-Na) concentration (ng/mL). The correlation coefficient of the calibration plot was 0.9988. Therefore, the limit of quantitation (LOQ) was 1 ng/mL, much lower than that of other reported analytical procedures. The linear plot obtained was suitable for quantification of derived MFA during the inter-assay and intra-day validations.

Detection limits are an important consideration when the components of biological matrices are analyzed; particularly when components are present at low or trace concentrations. When the signal-to-noise ratio of three was used, the detection limit (LOD) of this method was 0.25 ng/mL. It is conceivable that if a larger sample volume was used, the lower detection limit could be decreased further.

The linear range and limits of quantitation and detection in other types of samples, like rice and meat could be similarly determined when necessary. We focused on serum samples in this report because of the need to analyze the serum samples of patients quantitatively.

### 3.4.5. Precision and accuracy

Serum samples were spiked with 10, 50, and 100 ng/mL MFA-Na. Each of the three samples were processed and analyzed by HPLC three times on the same day and on different days. Repeatability of the method was expressed as the inter-assay precision (RSD%, $n = 5$) and intra-day (6 days) precision (RSD%, $n = 3$). For all the biological samples RSD values were found to be lower than 5%. Table 2 indicates that the method

Table 2
Inter-assay and intra-day precisions and accuracy for analysis of MFA in serum

| Spike concentration (ng/mL) | Inter-assay ($n = 5$) | | Intra-day ($n = 3$) | |
|---|---|---|---|---|
| | Determined concentration (ng/mL) | RSD (%) | determined concentration (ng/mL) | RSD (%) |
| 10 | 8.3 ± 0.4 | 3.0 | 8.7 ± 0.8 | 4.5 |
| 50 | 48.1 ± 0.7 | 3.9 | 45.4 ± 1.2 | 3.9 |
| 100 | 92.7 ± 1.5. | 2.2 | 90.4 ± 1.5 | 4.8 |

The concentrations are given as mean ± 1 standard deviation.

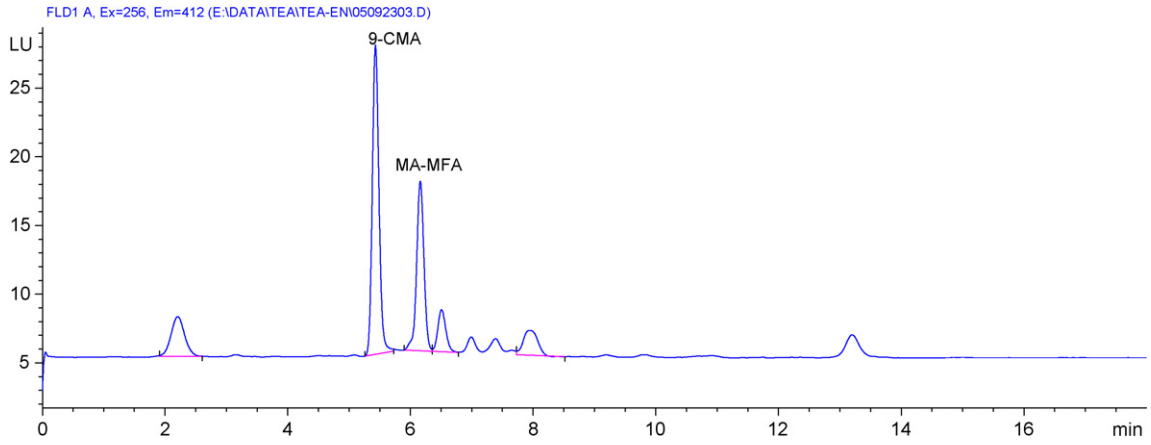



Fig. 7. Chromatogram of the derivatized serum sample from an individual after MFA-Na ingestion.

has high precision to quantitatively measure the MFA-Na concentrations in serum samples. The determined concentrations were within 6.5% of the theoretical concentrations, indicating that this method can reasonably and accurately determine the concentration of MFA-Na in serum samples.

### *3.5. Analysis of clinical samples*

Serum samples were obtained from a poisoned individual in an actual criminal case that happened in Guangdong Province, China. Serum (200 μL) was extracted, treated with 9-chloromethylanthracene according to the method described, and analyzed with HPLC. Fig. 7 shows the chromatogram of one of the samples. From the calibration plot equation, the concentration of MFA-Na in this serum sample was determined at 175 ng/mL. Method development, validation study, and the application of the method in one real criminal case demonstrated that our method for the quantitative determination of MFA-Na in serum is ready to be used in the analysis of clinical and toxicological samples.

## 4. Conclusion

We have developed a selective, sensitive and convenient HPLC method to quantitatively determine the concentration of MFA or MFA-Na in serum samples. The fluorescent reagent 9-chloromethylanthracene was used as the derivatizing agent for the determination of sodium monofluoroacetate in biological samples. The optimal reaction conditions, physical properties and spectral characteristics of the derivative product, 9-methyleneanthracene fluoroacetate, were investigated. The developed analytical method was selective and showed a lower limit of quantitation of 1 ng/mL and a lower limit of detection of 0.25 ng/mL, which were much lower than that of previously reported procedures. This method was successfully used for the quantitative determination of MFA-Na in serum samples of an individual who consumed MFA-Na. MFA-Na was also recovered in the similar yield from meat and rice samples as from serum samples; a similar method could be developed and validated for the quantitative determination of MFA (MFA-Na) in meat and rice samples.

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