Title

ANALYSIS OF TRYPTOPHAN AND ITS METABOLITES BY REVERSE-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY



Journal

New Phytologist, 88(4)

ISSN

0028-646X

Authors

TARR, JOHN B

ARDITTI, JOSEPH

Publication Date

1981-08-01

DOI

10.1111/j.1469-8137.1981.tb01737.x

*New Phytol.* (1981) **88**, 621–626



# ANALYSIS OF TRYPTOPHAN AND ITS METABOLITES BY REVERSE-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

By JOHN B. TARR and JOSEPH ARDITTI

*Department of Developmental and Cell Biology, University of California, Irvine, CA 92717, U.S.A.*

(*Accepted 24 November 1980*)

## Summary

Rapid separation and determination of tryptophan, *N*-formylkynurenine, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, nicotinic acid, nicotinamide, trigonelline, kynurenic acid and xanthurenic acid from pea seedling using reverse-phase high-pressure liquid chromatography is described. Accuracy of the determinations was better than 5 % and less than 50 pmol of each metabolite could be measured reliably.

## Introduction

Tryptophan is a precursor of niacin in animals and many micro-organisms. Therefore, its catabolism is of considerable interest as a pathway for the biosynthesis of the vitamin and its coenzymes. The role of tryptophan and that of other potential precursors in the biosynthesis of niacin in plants (especially angiosperms) is still unclear however (Arditti and Tarr, 1979), and requires further research. Therefore, a rapid and sensitive assay for the intermediates of tryptophan catabolism in plants, suitable for use with small samples, is highly desirable.

Several methods for the analysis of tryptophan and its metabolites in biological materials have been developed. The most comprehensive procedure (Price, Brown and Yess, 1965) involves many steps of ion-exchange chromatography and spectrophotometric and colorimetric assays, and requires a great deal of effort, time and laboratory space. A variety of methods using ion-exchange (Chilcote and Mrochek, 1972; Chen and Gholson, 1972; Morris and Moon, 1974; Yeh and Brown, 1975), thin layer (Morris and Moon, 1974; Anderson, 1975; Baumann, 1975), and gel permeation (Stratakis, 1976) chromatography, as well as spectroscopic (Hood and Winefordner, 1969; Buxto and Guilbault, 1974) and fluorometric (Nakken, 1963; Gaitonde, Evans and Hartmann, 1979) methods, have been described; these are difficult to use quantitatively, require large samples, or are applicable to only a few compounds. A separation of four compounds by reverse-phase high-pressure liquid chromatography (HPLC), suitable for determination of tryptophan and kynurenine in urine samples, has been reported (Grushka, Kikta and Naylor, 1977), and a method for determination of nicotinic acid in urine by ion-pair reverse-phase HPLC has been developed (Hengen, Seiberth and Hengen, 1978). Quantitative analysis of tryptophan, kynurenine, 3-hydroxykynurenine and kynuramine in rat brain by a combination of ion-exchange HPLC and electron-capture gas–liquid chromatography (GLC) has also been described (Gal and Sherman, 1978). This procedure requires two HPLC steps followed by derivatization and GLC of each effluent fraction. The present

0028-646X/81/080621+06 $02.00/0

report describes the separation and measurement of ten tryptophan metabolites using reverse-phase HPLC. The advantages of the method in analysis of plant materials are discussed.

## MATERIALS AND METHODS

### *Apparatus*

A Waters high-pressure liquid chromatograph consisting of a Model 6000A pumping system, Model 440 ultraviolet absorbance detector set at 254 nm and Model R 401 differential refractometer was used. Injections were made via a Waters U 6 K injector. Monitor outputs were recorded on a Houston Instruments Omni-Scribe dual-pen chart recorder. The column was a Waters μBondapak $C_{18}$, 0·39 × 30 cm, consisting of octadecyl groups covalently attached to a silica gel matrix of particle size 10 μm. It was stored in 50 % methanol and was equilibrated to new solvents for at least 30 min before use. The eluent, a 10 mM sodium acetate buffer, pH 4·8, was prepared from a 1 M stock solution, containing 2·50 g glacial acetic acid and 4·80 g anhydrous sodium acetate in 100 ml immediately before use, degassed *in vacuo* for at least 10 min and filtered. The flow rate was 1·0 ml $min^{-1}$, maintained by a pressure of 900 pounds per square inch (6·21 MPa). After 40 min of sample elution, the flow rate was increased to 2·0 ml $min^{-1}$ at 1800 pounds per square inch (12·42 MPa). All chromatograms were obtained at ambient temperature (*c.* 20 °C).

### *Chemicals*

L-Tryptophan, L-kynurenine, 3-hydroxy-DL-kynurenine, 3-hydroxyanthranilic acid, quinolinic acid, nicotinamide, trigonelline hydrochloride, kynurenic acid and xanthurenic acid were purchased from Sigma. *N*-Formyl-L-kynurenine and nicotinic acid were purchased from Calbiochem. Acetic acid, anhydrous sodium acetate and methanol (AR) were Mallinckrodt products. All water used was deionized and glass-distilled.

Standards were prepared as 1·0 mM solutions, 3-hydroxykynurenine and 3-hydroxyanthranilic acid in 0·01 N HCl, kynurenic acid and xanthurenic acid in 20 % methanol containing 10 mM sodium acetate, pH 4·8, and all others in 10 mM sodium acetate, pH 4·8. The concentration of each solution was standardized by u.v. absorbance measurements. Solutions were mixed in the desired combinations in the injector.

Solvents were filtered through 0·22 μm Millipore filters and samples passed through 0·45 μm Metrical GN-6 membranes (Gelman). Sample injections were made with Hamilton syringes nos 801 (10 μl), 802 (25 μl) and 725 (250 μl).

### *Quantification*

Quantitative analysis of samples was by preparation of standard curves of u.v. peak area (peak height × width at half of peak height, or by integration) *vs.* amount of each compound injected. Recovery of a compound in the column effluent was tested by injecting a large amount (0·5 to 5·0 μmol, 10 to 100 mM), collecting the peak effluent in a volumetric flask (2 to 10 ml) and diluting to volume. An aliquot (25 to 100 μl) of the resulting solution was reinjected, and the amount of the compound present was determined from the peak area. Recovery is the amount reinjected expressed as a percentage of that expected in the diluted portion. The system was tested extensively with a standard mixture before analysis of samples.

*Plant materials.*

Pea and corn seeds were surface-sterilized by soaking for 40 and 30 min respectively in 5% (v/v) commercial sodium hypochlorite (domestic laundry bleach, Iris brand), and germinated and grown for 4 and 7 days under aseptic conditions. Orchids were germinated by standard procedures (Harrison and Arditti, 1970). Tissue samples were homogenized and extracted four times with methanol:chloroform:water (12:5:3, v/v/v). Extracts were prepared by a previously described method (Bieleski and Turner, 1966). Identical samples were supplemented with 100 nmol of each standard and extracted in parallel. Extract residues were diluted to 2 ml with 10 mM sodium acetate, pH 4·8.

## Results

Several eluants for the separation of tryptophan and its metabolites were tested before adequate separation of the compounds was achieved. Distilled, deionized water was unsuitable because most of the peaks were too broad for complete resolution of mixtures. Peaks were much sharper in all buffered eluants tested and 10 mM sodium phosphate, pH 7·2, and sodium 2-morpholinoethanesulphonate, pH 6·0 and 5·5, gave insufficient separation of the compounds eluting in the first few minutes. The elution time of 3-hydroxyanthranilic acid was strongly affected by eluant pH, being 4 min 20 s at pH 7·2, 5 min 40 s at pH 6·0, 9 min 15 s at pH 5·5 and 20 min at pH 4·8. Elution times of tryptophan, and kynurenine and its derivatives decreased below pH 5·5, and that of nicotinic acid increased.

Separation of eight compounds from a synthetic mixture of 10 tryptophan metabolites with 10 mM sodium acetate, pH 4·8, as eluant was complete after 38 min (Fig. 1). Xanthurenic acid and kynurenic acid were separated by about 50 min when the flow rate was increased after 40 min, whereas complete separation required about 68 min if the flow rate was maintained at 1·0 ml $min^{-1}$. Quinolinic

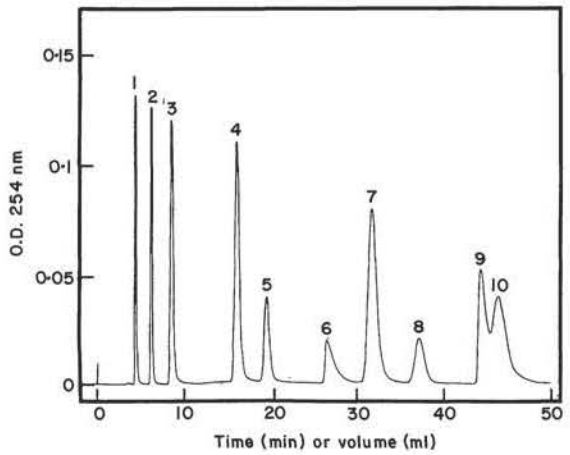


Fig. 1. Separation of a mixture of tryptophan metabolites, containing 10 nmol of each compound, on a $\mu$Bondapak $C_{18}$ column, with 10 mM sodium acetate, pH 4·8, as eluant. Flow rate: 1·0 ml $min^{-1}$, increased to 2·0 ml $min^{-1}$ at 40 min. Explanation of numbers: (1) trigonelline; (2) nicotinic acid; (3) 3-hydroxykynurenine; (4) kynurenine; (5) 3-hydroxyanthranilic acid; (6) nicotinamide; (7) *N*-formylkynurenine; (8) tryptophan; (9) xanthurenic acid; (10) kynurenic acid.

Table 1. *Recovery of injected compounds in column effluent*

| Compound | 1st injection | | 2nd injection | | | Recovery (%) |
|---|---|---|---|---|---|---|
| | Amount (μmol) | Volume collected (ml) | Volume (μl) | Amount expected (nmol) | Amount detected (nmol) | |
| Tryptophan | 5·0 | 10 | 50 | 25 | 26·0 | 104 |
| Kynurenine | 0·5 | 5 | 100 | 10 | 10·4 | 104 |
| 3-Hydroxyanthranilic acid | 0·5 | 10 | 200 | 10 | 9·7 | 97 |
| Nicotinic acid | 5·0 | 2 | 20 | 50 | 50·8 | 102 |
| Nicotinamide | 5·0 | 10 | 50 | 25 | 31·1 | 104 |
| Trigonelline | 2·0 | 2 | 25 | 25 | 26·2 | 105 |

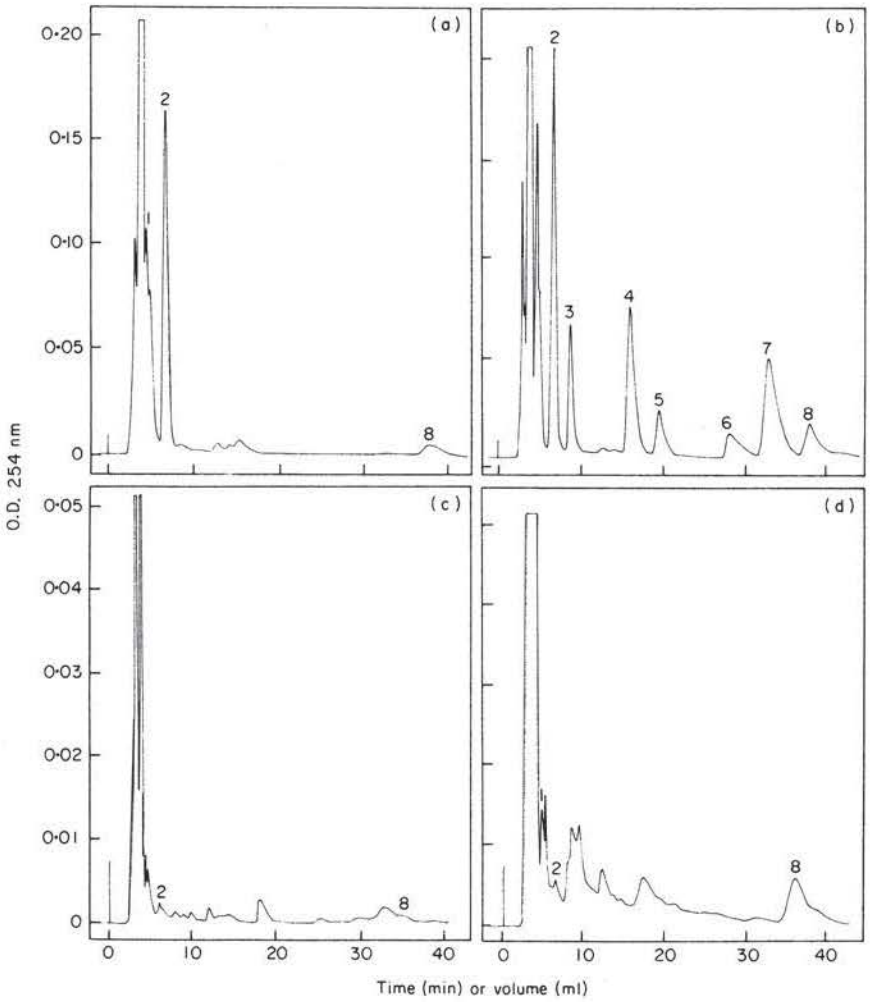


Fig. 2. Chromatograms of (a) an extract of 10 mg pea seedling epicotyls; (b) an extract of 10 mg pea seedling epicotyls, supplemented with 10 nmol each of tryptophan and metabolites prior to extraction; (c) an extract of 10 mg corn seedling epicotyls; (d) an extract of 10 mg *Cattleya skinneri* seedlings. Conditions and peaks are as in Figure 1.

Table 2. *Recovery of 100 nmol each of added standards in extracts of pea seedlings**

| Compound | Amount in 200 $\mu$l aliquot (nmol) | Total amount in extract (nmol) | Recovery (%) |
|---|---|---|---|
| Tryptophan | 9·1 | 91 | 91 |
| *N*-Formylkynurenine | 9·2 | 92 | 92 |
| Kynurenine | 8·6 | 86 | 86 |
| 3-Hydroxykynurenine | 7·4 | 75 | 75 |
| 3-Hydroxyanthranilic acid | 6·6 | 66 | 66 |
| Nicotinic acid | 8·6 | 86 | 86 |
| Nicotinamide | 10·6 | 106 | 106 |

* Average of three to eight extractions.
† Endogenous peaks subtracted.

acid was not resolved under the conditions used in this analysis, and its elution was too broad to be of practical analytical use.

Retention times of all compounds showed daily variations of up to $\pm 5\%$ due to changes in column state, buffer composition, temperature and other factors. Detection by u.v. absorbance was found to be more sensitive than refractive index detection in all cases.

Standard curves of peak area *vs.* amount injected were linear at least in the range of 0 to 50 nmol; measurements were not made with larger amounts. In recovery tests, no changes in the asymmetry of the u.v. or refractive index peaks were observed, indicating that the column was not overloaded at the levels initially injected. Recoveries of all compounds tested were quantitative (Table 1).

Tryptophan and nicotinic acid were found in pea seedlings at mean levels of 0·57 and 3·9 $\mu$mol $g^{-1}$, respectively [Fig. 2(a), (b)]. Trigonelline was present but not measurable due to interfering peaks. Other tryptophan metabolites were not detected even at maximum sensitivity, indicating that the tissues contain less than 2 nmol $g^{-1}$. The amount of each compound in the supplemented sample [Fig. 2(b)] was used after subtracting endogenous peaks, to determine recovery of the added standard in the extraction procedure. Mean recoveries ranged from 66 to 106% (Table 2). Tryptophan, nicotinic acid and trigonelline were also found in corn and orchid seedlings [Fig. 2(c), (d)].

## Discussion

The method of extraction described in this paper is more effective than a procedure using water-immiscible solvents, since nicotinic acid and trigonelline are very hydrophilic. It extracts all of the compounds to be separated almost quantitatively, despite the wide range of structure and hydrophilic character. This method also removes most tissue solutes that would interfere with the analysis and shorten the life of the column.

It was generally difficult to obtain quantitative estimates of trigonelline in extracts due to the presence of interfering peaks in the chromatograms. The other compounds were free of interfering peaks and their levels were readily measured. Similar results were obtained with equivalent extracts of corn and orchid seedlings.

The procedure described here is much simpler and faster than previous methods (a sample can be extracted and analyzed in a few hours) and is suitable for analysis of very small samples. It is as reliable as most other methods currently in use (standard curves indicate that the measurements are accurate to 5 % or less). This method has been used with complete reliability to analyze samples containing 50 pmol of each compound, and is able to detect less than 20 pmol. Since recovery of injected tryptophan metabolites is quantitative, the purified compounds can be collected in the effluent for determination of radioactivity or other analytical procedures. The method is being used at present in studies of niacin biosynthesis by peas, corn and orchids utilizing [$^{14}$C] and [$^{3}$H]tryptophan as well as [$^{14}$C]glycerol and aspartate.

## ACKNOWLEDGEMENTS

We thank Dr E. Rodriguez for the use of his liquid chromatograph, Ms J. L. Cooper for technical assistance, and Mr G. Reynolds and Dr R. Ernst for helpful discussions. This work was supported by NSF grant PCM 76-21483.

## REFERENCES

ANDERSON, G. M. (1975). Quantitation of tryptophan metabolites in rat feces by thin-layer chromatography. *Journal of Chromatography*, **105**, 323–328.

ARDITTI, J. & TARR, J. B. (1979). Niacin biosynthesis in plants. *American Journal of Botany*, **66**, 1105–1113.

BAUMANN, P. (1975). Quantitative determination of indolic compounds in the rat brain using *P*-dimethylaminocinnamaldehyde as reagent. *Journal of Chromatography*, **109**, 313–324.

BIELESKI, R. L. & TURNER, N. A. (1966). Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Analytical Biochemistry*, **17**, 278–293.

BUXTO, J. & GUILBAULT, G. G. (1974). Fluorometric analysis for *N'*-formylkynurenine in plasma and urine. *Clinical Chemistry*, **20**, 765–768.

CHEN, N. C. & GHOLSON, R. K. (1972). An improved column chromatography method for isolation of tryptophan metabolites. *Analytical Biochemistry*, **47**, 139–145.

CHILCOTE, D. D. & MROCHEK, J. E. (1972). Chromatographic analysis of naturally fluorescing compounds. I. Rapid analysis of nanogram amounts of indoles in physiologic fluids. *Clinical Chemistry*, **18**, 778–779.

GAITONDE, M. K., EVANS, G. & HARTMAN, M. K. (1979). Determination of tryptophan by manually operated and autolyzer methods on the formation on norharman. *Analytical Biochemistry*, **92**, 338–344.

GAL, E. M. & SHERMAN, A. D. (1978). Synthesis and metabolism of 1-kynurenine in rat brain. *Journal of Neurochemistry*, **30**, 607–613.

GRUSHKA, E., KIKTA, E. J. & NAYLOR, E. W. (1977). Tryptophan and kynurenine determination in untreated urine by reversed phase high-pressure liquid chromatography. *Journal of Chromatography*, **143**, 51–56.

HARRISON, C. R. & ARDITTI, J. (1970). Growing orchids from seed. *Orchid Digest*, **34**, 199–204.

HENGEN, N., SEIBERTH, V. & HENGEN, M. (1978). High-performance liquid-chromatography determination of free nicotinic acid and its metabolite, nicotinuric acid, in plasma and urine. *Clinical Chemistry*, **24**, 1740–1743.

HOOD, L. V. S. & WINEFORDNER, J. D. (1969). Phosphorimetric investigation of several tryptophan metabolites: determination of kynurenic acid in urine. *Analytical Biochemistry*, **27**, 523–529.

MORRIS, K. M. & MOON, R. J. (1974). Microanalysis of tryptophan metabolites in mice. *Analytical Biochemistry*, **61**, 313–327.

NAKKEN, K. F. (1963). Quantitative fluorometric determination of anthranilic acid, 3-hydroxy- and 5-hydroxyanthranilic acid in the urine. *Scandinavian Journal of Clinical and Laboratory Investigation*, **15** (Suppl. 76), 78–79.

PRICE, J. M., BROWN, R. R. & YESS, N. (1965). Testing the functional capacity of the tryptophan–niacin pathway in man by analysis of urinary metabolites. *Advances in Metabolic Disorders*, **2**, 159–225.

STRATAKIS, E. (1976). Säulenchromagraphische trennung von tryptophan, kynurenin und 3-kynurenin aus biologischem material mittels Sephadex G-25. *Insect Biochemistry*, **6**, 149–151.

YEH, J. K. & BROWN, R. R. (1975). Urinary tryptophan metabolites: assay of free and acid-hydrolyzable conjugates of 3-hydroxykynurenine and 3-hydroxyanthranilic acid. *Biochemical medicine*, **14**, 12–23.