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# Method for the determination of indole-3-acetic acid and related compounds of L-tryptophan catabolism in soils

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## ABSTRACT

An optimized method for the determination of substances occurring in auxin metabolism and L-tryptophan (TRP) catabolism was developed. It is based on solid-phase extraction (SPE), two isocratic reversed-phase high-performance liquid chromatographic (HPLC) separations at different liquid phase conditions and the simultaneous detection of fluorescence and UV absorbance at different wavelengths. Advantages of the proposed method are: the solvent (ethanol) and liquid phase (containing 2-propanol) provide optimum stability and selectivity; almost no toxic wastes are produced; no time-consuming liquid–liquid extractions (LLE), derivatization procedures or column re-equilibration (obligatory for gradient systems) are necessary, no need for antioxidants, ion-pair or derivatization reagents; recovery rates of the SPE system are superior to LLE efficiencies; high sensitivity, selectivity and identification capacity are provided by the proposed HPLC and detection system. By measuring various chromatographic and spectral parameters simultaneously, the determination reliability is improved. The characteristic chromatographic and spectral data for selected indole derivatives and TRP catabolites are presented. In samples from two different soils that were tested with the proposed method, the actual contents of TRP were 1.4 and 5.8 μg g dry soil. In addition, traces of indole-3-acetic acid (IAA) could be detected. When TRP was added, IAA was the predominant catabolite in both soils, and reached values of 2.9 and 8.0 μg g dry soil. In addition to IAA, indole-3-ethanol, indole-3-aldehyde, indole-3-carboxylic acid, indole-3-lactic acid, anthranilic acid and traces of indole-3-acetamide were identified and determined.

## INTRODUCTION

Indole-3-acetic acid (IAA) and other indole derivatives with auxin-like phytohormone activity are synthesized by plants and many soil microorganisms. Originating from the central auxin precursor L-tryptophan (TRP), different pathways of microbial auxin biosynthesis have been described: the tryptamine (TAM) [1,2], the indole-3-pyruvic acid (IPA) [1–3], the indole-3-acetamide (IAM) [1,2,4] and the tryptophan side-chain oxidase (TSO) [5] pathways. Many plant growth promoting rhizobacteria [3,5–9] seem to catabolize TRP via the IPA pathway. The occurrence of indole-3-lactic acid (ILA), a side product of the unstable intermediate IPA, points to the IPA pathway of IAA biosynthesis. IAM, the characteristic intermediate of the IAM pathway, has been detected in several plant pathogens [4,10–12].

Enzymatic IAA catabolism and autoxidative auxin degradation can result in many different products [2,13]. Indole-3-methanol (IM), indole-3-aldehyde (IAld) and indole-3-carboxylic acid (ICA), products of decarboxylative IAA degradation by "IAA oxidase" catalysis, have often been detected [2,7,13].

In addition to auxin biosynthesis, TRP can be catabolized via the "aromatic pathway" [14] with anthranilic acid (AnthrA) as an intermediate.

Although many soil microorganisms have been tested for IAA biosynthesis, very few reports have dealt with auxin metabolism in soil. Actual auxin

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contents seem to be very low in soil, but microbial auxin production is greatly enhanced by adding TRP (potential for auxin biosynthesis) [1].

The preparation of samples containing different indole derivatives is usually performed by partition techniques that require several liquid–liquid extraction (LLE) steps [1,2,15–17]. LLE methods, however, are very time consuming, and produce considerable amounts of toxic organic wastes. Compared with LLE procedures, solid-phase extraction (SPE) provides several advantages: SPE produces less toxic organic waste, is less time consuming and can be applied easily to routine assays. For the purification and concentration of several plant hormones, Amberlite SPE resins have already been used successfully and provided excellent recoveries [18,19].

Immunoassays (*e.g.*, enzyme-linked immunosorbent assay, radioimmunoassay) [2] with highly specific antibodies provide the most sensitive methods to measure single phytohormones. However, if an unknown spectrum of various phytohormone derivatives in a sample is to be analysed, immunological methods cannot be applied. Derivatization procedures that focus on selected targets [16,17] are also of no use.

For the separation of several phytohormones and their derivatives, reversed-phase high-performance liquid chromatography (RP-HPLC) is a practical method [2,13]. The chromatographic process can be influenced by ion-pair reagents. Ion-pair RP-HPLC with gradient elution has been employed to determine the potency for auxin biosynthesis in TRP-spiked soil samples [1]. Many ion-pair reagents however, are expensive and toxic. Additionally, as the chromatographic behaviour of standard, investigated and unknown substances is modified at the same time, ion-pair reagents can impede identification and quantification. Therefore, chromatography should be performed with the original substances, as far as possible.

Most of the proposed HPLC methods for the separation of indole derivatives use gradient systems and a mixture of methanol, water and acetic acid as the mobile phase [1,7–9]. Gradient elution, however, requires time- and solvent-consuming column re-equilibration after each run. This can be avoided by an optimized isocratic system and present advantages for routine analyses. According to our results, methanol neither provides optimum selectivity as an organic modifier in RP-HPLC for indole derivatives, nor prevents unstable indoles (*e.g.*, IPA) from autoxidative breakdown.

To reduce the breakdown of indole derivatives, the use of antioxidants has been proposed [2,13]. In our experience, they give rise to interfering peaks when they are present in the sample. As components of the liquid phase, they cause a high background noise.

Fluorescence monitors can detect as little as 1 pg of IAA, whereas UV absorbance monitors are less sensitive, with detection limits in the low nanogram range [2]. Concerning sensitivity and specificity, both are superior to spectrophotometric methods [15] and biotests used for auxin determination. In spite of its lower sensitivity, UV absorbance monitoring is necessary in addition to fluorimetry because some important indoles, such as IAld, IPA, indole-3-acetaldehyde (IAAld), indole-3-acrylic acid (IAcrA) and indole-3-glyoxylic acid (IGA), show no fluorescence [2]. The combination of a fluorimeter and a UV absorbance monitor has been used to determine indole derivatives excreted by bacteria [9].

An improved alternative system for the extraction, separation and simultaneous determination of different indole derivatives was still needed. We developed a simple, reliable, highly specific and sensitive routine method for the simultaneous determination of characteristic substances occurring in the different proposed pathways of TRP catabolism, auxin biosynthesis and IAA catabolism. This method combines the advantages of SPE, isocratic RP-HPLC, UV absorbance and fluorimetry. It was optimized to prevent indole derivatives from decomposing and to provide a high resolving power. The method was tested with soil samples, but it can also be applied to microorganisms. We determined actual concentrations of TRP and TRP catabolites and the potency for auxin biosynthesis and TRP catabolism in two different fallow agricultural fields.

## EXPERIMENTAL

### *Chemicals and materials*

As standard substances we tested L-tryptophan (TRP), 5-OH-tryptophan (5-OH-TRP), indole-3-acetic acid (IAA), indole-3-acetamide (IAM), in-

dole-3-pyruvic acid (IPA), indole-3-lactic acid (ILA), indole-3-ethanol (TOL, tryptophol), tryptamine (TAM), 5-OH-tryptamine (5-OH-TAM, serotonine), indole-3-glyoxylic acid (IGA), indole-3-acrylic acid (IAcrA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPropA), indole-3-aldehyde (IAld), indole-3-acetaldehyde (hydrogensulphite compound, IAAld), indole-3-carboxylic acid (ICA), indole-3-methanol (IM), anthranilic acid (AnthrA), 3-OH-anthranilic acid (3-OH-AnthrA), kynurenine (Kynur), 3-OH-kynurenine (3-OH-Kynur), kynurenic acid (KynurA), phenylalanine (PAla), phenylacetic acid (PAA), phenyllactic acid (PLA), phenylacetamide (PAM), phenylpyruvic acid (PPA) and phenylethanol (POL). These compounds and sodium diethyldithiocarbamic acid (all of analytical-reagent grade) were purchased from Sigma.

The remaining chemicals (all of analytical-reagent grade), 2-propanol and ethanol (both of HPLC grade) were obtained from Merck or Sigma. Standard buffers (pH 2.00, 4.005 and 6.865) came from Ingold Messtechnik (Steinbach, Germany), and pH 523 pH meter and E 56 electrode from WTW (Weilheim, Germany).

Water for HPLC purposes and reference standard solutions was distilled twice. Contact of hot doubly distilled water with plastic materials was avoided.

*Preparation of soil samples and extraction of indole derivatives and other TRP catabolites*

Soil samples were taken early in March from the $A_p$ horizons (5–20 cm) of two fallow agricultural wheat fields (Scheyern, Bavaria, Germany, sandy loam, water content 23%, total carbon 1.23%, total nitrogen 0.12%, $n = 3$, and Neumarkt, Bavaria, Germany, loamy sand, water content 13%, total carbon 0.78%, total nitrogen 0.07%, $n = 3$).

To determine the potency for auxin biosynthesis and TRP catabolism in soils, we modified the method of Frankenberger and Brunner [1] to incubate soil samples for 24 h with TRP. The actual contents of TRP and TRP catabolites were determined without TRP addition.

The sieved (<2 mm) soils were adjusted to 50% of the maximum water-holding capacity and equilibrated for at least 3 days at 22°C. A 3-g amount of equilibrated soil was incubated with 10 mg of TRP [dissolved freshly in 1 ml of a 0.1 *M* $NaH_2PO_4$–$Na_2HPO_4$ buffer (pH 7.0)] in 50-ml polypropylene centrifugation vials (Falcon 2070; Becton Dickinson, Heidelberg, Germany). The soil aggregates were stirred well and disrupted by ultrasonication for 20 s (Sonorex RK 100 ultrasonication bath, HF power 80/160 W; Bandelin, Berlin, Germany). This allows the endogenous microflora to effect a better contact with the applied TRP. Control experiments showed that this ultrasonication treatment slightly enhanced TRP catabolism.

After incubation for 24 h at 22°C in the dark, the soil samples were suspended in 4 ml of phosphate buffer (pH 7.0), stirred well and sonicated for 1 min. Ultrasonication after the incubation caused a higher extraction efficiency. This is probably due to a better desorption from clay, humic substances and other organic material.

The soil suspension was centrifuged in a swing-out rotor for 30 min at 5800 *g* (Heraeus Sepatech Omnifuge 2.0 RS, Model 3360 rotor). The supernatant was carefully removed and filtered (SM 16315 filtration equipment and 11106-25-N cellulose acetate filters, pore size 0.45 μm, from Sartorius). The pellet surfaces and filtration equipment were washed twice with 1 ml of phosphate buffer (pH 7.0).

The filtrate (including the filtered washing solutions) was adjusted to pH 2.0–2.5 with $H_3PO_4$ to protonate and enhance the lipophilicity of carboxyl groups. This solution was applied under partial vacuum to SPE columns (fixed on a Supelco Visiprep SPE 50730 vacuum manifold) prefilled with Amberlite XAD-2 (Bio-Rad SM2 Bio-Beads 731-6315, 200–400 mesh). The SPE columns had been equilibrated for at least 1 h with 0.1 *M* $NaH_2PO_4$–$Na_2HPO_4$ buffer, adjusted to pH 2.25 with $H_3PO_4$. The filtrate glass vial was washed twice with 1 ml of phosphate buffer (pH 2.25) and the washing solution was passed through the SPE columns. Until the elution step, the columns were not allowed to run dry.

For the solvent exchange, the columns were run dry under partial vacuum. The adsorbed purified TRP catabolites were eluted with 5 ml of ethanol and measured immediately or stored at −30°C in the dark.

Instead of the phosphate buffers (pH 7.0 and 2.25), 0.1 *M* Tris–HCl buffers (adjusted to the same pH values) can be used.

*Determination of indole derivatives and TRP catabolites*

For HPLC, a 5-μm Hyperchrome Spherisorb ODS II $C_{18}$ column (250 × 4 mm I.D.) with a 20 mm precolumn, obtained from Bischoff Chromatography (Leonberg, Germany), was used. Two reversed-phase separations with different mobile phases were carried out for every sample. The first separation was performed with 2-propanol–doubly distilled water (13:87), with 1.80 m*M* citrate added, pH 4.20–4.30 adjusted with 32% NaOH, and the second with 2-propanol–doubly distilled water (14:86), containing 5.0 m*M* $NaH_2PO_4$, pH 2.15–2.35 adjusted with 85% $H_3PO_4$. The mobile phases were degassed under partial vacuum by ultrasonication for 5 min.

Volumes of 20 μl of the ethanolic SPE eluates were injected under isocratic [1.1 ml/min, Model 300 B high-precision pump from Gynkotek (Germering, Germany)] and isothermal conditions (22°C).

The UV absorption at 233 (or 280) nm and 316 nm and fluorescence emission at 360 (408) nm after excitation at 280 (340) nm were measured with a Pharmacia-LKB VWM 2141 variable-wavelength monitor and a Shimadzu RF-535 fluorescence monitor simultaneously by connecting the monitors in series. Data were processed with a Merck Hitachi D-2500 chromato-integrator (with two-channel option) and a Hewlett-Packard 3390A integrator.

For the calculation of actual contents of TRP and TRP catabolites and the potency for auxin biosynthesis in a soil, the data from the soil samples were corrected for the non-extractable part of the respective references. A defined mixture of indole derivatives and TRP catabolites was incubated for 11 min with the tested soil and stirred well. Sonication, extraction and determination were carried out as described above for the soil samples. The concentrations of the reference substances were about 50% higher than those present in the soil extract after the incubation with TRP. The extracted references represented the extractable part of the indole derivatives and TRP catabolites which were produced in the tested soil. The data for the soil samples were determined relative to these references.

For the determination of the potential for microbial auxin biosynthesis, the results from the TRP-spiked soil samples had to be corrected for the products that resulted from the autoxidation of TRP. The peak integrals of the extractable TRP breakdown products that were formed during 24 h of incubation of TRP in sterile filtered phosphate buffer (pH 7.0) (Sartorius Minisart NML sterile filtration equipment, SM 16534, pore size 0.2 μm) were determined as described above for the extractable references. The peak areas that resulted from TRP autoxidation were taken as the background for the microbial TRP catabolism in the soil samples.

A scheme of the important experimental steps for the determination of auxin biosynthesis and TRP catabolism in soils is presented in Fig. 1.

Heating the TRP, contamination of sterile TRP and sterile soil with microorganisms and exposure of indole derivatives to daylight were avoided. Samples containing indole derivatives must not come into contact with polystyrene.

## RESULTS

*Stability of the indole derivatives in different solvents*

Different aqueous buffers, methanol, ethanol, 1-propanol, 2-propanol, butanol, acetonitrile and ethyl acetate were tested as solvents. Every tested indole derivative and TRP catabolite could be dissolved only in aqueous solutions, methanol and ethanol. Ethanol showed remarkable antioxidative effects.

When dissolved in aqueous solutions, indole-3-pyruvic acid (IPA) decomposed spontaneously. Different peaks with shifting UV absorbance maxima, depending on the pH and age of the sample, were observed. IAA and IAld were always present as breakdown products. In contrast, IPA was stable in ethanol and in the mobile phase at pH values below 2.75. Indole-3-methanol (IM) did not break down in ethanol and in the mobile phase at pH values higher than pH 4.0. Because of its rapid autoxidation, IAAld could only be determined as the hydrogensulphite adduct in the presence of excess of antioxidant ($Na_2S_2O_5$) in the liquid phase at low pH values when it was dissolved in ethanol. Samples of the standard compounds dissolved in ethanol could be stored as liquids at −30°C in the dark for more than 6 months without obvious degradation

We tested $Na_2S_2O_5$, sodium diethyldithiocar-

**SOIL SAMPLE** (sieved < 2 mm, 3 g wet weight);

**INCUBATION** without and with **TRP**
(10 mg / ml 0.1 M phosphate buffer, pH 7.0)
24 h at 22°C in the dark, after 20 s ultrasonication;

suspend samples in phosphate buffer, pH 7.0,
1 min ultrasonication;

**CENTRIFUGATION** 30 min at 5800 g,
**FILTRATION** of the supernatant (0.45 μm),
adjust filtrate to **pH 2.0 - 2.5** with $H_3PO_4$;

**SOLID PHASE EXTRACTION** (Amberlite XAD-2, elution with **ethanol**);

**ISOCRATIC REVERSED-PHASE HPLC**
1) pH 4.2 - 4.3,
2) $COO^-$-suppressive conditions (pH 2.15 - 3.50);

**DETECTION** (simultaneous)
1) Fluorescence 280/360 nm (340/408 nm),
2) UV absorbance 233 or 280 nm, and 316 nm (340 nm);

Fig. 1. Scheme for the determination of auxin biosynthesis and TRP catabolism in soils.

bamic acid and 2,6-di-*tert*.-butyl-4-methylphenol as antioxidants. They had no significant preventive effect on the breakdown of IPA at high and of IM at low pH values of the mobile phase during HPLC, when IPA and IM had been dissolved in ethanol. However, the antioxidants impeded the chromatography. When present in the sample, they gave rise to peaks (partially negative). Being part of the mobile phase, the antioxidants modified the chromatographic conditions by enhancing the ionic strength and caused an increased background noise. Therefore, their use was avoided.

If IPA, IAAld or IM was to be determined, the composition of the solvent and the pH of the mobile phase have to be considered. For the detection of IAAld, excess of $Na_2S_2O_5$ should be present in each experimental step.

*HPLC separation conditions*

The best separations were obtained with 2-propanol–water as mobile phase and the Hyperchrome column. With a 5-μm LiChrospher 60 RP-select B column (250 × 4 mm I.D.) from Merck, the results were not as good as with the Hyperchrome column

(data not shown). We carried out two isocratic HPLC runs under different mobile phase conditions using the fact that the investigated compounds reacted differently in their chromatographic behaviour to changes in pH and ionic strength of the mobile phase (see Table I and Figs. 2 and 3). To ensure the identity of the sample peaks, their spectral properties and their chromatographic behaviour were compared with those of the mixed standard substances under both chromatographic conditions.

The first run was performed at pH 4.23 (adjusted with NaOH) of the mobile phase 2-propanol–water (13:87), with 1.80 m*M* citrate added, which provided the best separation conditions. The second run was carried out at a lower pH of the mobile phase [*e.g.*, 2-propanol–water (14:86) containing 5.0 m*M* $NaH_2PO_4$, pH 2.31 adjusted with $H_3PO_4$]. Ionic strength and pH were chosen specifically to resolve remaining doubts on peak identities and to verify the results from the first run.

The higher $H^+$ concentration in the second mobile phase changed the retention times by suppressing carboxyl ions and by enhancing the ionic character of amino groups. Compounds with a carboxyl group became more lipophilic, and were retarded by the reversed-phase column. Substances with an amino group became more ionic and were eluted faster by the hydrophilic liquid phase (Table I, Figs. 2 and 3). The retention times of amino compounds became shorter on raising the ionic strength in the mobile phase. A higher concentration of 2-propanol accelerated the passage of all the tested substances, with slight differences according to their lipophilicity (data not shown). For reproducible results, the pH and ionic strength of the mobile phase must be adjusted precisely because several compounds react very sensitively to these parameters.

TABLE I

SPECTRAL AND CHROMATOGRAPHIC CHARACTERISTICS OF TRP CATABOLITES AND INDOLE DERIVATIVES

Symbols: +, strong; (+), weak; and −, no fluorescence or UV absorbance. The compositions of the pH 4.23 and pH 2.31 mobile phases are described in under *HPLC separation conditions*.

| Substance | Fluorescence (excitation/emission) | | UV absorbance | | Retention time (min) in the mobile phase at | |
|---|---|---|---|---|---|---|
| | 280/360 nm | 340/408 nm | 280 nm | 316 nm | pH 4.23 | pH 2.31 |
| Kynurenic acid | − | + | (+) | + | 2.10 | 4.54 (slight decomposition) |
| 3-Hydroxykynurenine | − | − | (+) | + | 2.65 | 2.61 |
| Kynurenine | − | − | (+) | + | 2.75 | 2.90 |
| 5-Hydroxytryptophan | + | − | + | − | 2.80 | 2.88 |
| Indole-3-glyoxylic acid | − | − | + | + | 2.80 | 8.82 |
| L-Tryptophan | + | − | + | − | 3.50 | 4.31 |
| 3-Hydroxyanthranilic acid | − | + | (+) | + | 3.96 | 3.48 |
| Indole-3-lactic acid | + | − | + | − | 5.35 | 9.27 |
| Indole-3-acetamide | + | − | + | − | 6.96 | 6.60 |
| 5-Hydroxytryptamine | + | − | + | − | 7.26 | 2.61 |
| Indole-3-methanol | + | − | + | − | 7.93 | Decomposition |
| Anthranilic acid | − | + | (+) | + | 8.50 | 5.71 |
| Indole-3-acetic acid | + | − | + | − | 12.80 | 13.92 |
| Indole-3-ethanol | + | − | + | − | 16.16 | 13.92 |
| Indole-3-aldehyde | − | − | + | + | 18.70 | 16.35 |
| Indole-3-carboxylic acid | (+) | − | + | − | 18.84 | 16.59 |
| Tryptamine | + | − | + | − | 23.06 | 5.16 |
| Indole-3-puruvic acid | − | − | + | + | Decomposition | 40.59 |
| Indole-3-propionic aicd | + | − | + | − | 27.34 | 29.03 |
| Indole-3-acrylic acid | − | − | + | + | 45.50 | 46.16 |
| Indole-3-butyric acid | + | − | + | − | 55.51 | 53.32 |

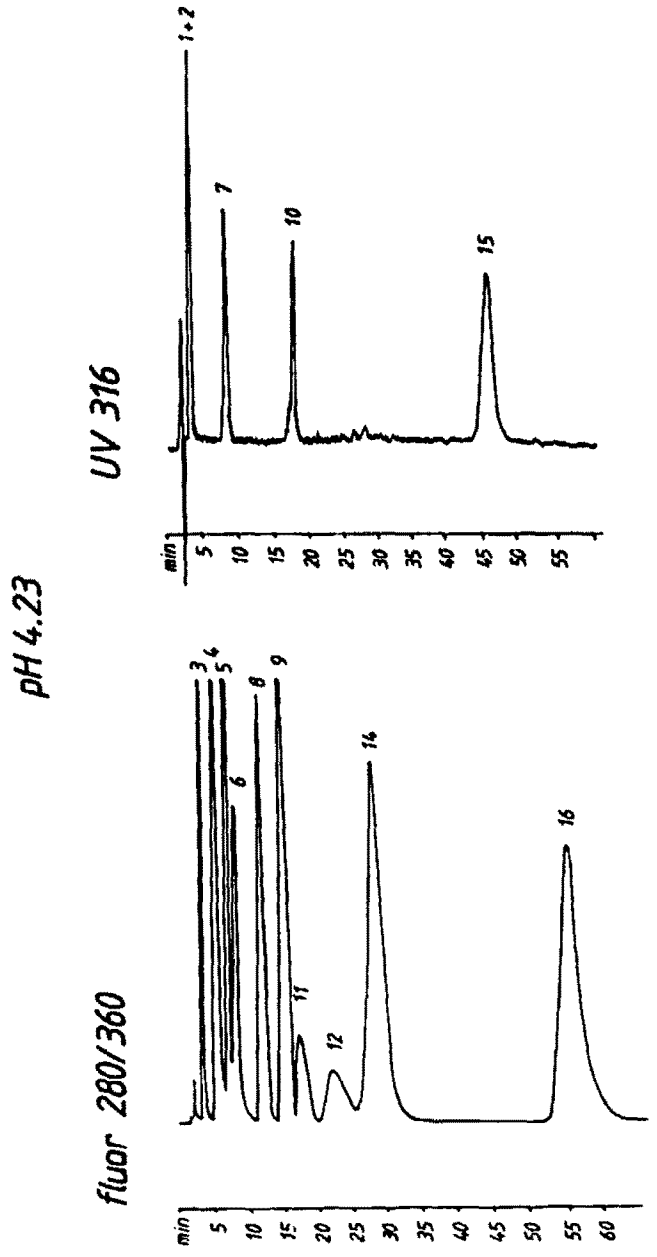


Fig. 2. Chromatogram of sixteen indole derivatives and TRP catabolites with the pH 4.23 mobile phase (for composition see *HPLC separation conditions*). Fluorescence was measured at 280 nm excitation and 360 nm emission (fluor 280/360) and UV absorbance at 316 nm (UV 316). Peaks: 1 = kynurenine; 2 = indole-3-glyoxylic acid; 3 = L-tryptophan; 4 = indole-3-lactic acid; 5 = indole-3-acetamide; 6 = indole-3-methanol; 7 = anthranilic acid; 8 = indole-3-acetic acid; 9 = indole-3-ethanol; 10 = indole-3-aldehyde; 11 = indole-3-carboxylic acid; 12 = tryptamine; 13 = indole-3-pyruvic acid; 14 = indole-3-propionic acid; 15 = indole-3-acrylic acid; 16 = indole-3-butyric acid.

*Spectral characteristics and chromatographic behaviour of the indole derivatives and TRP catabolites*

In spite of the much higher sensitivity of fluorescence detection, UV absorbance monitoring of two wavelengths was necessary, because several compounds showed no or only weak fluorescence and differed in their UV absorbance spectra. Table I summarizes the spectral characteristics and the retention times in the mobile phase of pH 4.23 and 2.31 which are relevant for the determination of the indole derivatives and TRP catabolites.

Figs. 2 and 3 show typical chromatograms of sixteen selected standard substances of indole derivatives and TRP catabolites which may be important

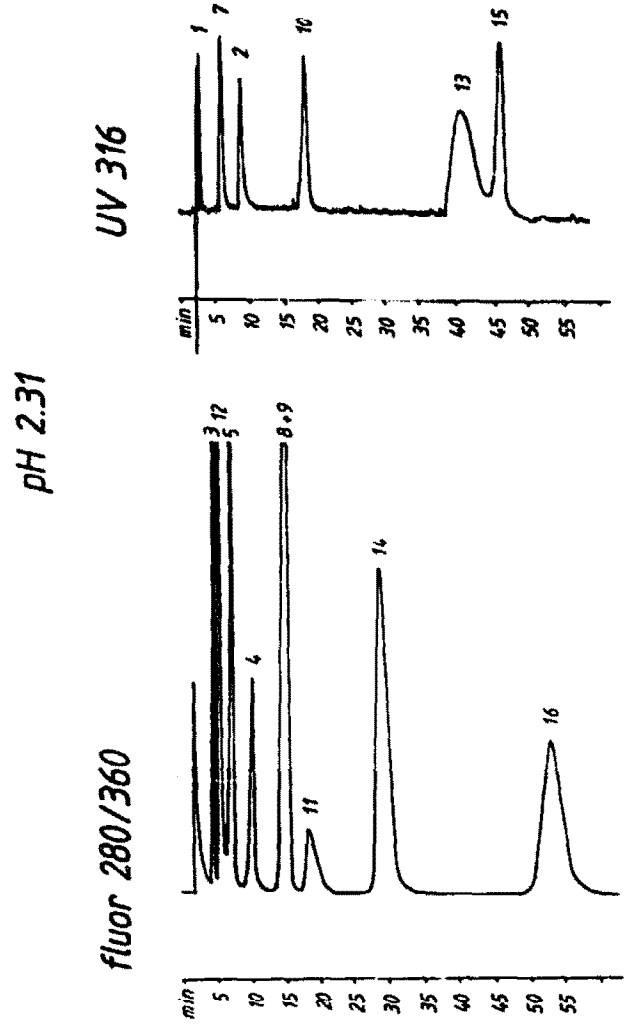


Fig. 3. As Fig. 2 with the pH 2.31 mobile phase. Peak numbers as in Fig. 2.

in the microbial turnover of TRP and auxin metabolism.

IAld and ICA co-chromatographed at all the chosen pH values. Their relative contributions to the total peak area at 233 (or 280) nm could be calculated, because IAld absorbs at 233, 280 and 316 nm and ICA absorbs at 233 and 280 nm but not at 316 nm (Table I, Figs. 2 and 3). Therefore, the factor IAld peak area at 316 nm divided by IAld peak area at 233 (or 280) nm was measured with an IAld standard sample. This allowed the ICA to be determined by difference calculation: IAld peak area measured at 316 nm × factor 1.26 resulted in the calculated IAld peak area at 233 nm. The difference between the total IAld plus ICA peak area measured at 233 nm and the calculated IAld peak area at 233 nm represented the ICA peak area at 233 nm. This calculation was supported by the data from the fluorescence determination of ICA. Similar calculations can be applied to other co-eluted substances, if they differ in their spectral properties. The spontaneous decomposition of IPA in the pH 4.23 mobile phase gave rise to IAA and two further peaks. The minor peak eluted at 12.7 min, and the major peak at 18.7 min. Both substances absorbed 316-nm UV radiation and were not fluorescent. The second peak represented IAld. The identity of the first peak could not be confirmed. IPA could be determined without decomposition at mobile phase pH values below 2.75. IM, which is unstable at pH values below 4.0, was completely destroyed in the pH 2.31 mobile phase and could not be detected.

Using the proposed combined chromatographic and spectral approach, it was possible to identify and determine simultaneously each tested compound of TRP catabolism. The identifications were confirmed by additional spiking experiments.

*Recoveries provided by solid-phase extraction (SPE)*

For the determination of the SPE efficiency, a mixture of standard compounds was dissolved in the incubation media used for soils [0.1 *M* phosphate buffer (pH 7.0) and 0.1 *M* Tris–HCl (pH 7.0)]. The concentrations of the references were in the range of the concentrations of the respective TRP catabolites which were detected in the experiments with soil. Titration to pH 2.25 and extraction of the standard substances at pH 2.25 were carried out as described for soil samples.

The highest recoveries were obtained with Amberlite XAD-2 (Bio-Rad SM2 Bio-Beads 731-6315, 200–400 mesh). They were higher than 90% (TAM and IAld 85%) for each tested standard compound. Compared with the LLE efficiencies [2] of indole derivatives, SPE with Amberlite XAD-2 provided better recoveries (data not shown). Particularly the aminoindoles TRP and TAM were recovered by XAD-2 with much higher efficiencies. $C_{18}$ columns (Supelcoclean LC18 SPE columns 5-7012) yielded comparable results for carboxylic indoles, but they were less efficient for indoles with an amino group (TRP 90% and TAM only 40%). The recoveries with Amberlite XAD-7 material (SM7 Bio-Beads 731-6335, 200–400 mesh) were generally about 5–10% lower than those obtained with XAD-2. Amberlite XAD-4 (SM4 Bio-Beads 731-6244, 100–200 mesh), a more lipophilic resin, retained the aminoindoles only very poorly.

If IPA was present in aqueous samples at pH 7.0, only the breakdown products could be extracted and determined. IM decayed when the sample was acidified to pH 2.2–2.3. Therefore, the acidification step must be omitted if the presence of IM is to be investigated. Without the acidification, however, the SM2 SPE recovery rates were generally about 5–15% less than at pH 2.25. For our purposes, IM was not an important substance, being an intermediate in IAA oxidation [2,13]. As it left no interfering decay products, IM was disregarded.

The SPE recoveries with 0.1 *M* Tris–HCl (pH 7.0 and 2.25) were the same as with the respective 0.1 *M* phosphate buffers.

*Binding of indole derivatives and other TRP catabolites to soil constituents*

For the determination of the binding capacity of soils to indole derivatives and TRP catabolites, we added one part of a mixture of standard substances to soil and left the other without soil contact. After SPE extraction and HPLC, the peak areas were compared. The results are summarized in Table II.

The individual reference compounds were bound to soil constituents to different extents. Some of them could be extracted only partially. The losses due to adsorption to soil were generally greater in the soil from Scheyern (Table II). The total C and total N contents were higher in the sandy loam from Scheyern than in the loamy sand from Neumarkt

TABLE II

BINDING OF INDOLE DERIVATIVES AND TRP CATABOLITES TO DIFFERENT SOILS

| Soil | Mean recoveries ($n$ = 2) of indole derivatives and TRP catabolites (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TRP | ILA | IAM | IAA | TOL | IAld | ICA | TAM | AnthrA |
| Neumarkt | 82.0 | 83.5 | 80.4 | 84.2 | 76.1 | 57.0 | 85.8 | 51.0 | 90.4 |
| Scheyern | 64.9 | 71.1 | 60.2 | 63.9 | 38.9 | 44.1 | 92.3 | 33.5 | 91.3 |

(see Experimental). This suggested that the non-extractable amount of the standard substances depended on soil structure and composition, probably mainly on the content of organic material. Compared with substances with a carboxyl group (*e.g.*, IAA, ICA, AnthrA and ILA), more lipophilic compounds (*e.g.*, TAM, TOL, and IAld) were adsorbed very efficiently, and could hardly be extracted from soil (Table II).

The capacity of the tested soils to bind indole derivatives and other TRP catabolites was checked separately in each soil experiment, obtaining the extractable and non-extractable fractions. The actual contents of TRP and TRP catabolites and the potential for TRP catabolism and auxin biosynthesis were calculated by correcting the data from the soil samples for the losses due to soil contact.

*Autoxidation of TRP in aqueous solutions*

Sterile aqueous solutions of TRP (Sartorius minisart NML sterile filtration equipment, SM 16534, pore size 0.2 μm), showed autoxidation. The pattern of the breakdown products depended on the incubation conditions and their amount increased with increasing incubation time.

In 24 h of incubation at 22°C in the dark, the breakdown of 10 mg of TRP, dissolved in phosphate buffer (pH 7.0) created 0.12 ± 0.03 μg of IAA, 0.10 ± 0.05 μg of TOL and traces of ILA. In addition, two substances were detected. In the pH 4.23 mobile phase, the first was strongly fluorescent and co-eluted with ILA at 5.35 min. The second was weakly fluorescent and eluted separately between ILA and IAM at 6.1 min. Spectrally, neither was different from ILA and IAM. At pH 2.31 of the mobile phase, the unknown substances eluted well separated prior to ILA. After exposure of the TRP decay products for 1 min to soil (as described above for the standard substances), only traces of IAA and TOL could be extracted, but the peak eluting at 5.35 min in the pH 4.23 mobile phase was still strong. Therefore, authentic ILA in TRP-treated samples was determined at liquid phase pH values below pH 2.75.

The peak areas that were due to TRP autoxidation were corrected for the losses due to binding to soil as described above, obtaining the extractable and non-extractable peak areas of TRP autoxidation. These corrected peak areas were taken as the background for the enzymatic TRP degradation by subtracting them from the areas of the corresponding soil sample peaks.

*Determination of indole derivatives and TRP catabolites extracted from TRP-treated soil samples (potency for auxin biosynthesis) and actual contents of TRP and TRP catabolites*

In the soils that were not treated with TRP, TRP was predominant. The actual TRP contents (corrected for the loss due to binding to soil) were 5.8 ± 0.2 μg/g dry soil from Scheyern and 1.4 ± 0.3 μg/g dry soil from Neumarkt. Without TRP addition, only traces of IAA could be detected in both soils.

Table III shows the potential for auxin biosynthesis and TRP catabolism in the soil samples from Neumarkt and Scheyern. The data were corrected for the non-extractable contents due to binding to soil constituents and for the amount of TRP autoxidation. They represent the total microbial substrate-induced biosynthesis.

The identification of the detected peaks was confirmed as described by performing two HPLC runs

TABLE III

POTENTIAL FOR AUXIN BIOSYNTHESIS AND TRP CATABOLISM IN DIFFERENT SOILS

| Compound | Concentration (μg/g)[a] | |
|---|---|---|
| | Scheyern soil | Neumarkt soil |
| Indole-3-lactic acid | 1.8 ± 0.5 | 1.0 ± 0.4 |
| Indole-3-acetamide | 0 | Traces |
| Indole-3-acetic acid | 8.0 ± 1.1 | 2.9 ± 0.2 |
| Indole-3-ethanol | 1.0 ± 0.4 | Traces |
| Indole-3-aldehyde | 1.3 ± 0.3 | 1.2 ± 0.4 |
| Indole-3-carboxylic acid | 2.4 ± 0.6 | 2.8 ± 0.3 |
| Anthranilic acid | 1.4 ± 0.2 | 1.7 ± 0.1 |

[a] Data are expressed in μg g dry soil ± S.D. ($n$ = 3) after 24 h of incubation with 10 mg of TRP per 3 g of fresh soil at 22°C.

at different mobile phase pH values and by spiking the soil samples with the corresponding standard substances.

Both soils were autoclaved and/or irradiated (22 h at 35.1 Gy) and spiked with sterile TRP. No TRP conversion apart from TRP autoxidation could be monitored after 24 h of incubation. Without TRP addition, however, the actual contents of TRP were enhanced in the sterilized soils, indicating lysis of the endogenous microflora (data not presented).

## DISCUSSION

The proposed method of SPE, isocratic HPLC separation and detection by combined fluorescence spectrometry and UV absorbance was developed for the simultaneous determination of the different indole auxins and related derivatives, central intermediates and side products of their different biosynthetic pathways [1–5,13], characteristic IAA catabolites [2,7,13] and TRP catabolites of the "aromatic pathway" [14]. It was tested with a mixture of the standard compounds and with samples from two different agricultural soils.

Two different mobile phase conditions in the HPLC separation were used in order to confirm the results that were obtained from the simultaneous measurements of UV absorbance and fluorescence emission at different characteristic wavelengths. The best separation conditions existed at pH 4.2–4.3 of the mobile phase. For the second run, pH values below 2.75 ($COO^-$-suppressive conditions) were chosen which changed the retention times (see Results).

According to their different UV absorbance spectra and fluorescence properties, the investigated substances were simultaneously detected by their UV absorbance at 233 (280) and 316 nm, and their fluorescence at 360 (408) nm, with UV excitation at 280 (340) nm. In spite of its lower sensitivity, UV absorbance monitoring was necessary because several compounds showed no fluorescence (Table I, Figs. 2 and 3).

Compared with other proposed HPLC-based auxin determination systems [1,2,7–9,13], this combined chromatographic and spectral approach offers several advantages. Being isocratic, no time consuming column re-equilibration (necessary with gradient systems) was needed. Ethanol was used as the sample solvent and small amounts of 2-propanol as modifier of the mobile phase. They provided stability of the tested compounds and high selectivity and helped to minimize the organic wastes. No reduction reagents were needed. The use of ion-pair reagents and derivatization procedures could be avoided. For the determination, various chromatographic and spectral parameters were tested (Table I, Figs. 2 and 3). The simultaneous detection of fluorescence and UV absorbance at different wavelengths combined high sensitivity with high detection capacity. The highest recoveries were obtained with the described SPE system (using columns with prefilled Amberlite XAD-2). In addition, SPE was less time consuming than LLE procedures, and helped to reduce the production of toxic solvent wastes.

The reference compounds IPA and IAAld decomposed spontaneously in aqueous solvents. Only the decay products could be extracted. Therefore, IPA and IAAld probably cannot be detected in aerobic soil ecosystems and aqueous suspensions of microorganisms as free substances. Nevertheless, they can be important as intermediates in the auxin metabolism of living cells. If IPA, IAAld and IM are to be determined, special conditions (see Results) have to be chosen.

Standards of indole derivatives and TRP catabolites added to soil samples were bound by soil con-

stituents to individually different extents, and remained non-extractable (Table II). The losses seemed to depend mainly on the content of organic material in the soil and on the lipophilicity of the introduced compounds. The TRP catabolism in the soil samples was corrected for these losses.

TRP autoxidized to several substances including IAA in aqueous solvents. The peak areas of the decay products which were created during the incubation of soil samples with TRP were determined, and taken as the background for the microbial TRP catabolism. The main breakdown product of TRP could not be identified. It was very similar but not identical with ILA. ILA, however, is a critical compound if the pathways of IAA biosynthesis should be determined [1,2]. With the presented method, we were able to check with the second HPLC separation if detected substances (*e.g.*, ILA) were identical with the respective standard compounds.

As an application example, soil samples from different agricultural fields were tested for TRP-induced auxin biosynthesis and actual contents of indole derivatives. The fields lay fallow, so exudation of auxins by plants was excluded.

In the soils, stimulation of auxin biosynthesis by TRP addition was necessary because as actually occurring indole derivatives, only TRP could be detected in considerable amounts apart from traces of IAA. This may indicate that in non-rhizosphere soil, the major part of the microflora is inactivated or metabolizes at reduced physiological status. In contrast, we could detect endogenous and excreted auxins in non-TRP-supplemented cultures of *Azospirilla* and *Rhizobia* which colonize the rhizosphere of crop plants (data not presented). Therefore, higher auxin contents may be found in rhizosphere soils.

In the TRP-spiked soil samples (Table III), we identified IAA as the major TRP catabolite. TOL and ILA were detected as representatives of the IPA pathway of auxin biosynthesis [1–3], and IAld and ICA as IAA catabolites [2]. Traces of IAM, the characteristic IAA intermediate in the IAM pathway of auxin biosynthesis [1–3], could only be monitored in the soil from Neumarkt. The detection of AnthrA in both soils revealed the presence of the "aromatic pathway" [14] of microbial TRP catabolism, and might indicate that TRP was converted as a source of carbon and nitrogen.

The higher total contents of C and N in the Scheyern soil (see Experimental) suggest, that the higher auxin biosynthesis in this soil (Table III) might be due to a higher microbial biomass. Clear evidence for microbial TRP catabolism was given by axenically performed experiments. In both sterilized soils, no TRP conversion was observed apart from the autoxidative TRP decay. The conversion of TRP to IAA and TOL via IPA in both agricultural soils agrees with the findings for *Azospirilla* [8,20,21], several *Rhizobia* [7], *Enterobacter cloacae* [3], *Pseudomonas putida* [9] and *Pseudomonas fluorescens* [5]. These bacteria are reported to promote plant growth and can be isolated from the rhizosphere of crop plants. We assume that the production and excretion of the auxins IAA and TOL are important factors in microbial plant growth promotion [20–22].

In our experimental design, the IAA concentrations ranged from 2.9 to 8.0 μg/g dry soil. Frankenberger and Brunner [1] reported IAA production of 200–800 μg/g soil. However, they used 37°C as the incubation temperature, a higher TRP supply of 40 mg, plant root-influenced soil from grassland and a different extraction and HPLC system with an ion-pair reagent. Detection of UV absorbance was at 254 nm, where indole derivatives show very poor absorbance. We assume that TRP autoxidation was not taken in account.

Owing to the influence of plants, especially in the rhizosphere, and to modified soil conditions (*e.g.*, water content), TRP catabolism and the pattern of detected substances may be different. Especially the occurrence of TAM, TOL and IAM in soils and microorganisms [1,2] needs further investigation.

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