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Short communication

Pipecolic acid methyl esters as artefacts from the ion-exchange chromatography of *Inga punctata* foliar extracts

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Abstract

Methyl esters of hydroxypipecolic acids can be produced as artefacts when cation-exchange resin is used in the sample preparation of plant extracts for chromatographic analysis. Esterification occurs when extracts containing methanol are applied directly to the resin and subsequently washed through with methanolic solutions.

Keywords: *Inga punctata*; Pipecolic acid methyl esters; Hydroxypipecolic acid

1. Introduction

Pipecolic acid is a common non-protein amino acid in plants and occurs with its hydroxylated derivatives in several families [1]. Most structural variations, however, are found in the Leguminosae [2]. Recent chemotaxonomic studies of pipecolic acid derivatives have used a two-dimensional combination of high voltage paper electrophoresis and paper chromatography of crude extracts to propose phytochemical groupings in some of the genera of the tribe Ingeae [3,4]. Such analyses, however, are time-consuming and suffer from poor reproducibility. High resolution techniques, such as liquid chromatography–mass spectrometry or gas chromatography–mass spectrometry (GC–MS), offer a more accurate and rapid alternative but require sample clean-up on ion-exchange resins. Sample preparation for GC–MS is hindered by the necessity to remove methanol from absolute or aqueous methanol extracts before applying samples to the resin. We have investigated whether this stage is necessary and report that methyl esters of pipecolic acids can be produced as artefacts if methanol removal is omitted.

2. Experimental

2.1. Sample preparation

Methanol, methanol–water (70:30, v/v) or water extracts from 40 mg of dried leaves of *Inga punctata* Willd. were each loaded onto Pasteur pipette ion-exchange columns (0.75 g of 100–200 mesh Dowex 50 X8 [H^+]; Merck no. 55162), equilibrated with either the extraction solvent or water (for water extracts, the second column was equilibrated with methanol). Columns were then washed with 10 ml of the equilibration solvent and eluted with 5 ml of 2 *M* aqueous ammonia. Replicates of the purification of the methanol extract on columns washed with metha-

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nol were performed in which elution with 2 *M* ammonia was delayed for fixed periods of up to 2 h.

2.2. Sample analysis

Aliquots of the eluents were freeze-dried and derivatised in Sigma Sil A (Sigma, St. Louis, MO, USA) for 30 min at 70°C. Reaction mixtures were analysed by GC–MS using a 25 m×0.2 mm I.D.× 0.25 μm BPX5 column (SGE) and compounds were identified by comparison with authentic standards or by mass spectral interpretation, as described previously [5].

3. Results and discussion

trans-4-Hydroxypipecolic acid, *cis*-5-hydroxypipecolic acid and 2,4-*trans*-4,5-*trans*-4,5-dihydroxypipecolic acid were detected as their trimethylsilyl (TMS) derivatives in the GC–MS analyses of extracts of *I. punctata*, in accordance with previous studies [3]. However, in analyses of methanolic extracts that had been subjected to ion-exchange in methanol, three additional compounds were present (Fig. 1). Their mass spectra indicated that they were TMS derivatives of the methyl esters of hydroxypipecolic acids; their initial fragmentation produced $[M-59]^+$ ions (i.e., loss of $COOCH_3$) at the same *m/z* values as the $[M-117]^+$ ions (loss of COO–TMS) of the corresponding acid (Fig. 1).

The production of methyl esters was found to be dependent on the length of time the sample was left bound to the resin in methanol before elution: Ratios of ester–acid were generally less than 1:20, if the sample was eluted immediately after washing, but exceeded 1:1, if the sample was left on the column for more than 1 h. The methyl esters were not detected in methanolic extracts purified on ion-exchange resin that was equilibrated with water but were present in water extracts purified on columns equilibrated with methanol, where they constituted 4.0% of the total hydroxypipecolic acid pool. It is likely that the acidic conditions on the column, caused by the displacement of H^+ counter-ions during sample binding, create the conditions for ester formation. Methyl esters were also detected if aqueous methanol was used as the extractant and column wash, but not if the column was washed with water after sample loading.

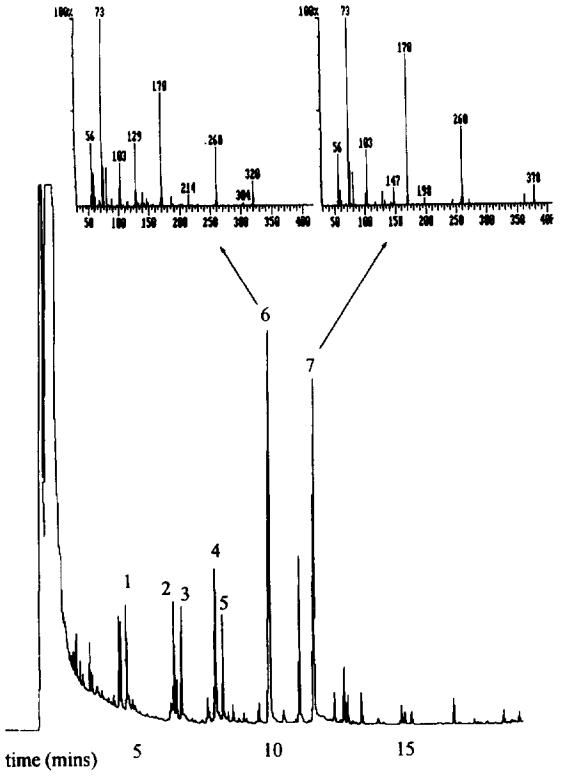

Fig. 1. Gas chromatography trace of a 70% methanol extract of *Inga punctata* prepared by ion-exchange chromatography on Dowex 50 (H^+) and derivatised using Sigma Sil A. Numbered peaks are TMS derivatives. 1=Pipecolic acid; 2=*trans*-4-hydroxypipecolic acid methyl ester; 3=*cis*-5-hydroxypipecolic acid methyl ester; 4=*trans*-4-hydroxypipecolic acid; 5=*cis*-5-hydroxypipecolic acid; 6=*trans*-4-*trans*-5-dihydropipecolic acid methyl ester and 7=*trans*-4-*trans*-5-dihydroxypipecolic acid. Peaks 6 and 7 are accompanied by their mass.

In view of the confusion to the analysis of methyl esters formation, it is suggested that the application of methanolic extracts to ion-exchange resin should be avoided. If the direct application of crude methanolic extracts to resin is necessary to reduce sample preparation times, then it is preferable to wash the resin with water after sample loading; however, this may cause column blockage due to the precipitation of apolar compounds. If analyses do not require the simultaneous extraction of less polar compounds than pipecolic acid derivatives, an alternative would be to make all extracts in water because water and

methanol–water (70:30, v/v) extract similar absolute quantities of hydroxypipecolic acids, thus reducing sample preparation time whilst avoiding extract precipitation on the ion-exchange columns and the formation of methyl ester artefacts.

Acknowledgments

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