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# Detection and determination of reticuline and *N*-methylcoculaurine in the *Annonaceae* family using liquid chromatography-tandem mass spectrometry

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**Abstract**

In Guadeloupe, the French West Indies, there is a high incidence of atypical parkinsonism or progressive supranuclear palsy, and all of the investigated patients had taken herbal tea or tropical fruits of the *Annonaceae* family. Local inhabitants consume the fruits, and also drink tea made from the leaves. In the present study, we used liquid chromatography-tandem mass spectrometry (LC/MS/MS) with multiple reaction monitoring (MRM) to detect low-molecular-weight neurotoxic benzylisoquinoline derivatives in the *Annonaceae* family. We detected reticuline and *N*-methylcoculaurine in every *Annona muricata* sample examined, except for pulp and seed. They were not detected in sweetsop fruits. Norreticuline was not detected in any sample. These three compounds were toxic to SH-SY5Y neuroblastoma cells and inhibited mitochondrial respiratory complex I. It is possible that uptake of the benzylisoquinoline derivatives reticuline and *N*-methylcoculaurine and their accumulation in the brain may be related to the pathogenesis of the local endemic disease.


*Keywords:* Reticuline; *N*-Methylcoculaurine

## 1. Introduction

In Guadeloupe, the French West Indies, the incidence of atypical parkinsonism or progressive supranuclear palsy is high, and all the investigated patients had taken herbal tea or tropical fruits of the *Annonaceae* family without exception [1]. Local inhabitants consume the fruits and drink tea made from the leaves.

A synthetic heroin analog contaminant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induces parkinsonism in primates by injuring dopaminergic neurons [2,3]. This finding led to the proposal that naturally occurring, low-molecular-weight compounds with similar chemical structure to MPTP are candidates for the endogenous neurotoxins that cause Parkinson's disease (PD) [4–6]. Such compounds might also cause neuronal degeneration in neurological diseases other than PD. Investigations of endogenous neurotoxins identified 1,2,3,4-tetrahydroisoquinoline (TIQ) and β-carboline derivatives as PD-related compounds [7–12]. We have reported that 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1BnTIQ) and 1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline (3′,4′DHBnTIQ) are endogenous neurotoxic brain amines which are related to PD [13–17] (Fig. 1).

In the present study, we measured the levels of reticuline, norreticuline, and *N*-methylcoculaurine, as candidate low-molecular-weight neurotoxic substances, in *Annona muricata* (soursop), *Annona reticulata* (sweetsop), and *Annona squamosa* (custard apple) of the *Annonaceae* family (Fig. 1). For quantitative determination, liquid chromatography-tandem mass spectrometry (LC/MS/MS) with multiple reaction monitoring (MRM) was used.

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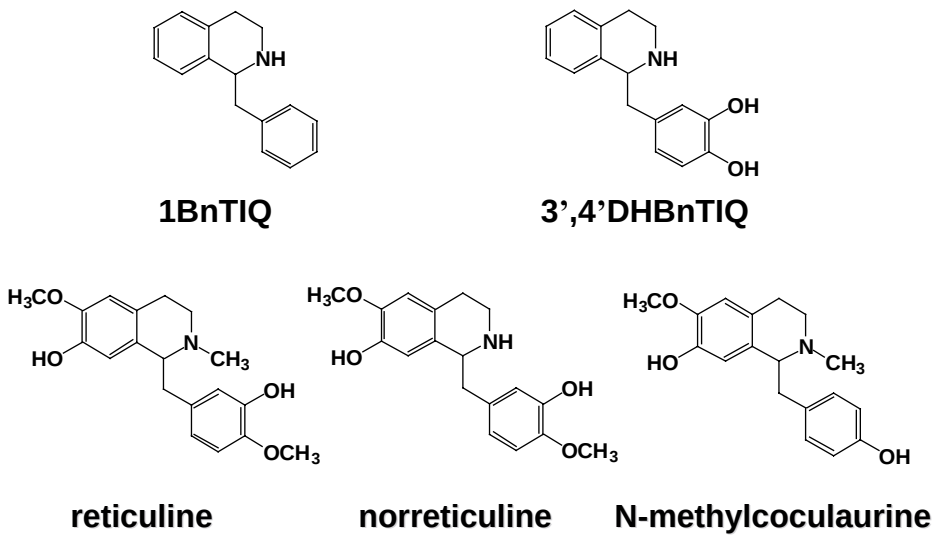


Fig. 1. Chemical structures of benzylisoquinoline derivatives.

This is a powerful technique with high selectivity and sensitivity.

## 2. Materials and methods

### 2.1. Chemicals

Authentic reticuline, norreticuline, and *N*-methylcoculaurine were synthesized by the reported methods [18,19]. Nicotinamide was purchased from Tokyo Kasei (Tokyo, Japan). Ammonia water (28%) was purchased from Sigma (St. Louis, MO). Hydrochloric acid, methanol, and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). Anhydrous sodium sulfate was purchased from Nacarai Tesque Inc. (Kyoto, Japan). All chemicals and solvents used were of the highest analytical grade commercially available.

### 2.2. Sample preparation for LC/MS/MS

*A. muricata* (soursop), *A. reticulata* (sweetsop), and *A. squamosa* (custard apple) of the *Annonaceae* family were gathered in Guadeloupe island, the French West Indies. The total alkaloid liquid-liquid extraction method was employed for determination of these compounds [20]. Portions (50 g) of pulps, pericarps, seeds, and leaves of the fruits were homogenized in a blender, and refluxed with 1 l of *n*-hexane for 1 h. The *n*-hexane was evaporated, and the residue was refluxed with methanol. The methanol extract was dissolved in 200 ml of ammonia water (28%), and the solution was extracted with 200 ml of chloroform. The chloroform layer was extracted with 5% hydrochloric acid. The water layer was added to *n*-hexane, made alkaline and extracted with chloroform. The chloroform layer was dried over anhydrous sodium sulfate, then evaporated, and the residue was dissolved in 3 ml of 50% methanol containing 0.1% acetic acid. Nicotinamide (3 μM) was added to the solution as an internal standard, and the sample was subjected to LC/MS/MS.

Table 1
Parameters of mass spectometric conditions

| Parameters | Compounds | | |
|---|---|---|---|
| | Reticuline | Norreficuline | *N*-methylcoculauline |
| Q1 mass | 329.8 | 315.8 | 299.9 |
| Q3 mass | 136.8 | 178.0 | 106.9 |
| DP | 46 | 21 | 41 |
| EP | 8 | 8 | 8 |
| FP | 340 | 340 | 350 |
| CEP | 16.77 | 16.42 | 16.02 |
| CE | 41 | 25 | 39 |
| CXP | 18 | 10 | 16 |

DP, declustering potential; FP, focusing potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

### 2.3. LC/MS/MS conditions

LC/MS/MS analysis was done with an API2000 (Applied Biosystems) triple-stage quadrupole mass spectrometer coupled to an Agilent 1100 HPLC system (Agilent, CA), and the electrospray ionization method was used for measurement. Reversed-phase Mightysil RP-18 GP (10 cm × 4.6 mm i.d., 5 μm particle diameter, Kanto Chemicals, Tokyo, Japan) was used as the separation column, and the mobile phase was 10% acetonitrile and 0.1% acetic acid in water. Analytical conditions were automatically determined by Analyst® (application software for quantitative determination with the API2000) and indicated in Table 1.

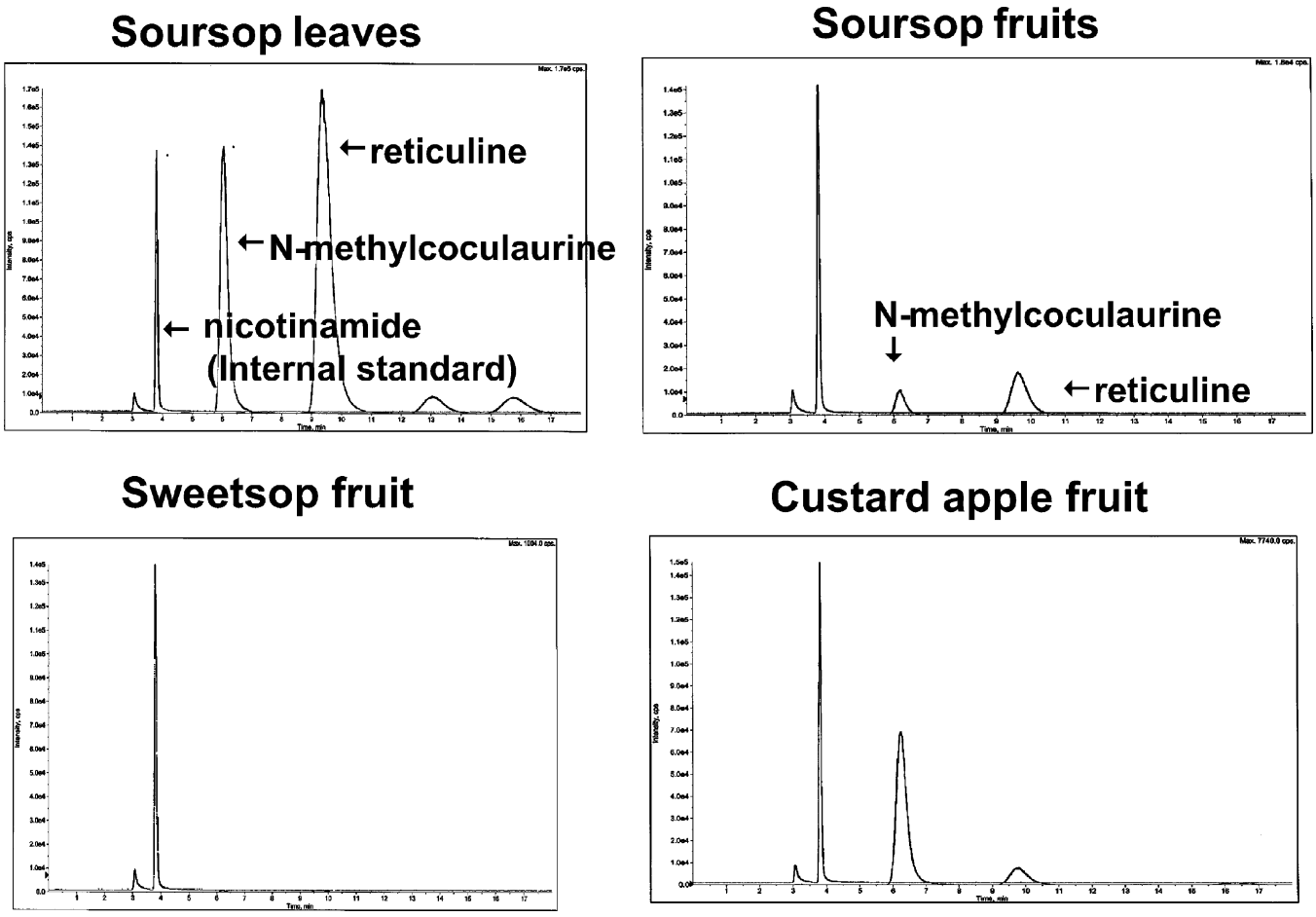


Fig. 2. Typical MRM chromatograms of samples from *Annona* species.

## 3. Results and discussion

First of all, we tried to detect reticuline, norreticuline, and *N*-methylcoculaurine in soursop. Automatic optimization by Analyst® indicated that the product ion *m*/*z* 137 of *m*/*z* 330 of reticuline was the most suitable ion for quantitative determination by MRM. Similarly, the product ions *m*/*z* 178 of *m*/*z* 316 of norreticuline, *m*/*z* 107 of *m*/*z* 300 of *N*-methylcoculaurine and *m*/*z* 80 of *m*/*z* 123 of nicotinamide were selected for quantitative determination by MRM. The typical chromatograms are shown in Fig. 2. MRM chromatograms of four compounds were superimposed. We identified each peak based on corresponding retention times of peaks in the extract sample and the authentic standard in each MRM chromatogram. Reticuline and *N*-methylcoculaurine were detected in every soursop sample except for pulp and seed. They were never detected in sweetsop fruits. Norreticuline was not detected in any sample. It has already been reported that reticuline is present in soursop [21], though its content was not determined.

Next, we determined the contents of these compounds. The calibration curves for the three compounds using MRM were obtained by plotting the peak area ratio of reticuline, norreticuline, and *N*-methylcoculaurine against nicotinamide, the internal standard. $R^2$ values were 0.9868, 0.9882, 0.9892, respectively, and good linearities were observed over the measured concentration range. Reticuline contents were $57.6 \pm 8.6\,\mu g/g$ tissue ($n = 4$) in leaves of soursop, $0.91 \pm 0.57\,\mu g/g$ tissue ($n = 4$) in soursop fruits and $1.0\,\mu g/g$ tissue ($n = 1$) in a custard apple fruit (Table 2). *N*-Methylcoculaurine contents were $9.7 \pm 2.0\,\mu g/g$ tissue ($n = 4$) in leaves of soursop, $0.088 \pm 0.030\,\mu g/g$ tissue ($n = 4$) in soursop fruits and $3.2\,\mu g/g$ tissue ($n = 1$) in a custard apple fruit (Table 2). Reticuline and *N*-methylcoculaurine were most abundant in leaves of soursop.

These compounds have the 1BnTIQ structure in common. 1BnTIQ, which was designed as a possible PD-eliciting neurotoxin, is an endogenous amine in the brain and its content is increased in the cerebrospinal fluid of patients with PD [13]. Repeated administration of 1BnTIQ induced PD-like symptoms in monkeys and mice [13,22]. 1BnTIQ was synthesized from 2-phenylethylamine and phenylacetaldehyde, which is a metabolite of 2-phenylethylamine, in in vivo and in vitro studies [23]. 3′,4′DHBnTIQ is also an endogenous parkinsonism-inducing 1BnTIQ derivative with a catechol structure, and it was reported to be taken up by the

Table 2
Reticuline, norreticuline, and *N*-methylcoculaurine contents (μg/g tissue) in the *Annonaceae* family

| Target compounds | | Reticuline | Norreticuline | *N*-methylcoculaurine |
|---|---|---|---|---|
| Soursop | Pulp | 0.91 ± 0.57 | nd | 0.088 ± 0.080 |
| | Pericarp | nd | nd | nd |
| | Seed | nd | nd | nd |
| | Leaf | 57.6 ± 8.6 | nd | 9.7±2.0 |
| Sweetsop | Pulp | nd | nd | nd |
| | Pericarp | – | – | – |
| | Seed | – | – | – |
| | Leaf | – | – | – |
| Custard apple | Pulp | 1.0 | nd | 3.2 |
| | Pericarp | – | – | – |
| | Seed | – | – | – |
| | Leaf | – | – | – |

nd, not detected; –, not determined.

dopamine transporter [14,15]. Most TIQ derivatives have the potential to inhibit complex I, like $MPP^+$, and 1BnTIQ derivatives have an especially potent action [24]. The three compounds examined in the present study were toxic to SH-SY5Y neuroblastoma cells and inhibited mitochondrial respiratory complex I (unpublished data). It seems possible that these neurotoxic TIQ derivatives are taken up into the human body and accumulated in the brain, and they might be related to the pathogenesis of the local endemic disease.

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