Analysis of Interfering Substances in the Measurement of Malondialdehyde Content in Plant Leaves

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The thiobarbituric acid reactive substances assay is an easy and quick assay for the assessment in plants of lipid peroxidation, in which malondialdehyde is derivatized. To analyze the applicability of this method, the malondialdehyde concentrations in the leaves of different plants were measured by the thiobarbituric acid method. For further separation of the interfering substances, fractional extraction and gas chromatography-mass spectrometry were used in this research. The results suggest that the lipid peroxidation is accurately measured by the thiobarbituric acid method with trichloroacetic acid as the extraction solution in most plants. However, the method was not suitable for measuring the malondialdehyde concentration in golden privet (*Ligustrum × vicaryi*) leaves. Negative malondialdehyde concentrations were obtained in golden privet leaves by this method.

Key Words: Gas chromatography-mass spectrometry, Interfering substance, Lipid peroxidation, Malondialdehyde, Thiobarbituric acid.

INTRODUCTION

In plants, the toxic effects of abiotic and biotic stresses include a decrease in photosynthetic activity[1], a reduction in water and nutrient uptake, growth inhibition[2,3], damage to proteins[4] and changes in lipid composition[5] and lipid peroxidation[6]. Lipid peroxidation has been suggested to be responsible for the developmental processes of plants, including the juvenile stages, production of volatile odors, senescence and formation of compounds such as jasmonic acid[7]. This process can be initiated by free radicals or enzymatic activities[8]. Therefore, the detection of lipid peroxidation is important in research into plant senescence and environmental stress.

Malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, is the important indicator for the process[9]. The thiobarbituric acid (TBA) reactive substances assay is an easy and quick assay for the assessment of malondialdehyde concentration in plants. In this assay, malondialdehyde reacts with thiobarbituric acid to form a pink pigment, a condensation product of thiobarbituric acid and malondialdehyde in a 2:1 molar ratio, which has an absorption maximum at 532 nm[10]. In plant tissue, however, certain compounds (anthocyanins and carbohydrates) may interfere with measurements at this wavelength. Furthermore, the

presence of barbituric acid impurities in the thiobarbituric acid reagent has been found to produce 1:1:1 TBA/MDA/barbituric acid and 2:1 barbituric acid/MDA adducts that absorb at 513 and 490 nm[11], respectively.

The aim of this work is to analyze the applicability of the thiobarbituric acid method for measuring malondialdehyde concentrations in various plants. As a further check of the malondialdehyde extraction rate, two frequently used solutions, phosphate buffer and trichloroacetic acid, were compared. In golden privet (*Ligustrum × vicaryi*) leaves, substances were found that interfered with the measurement of malondialdehyde concentration using the thiobarbituric acid method. To separate the interfering substances, fractional extraction and gas chromatography-mass spectrometry were used.

EXPERIMENTAL

The functional and the senescent leaves of 12 plants grown at the botanical gardens of the Anhui Agricultural University (eastern China, 32º N) were selected as the materials (Fig. 1). The samples were collected, placed immediately into an ice box, weighed, quickly frozen in liquid nitrogen and stored at -80 ºC.

Extraction of malondialdehyde: Fresh functional or senescent leaves (0.5 g) from at least seven plants were taken
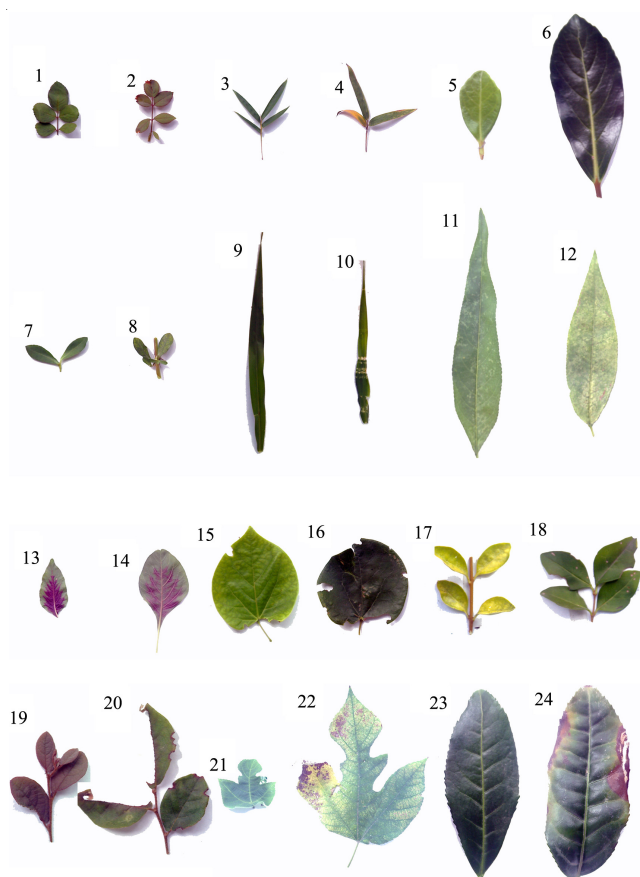
Fig. 1. Leaves of various plants sampled for the analysis of MDA concentrations. Leaves shown are: 1,2: multiflora rose (*Rosa multiflora*); 3,4: bamboo (*Phyllostachys bambusoides*); 5,6: sweet viburnum (*Viburnum odoratissimum*); 7,8: alligator weed (*Alternanthera philoxeroides*); 9,10: green bristlegrass (*Setaria viridis*); 11,12: peach tree (*Prunus persica*); 13,14: amaranth (*Amaranthus gangeticus*); 15,16: Chinese redbud (*Cercis chinensis*); 17,18: golden privet (*Ligustrum ×vicaryi*); 19,20: red-flowering loropetalum (*Loropetalum chindensevar*); 21,22: paper mulberry (*Broussonetia papyrifera*); 23,24: tea plant (*Camellia sinensis*). For each species, odd numbers indicate functional leaves and even numbers indicate senescent leaves

and ground in 5 mL extraction solution (separately using 0.05 mol L^{-1} pH 7.8 phosphate buffer or 5 % trichloroacetic acid as extraction solution), followed by centrifugation at 5000 g for 15 min. The supernatant is the malondialdehyde extraction solution, which was stored at 4 ºC.

Measurement of malondialdehyde: Malondialdehyde levels were estimated according to the corrected thiobarbituric acid method[12]. Two milliliters of extraction solution and 3 mL 0.5 % thiobarbituric acid including 5 % trichloroacetic acid were mixed vigorously. The mixture was heated at 95 ºC in a constant temperature water bath for 0.5 h and then cooled in ice to room temperature. After centrifuging at 5000 gn for 15 min, the supernatant was detected at 450, 532 and 600 nm. The concentration of malondialdehyde was determined using the formula:

CMDA (µmol mL^{-1}) = 6.45 × (D$_{532}$ - D$_{600}$) - 0.56 × D$_{450}$

where D$_{450}$, D$_{532}$ and D$_{600}$ are the absorbencies at 450, 532 and 600 nm, respectively.

Fractional extraction for the interfering substances: The extraction of malondialdehyde was performed using the thiobarbituric acid method. To separate the interfering substances, equal volumes of petroleum ether, ether, ethyl acetate and *n*-butyl alcohol were successively used as extracting solvents. Products of interfering substances in partial extracts that reacted with thiobarbituric acid were detected and scanned by absorption spectroscopy at 400-700 nm.

GC-MS conditions: The ether phase extracted from golden privet and paper mulberry leaves was evaporated to dryness with nitrogen. Then the dried sample was re-dissolved using 2 mL ether for GC-MS analysis. Auto system Shimadzu QP 2010 GC-MS was used to identify the interfering substances. The temperature programming of GC separation was as follows: initial oven temperature was set at 150 ºC and maintained at that temperature for 2 min, then raised to 210 ºC at a ramp of 4 ºC/min and maintained for 2 min and to 280 ºC at 5 ºC min^{-1} and maintained for 7 min. Nitrogen was used as carrier gas with column head pressure at 12.26 kPa in constant pressure mode. The injection volume was 2 µL, the programming split/splitless injection temperature was set at 280 ºC with a split ratio of 5:1 and the electron capture detector was set at 300 ºC. The mass spectrometer was operated at 200 ºC in electron impact mode (70 eV), scanning from *m/z* 40 to 600 in 0.3 s with a 0.2 s interval time of the scan; the temperature of the GC-MS interface was 300 ºC and the voltage of the photo-electric multiplier tube was 200 V. Mass spectra identification was carried out by comparing with the NIST 107 (National Institute of Standards and Technology, Gaithersburg, USA) and Wiley 6.0 (Wiley, New York, NY, USA) mass spectral libraries.

Statistical analysis: Data were expressed as the mean ± SD. The statistical significance of differences between groups was determined with Student's *t*-test using SPSS software (Chicago, IL, USA). Values of p < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of malondialdehyde extraction with two different solutions: The extraction solutions used were 0.05 mol L^{-1} pH 7.8 phosphate buffer and 5 % trichloroacetic acid solutions. To compare the extraction efficiency with different solutions, malondialdehyde concentrations were measured by the thiobarbituric acid method in 12 different plant species. The malondialdehyde concentrations obtained from the two different extraction solutions differed significantly (P = 0.031) from each other (Table-1). The malondialdehyde concentrations with 5 % trichloroacetic acid as the extraction solution were on average 32 % higher than those with phosphate buffer as the extraction solution. The solutions extracted with 5 % trichloroacetic acid were clearer than those extracted with phosphate buffer (data not shown). The malondialdehyde concentrations in senescent leaves were higher than those in young or mature leaves and the difference between them was significant (P = 0.025), except for amaranth and golden privet. The average concentration in senescent leaves was 5.41 µmol g^{-1} fresh weight, whereas in mature leaves it was 3.90 µmol g^{-1} fresh weight. These data indicate that the thiobarbituric acid method with trichloroacetic acid extraction solution was appropriate for analyzing malondialdehyde concentrations in most plants tested.

TABLE-1
COMPARISON OF MDA EXTRACTION EFFECT BY TWO DIFFERENT SOLUTIONS

Plant names	MDA concentration (μmol g^{-1} fresh weight)			
	PB		TCA	
	Functional leave	Senescent leave	Functional leave	Senescent leave
Multiflora rose	4.45 ± 0.99	4.99 ± 1.00	8.10 ± 1.03	8.38 ± 1.21
Bamboo	2.49 ± 0.89	3.61 ± 0.89	5.67 ± 0.96	7.68 ± 0.94
Japan arrowwood	3.44 ± 1.07	1.21 ± 0.77	4.41 ± 1.09	5.44 ± 0.66
Alligator weed	1.22 ± 0.22	1.45 ± 0.19	1.79 ± 0.69	2.15 ± 0.19
Green bristlegass	2.34 ± 0.20	3.35 ± 0.94	2.33 ± 1.00	2.67 ± 0.34
Peach plant	1.09 ± 0.77	2.76 ± 0.97	5.45 ± 0.85	4.13 ± 0.88
Amaranth	1.67 ± 0.91	1.51 ± 0.36	0.83 ± 0.13	0.60 ± 0.59
Chinese redbud	4.70 ± 1.08	4.88 ± 0.88	4.64 ± 0.66	6.12 ± 0.98
Golden privet	-0.69 ± 0.19	-2.26 ± 0.34	-5.32 ± 0.91	-18.74 ± 1.99
Redflowered loropetalum	5.63 ± 1.11	9.76 ± 0.93	2.28 ± 0.83	12.9 ± 0.92
Paper mulberry	2.09 ± 0.35	5.38 ± 0.99	4.09 ± 1.92	4.11 ± 1.00
Tea plant	2.45 ± 0.11	4.07 ± 0.54	3.28 ± 0.97	4.31 ± 0.90

PB means MDA solutions extracted by 0.05 mol L^{-1} pH 7.8 phosphate buffer; TCA means MDA solutions extracted by 5 % TCA. The MDA concentration values represent the mean value ± SD.

The solution extracted from young leaves of amaranth was redder than the solution from senescent leaves (data not shown). We suggest that the anthocyanins in amaranth leaves may be the main interfering substances that influence the accuracy of the thiobarbituric acid method; amaranth leaves have a higher anthocyanin content than leaves of other species. Unexpectedly, the malondialdehyde concentrations in golden privet leaves as measured by this method were negative and there was little red residue in the solutions extracted from these leaves. The solutions of these samples had higher absorption at 600 nm than at 532 nm (Fig. 2C).

Fractional extraction for interfering substances: To further clarify the reason for the apparent negative malondialdehyde concentrations detected by the thiobarbituric acid method in golden privet leaves, the interfering substances were separated by successive liquid-liquid extraction. Paper mulberry leaves were used as control material. Petroleum ether, ether, ethyl acetate and *n*-butyl alcohol were used as extraction solvents. Compared with the red products of malondialdehyde (solution extracted from paper mulberry) following reaction with thiobarbituric acid, the products of the interfering substances (solution extracted from golden privet) following reaction with thiobarbituric acid appeared dark green (Fig. 2A and B). The extraction results show that the concentration of the dark green interfering substance was highest in the ether phase and lowest in the ethyl acetate phase (Fig. 2A), whereas the red product of control material (paper mulberry) was highest in the water phase and lowest in the ether phase (Fig. 2B).

The absorption spectroscopy scanning results showed that the dark green substance had a characteristic absorption peak at 615 nm, whereas the red product of the control had a characteristic peak at 500 nm (Fig. 2C). Detection of the interfering substance in different tissues showed that this substance was mainly found in leaves but was almost undetectable in the stems and roots (Fig. 2D).

GC-MS analysis: To determine the structure of these malondialdehyde-interfering compounds, the ether phases extracted from the golden privet leaves and stems were analyzed by GC-MS (Fig. 3). Compared with the gas chromatogram of

stems, the gas chromatogram of the ether phase of golden privet leaves showed a few different peaks, such as peaks 8, 9, 10 and 11. The analysis of mass spectrum predicted that peaks 8, 9, 10 and 11 were similar to diethyl hydroxybutanedioate (similarity: 94 %), 3,4-dimethoxybenzoic acid (72 %), quinuclidine-2-carboxylic acid, 2,3-dehydro-3-amino-ethyl ester (63 %) and tyramine, *N*-formyl-2-(4-hydroxyphenyl) ethylformamide (82 %), respectively.
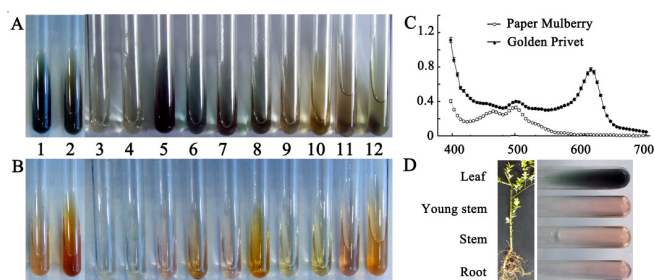

Fig. 2. Separation of the interfering substances by successive liquid-liquid extraction in golden privet and paper mulberry leaves. A: solutions extracted from golden privet; B: solutions extracted from paper mulberry; C: visible light spectra of ether extract solution; D: the accumulation of interfering substances in the different tissues of golden privet. MDA solutions were successively liquid-liquid extracted by: 1,2 5 % TCA; 3,4 petroleum ether; 5,6 ether; 7,8 ethyl acetate; 9,10 n-butyl alcohol. 11,12 show the water phase. For each solution, odd numbers indicate extraction from functional leaves and even numbers indicate extraction from senescent leaves

The rationale and methodology of the thiobarbituric acid reactive substances assay have been discussed in detail elsewhere[10,11]. The method has been rightly criticized for low specificity and artifact formation[13-15]. In recent years, several HPLC-based thiobarbituric acid assays have been developed with increased specificity[16]. Despite these facts, the spectrophotometric method of thiobarbituric acid remains one of the most useful and commonly used measurements of lipid peroxidation because of its simplicity.

Phosphate buffer is used as a common extraction solution for activity analysis of the enzymes in activated oxygen metabolism[17]. It can also be used in lipid peroxidation detection, which is very useful in rare plant studies to save
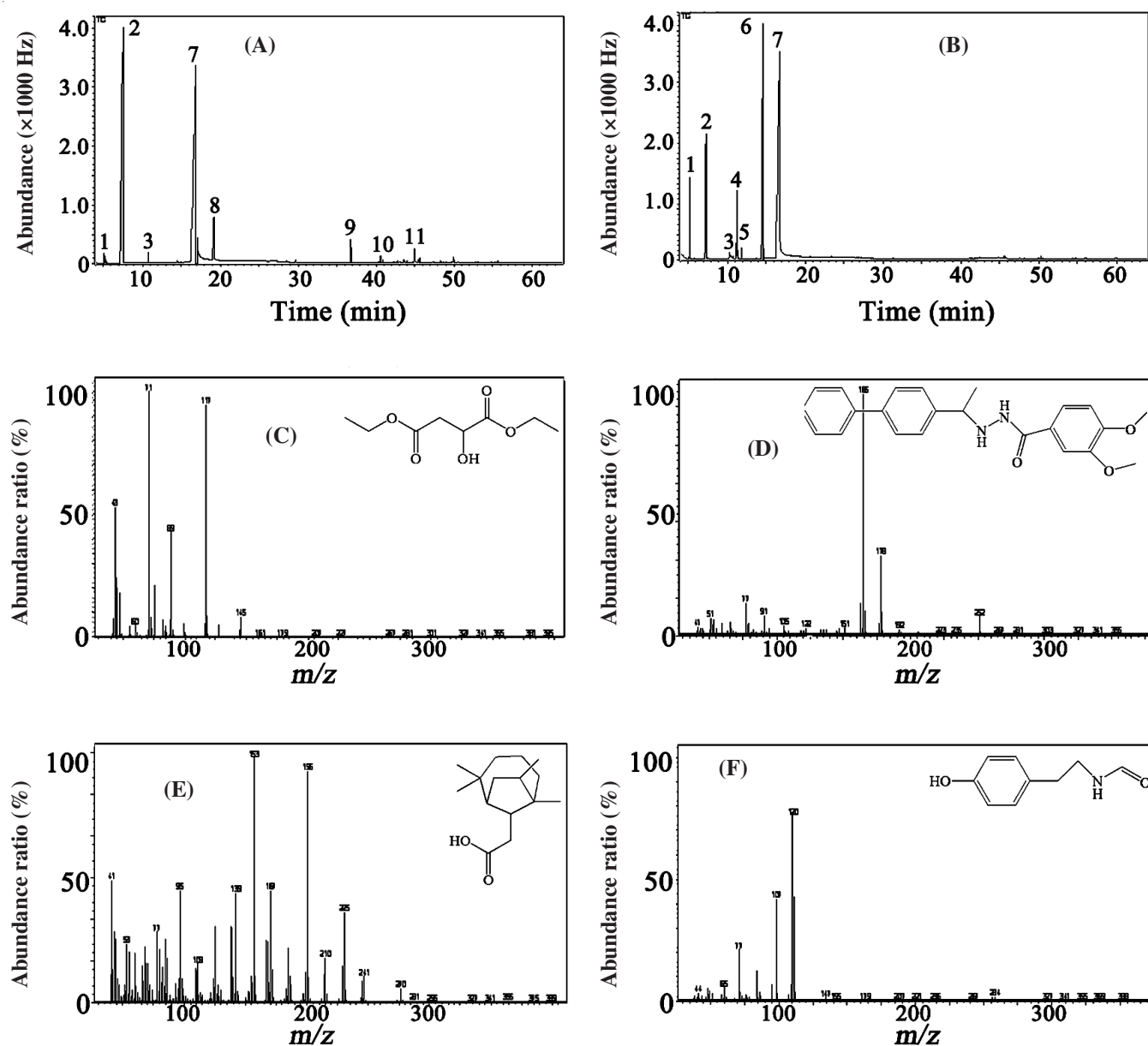
Fig. 3. GC-MS analysis of the MDA-interfering substances in golden privet leaves. Gas chromatograms of ether phase extracted from golden privet leaves (A) and stems (B) and the mass spectra of peaks 8(C), 9(D) and 10 (E) and 11 (F). The mass spectra show that the peaks are most similar to the following compounds: peak 8, diethyl hydroxybutanedioate (similarity: 94 %); peak 9, 3,4-dimethoxy-benzoic acid (similarity: 74 %); peak 10, quinuclidine-2-carboxylic acid, 2,3-dehydro-3-amino-ethyl ester (similarity: 63 %); peak 11, tyramine, N-formyl- 2-(4-Hydroxyphenyl) ethylformamide (similarity: 82 %)

experimental material. The malondialdehyde concentrations measured by the thiobarbituric acid method with phosphate buffer and trichloroacetic acid as extraction solutions differed significantly from each other. The malondialdehyde concentrations with 5 % trichloroacetic acid as the extraction solution were on average higher than those with phosphate buffer solution. In agreement with some other studies[18,19], the results suggest that lipid peroxidation should be corrected for interfering compounds when measured by the thiobarbituric acid method with trichloroacetic acid as the extraction solution in most experimental plants.

Unexpectedly, an interesting result was detected that in golden privet leaves the malondialdehyde concentration was negative when measured by this method. Golden privet, also known as golden vicary privet or golden Ligustrum, is a vase-

shaped shrub often used for shrub borders and hedging. Leaves of golden privet are golden yellow and lance-shaped. To further clarify the reason for the negative malondialdehyde concentrations acquired by the thiobarbituric acid method in golden privet leaves, the interfering substance was separated by successive liquid-liquid extraction. The results indicated that the interfering substances were highly lipid soluble and were present at the highest levels in the leaves and almost undetectable in stems and roots.

The product of malondialdehyde following reaction with thiobarbituric acid was measured at 532 nm with 600 nm as the reference wavelength. The latter wavelength is used merely for baseline correction[16]. The products of the interfering substances following reaction with thiobarbituric acid had a characteristic absorption peak at 615 nm, which is the reason

for the negative malondialdehyde concentrations measured by the thiobarbituric acid method in golden privet leaves.

Reaction with thiobarbituric acid yielding coloured derivatives is a characteristic of conjugated aldehydes. Futterman and Saslaw[20] found that vitamin A aldehyde reacts with aqueous thiobarbituric acid to yield a black derivative, which imparts various colours to different organic solvents. Our GC-MS data indicated that a few different substances found in the ether phase of golden privet leaves, in which peak 11 (similar with *N*-formyl-2-(4-hydroxyphenyl) ethylformamide) might be one of aldehyde compounds.

Conclusion

In conclusion, comparison of the malondialdehyde extraction rate between two commonly used solutions, phosphate buffer and trichloroacetic acid, suggested that lipid peroxidation should be corrected for interfering compounds when measured by the thiobarbituric acid method with trichloro-acetic acid as extraction solution in most experimental plants. However, the thiobarbituric acid method is not suitable for measuring the malondialdehyde concentration in golden privet (*Ligustrum × vicaryi*) leaves, because of the existence of interfering substances. The extraction results indicate that the interfering substance was highly lipid soluble and was present at the highest levels in the leaves. Absorption spectroscopy scanning results showed that the products of the interfering substance reacted with thiobarbituric acid had a characteristic absorption peak at 615 nm. The comparison of GC-MS profiles indicated that a few different substances were found in golden privet leaves, in which the peak 11 might be one of aldehyde compounds, similar with *N*-formyl-2-(4-Hydroxyphenyl) ethylformamide. Further exploration and a more detailed explanation of the interfering substances will be carried out in a future study.

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