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Determination of nitric oxide in hydrophytes using poly(methacrylic acid-ethylene glycol dimethacrylate) monolith microextraction coupled to high-performance liquid chromatography with fluorescence detection

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Abstract

Nitric oxide (NO) is a bioactive molecule that has recently emerged as a cellular messenger in numerous physiological processes in plants. A novel high-performance liquid chromatography (HPLC) method combined with poly(methacrylic acid-ethylene glycol dimethacrylate) (MAA-EGDMA) monolith microextraction (PMME) is developed for sensitive determination of NO in hydrophytes. NO is derivatized using a fluorescent probe, 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacene (DAMBO), and then the derivatives are extracted with PMME and analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection. The conditions for the derivatization and the subsequent extraction of NO derivatives are optimized in detail. The detection limit (S/N = 3) of NO is determined to be 2×10^{-12} mol L^{-1}. Close correlation coefficient and excellent method reproducibility are obtained for the analyte over a linear range of 9×10^{-11}–4.5×10^{-8} mol L^{-1}. The inter- and intraday relative standard deviations (R.S.D.s) are less than 5%. The proposed method is successfully applied to the determination of NO levels in hydrophytes samples.
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Keywords: High-performance liquid chromatography; Nitric oxide; Poly(methacrylic acid-ethylene glycol dimethacrylate) monolith microextraction; 1,3,5,7-Tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacene

1. Introduction

Nitric oxide (NO) is a diffusible gaseous free radical and is produced from L-arginine by NO synthase in mammalian cells [1]. Now extensive research about the role of NO in animals has demonstrated that it is a key signal molecule involved in a wide variety of processes, including vasorelaxation, neurotransmission and the innate immune response [2]. In contrast, the role of NO in plants is less well understood. Evidence is emerging to support that NO may also play important role in plant. Its emission from plants has been reported several years ago in soybean and peas [3,4]. Recently, in vivo and in vitro NO production have been found in other plants, such as sunflower and maize [5]. Its involvement in regulation of plant growth [6], interactions with pathogenic microorganisms [7], cell differentiation [8], root growth [9], apoptosis in callus cells and foliar tissues [10] and phytoalexin accumulation [11] has been suggested. Also, nitric oxide may function to mitigate or trigger stressors in diverse plant species [12]. Whereas some authors considered NO as a stress-inducing agent [13], others have reported its protective role [14], depending on its concentration, the plant tissue or age, and the type of stress. Furthermore, Laxalt et al. [15] have reported that NO is able to partially prevent the chlorophyll decay produced by phytophtorainfestans in potato leaves, and postulated the capability of NO to scavenge ROS. Hung et al. [16] have reported that NO is able to counteract the toxicity of diquat and paraquat on potato and rice leaves, respectively. Since NO plays important role in plants, developing sensitive, specific, simple and low-cost methods for the determination of NO is of great importance.

Since NO is volatile and has a very short half-life (<10 s), the customary method of determination of NO is a challenging

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problem, especially under physiological conditions where NO production is relatively low. Several methods have already been tried to this end, including spectrophotometry [17], electron paramagnetic resonance (EPR) spectroscopy [18], chemiluminescence [19], oxyhemoglobin method [20], and NO electrode [21]. However, none of these methods has ever succeeded in providing adequate sensitivity and/or specificity for NO detection [22]. Derivatization has usually been adopted to enhance the sensitivity and selectivity of analyses based on chromatographic techniques. Recently, a variety of fluorescent reagents has been developed to label NO, such as diaminonaphthalene (DAN) [23], diaminorhodamines (DARs) [24] and diaminofluoresceins (DAFs) [25] and so on. However, some drawbacks are present in these reagents, including poor photostability and/or their serious pH dependence, or the poor stability of the derivatives determined. A novel fluorescent probe, 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacene (DAMBO), has been developed for NO labeling [26]. A fluorophore of this reagent, difluoroboradiaza-*s*-indacene (BODIPY), has a high fluorescence quantum efficiency, excellent photo stability and good stability over a wide pH range [27]. Thus, derivative of NO has good stability. This has been proved in our previous work [28]. Accordingly, a sensitive and specific method can be developed for NO detection based on the probe DAMBO. In this study, DAMBO was used to label NO, which enabled their determination of trace-level NO in plant samples by HPLC with fluorescence detection.

Due to its advantages, such as the simplicity of the procedure, solvent-free characteristics and ease of automation compared with conventional extraction methods [29], solid-phase microextraction (SPME) has become a popular pretreatment method that can be applied for the extraction of organic compounds in different fields, including environmental, pharmaceutical, biomedical and food analysis. As a member of it, the monolithic capillary column by in situ polymerization recently shows several more attractive features like in-tube configuration can protect the extraction material to avoid the outside breakage; compared to the coated capillaries, the monolithic column exhibits high extraction capacity; also, the monolithic material has emerged as more popular alternatives to packed columns due to the simplicity of their preparation as well as the diverse surface chemistry; furthermore, a convective mass transfer procedure offered by the porous structure can facilitate the extraction process [30,31].

In our previous study, poly(MAA-EGDMA) monolithic capillary column has been applied to in-tube SPME of several basic analytes [32,33]. Based on these investigations, a novel polymer monolith microextraction (PMME) has been developed and successfully used to determine analytes in human urine [34] and rabbit retina [35]. In this study, PMME using a poly(MAA-EGDMA) monolithic capillary for NO derivative is developed. The productions of extraction are sensitively analyzed using HPLC with fluorescence detection. The changes in peak area are linear in the concentration range of 0.09–45 nmol L^{-1} of NO. The detection limit can reach 2 pmol L^{-1}. The proposed method is successfully applied to determine trace-level NO in hydrophytes samples, including water plant foxtail, wild water bamboo root, potamogeton maackianus and Ludwigia X taiwanensis Peng.

2. Experimental

2.1. Chemicals and materials

Ethylene glycol dimethacrylate (EGDMA) was purchased from Acros (Stockholm, Sweden). Methacrylic acid (MAA), 2,2′-azobis (2-methylpropionitrile) (AIBN), dodecanol and toluene were obtained from Shanghai Chemical Co. Ltd. (Shanghai, China) and were of analytical reagent grade.

DAMBO and its NO derivative 1,3,5,7-tetramethyl-8-(5-benzotriazolyl)-difluoroboradiaza-*s*-indacene (DAMBO-T) were synthesized in our lab according to Ref. [26]. Dimethyl sulfoxide (DMSO), calcium- and magnesium-free phosphate buffered saline (PBS) were purchased from Sigma (St. Louis, MO, USA).

NO gas was generated by slowly dropping a 2 mol L^{-1} H_2SO_4 into a glass flask containing saturated $NaNO_2$ solution, NO being formed in this disproportional reaction [36]. The gas generated was forced to twice the bubble in a 30% NaOH solution and once in water in order to trap any NO_2 formed as a result of oxidation of NO from traces of oxygen. Before the addition of H_2SO_4, all apparatus was degassed meticulously with nitrogen for 30 min to exclude O_2, as NO is rapidly destroyed by O_2. To produce a saturated NO solution, 10 mL deoxygenated distilled water was bubbled with pure NO gas for 30 min and kept under NO atmosphere until use. NO gas is toxic at concentration higher than 100 ppm, so the bubbling procedure was carried out in a fume hood. Standard solutions were freshly prepared for each experiment and kept in a glass flask with a rubber septum. Dilutions of the saturated solution were made using deoxygenated water samples.

All other reagents were of analytical grade; the water used in the experiment was purified in a Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Instrumental and analytical conditions

A CP 2000 syringe infusion pump (Silugao High-Technology Development, Beijing, China), which supplies the drive to smoothly and regularly push the mobile phase through the monolithic capillary in a high reproducible manner, automatically, as described in detail in previous papers [34], was employed to perform the extraction procedure. The extraction device is shown in Fig. 1. It is mainly composed of an extraction pinhead, a syringe barrel and a 3 cm-long monolithic capillary.

An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) that composed with a quaternary pump, a micro-vacuum degasser, a six-port valve and a fluorescence detector was employed to separate analytes. The analytical column was a ZORBAX Eclipse XDB-C_{18} column (150 mm × 4.6 mm, i.d., 5 μm). The optimized mobile phase consisted of 50 mmol L^{-1} ethanolamine/methanol; the

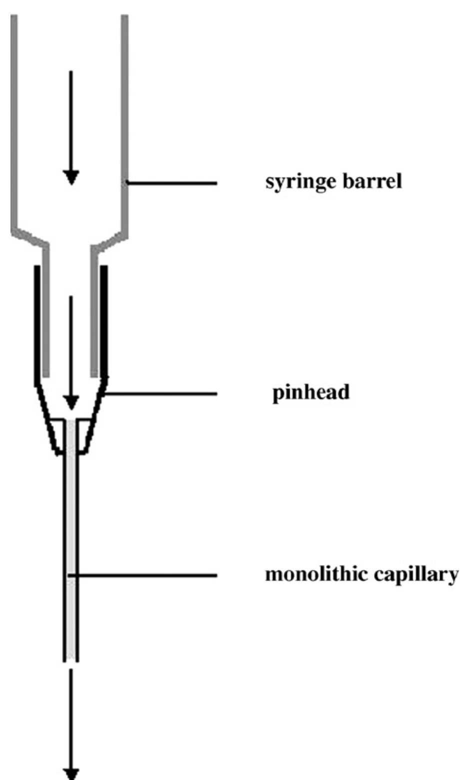

Fig. 1. Scheme of the PMME.

flow rate was 1.0 mL min^{-1}. The fluorescence detector was set at $\lambda_{ex}/\lambda_{em}$ = 498/507 nm for NO derivative detection.

2.3. Poly(MAA-EGDMA) monolith microextraction procedure

The poly(MAA–EGDMA) monolith was synthesized inside a fused silica capillary (20 cm × 530 m i.d., Yongnian, Hebei, China). The polymerization method was described in detail previously [37]. After the derivatization process was finished, the extraction procedure was processed as shown in Fig. 2. In brief, 0.3 mL methanol was first introduced into the syringe and expelled to pass through the monolithic capillary tube at a speed of 0.075 mL min^{-1} for preconditioning of the monolithic capillary tube, and then 0.2 mL PBS solution was employed to wash the tube at 0.2 mL min^{-1}. After that, 2 mL sample solution was added in the syringe and passed through the monolithic capillary tube at 0.2 mL min^{-1}. In order to avoid the contamination of the eluate, the residual solution in the pinhead and the monolithic capillary tube was removed with a clean syringe. Subsequently, 0.05 mL methanol was let to pass through the monolithic capillary tube at a speed of 0.075 mL min^{-1}, and the methanol eluate was collected for the HPLC analysis.

2.4. Sample preparation

Hydrophytes samples were supplied by Wuhan Institute of Botany, Chinese Academy of Science. Hydrophytes (including water plant foxtail, Wild water bamboo root, potamogeton maackianus and Ludwigia X taiwanensis Peng) were clean and dried at room temperature, and then 0.4 g of the samples was weighed. It was transferred into a 10 mL volumetric flask that contained 5 mL PBS solution (pH 7.4) and 4 mL 2.5×10^{-5} mol L^{-1} DAMBO. After homogenized, the mixture was diluted to mark with water and then was incubated at 37 °C for 40 min. The whole solution was then centrifuged at 5000 rpm for 10 min. Subsequently, the supernatant was taken out and filtered through a 0.22 μm membrane filter and was collected for analytical samples. After PMME procedure, triplicate injections of the sample were performed and peak areas were used for quantification.

3. Results and discussion

3.1. Reaction of DAMBO and NO

The reaction of DAMBO and NO is shown in Fig. 3. The fluorescence properties of DAMBO and its derivative DAMBO-T in neutral conditions have been investigated [26]. The results indicated that the maximum excitation and emission wavelengths of DAMBO-T were 498 and 507 nm, respectively.

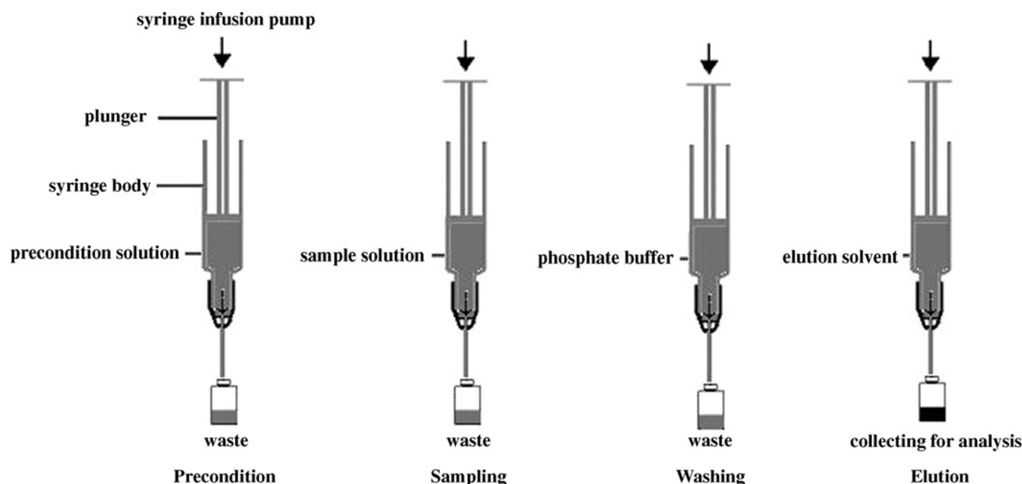

Fig. 2. Scheme of the PMME process.

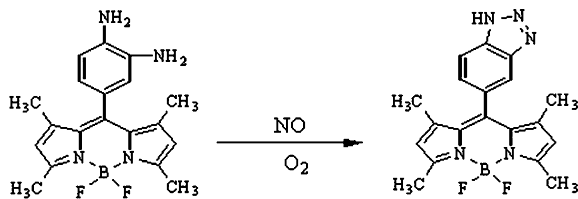

Fig. 3. Reaction of DAMBO with nitric oxide.

3.2. Optimization of derivatization

In order to achieve the maximum derivatization yield, the derivatization conditions including the concentration of DAMBO, the reaction time and the reaction temperature were carefully optimized. 0.4 g leafage of hydrophytes samples were used and the experimental procedure was performed as described in Section 2.4.

The effect of DAMBO concentration on the derivatization yield was evaluated from 4.0×10^{-6} to 1.4×10^{-5} mol L^{-1}. The subsequent HPLC analysis showed that the peak areas of derivative were maximum and stable when the concentration of DAMBO was 1.0×10^{-5} mol L^{-1}. Then this concentration was used in the following experiments.

The effect of the temperature on derivatization yield was also studied and the results showed that the yield of NO derivative was fairly stable in the range of 35–40 °C. Then 37 °C was selected as the optimum temperature value for the derivatization.

With respect to the reaction time, optimization was performed in the range of 5–60 min. The results showed that derivatization yield reached the largest and invariable when the reaction time was 40 min. Therefore, 40 min was selected in this work.

3.3. Desorption of the analytes from the poly(MAA-EGDMA) monolithic capillary

The desorption procedure after extraction was optimized carefully in order to achieve high recovery of the analytes from the monolithic column. The results showed that 0.05 mL of separation mobile phase was enough to elute more than 90% of analytes from the poly(MAA-EGDMA) monolithic column. The further increasing the volume of desorption solution would lower the detection sensitivity. Therefore, 0.05 mL separation mobile phase was chosen to desorb the analytes from the sorbent. The influence of the flow rate of desorption solution was also evaluated between 0.05 and 0.2 mL min^{-1}. The results showed that the faster desorption and higher desorption efficiency were both satisfied when the flow rate was 0.075 mL min^{-1}.

3.4. Optimization of the poly(MAA-EGDMA) PMME conditions

In order to obtain the best efficiency of the PMME, several parameters including the extraction equilibrium profiles, extraction flow rate and the pH value, which affected the extraction efficiency of the poly(MAA-EGDMA) monolithic column for NO derivative were optimized.

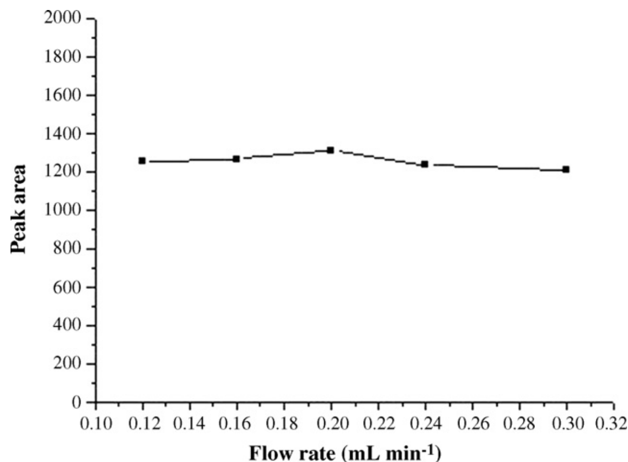

Fig. 4. The extraction flow rate profile of DAMBO-T for the PMME. The mobile phase of HPLC was methanol that contained 50 m mol L^{-1} ethanolamine. The flow rate for chromatographic separation was 1 mL min^{-1}. The detection wavelengths were $\lambda_{ex}/\lambda_{em}$ = 498/507 nm. PMME conditions were as outlined in Section 2.

The flow rate was investigated in the range of 0.12–0.3 mL min^{-1}. As shown in Fig. 4, changing the flow rate had no significant influence on the extraction efficiency in the tested range. Then 0.2 mL min^{-1} was selected for the following experiments, considering the extraction time and the pressure of the monolithic column.

The extraction equilibrium profiles of the poly(MAA-EGDMA) monolithic capillary column were evaluated by increasing the volumes of the extracted sample from 2 to 8 mL at a constant flow rate of 0.2 mL min^{-1}. As shown in Fig. 5, with the increasing sample volume, the yield of derivative extracted increased rapidly. The extraction equilibrium of the NO derivative was not reached even when 8 mL sample solution was used. The sample volume was not desirable for routine analysis because the total time needed for one analysis would be prolonged, a 2 mL of sample volume was selected for subsequent analysis with satisfactory sensitivity achieved.

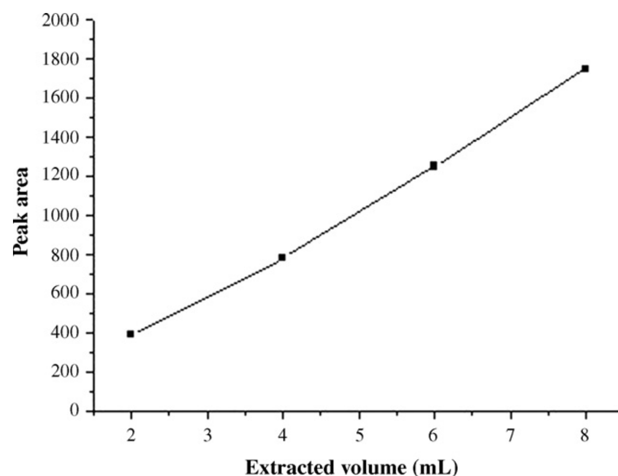

Fig. 5. The extracted sample volume profile of DAMBO-T for the PMME. PMME conditions outlined in Section 2 and HPLC conditions were the same as depicted in Fig. 3.

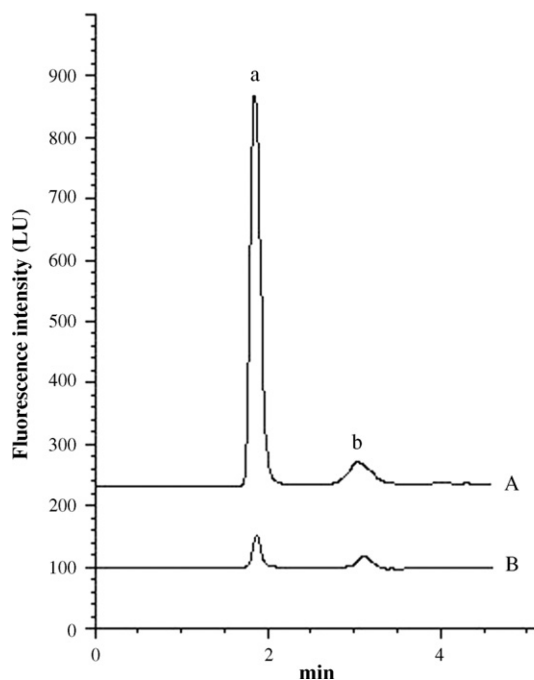

Fig. 6. Chromatograms for DAMBO-T obtained by PMME (A) and direct HPLC analysis (B). Peaks: (a) DAMBO-T; (b) DAMBO. PMME conditions outlined in Section 2 and HPLC conditions were the same as depicted in Fig. 3.

The effect of pH in the range of 5–9 on the extraction efficiency was investigated. The results showed that the extraction efficiency increased over the pH range of 5–7. However, it decreased slightly when pH was above 7. This can be explained that the interaction between the analyte and the monolithic column was mainly based on the hydrophobic interactions and the ion-exchange sites. In the alkaline matrix, the amount of the polymer extraction phase increased, causing the decreased hydrophobic interaction between derivative and the extraction phase, a slight decrease was then observed in the high pH value range. Thus, the analytical samples were adjusted to pH 7.0 in the microextraction process.

The chromatograms obtained by PMME-HPLC and direct HPLC analysis under the optimized conditions are shown in Fig. 6. Comparing the chromatograms obtained by PMME-HPLC (Fig. 6A) to that of the direct injection (Fig. 6B), a dramatic peak enhancement was presented. This indicated the remarkable preconcentration ability of the monolithic column to DAMBO-T.

3.5. Hydrophytes samples analysis

Under the optimized conditions, the proposed method was applied for determination of NO in hydrophytes samples. Fig. 7 shows the chromatograms obtained by PMME-HPLC of the Ludwigia X taiwanensis Peng samples (including leafage, bud, stalk and root) with DAMBO-T spiked at 5×10^{-8} mol L^{-1}. Peaks of DAMBO and DAMBO-T can be seen very clearly on the chromatograms, with no significant interference from any Ludwigia X taiwanensis Peng components. Thus, quantification of the NO in Ludwigia X taiwanensis Peng could be successfully achieved.

It has been reported that NO was of ubiquitous occurrence in higher plants, including foliage, intact seeds, individual seed components and in roots [38–40]. The interplay between NO and ethylene in the regulation of maturation and senescence of plant tissue has been already evidenced [41]. Plant growth regulator action amongst other criteria abrogates both growth promotion at low concentrations and inhibition at higher ones. The work of Leshem et al. [41] and Leshem and Pinchasov [42] has indicated that in a wide variety of immature or unripe

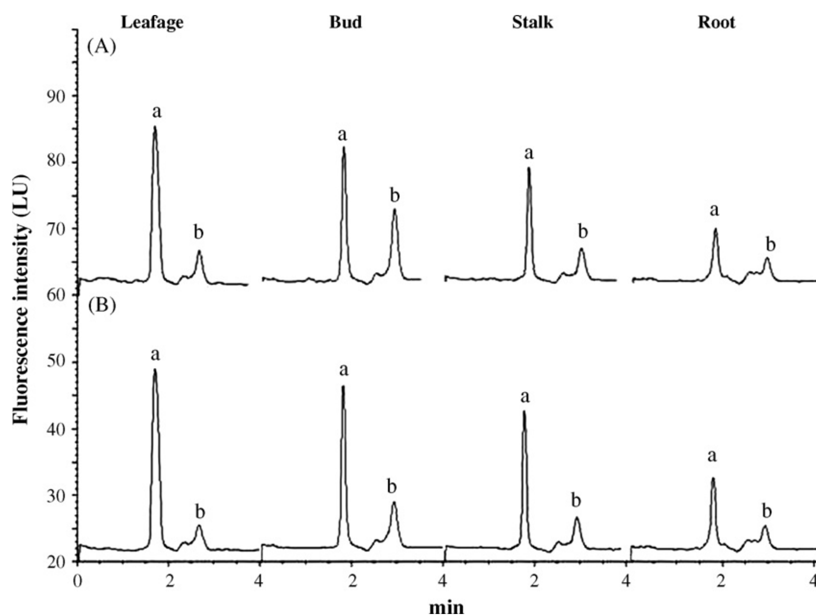

Fig. 7. HPLC profiles from PMME performed on Ludwigia X taiwanensis Peng samples from leafage, bud, stalk and root, respectively. (A) Chromatograms obtained from Ludwigia X taiwanensis Peng samples; (B) the same samples as (A) spiked with 5×10^{-8} mol L^{-1} DAMBO-T. Peaks: (a) DAMBO-T; (b) DAMBO. PMME conditions outlined in Section 2 and HPLC conditions were the same as depicted in Fig. 3.

Table 1
Precisions and recoveries obtained in hydrophytes samples

Samples	Concentration (nM)	Recovery (%)	R.S.D. (%) ($n = 6$)	
			Intraday	Interday
Ludwigia X taiwanensis Peng				
Leafage	70	92.9	1.42	2.11
	140	92.7	1.35	2.23
	180	91.9	1.52	3.21
Bud	60	90.0	1.36	2.16
	120	91.1	1.45	2.45
	180	89.6	1.63	2.45
Stalk	50	92.0	1.75	2.71
	100	90.9	1.82	2.51
	150	91.0	1.92	2.62
Root	30	90.0	1.46	2.32
	60	91.2	1.56	2.52
	90	92.3	1.35	2.51
Foxtail	60	91.7	1.92	2.71
	120	92.1	1.87	2.62
	150	92.5	1.65	2.67
Wild water bamboo root	50	90.0	1.91	2.42
	100	88.9	1.64	1.96
	150	90.1	1.74	1.89
Potamogeton maackianus	80	88.8	1.42	2.75
	160	90.1	1.88	2.66
	200	89.9	1.68	2.74

plant organs-fruits, vegetables and flowers-endogenous levels of NO emission significantly exceed that emitted in the mature or senescing condition. In the present work, it was found that the concentration of NO in root was far lower than that in the leafage (95–218 nM) (Table 1). Also, the content of NO in bud and stalk was more than that in the root (196 nM, 164–95 nM) (Table 1). All these well accorded with the finding of Leshem. It clearly showed that NO played a very important role in plant growth.

The proposed method was also successfully applied to determine NO in the samples of water plant foxtail, Wild water bamboo root and potamogeton maackianus. The chromatograms are shown in Fig. 8. As shown in Table 1, the concentrations of NO in these plant samples were 174, 153 and 252 nM, respectively.

In order to validate the linearity of the PMME-HPLC method, calibration curve was constructed by comparing peak area counts to the corresponding NO concentration under the optimized condition. A linear relationship was established in the range of 9×10^{-11}–4.5×10^{-8} mol L^{-1} ($R^2 = 0.998$). The linear regression equation was $Y = 72.67X + 1.12$ (Y was the chromatographic peak area of the triazole, X was the concentration of NO). The limit of detection (LOD) was found to be 2×10^{-12} mol L^{-1} at a signal-to-noise ratio of 3.

The recoveries of DAMBO-T from hydrophytes samples spiked with three concentrations (low, medium and high) were evaluated by comparing the actual amounts of DAMBO-T added to those obtained by calculation, respectively. As shown in Table 2, the recoveries were in the range of 88.8–92.9%.

The reproducibility of the developed method was determined by the interday and intraday precision. As shown in Table 2, three levels of sample concentration were studied. The intraday and interday precisions were below 2.0 and 3.0%, respectively. Both were calculated as relative standard deviation (R.S.D.) for six measurements. It could be concluded that the method for extraction of DAMBO-T from plant samples was robust and reliable.

Generally, a routine method dealing with biological samples requires acceptable reusability of the extraction sorbent. In this experiment, the decrease in the extraction efficiency of the monolithic column towards DAMBO-T was not observed even after hundreds of usages. Abnormal fluctuation of the column backpressure was also not observed. This can be attributed to the biocompatibility of the monolithic column. Considering the convenient in situ polymerization method with satisfactory

Table 2
The concentration of NO in hydrophytes samples

Samples	Concentration (nM)
Ludwigia X taiwanensis Peng	
Leafage	218
Bud	196
Stalk	164
Root	95
Foxtail	174
Wild water bamboo root	153
Potamogeton maackianus	252

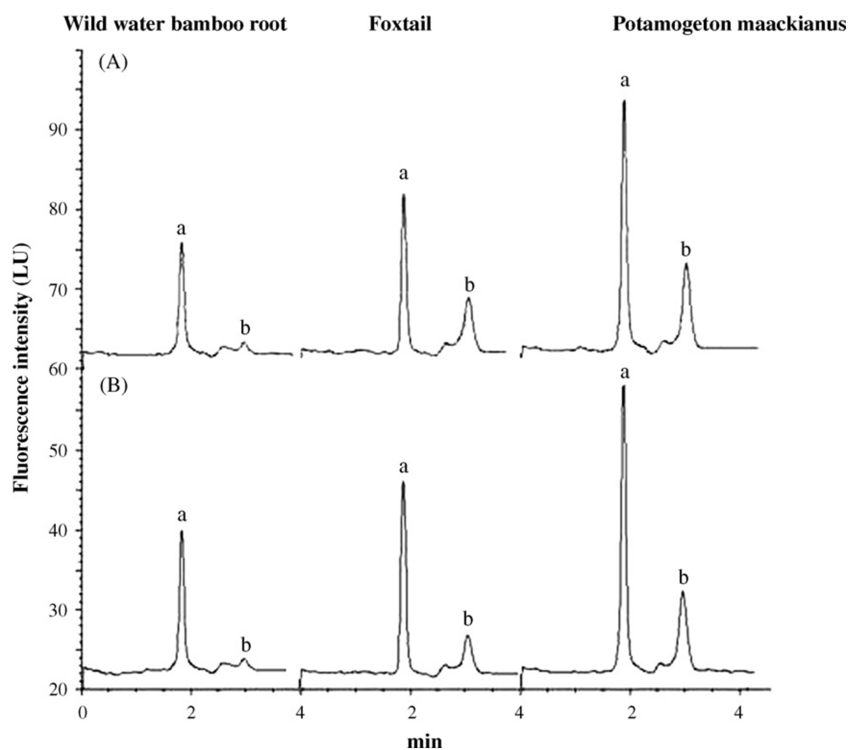

Fig. 8. HPLC profiles from PMME performed on hydrophytes samples from water plant foxtail, wild water bamboo root and potamogeton maackianus, respectively. (A) Chromatograms obtained from hydrophytes samples; (B) the same samples as (A) spiked with 5×10^{-8} mol L^{-1} DAMBO-T. Peaks: (a) DAMBO-T; (b) DAMBO. PMME conditions outlined in Section 2 and HPLC conditions were the same as depicted in Fig. 3.

reproducibility (the R.S.D. of the extraction efficiency <3.5% from column to column) and low cost, one monolithic column can be used only for one routine analysis to shorten analysis time and to reduce contamination.

4. Conclusion

Poly(methacrylic acid-ethylene glycol dimethacrylate) PMME-HPLC was successfully applied to the specific and sensitive determination of NO in hydrophytes samples based on a fluorescent probe, 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacene (DAMBO), derivatization. In comparison to the methods as reported previously, the proposed method is environmentally friendly, inexpensive, simple and sensitive. Also, the proposed method has potential for determination of trace-level NO in other samples, such as cell, tissue and body fluid and in studying NO-related pathology and physiology.

Acknowledgments

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