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# On-Line Solid-Phase Extraction of Soil Auxins Produced from Exogenously-Applied Tryptophan with Ion-Suppression Reverse-Phase HPLC Analysis

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## Key Words

Column liquid chromatography  
Plant growth regulators  
Rhizosphere bacteria  
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## Summary

The isolation and purification of plant hormones such as auxins from biological matrices has long been complicated by the need to use liquid-liquid solvent partitioning to isolate and concentrate the neutral, basic and acidic auxin compounds from matrix interferences. To eliminate this solvent partitioning step, a column switching method was devised for use with on-line solid phase extraction and separation by ion-suppression reverse-phase high performance liquid chromatography (IS-HPLC) with ultra-violet (UV) detection (280 nm). The on-line purification of auxins with isocratic HPLC separation is applicable to neutral, basic and acidic auxins and auxin conjugates in a single 40-minute analysis. The method allows direct injection of aliquots from 5 to 100  $\mu$ l with the same precision as loop injection. The method also allows for the analysis of tryptophan and its transformed intermediates into secondary auxin products in a single chromatographic run. Bacterial fermentation broths and soil extracts were analyzed by this method to monitor the conversion of exogenously-applied tryptophan into auxin derivatives.

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## Introduction

Microbial production of auxin derivatives from exogenous sources of tryptophan (TRP)<sup>1</sup> has been demonstrated in both culture media and soils [1-7]. The role of soil auxins in plant development is not fully understood, but preliminary experiments have shown that TRP applications to soil have improved both plant growth and yield in a variety of

vegetable and fruit crops [8-9]. High performance liquid chromatography (HPLC) has been found to be an excellent technique for separation and isolation of auxins [10-11], but complex multicomponent samples such as bacterial broths or soil extracts requires a prefractionation step.

The purification of microbial-derived indole-3-acetic acid (IAA) from bacterial cultures or soils has been traditionally accomplished by the use of solvent partitioning with ethyl acetate to separate interferences from auxin derivatives. The distribution of ionizable molecules such as acidic auxin derivatives into the solvent is influenced by their  $pK_a$  and the pH of the extraction solution. Auxins tend to migrate into the organic phase when in an uncharged form. Neutral compounds are distributed between the two phases according to the partition coefficient,  $K_d = C_{org}/C_{aq}$  [12]. Amphoteric compounds such as the TRP derivatives will remain in the aqueous phase because they exist as dissociated compounds regardless of pH. IAA conjugates will remain in the aqueous phase because of their low solubility in ethyl acetate [13]. In addition, the labile nature of the auxin derivatives during purification has been well documented [12-13]. The necessity of the liquid-liquid partitioning step has limited the scope of investigations focusing on the production of microbial-derived IAA from exogenous TRP sources.

Numerous purification techniques involving the use of ion-exchange resins, Sephadex LH-20, immunoaffinity chromatography, and solid phase extraction have been successfully utilized for purification and concentration of auxin compounds in place of liquid-liquid partitioning [12], but they are of limited use when a large number of samples need to be analyzed.

Recent reviews on the aspects and potential applications of column switching for purification and quantification of organic compounds with HPLC analysis have been published [14-16]. Column switching allows on-line cleanup of biological samples by isolation of the analyte on a precolumn placed in the sample loop port followed by on-line elution of the analyte onto an analytical column. This direct application eliminates errors introduced in the clean-up process and transfer steps and limits possible decomposition of labile analytes by O<sub>2</sub> and light. On-line solid phase procedures with elution onto the analytical column has

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<sup>1</sup>For abbreviations used please refer to list at the end of this paper.

been found to be useful in pharmaceutical applications where labile analytes are directly analyzed from urine and plasma [17–18].

The objective of this work was to evaluate the potential of on-line cleanup in place of liquid-liquid partitioning and subsequent analyses of auxins produced in soils and bacteria fermentation by IS-HPLC. The method was compared with liquid-liquid partitioning for analyses of auxins synthesized from exogenous applications of TRP in soil extracts.

## Experimental

### Reagents

The auxins were obtained from Sigma Chemical Co. (St. Louis, MO, USA) except for the 3-indoleacetyl-aspartic acid and 3-indoleacetyl-glycine which were obtained from Research Organics (Cleveland, OH, USA).

### Chromatographic Instrumentation

Ion suppression-high performance liquid chromatography (IS-HPLC)

The IS-HPLC analysis was performed on a Beckman Model 330 HPLC system including a Model 110A pump, a Model 210 sample injector equipped with a BioRad (BioRad Lab., Richmond, CT, USA) ODS-5  $\mu\text{m}$  guard column (30  $\times$  4.6 mm) as the primary column, a R-Sil (250  $\times$  4.6 mm) reverse phase column (Alltech Associates, Deerfield, IL, USA) as the secondary column and a Beckman Model 165 absorbance monitor set at 280 nm (0.01 absorbance units full scale, AUFS). The mobile phase was 45 % methanol : 55 % deaired HPLC-grade water adjusted to pH 2.53 with  $\text{H}_3\text{PO}_4$  [11]. Separations were conducted at ambient temperatures and the flow rate was 1 mL  $\text{min}^{-1}$ .

### Column Switching Procedure

A sample aliquot ranging from 5 to 100  $\mu\text{L}$  was obtained with a calibrated syringe (Hamilton, Co., Reno, NV, USA) and injected into a 1 mL syringe containing 0.4 mL water. The water-sample aliquot was then injected onto the primary column and washed with 0.4 mL water to remove ionic interferences (Figure 1a). The mobile phase (0.4 mL) was used to rinse the water from the guard column (reducing the water dip) and to remove all non-retained organic compounds. The sample was then eluted from the primary column to the secondary column by moving the injector handle from the load to inject position (Figure 1b) for reverse phase separation and UV detection.

### Detection of Auxins in Fluorescent *Pseudomonas* Culture and Soil

Soil bacteria were isolated on nutrient agar from field-plots which had received 100 Mg  $\text{ha}^{-1}$  of straw over a period of three years (1987–1990). The bacterium selected for this study was characterized as a fluorescent *Pseudomonas* sp. based on its morphological and biochemical properties. Isolated colonies were then streaked on minimal media which had the following composition: (g  $\text{L}^{-1}$ ) glucose, 10; L-

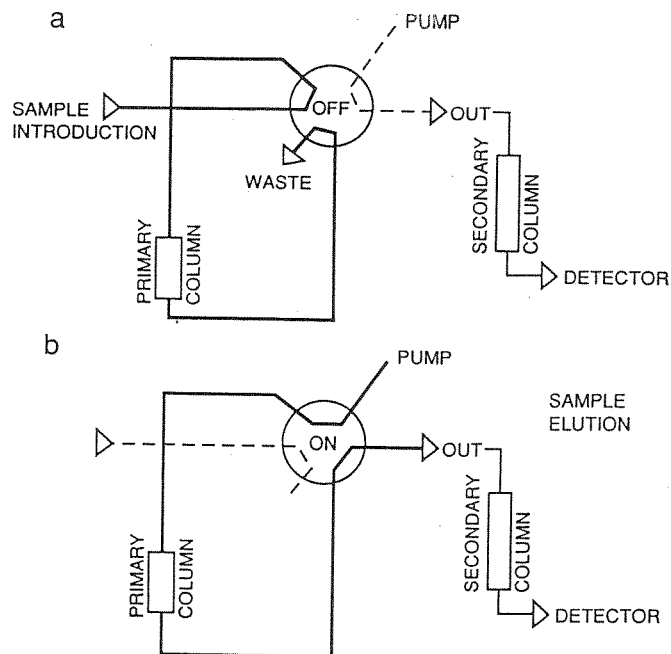


Figure 1

Diagram of the indirect column switching technique. A) Loading and purification of the sample onto the primary column; b) transfer onto the secondary column with secondary eluant. Sample introduction = primary eluant (water-sample) entrance to the primary column, Sample elution = secondary eluant (45 % methanol : 55 % water, pH 2.53) entrance to the primary and secondary column.

tryptophan, 1.0;  $\text{KH}_2\text{PO}_4$ , 1.36;  $\text{NaHPO}_4$ , 2.1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2; 10 mL of a trace element solution (mg  $\text{L}^{-1}$ )  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 700;  $\text{FeSO}_4$ , 200;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 20;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 40;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 700;  $\text{FeSO}_4$ , 200;  $\text{H}_3\text{BO}_4$ , 3;  $\text{CoCl} \cdot 6\text{H}_2\text{O}$ , 7;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 4; yeast extract, 0.10 g; agar, 15 g; and 990 mL of distilled water adjusted to pH 7.0. Isolated colonies were added to 100 mL of the minimal medium (minus agar) and agitated on a rotary shaker (100 rpm) and maintained at 25 °C. Cultivation was stopped at the end of log phase (48 h) and the cells were removed from the broth by centrifugation at 10,000  $\text{rev min}^{-1}$  for 10 min. The supernatant was passed through a 0.22  $\mu\text{m}$  Millipore GS filter (Bedford, MA, USA). An aliquot (10–40  $\mu\text{l}$ ) was directly injected into the HPLC system utilizing the previously described on-line purification method. An aliquot of auxin standards was diluted with the minimal medium for area and retention time analysis to compensate for the effects of the growth medium on auxin chromatography.

Six California soil surface samples (0–25 cm) were selected to obtain a wide range in physical properties (Table I). The following methods were used for soil analysis: pH was determined by a glass electrode (soil : water, 1 : 2.5), organic C [19], total N [20], cation exchange capacity [21] and particle size distribution [22]. Synthesis of soil auxins upon the addition of TRP was monitored as follows: L-TRP (225  $\mu\text{g g}^{-1}$  soil) was added to 2.5 g soil in 1 mL  $\text{H}_2\text{O}$  and incubated at 30 °C for various times. The auxins produced by the soil microbiota were extracted with 4 mL 0.5 M  $\text{KH}_2\text{PO}_4$  (pH 7.0), shaken on a rotary shaker (200  $\text{rev min}^{-1}$ ; 4 °C; 10 min) and filtered through a 0.22  $\mu\text{m}$  Millipore GS filter. An aliquot (10–40  $\mu\text{l}$ ) was directly injected into the HPLC system utilizing the on-line purification proce-

**Table I** Properties of soils used

Soil	pH	Organic C	Total N	Clay	Sand	CEC <sup>a</sup>
		g kg <sup>-1</sup>				
Sheephead	6.87	2.28	0.132	26	56	13.9
Altamont	6.45	1.16	0.168	46	21	26.8
Ramona	6.31	0.78	0.169	26	48	13.1
Domino	8.55	0.44	0.104	29	43	16.8
Redding	6.56	0.38	0.068	64	25	25.4
Hesperia	7.22	0.28	0.066	18	74	6.6

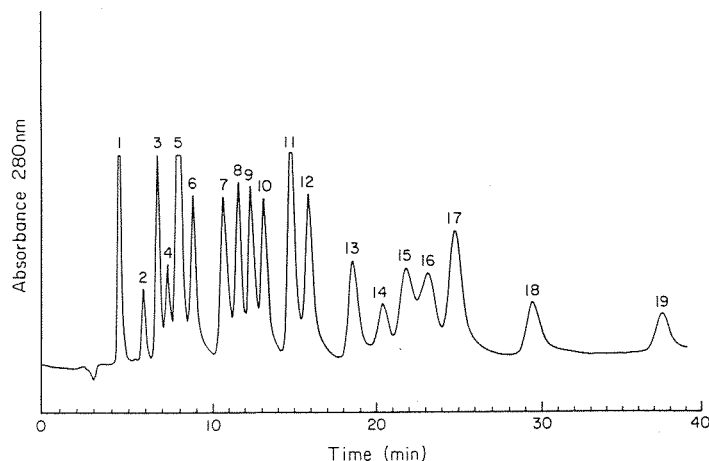
<sup>a</sup>CEC, cation exchange capacity (cM<sup>+</sup> kg<sup>-1</sup> soil).

cedure previously described. In addition, a 2-mL aliquot of the sample was pH adjusted to pH 2.8 with H<sub>3</sub>PO<sub>4</sub> and extracted with three 10-mL portions of ethyl acetate. The ethyl acetate fractions were pooled, reduced in volume to a residue under N<sub>2</sub> gas and resuspended in 2 mL of mobile phase. The ethyl acetate fraction contained primarily the acidic indoles [12]. An aliquot of auxin standards was diluted with the 0.5 M KH<sub>2</sub>PO<sub>4</sub> extraction solution (pH 7.0) for area and retention time analysis to compensate for effects of the extraction solution on auxin chromatography.

## Results and Discussion

### Column Switching

Direct HPLC analysis of biological extracts is often subject to interferences from numerous endogenous compounds in the sample when detecting a low analyte concentration. Fractionation and concentration steps in preparation of auxin derivatives for HPLC analysis by solvent partitioning are labor intensive, time consuming, requires the use of expensive solvents and can introduce additional sources of errors. Figure 1 shows the switching valve configuration for executing the indirect sample transfer technique used in the

**Figure 2**

Chromatogram of tryptophan and auxin derivatives detected by IS-HPLC. 1 = 5-OH-IAA; 2 = IAA-Asp; 3 = IAA-Gln; 4 = 5-OH-TRP; 5 = IAM; 6 = ILA; 7 = 5-OH-TAM; 8 = IAA; 9 = IAALD; 10 = TOL; 11 = IALD; 12 = IAN; 13 = IPA; 14 = IPyA; 15 = TRP; 16 = IM; 17 = IAcry; 18 = IBA; 19 = TAM.

work reported here [14]. The sample-water aliquot loads the analytes on the primary column (Figure 1a). The additional wash with water and then the mobile phase separates the analytes present from other interfering compounds, but TRP and auxin derivatives of interest do not elute from the column. By rotating the switching valve (Figure 1b) into the transfer position, the secondary mobile phase (45 % methanol : 55 % water, pH 2.53) with a higher elution power elutes the analytes onto the secondary column (R-Sil) for detection while the primary column is conditioned with the mobile phase for the next sample. The indirect sample transfer method was used in order to maintain the same direction of flow of both sample and mobile phase through the primary column. The main advantages of this extraction method is that the on-line sample preparation is highly specific for the analyte, it significantly reduces sample preparation time from ~ 1 h to < 2 min and also eliminates the need for solvent partitioning.

### Liquid Chromatographic Analysis

A standard chromatogram of selected TRP and auxin derivatives analyzed by the on-line elution method is shown in Figure 2. The time of analysis for detection of the 19 TRP and auxin derivatives was < 40 min. The retention time of TRP is a direct result the eluant pH and ionic concentration. Tryptophan (pK<sub>1</sub> = 2.38) is not fully ion suppressed at pH 2.5 as are the auxin derivatives and responds to the phosphate concentration in the eluant and extraction solution. The TRP retention time at ≤ pH 2.50 was 15 min and by increasing the pH to 2.53, the TRP retention time was increased to 23 min. Degassing of the mobile phase and pH selection resulted in reproducible TRP retention times.

The column capacity factor (*k'*), resolution (*R<sub>s</sub>*) and the efficiencies (*N* and HETP) for separation of the TRP and auxin derivatives by the R-Sil column and the mobile phase employed here have been previously reported elsewhere [9]. The precision and limits of detection (LODs) for the on-line and loop injections are given in Table II. Precision was determined based upon repeated 20 μL injections (*N* = 10) of the combined standard at 5 mg L<sup>-1</sup>. The relative standard deviations in detection of TRP and auxin derivatives ranged from 0.10 to 0.54 % for the loop and on-line injections. On-line elution of auxin derivatives had the same precision as loop injection. The LODs of the TRP and auxin derivatives were examined by spiking the extraction solution with a known amount of compound to be determined. The LODs ranged from 0.05 to 0.2 mg L<sup>-1</sup> (100 μL sample) with on-line solid phase concentration. The linearity of the on-line elution method for the analysis of IAM, IAA, IPyA and TRP is shown in Figure 3.

### Detection of auxins in Soils and Bacterial Broths

Figure 4 shows the comparison between the liquid-liquid solvent partitioning step with ethyl acetate and the on-line column switching technique. The peak area determined upon analysis of the sample by the liquid partitioning method (< 1 h sample preparation) was only 75 % of the IAA area determined by the on-line method. The unknown peak (number 1) was determined to be a breakdown product of IAA (Figure 4b). This peak became larger with the

**Table II** Selected chromatographic parameters in detection of tryptophan and auxin derivatives by IS-HPLC<sup>a</sup>.

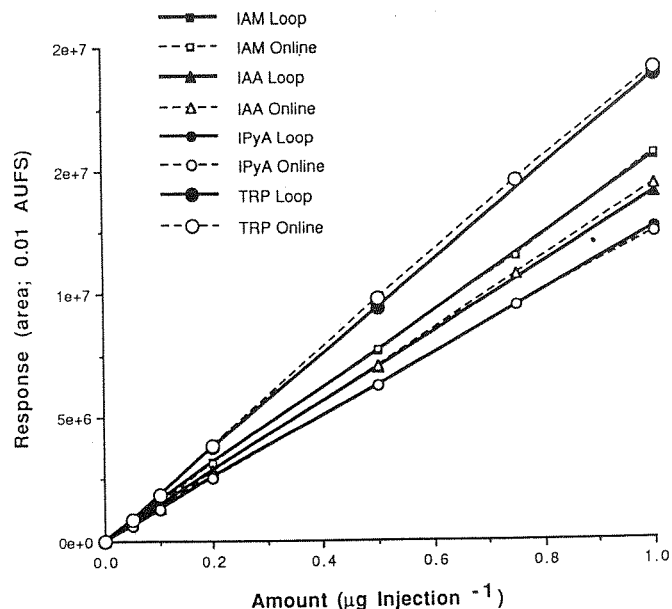
Compound	$t_R$ (min)		LOD (mg L <sup>-1</sup> )		RSD (%)	
	On-line elution	On-line <sup>b</sup> elution	On-line elution	On-line Loop injection	On-line elution	Loop injection
5-OH-IAA	4.70	0.07	0.10	0.10		
IAA-Asp	6.30	0.15	0.18	0.19		
IAA-Gln	7.10	0.08	0.25	0.23		
5-OH-TRP	7.60	0.18	0.18	0.19		
IAM	8.40	0.05	0.11	0.11		
ILA	9.30	0.15	0.13	0.13		
5-OH-TAM	11.20	0.10	0.24	0.24		
IAA	12.20	0.12	0.15	0.15		
IAALD	13.10	0.15	0.18	0.18		
TOL	13.90	0.08	0.18	0.18		
IALD	15.50	0.60	0.15	0.16		
IAN	16.60	0.10	0.25	0.24		
IPA	19.40	0.18	0.30	0.30		
IPyA	21.30	0.10	0.55	0.54		
TRP	22.80	0.10	0.40	0.39		
IM	24.10	0.12	0.37	0.37		
IAcry	25.90	0.09	0.28	0.28		
IBA	30.70	0.13	0.35	0.36		
TAM	38.70	0.20	0.50	0.50		

<sup>a</sup> $t_R$ , retention time; LOD, limits of detection; RSD, relative standard deviation.

<sup>b</sup>Absorbance unit full scale = 0.01.

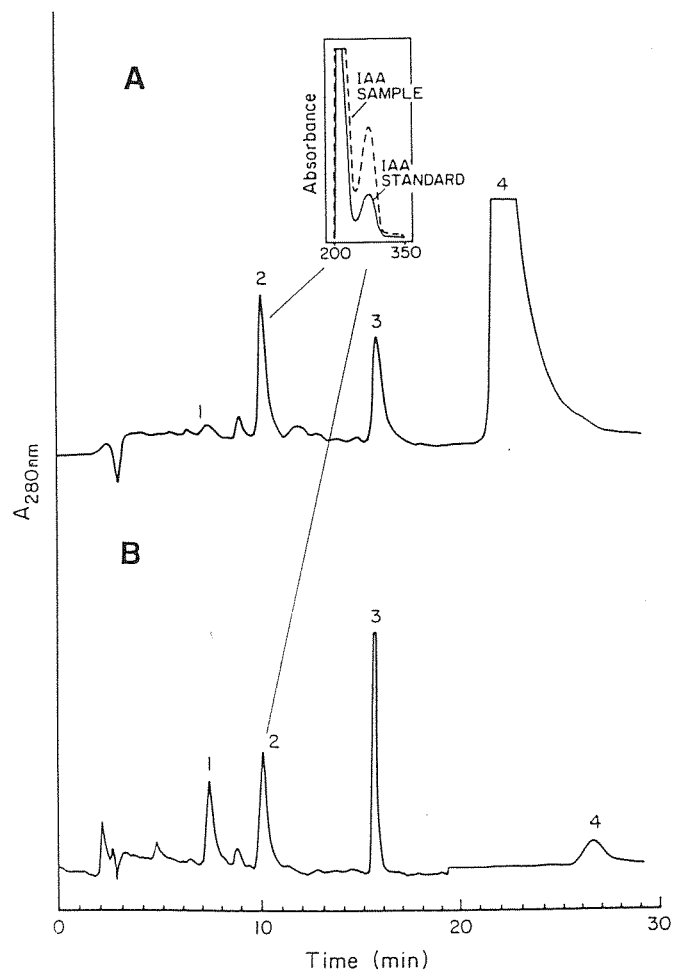
subsequent IAA peak area are smaller as the evaporation time for removal of the ethyl acetate was increased (< 1 to 2 h). Indole-3-propionic acid was used as an internal standard in the analysis of the TRP-amended soil extracts. The phosphate content of the extraction solution affected the chromatography of TRP by decreasing the retention time of TRP from 27 min (Figure 4b) to 23 min (Figure 4a). Tryptophan recovery was very low in the ethyl acetate fraction when compared with the column switching method due to the lack of partitioning of the amphoteric compound into the solvent.

The amounts of IAA produced in the six soils employed in this study upon incubation with TRP are shown in Table III. Indole-3-acetic acid is considered to be one of the most active auxins produced by soil microflora. Synthesis of IAA was found within 1 day of incubation in the Domino soil and after incubation for 3 days, IAA concentrations (6 soils) ranged from 1  $\mu\text{g}$  to 13  $\mu\text{g g}^{-1}$  soil in the six soils. The average soil IAA concentration reached a maximum after incubation for 5 days and then decreased with increasing incubation time. At time zero, the soils used in this study had adsorbed 26.9 to 81.1  $\mu\text{g TRP g}^{-1}$  soil. Tryptophan adsorption was correlated with total N content ( $r = 0.84^*$ ) but was not significantly correlated with the other measured soil chemical and physical properties. Amphoteric compounds such as amino acids have been found to be adsorbed to soil clay materials by cation and anion exchange [23]. The total N content measured is an index of organic matter in



**Figure 3**

Linearity of the on-line column switching technique for detection of IAM, IAA, IPyA and TRP.



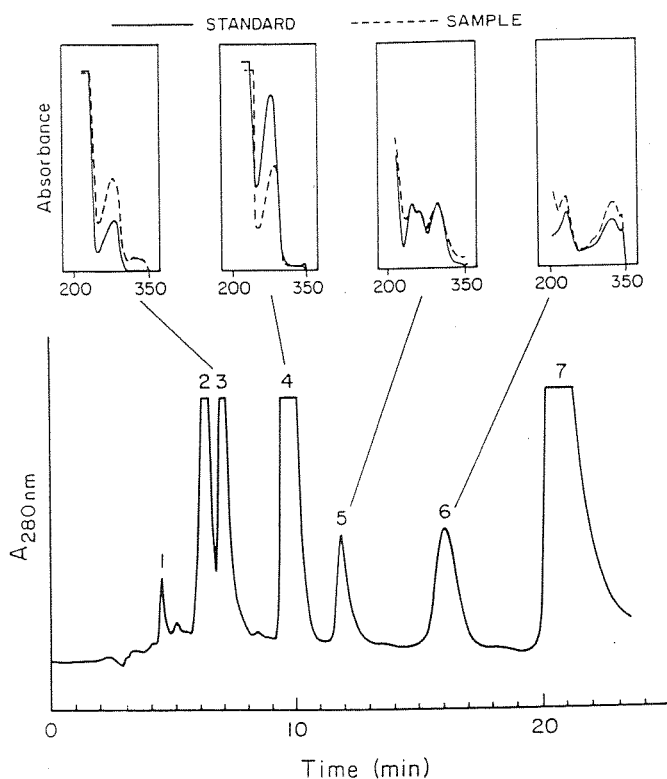
**Figure 4**

Comparison of a) on-line column switching technique and b) solvent partitioning for purification of IAA produced in soil from TRP application. 1 = unknown; 2 = IAA; 3 = IPA (international standard); 4 = TRP.

**Table III** Metabolism of TRP and synthesis of IAA in soils amended with TRP<sup>a</sup>.

Soil	Time (days)									
	0	2	3	5	7	0	2	3	5	7
	μg g <sup>-1</sup> soil									
	TRP					IAA				
Sheephead	159.7	61.3	8.1	0.0	0.0	0.0	0.0	1.2	8.3	2.4
Altamont	149.2	85.1	16.1	1.1	0.0	0.0	0.6	3.1	5.6	2.4
Ramona	143.9	96.1	30.2	11.0	0.0	0.0	3.0	8.1	11.3	0.0
Domino	181.8	34.1	8.4	0.0	0.0	0.0	16.8	13.1	12.3	1.5
Redding	197.4	41.1	6.6	0.0	0.0	0.0	0.8	1.3	0.8	0.0
Hesperia	167.1	58.4	15.0	2.4	0.0	0.0	2.6	4.1	12.1	0.0

<sup>a</sup>Soil samples were incubated with 225 μg TRP g<sup>-1</sup> soil for various times at 30 °C.



**Figure 5**

IS-HPLC chromatogram of auxins produced in a *Pseudomonas* sp. fermentation broth amended with TRP and separated with on-line column switching. 1 = 5-OH-IAA; 2 = 5-OH-TRP, 3 = IAM; 4 = IAA; 5 = IALD; 6 = IPyA; 7 = TRP.

soils. The hydrophobic nature of the indole nucleus in TRP may influence its adsorption to the organic fraction of soils. The six soils with the exception of the Ramona soil had a very high rate of TRP metabolism with little or no TRP remaining after incubation for 5 days.

The *Pseudomonas* sp. used here was selected from over 50 isolates due to its relatively large production of auxins upon the addition of TRP. The auxin derivatives produced by the *Pseudomonas* sp. when supplied with an exogenous source

of TRP are shown in Figure 5. The identity of IAA and the other auxins were confirmed by UV spectrometry as they eluted from the C<sub>18</sub> column. On-line elution and detection of neutral and acidic auxins and TRP as a substrate in a single chromatographic analysis is a major advantage over solvent partitioning for auxin analysis.

## Conclusion

The described on-line solid phase extraction procedure for the quantitation of TRP and auxin derivatives allows separation by IS-HPLC with UV detection in complex biological matrices such as soils and bacteria fermentation broths. The advantages of the on-line concentration method include reduced sample preparation and chromatographic analysis time; limits operator exposure to solvents; allows many sample (> 100) injections to be processed on one primary column; eliminates photo-oxidation of labile auxins; allows analysis of both the substrate and products and this on-line sample clean-up procedure can be automated with the addition of an autosampler.

## Abbreviations Used

IAA,	indole-3-acetic acid;
IS-HPLC,	ion suppression reverse phase high performance liquid chromatography;
TAM,	tryptamine;
IAALD,	indole-3-acetaldehyde;
IAM,	indole-3-acetamide;
IAN,	indole-3-acetonitrile;
IAcry,	3-β-indoleacrylic acid;
IALD,	indole-3-aldehyde;
IBA,	indole-3-butyric acid;
TOL,	indole-3-ethanol;
ILA,	indole-3-lactic acid;
IM,	indole-3-methanol;

IPA, indole-3-propionic acid;  
 IPyA, indole-3-pyruvic acid;  
 IAA-Asp, 3-indoleacetyl-aspartic acid;  
 IAA-Gln, 3-indoleacetyl-glycine;  
 5-OH-IAA, 5-hydroxyl-indole-3-acetic acid;  
 5-OH-TAM, 5-hydroxyltryptamine;  
 5-OH-TRP, 5-hydroxyltryptophan;  
 TRP, tryptophan.

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