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Short Communication

Rapid determination of free tryptophan in plant samples by gas chromatography–selected ion monitoring mass spectrometry

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ABSTRACT

An isotope dilution assay for plant tryptophan is described. The method consists of solid-phase extraction techniques, the one-step formation of the N-acetyl methyl ester derivative, followed by purification by C_{18} high-performance liquid chromatography and analysis by gas chromatography–selected ion monitoring mass spectrometry. The method can be used effectively to measure free tryptophan in plant samples as small as 10 mg fresh weight.

INTRODUCTION

The biosynthetic pathway to the amino acid tryptophan is closely linked to the metabolism of an important plant growth regulator, indole-3-acetic acid (IAA). In order to effectively study IAA biosynthesis in plants, it is essential to have effective methods for quantification and mass spectral analysis of low levels of both IAA and tryptophan. We have previously described methods for the analysis of plant IAA by quantitative mass spectrometry (MS) [1,2]. However, only a few methods for amino acid analysis by gas chromatography (GC)–MS have considered the difficult problem of sample preparation from plant tissues. Most prior efforts have tended to stress derivatization procedures and conditions for separation of mixtures of amino acids by GC [3–7]. Samples of vegetative plant tissues such as leaves, shoots, or roots contain a multitude of native compounds which coextract with tryptophan, thus necessitating extensive purification. Since, like other indolic compounds, tryptophan is a notably labile compound, purification procedures often cause significant losses [8]. Indeed, recoveries of less than 5% have been noted [3]. For our work on indole metabolism in plant tissues we have developed a quantitative mass spectral isotope dilution assay which utilizes disposable sample preparation columns and a single step of high-performance liquid chromatography (HPLC) prior to GC–selected ion monitoring (SIM) MS analysis. The procedure provides a quick and efficient way to

accurately measure tryptophan levels in a few mg (fresh weight) of plant material.

EXPERIMENTAL[a]

Leaf tissue from a Rock Elm (*Ulmus thomasii* Sarg.), 0.01 to 1.0 g, was frozen with liquid nitrogen and ground, while frozen, with a mortar and pestle. The ground sample was then homogenized in 5 to 8 times the tissue volume of isopropanol–0.2 *M* imidazole buffer, pH 7.0 (65:35). A radioactive tracer, [5-^{3}H]tryptophan (0.5 kBq; Amersham, 1 TBq/mmol) and an internal standard of [2,4,5,6,7-$^2H_5$]-tryptophan (98.5% isotope enrichment, MSD Isotopes; 0.01 to 1.0 μg), were added and the sample was left at 4°C for 1 h for isotope equilibration. After 1 h, the extract was centrifuged for 5 min at 10 000 *g*. The pellet was washed three times with isopropanol–imidazole buffer and the pellet discarded. The combined supernatants were then evaporated *in vacuo* to approximately one third of the initial volume in order to remove the organic phase. Water-insoluble compounds which precipitated were removed by centrifugation. In experiments where the full spectrum of endogenous tryptophan was to be obtained for confirmation of identity, the addition of the deuterated standard was omitted. The supernatant was applied to an 8-ml bed volume column of Dowex 50W-X2, 200–400 mesh, equilibrated with 1 *M* HCl. After the sample was applied, the column was washed with three volumes of distilled water. The sample was then eluted with three bed volumes of 2 *M* NH_4OH. The alkaline eluate was evaporated *in vacuo* to approximately one third of the initial volume and was passed through a BakerBond spe amino disposable extraction column (J.T. Baker, pre-equilibrated with 2 ml of methanol followed by 2 ml of distilled water). Under these conditions, tryptophan was not retained on the column. The column was washed with 3 ml of distilled water and the combined eluent was evaporated to dryness *in vacuo* on a rotary evaporator in a 10-ml evaporation flask. The oily residue was dehydrated by multiple azeotropic distillations *in vacuo*: first with absolute ethanol, then with dichloromethane. Immediately after the last distillation from dichloromethane, 1 ml of anhydrous methanol (dehydrated by distillation from magnesium activated with iodine) and 0.5 ml of acetic anhydrice (Supelco) were added and the evaporation flask was closed tightly with a PTFE-sleeved glass stopper and clip. The contents were mixed for 1 min on a vortex mixer and put into a heating block set at 65°C. After 1 h the methanol and acetic anhydride were evaporated under vacuum. The residue was dissolved in 1 ml of distilled water and applied to a Fisher PrepSep C_{18} disposable column. The column was washed with 5 ml of distilled water and the sample was eluted with 2 ml of acetonitrile. The acetonitrile was evaporated to dryness. The residue was dissolved in 100 μl of methanol–water (50:50) and injected onto a Waters NovaPak C_{18} column (15 cm × 3.9 mm) connected to a Waters 600MS HPLC pumping system. The column was eluted with methanol–water (30:70) and the retention time for the N-acetyl methyl ester of tryptophan was 10.4 min. Fractions containing radioactivity were collected, evaporated to dryness and redissolved in 10 μl of ethyl acetate. GC–MS analysis was done on a Hewlett-Packard 5890 gas chromatograph connected to a 5917A mass selective detector. The gas chromatograph was equipped with a 15 m × 0.237 mm DB-1701 fused-silica capillary column (J & W Scientific) and helium was used as carrier gas at 1 ml/min. A 1-μl injection was made in the splitless mode with GC conditions as follows: injector temperature 250°C, initial oven temperature 140°C for 1 min, followed by a ramp at either 20 or 30°C/min up to 280°C and hold for 2 min. Under these conditions the retention time for the N-acetyl methyl ester of tryptophan was 6.07 or 7.83 min, depending on the temperature ramp rate. For quantitative analysis of tryptophan, ions at *m/z* 130 and 135 (unlabeled and labeled quinolinium ions) and at 260 and 265 (unlabeled and labeled molecular ions) were monitored.

RESULTS AND DISCUSSION

One objective of this work was to develop a relatively quick and efficient method for purification of tryptophan from small amounts of plant material

[a] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

for analysis by GC–MS. Crude extracts from different plant samples (leaves, carrot cell suspension, bean hypocotyls) contained from 20 to 100 mg of soluble solids per gram of extracted material. The two ion-exchange preparative columns removed the bulk of the impurities (with essentially quantitative recovery of radioactive tracer), but samples at this step were still too contaminated for high-resolution HPLC under the conditions necessary for elution of underivatized tryptophan. Thus, the partially purified sample was derivatized. Among the many possible amino acid derivatives showing improved properties for reversed-phase HPLC analysis described in the literature [9], the most promising for use on very small samples was the concerted formation of stable N-acyl alkyl esters. Such compounds are stable under the conditions necessary for further purification and are also directly suitable for analysis by GC. Derivatization decreased the polarity of the molecule such that reversed-phase chromatography using simple alcohol–water mixtures became an effective purification technique. In addition, the derivatives are relatively simple in structure, thus facilitating final interpretation of the spectral results. Using a microscale modification of the procedure described by Mee *et al.* [10] for the single step acetylation and methylation of several amino acids using a mixture of anhydrous methanol and acetic anhydride, we were able to obtain good yields of derivatized tryptophan even in complex plant extracts. A large excess of the reagents allowed the effective derivatization of crude samples and the excess of acetic anhydride consumed small traces of water retained in the oily residues. At 65°C, derivatization was essentially quantitative after 35–45 min (as determined by thin-layer chromatography of aliquots taken out during the course of the reaction). The reaction produced about 90% of N-acetyl methyl ester (Fig. 1, peak A) and about 10% of the N,N-diacetyl methyl ester of tryptophan (Fig. 1,

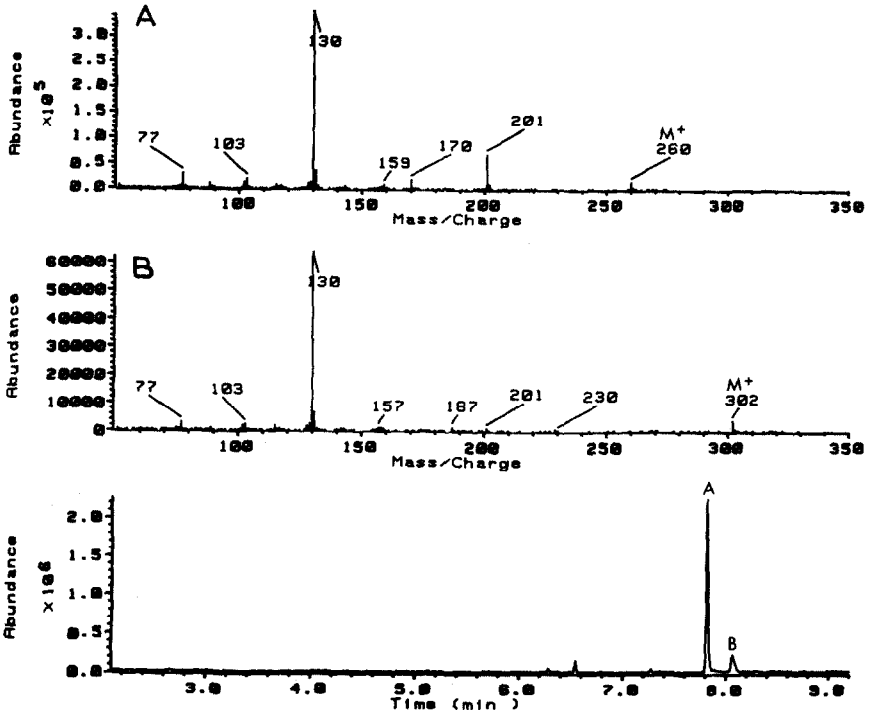

Fig. 1. Reconstructed ion chromatogram (lower frame) from the GC–MS analysis of tryptophan derivatized as described in text to form the mono- and diacetyl methyl ester. The chromatographic peak and the averaged spectrum of the primary reaction product, N-acetyl-tryptophan methyl ester, are labeled A. Under the conditions described only minor amounts of the diacetyl-tryptophan methyl ester are formed, as shown by the peak and averaged spectrum labeled B.

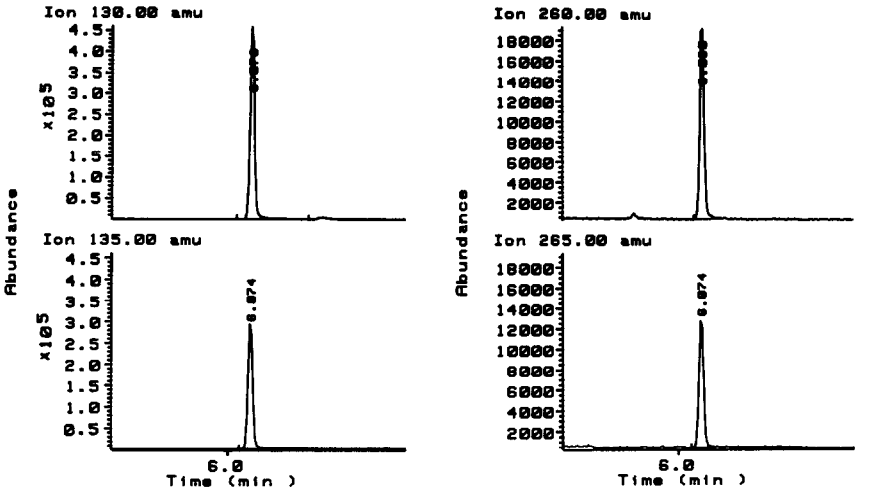

Fig. 2. Selected ion chromatograms for tryptophan isolated from leaf tissue. Ions at *m/z* 130 and 260 are the quinolinium and molecular ions from the native, plant derived, tryptophan while ions at 135 and 265 are from the [$^2H_5$]tryptophan internal standard.

peak B). After HPLC, the derivatized sample was sufficiently pure such that no major peaks, aside from derivatized tryptophan, were detectable by GC–SIM-MS.

A second objective of this study, once suitable purification and derivatization methods were developed, was the application of stable isotope dilution analysis to measure tryptophan in plant samples. For quantification of isolated and derivatized plant tryptophan by GC–SIM-MS two ion pairs were chosen: the quinolinium ion, characteristic for 3-substituted indoles (*m/z* 130 for the naturally occuring compound and *m/z* 135 for the internal standard) and the molecular ion (*m/z* 260 and 265 for the natural and deuterated compounds, respectively). Because the internal standard was labeled at high enrichment with five deuterium atoms on the indole ring, the ions derived from the endogenous compound and from the internal standard were separated by five atomic mass units and there was no overlapping of peaks derived from the endogenous compound and internal standard. Typical SIM traces, as shown for the sample of Rock Elm (*Ulmus thomasii* Sarg.) leaf tissue, are in Fig. 2.

The recovery of tryptophan, based on the radioactive tracer, was 50 to 60%, depending on sample size. The method has proven to be highly reproducible and has been applied to analyze low levels of tryptophan in a wide variety of plant samples including leaves from trees, seedlings of cereal grasses, and legume seedlings. In all of these plant materials, samples of 10 mg fresh weight were clearly sufficient for analysis. The precision of the technique is only limited by the accuracy of weighing the tissue, addition of the internal standard and integration of selected ion peaks. In our hands, variation of replicate samples was usually less than 2%. Thus, this method provides a convenient, sensitive and accurate technique for the routine analysis of tryptophan from complex plant samples.

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