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Determination of 1-deoxynojirimycin in *Morus alba* L. leaves by derivatization with 9-fluorenylmethyl chloroformate followed by reversed-phase high-performance liquid chromatography

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Abstract

A rapid and reliable method suitable for assays of a large number of *Morus alba* leaves for 1-deoxynojirimycin (DNJ) has been developed. DNJ in 0.1 g of freeze-dried leaves was double-extracted in 10 mL of aqueous 0.05 *M* HCl by vortexing for 15 s at room temperature, derivatized with 9-fluorenylmethyl chloroformate (FMOC-Cl), and analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with a fluorescence detector. The double extraction recovered >99% of extractable DNJ from the leaves. Stabilization of FMOC-derivatized DNJ (DNJ-FMOC) was achieved by diluting the reactant with aqueous acetic acid after derivatization. DNJ-FMOC was stable for at least 16 days under acidic conditions at room temperature (24 °C). Linearity ranged between 0.3 and 30 μg mL^{-1}. The intra- and inter-day precision for DNJ-spiked biological samples was between 0.6 and 1.8% and between 3.7 and 4.5%, respectively.
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Keywords: *Morus alba*; Plant materials; Derivatization, LC; Deoxynojirimycin; Alkaloids

1. Introduction

More than 100 kinds of polyhydroxylated alkaloids possessing piperidine, pyrrolidine, indolizidine, pyrrolizidine, and nortropane structures, and their glycosides, have been isolated from a wide range of plants and microorganisms since the late 1970s [1,2]. The structural similarity to sugars and the inhibitory activity against glycosidase means that most polyhydroxylated alkaloids are antihyperglycemic [3,4]. A piperidine alkaloid, 1-deoxynojirimycin (DNJ), is known to be one of the most potent α-glycosidase inhibitors [5].

Larvae of *Bombyx mori* (silkworm) and leaves of *Morus* species, the sole diet of the silkworm, are widely consumed in Korea and Japan as antihyperglycemic nutraceutical foods for patients with

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diabetes mellitus [6,7]. Twenty-one polyhydroxylated alkaloids have been identified in *M. alba* and larvae of *B. mori* [8–10]. Therefore, because the DNJ content can serve as an antihyperglycemic quality criterion for *M. alba* and *B. mori* products, a reliable method for the determination of DNJ in nutraceutical products such as powdery silkworm, mulberry extract, and other silkworm- and mulberry-based health foods for diabetic patients is necessary.

Trimethylsilyl (TMS) derivatization followed by GC–MS has been the general method for the determination of polyhydroxylated alkaloids by virtue of the high resolution of GC and the additional structural information obtained by MS [11–14], despite the disadvantage of the removal of water from samples for silylation. As an alternative, liquid chromatography has also been considered. However, the resolution of closely related polyhydroxylated alkaloids by liquid chromatography [15] and the lack of chromophores for detection of these compounds must first be overcome.

9-Fluorenylmethyl chloroformate (FMOC-Cl) reacts with primary and secondary amines under mild conditions to yield stable fluorescent derivatives. In the case of tertiary amines, the FMOC moiety is added with dealkylation, even though only limited examples have been reported [16]. Derivatization has been exploited to analyze *N*-containing compounds without a chromophore, such as amino acids [17–20], peptides [21,22], and other compounds in microbial broths, human urine and serum [23–25], by selective pre-column derivatization with FMOC-Cl, followed by reversed-phase high-performance liquid chromatography with UV or fluorescence detection. The FMOC derivatization utilized by Cole et al. to successfully separate six polyhydroxylated alkaloids, including DNJ [15], was applied to a tissue culture of *M. alba* [26]. However, the instability of DNJ-FMOC, as demonstrated in the present report, casts doubt on the credibility of the method.

We report the stabilization of DNJ-FMOC by reducing the pH and its subsequent application to a method for determining DNJ in *M. alba* leaves by RP-HPLC. We have also established a convenient quantitative extraction method for DNJ from *M. alba* leaves for the analyses of large numbers of samples.

2. Experimental

2.1. Chemicals and reagents

Authentic DNJ was purified from *B. mori* by the method described elsewhere [7], and confirmed by ^{1}H- and ^{13}C-NMR. Eight additional polyhydroxylated alkaloids from *M. alba* [8], 2-*O*-α-D-galactopyranosyl-1-deoxynojirimycin (2α-Gal-DNJ), 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), 1,4-dideoxy-1,4-imino-(2-*O*-β-D-glucopyranosyl)-D-arabinitol (2-β-Glc-DAB), fagomine (2-deoxy-DNJ), 3-*epi*-fagomine, calystegines B_1 and B_2 (tropane alkaloids), and *N*-methyl-1-deoxynojirimycin (*N*-Me-DNJ), were generously provided by Professor Naoki Asano (Hokuriku University, Japan). FMOC-Cl was purchased from Fluka (Buchs, Switzerland), and acetonitrile, methanol, and acetic acid for HPLC were from Merck (Darmstadt, Germany). Five millimolar FMOC-Cl in acetonitrile is stable for up to 2 weeks when stored at 4 °C [24]. The water used throughout the experiment was distilled and further purified using a Millipore Milli-Q Gradient system (Bedford, MA, USA).

2.2. Extraction of DNJ from M. alba *leaves*

M. alba leaves were gathered from the mulberry plantation of the Department of Sericulture and Entomology (Suwon, South Korea) on May 17, 2001 and immediately stored in a deep freezer (−70 °C). Frozen leaves were lyophilized (ISE Freeze Dryer, Il Shin Engineering, Seoul, South Korea) and ground to powder before analysis. One hundred milligrams of the powder was added to 10 mL aqueous 0.05 *M* HCl, vortexed for 15 s, and centrifuged at 21 690 *g* for 15 min (Supra 21K, Hanil Science Industrial, Incheon, South Korea). The supernatant was saved, and the pellet was extracted again as described above. The first and second supernatants were then pooled and diluted to 100 mL with water. The diluted extract was used for subsequent derivatization.

2.3. Derivatization

Ten microliters of DNJ standard solution or leaf

extract was mixed with 10 μL of 0.4 *M* potassium borate buffer (pH 8.5) in a 1.5-mL microtube. Twenty microliters of 5 m*M* FMOC-Cl in CH_3CN was added with immediate mixing and allowed to react at 20 °C for 20 min in a water circulator (Dasol Scientific, Suwon, South Korea). Ten microliters of 0.1 *M* glycine (Sigma, St. Louis, MO, USA) was added to terminate the reaction by quenching the remaining FMOC-Cl. The mixture was diluted with 950 μL of 0.1% (v/v) aqueous acetic acid (17.5 m*M*) to stabilize the DNJ-FMOC, and filtered through a 0.2-μm nylon syringe filter (Nalgene, Rochester, NY, USA). A 10-μL aliquot of the filtrate was injected into the HPLC system. The conditions used to optimize the derivatization by FMOC-Cl were the same as above unless stated otherwise.

2.4. HPLC and LC–MS

A SpectraSystem HPLC system (ThermoQuest, San Jose, CA, USA), consisting of an SCM1000 vacuum degasser, a P4000 quaternary gradient pump, an AS3000 autosampler fitted with a 100-μL loop, a stainless-steel Phenomenex Luna C18(2) column (250×4.60 mm I.D., 5 μm), an FL3000 fluorescence detector (excitation 254 nm, emission 322 nm), and ChromQuest (version 2.51) as data processing software was used for analysis. The analyte was eluted with a mobile phase of acetonitrile–0.1% aqueous acetic acid (1:1, v/v) at 1.0 mL min^{-1} for 14 min. After each analysis, 16 min of column washing with MeOH to remove impurities and 10 min of column equilibration with the analyzing mobile phase were necessary to obtain reproducible chromatograms. The column temperature was ambient. A Quattro LC single quadrupole mass spectrometer equipped with an HP-1100 HPLC (Agilent, Palo Alto, CA, USA) and an electrospray ionization (ESI) source (Micromass, Manchester, UK) operating in the positive-ion mode was used for the confirmation of DNJ-FMOC.

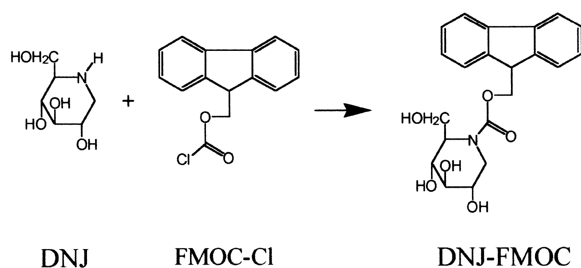

Fig. 1. Formation of DNJ-FMOC from the reaction of DNJ and FMOC-Cl.

3. Results and discussion

3.1. Preparation and LC separation of DNJ-FMOC

The formation of DNJ-FMOC is shown in Fig. 1. HPLC analysis of the reaction mixture revealed peaks for DNJ-FMOC, FMOC-derivatized glycine (Gly-FMOC), and hydrolyzed FMOC-Cl (FMOC-OH) [17–22,24,25] in the chromatogram (data not shown, refer to Fig. 2). Each peak was identified from a separate reaction with DNJ, glycine or water. The formation of DNJ-FMOC was further confirmed by LC–MS. The mass spectrum of the peak at t_R 3.8 min showed the $[M+H]^+$ ion of DNJ-FMOC at m/z 386. The excitation and emission maxima of DNJ-FMOC were 254 and 322 nm, respectively.

DNJ-FMOC was well resolved from the derivatives of the seven relatively abundant polyhydroxy-

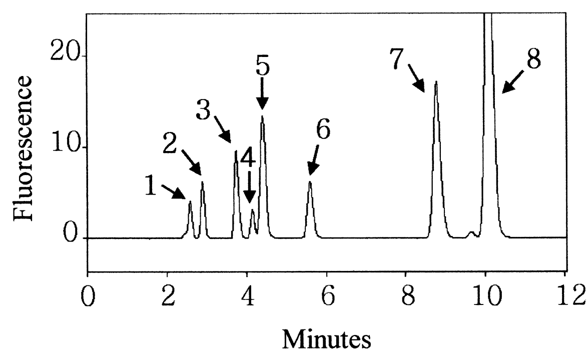

Fig. 2. HPLC separation of DNJ-FMOC from seven other FMOC-labeled polyhydroxylated alkaloids in *M. alba*. Conditions as reported in Section 2. One hundred nanograms of each compound was used for derivatization, except for 10 ng each for calystegine B_1 and 3-*epi*-fagomine. Numbers are FMOC derivatives: **1**, 2β-Gal-DNJ; **2**, 2β-Glc-DAB; **3**, DNJ; **4**, 3-*epi*-fagomine plus calystegine B_1; **5**, fagomine plus DAB; **6**, calystegine B_2; **7**, Gly-FMOC; **8**, FMOC-OH.

lated alkaloids in *M. alba*, whereas the peaks of FMOC-derivatized fagomine and DAB, and calystegine B_1 and 3-*epi*-fagomine overlapped at t_R 4.1 and 4.3 min, respectively (Fig. 2). Overlapping of the FMOC-derivatized fagomine and DAB peaks has been reported elsewhere [15]. *N*-Me-DNJ, a tertiary amine, was not derivatized with FMOC-Cl by demethylation under the derivatization conditions described in Section 2 [16]. Furthermore, the FMOC derivatives of 20 standard amino acids did not interfere with the detection of DNJ-FMOC (data not shown).

3.2. Stabilization of FMOC-labeled polyhydroxylated alkaloids

Because DNJ-FMOC is very unstable with a half-life of about 3 days, only 1.5% of its initial peak area remained after 16 days under non-acidic conditions at room temperature (24 °C) (Fig. 3). The peak area of DNJ-FMOC as measured by UV detection at 254 nm during storage correlated linearly with that measured by fluorescence detection. This suggests that the peak area of DNJ-FMOC is reduced by degradation. A search for stable conditions for DNJ-FMOC revealed that a 20-fold dilution with 0.1% aqueous acetic acid after derivatization stabilizes the DNJ-FMOC molecule with no detectable degradation for 16 days in the dark at room temperature. For reasons yet unknown, the HPLC peak area of DNJ-FMOC under acidic conditions increased by about 4–8% at 4 °C over 16 days of storage. DNJ-FMOC became stable when the reaction mixture was diluted more than two-fold with acetic acid solution. The dilution factor was selected as 20 so as not to exceed the detection limit of the fluorescence detector. Furthermore, DNJ-FMOC was stabilized regardless of the acid used for dilution; 17.5 m*M* each of acetic, formic, hydrochloric, sulfuric, and phosphoric acids showed the same stabilizing effect. However, the photo-stability of DNJ-FMOC was not studied further.

The stabilities of FMOC-labeled polyhydroxylated alkaloids in *M. alba*, other than DNJ-FMOC, were also tested after dilution with water at 24 °C storage temperature, a condition unstable for DNJ-FMOC (Fig. 4). FMOC derivatives of DAB, 2β-Glc-DAB, calystegines B_1 and B_2, and 3-*epi*-fagomine were stable, whereas FMOC derivatives of 2α-Gal-DNJ and fagomine were not. The half-life of FMOC-derivatized 2α-Gal-DNJ was similar to that of DNJ-FMOC. Notably, the FMOC derivative of fagomine (2-deoxy-DNJ) was considerably unstable, whereas that of 3-*epi*-fagomine was stable for 5 days under

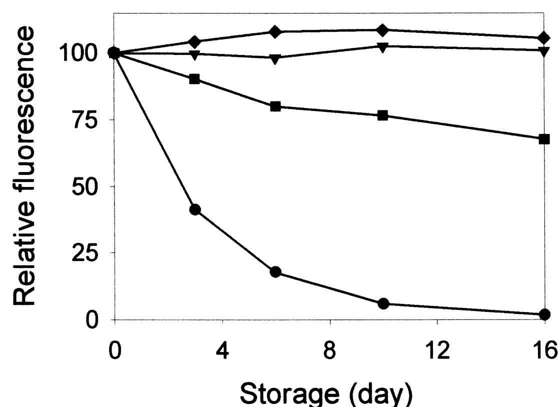

Fig. 3. Stability of DNJ-FMOC after dilution with various solvents and at different storage temperatures. (●) Water/24 °C; (■) water/4 °C; (▼) 0.1% acetic acid/24 °C; (♦) 0.1% acetic acid/4 °C. One hundred nanograms of DNJ was used. Conditions as reported in Section 2 except for the dilution solvent and storage temperature.

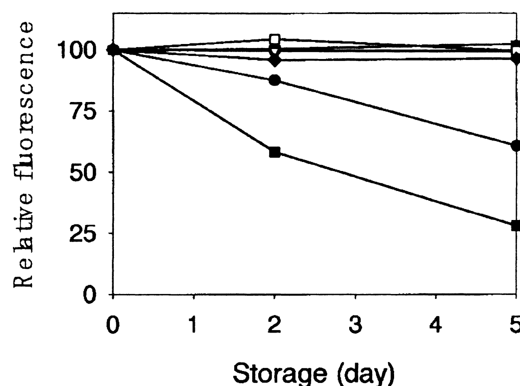

Fig. 4. Stability of FMOC derivatives of polyhydroxylated alkaloids in *M. alba*, other than DNJ-FMOC. FMOC derivatives of (●) fagomine; (○) 3-*epi*-fagomine; (▼) calystegine B_1; (▽) calystegine B_2; (■) 2α-Gal-DNJ; (□) 2β-Glc-DAB; (♦) DAB. One hundred nanograms of each compound was used for derivatization, except for 10 ng each for calystegine B_1 and 3-*epi*-fagomine. Conditions as reported in Section 2 except each derivative was diluted with deionized water (unstable condition for DNJ-FMOC) instead of 0.1% aqueous acetic acid.

non-acidic storage conditions. The stereochemistry of –OH at C-3 might play a role in the stability of the FMOC derivative of polyhydroxylated piperidine, although more examples are necessary to correlate the structure and stability. Under acidic conditions, all FMOC-labeled alkaloids tested, including DNJ-FMOC, were stable.

3.3. Optimization of reaction conditions

Optimal conditions for derivatization were investigated, including pH and concentration of borate buffer, FMOC-Cl and acetonitrile concentrations, and reaction temperature and time. The optimal ranges for pH and concentration for the derivatization of FMOC were pH 7.5–8.5 and 100–400 m*M*, respectively. To achieve maximum buffering capacity of the borate buffer, pH 8.5 and 400 m*M* were selected. The effect of FMOC-Cl concentration on derivatization was examined. The production of DNJ-FMOC increased between 0 and 3 m*M* FMOC-Cl, formed a plateau between 3 and 10 m*M*, and decreased over 10 m*M*. Five millimolar was chosen as the experimental concentration of FMOC-Cl to supply sufficient FMOC-Cl to DNJ during the reaction. When 10 m*M* FMOC-Cl solution was used for derivatization, the Gly-FMOC signal sometimes exceeded the limit of fluorescence detection. The consequence of an insufficient supply of FMOC-Cl in the reaction will be discussed further below. The effect of acetonitrile concentration was also considered. The highest reactivity was observed at 50 or 60% (v/v) acetonitrile concentration; thus 50% was selected for convenience.

As a subsequent step, a time course study of the derivatization of FMOC was performed at 15, 20, 25, and 30 °C under the optimal conditions reported above. Different reaction temperatures resulted in different maximum peak areas at different times. The peak area decreased slowly after reaching the respective maximum value. This suggests that the formation and decomposition of DNJ-FMOC proceed simultaneously from the beginning of the reaction and that the peak area of DNJ-FMOC starts to decrease when the rate of formation becomes slower than that of decomposition. Moreover, increasing reaction temperature resulted in a decreasing maximum peak area of DNJ-FMOC, an indication that the decomposition of DNJ-FMOC becomes greater at elevated temperature. Therefore, a lower temperature favors a higher yield of DNJ-FMOC. However, when the reaction temperature was below 15 °C, the formation of DNJ-FMOC was slow, and the plateau could not be reached in 30 min, although the optimal reaction times at reaction temperatures of 20 and 30 °C were 20 and 10 min, respectively. The reaction conditions were set at 20 °C and 20 min for efficiency.

3.4. Linearity and limit of detection

Various amounts of DNJ (3, 5, 10, 30, 50, 100, and 300 ng) in 10 μL were reacted with 20 μL of 5 m*M* FMOC-Cl in triplicate, corresponding to a 110–11 000 molar ratio of FMOC-Cl to DNJ, to show linearity. The linearity was evaluated by plotting the peak area against DNJ content in nanograms, resulting in a correlation factor, r^2, of 1.0000 and a slope of the regression of 116.2 with an intercept of 61.7. Variations between the replicates are not expressed, because they were negligibly lower than 1%. A calibration curve of DNJ-spiked spinach leaves (*Spinacia oleracea*), which do not contain DNJ, gave the same linearity. The limit of detection was 0.03 μg mL^{-1} for DNJ samples with a signal-to-noise ratio of 3 [27].

3.5. Extraction procedure

The extraction of polyhydroxylated alkaloids from natural samples generally employs aqueous methanol as the solvent at room temperature [11] or at 4 °C [12], or boiling acidic water [13,14] followed by solid-phase extraction (SPE). However, in the case of *M. alba*, good separation of the peak of DNJ-FMOC from those of other interfering materials and the high DNJ content of *M. alba* enable the fluorescence detection of DNJ-FMOC without further purification and concentration.

Rapid, quantitative extraction conditions for DNJ, including extraction temperature, time and solvent, were investigated. DNJ in 0.1 g of dried *M. alba* leaves was readily extracted by vortexing for 15 s at room temperature (24 °C) in 0.05 *M* HCl. Temperatures between 4 and 50 °C and extraction times longer than 15 s did not increase the extraction yield.

Table 1
Proportion of DNJ extracted at each step from 0.1 g of freeze-dried and pulverized *M. alba* leaves ($n=5$) in 10 mL of 0.05 *M* HCl by vortexing for 15 s. Subsequent derivatization conditions were as reported in Section 2

Extraction	DNJ extracted (μg)	RSD (%)	Proportion (%)
First	380.6	1.1	94.9
Second	20.5	15.5	5.0
Third	0.5	9.3	0.1
Total	401.6		100

Double extraction by 15 s vortexing at room temperature (24 °C) was sufficient to recover over 99% of the extractable DNJ (Table 1). Although the pH was lowered by about 0.1 in the extraction step, it was still within the optimal reaction pH range as described in Section 3.3. Only 93% of DNJ in the sample extractable by 0.05 *M* HCl was recovered by water. Although 70% aqueous MeOH gave an extraction yield similar to 0.05 *M* HCl, the presence of MeOH resulted in a slightly lower fluorescence development by 3%.

The chromatogram for DNJ analysis in *M. alba* leaves as described above is shown in Fig. 5. DNJ was the major compound in the extract, and the other compounds present did not interfere significantly with the analysis of DNJ.

3.6. Intra- and inter-day reproducibility for DNJ-spiked *M. alba* leaves

The intra- and inter-day reproducibilities were determined with DNJ-spiked *M. alba* leaves. One hundred micrograms and 1 mg of DNJ were spiked into 0.1 g of *M. alba* leaf powder by adding 100 μL of DNJ standard solution at 1 and 10 μg μL^{-1}, respectively. DNJ was extracted and quantified as described in Section 2. The results demonstrated that the optimized procedure provided satisfactory intra- and inter-day reproducibilities (Table 2). Inter-day reproducibility was obtained for 5 days, guaranteeing the stability of DNJ-FMOC in biological samples for at least 5 days.

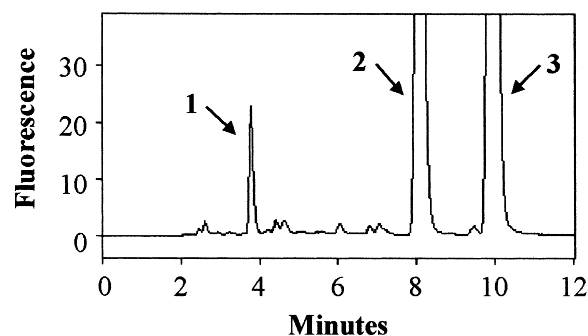

Fig. 5. High-performance liquid chromatogram of the derivatized alkaloids in *M. alba* **1**, DNJ-FMOC; **2**, Gly-FMOC; **3**, FMOC-OH.

Table 2
Intra- and inter-day imprecision for DNJ-spiked *M. alba* leaves ($n=5$)

	Estimated amount of DNJ (μg)	RSD (%)	Estimated addition of DNJ (μg)
Intra-day imprecision			
Non-spiked	372.9	0.6	–
100 μg	471.1	1.8	98.2
1000 μg	1346	1.5	973
Inter-day imprecision			
Non-spiked	383.2	3.7	–
100 μg	479.7	4.3	96.5
1000 μg	1362	4.5	979

4. Conclusion

Quantitative analysis of DNJ by RP-HPLC after derivatization with FMOC-Cl was established, and the stabilization of DNJ-FMOC is reported in detail. An extraction yield of >99% of DNJ from *M. alba* leaves was achieved by double-extraction with 0.05 *M* HCl at ambient temperature, negating the need for additional purification steps such as SPE. This method is valuable for the simple and reliable quantification of DNJ in large numbers of *M. alba* leaves. Moreover, this assay can easily be modified for the quantification of DNJ in powdery larvae and feces of silkworms, although the extraction of DNJ from feces requires a longer extraction time than for *M. alba* leaf powder and *B. mori* larvae powder (unpublished data).

When excessive leaf extract or over 3 mg of DNJ was allowed to react with 20 μL of 5 m*M* FMOC-Cl (molar ratio of FMOC-Cl to DNJ <11), the peak

area of Gly-FMOC decreased dramatically due to the exhaustion of FMOC-Cl, indicating that a sufficient supply of FMOC-Cl is important. By the same logic, if DNJ is present at a relatively low concentration and a large amount of interfering compound, such as a protein, is present in the derivatization with FMOC-Cl, DNJ will not be completely derivatized. In fact, DNJ in the hemolymph of silkworms was easily derivatized and analyzed just by mixing FMOC-Cl after the hemolymph was adequately diluted with distilled water even though FMOC-reactable proteins are present in plenty in hemolymph, which is by virtue of the relatively high concentration of DNJ. However, DNJ in the serum of mice fed orally with DNJ was difficult to quantify without additional sample treatment to remove interfering substances (unpublished results from our laboratory). Studies on the role of DNJ as a feeding deterent against herbivores other than *B. mori* is underway in our laboratory using this efficient analytical protocol.

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