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Note

Reversed-phase high-performance liquid chromatography of substituted indoleacetic acids

VOLKERT SJUT
University of Hohenheim, Department of Pomology and Vegetable Crops, 7000 Stuttgart 70 (G.F.R.)
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Methods using high-performance liquid chromatography (HPLC) for purification[1,2] and quantification[3] of indoleacetic acid (IAA) in plant extracts are now well established. In clinical chemistry HPLC is used for the determination of 5-hydroxyindoleacetic acid (5-OH-IAA)[4]. Recently, there has been considerable interest in substituted indoleacetic acids. 4-Chloroindoleacetic acid (4-Cl-IAA) and its methyl ester have been identified in immature seeds[5,6]. There are indications, although no conclusive evidence, that 5-OH-IAA is also present in plant tissues[7].

Both 5-OH-IAA and 4-Cl-IAA each show *ca.* 40% of the fluorescence intensity that IAA itself would show in the indolo-α-pyrone fluorescence determination after conversion into the respective α-pyrones[8]. This widely used procedure has proved to be a rapid, accurate[9] and reliable[10] method for quantification of IAA in plant extracts. However, 5-OH-IAA and 4-Cl-IAA must be excluded if the measured fluorescence is to be assigned only to IAA; this is not accomplished with the usual clean-up procedure[9]. Separation of IAA and 4-Cl-IAA seems to be difficult even by thin-layer chromatography (TLC) or gas–liquid chromatography (GLC)[8]. Thus, it was desirable to develop a quick, complete and easy separation of IAA, 5-OH-IAA, 4-Cl-IAA and its methyl ester in order to keep the fluorometric analysis of IAA reliable and to ease further research on substituted indoleacetic acids.

EXPERIMENTAL

Chemicals

IAA and 5-OH-IAA were purchased from Serva (Heidelberg, G.F.R.) and Sigma (St. Louis, MD, U.S.A.) respectively. 4-Cl-IAA and its methyl ester were a generous gift from K. C. Engvild, Risø National Laboratory, Department of Agricultural Research, Roskilde, Denmark. All chromatographic solvents were of high purity and redistilled before use.

Chromatographic equipment

Prepacked columns of 10-μm Spherisorb ODS (Phase Separations, Queensferry, Great Britain) and 7-μm LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.) were used, of a size suitable for either preparative (250 × 10 mm I.D.) or analytical purposes (250 × 4.6 mm I.D.). Chromatography was carried out using an HPLC

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system (Laboratory Data Control, Riviera Beach, FL, U.S.A.) consisting of two pumps (Constametric II G), a gradient programmer (Gradient Master) and a fixed wavelength UV monitor (Model 1203, UV III) operating at 254 nm. Sample introduction was via a Rheodyne 7010 sample injector, injections of 800 μl being made into a 1-ml loop or of 50 μl into a 100-μl loop.

RESULTS AND DISCUSSION

The solvent systems used were binary mixtures of acidic buffers in water (0.1 *N* acetic acid) and methanol (0.1 *N* acetic acid in methanol). The addition of an acidic buffer masks residual adsorption sites on the stationary phase[2]. Spherisorb ODS is a spherical, totally porous silica with a C_{18} bonded stationary phase and LiChrosorb RP-8 is a similar but irregularly shaped material with a C_8 bonded phase. Both reversed-phase materials were suitable for separation of the four compounds (Figs. 1–3). Even with a convex gradient of increasing concentration of methanol, the compounds were well separated (Fig. 1). This gradient was applied to ensure an acceptable elution time for the least polar compound, the methyl ester of 4-Cl-IAA. Use of the preparative RP column as in Fig. 1 provides a rapid clean-up procedure. With crude plant extracts, addition of a guard column is strongly recommended.

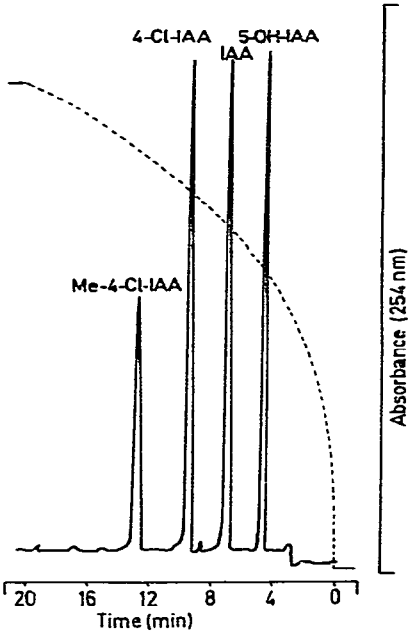

Fig. 1. Separation of IAA and substituted indoleacetic acids by reversed-phase HPLC. Column: 10-μm Spherisorb ODS (250 × 10 mm I.D.). Flow-rate: 5 ml/min. Mobile phase, convex gradient, 0.1 *N* acetic acid in water to 70% 0.1 *N* acetic acid in methanol over 20 min. Sample injected: 5-OH-IAA and IAA, 10 μg each; 4-Cl-IAA and Me-4-Cl-IAA, 20 μg each. Abbreviation: Me-4-Cl-IAA = methyl ester of 4-Cl-IAA.

The elution sequence was the same for LiChrosorb RP-8 columns (Figs. 2 and 3). With analytical columns, excellent separation was obtained with a linear gradient

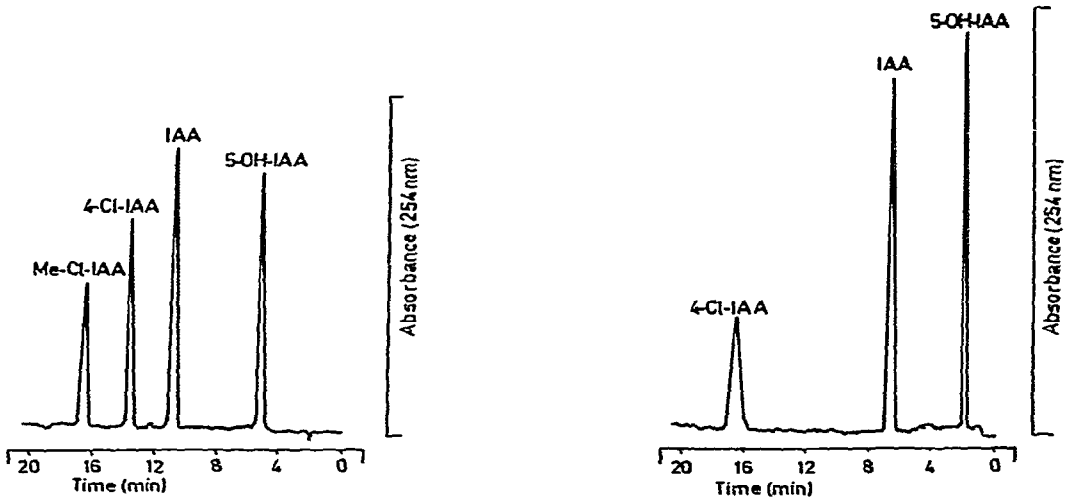

Fig. 2. Separation of IAA and substituted indoleacetic acids by reversed-phase HPLC. Column: 7-μm LiChrosorb RP-8 (250 × 4.6 mm I.D.). Flow-rate: 1.5 ml/min. Mobile phase, linear gradient, 20% 0.1 *N* acetic acid to 70% 0.1 *N* acetic acid in methanol over 15 min. Sample injected: 5-OH-IAA, 250 ng; others, 500 ng.

Fig. 3. Separation of IAA and substituted indoleacetic acids by reversed-phase HPLC. Separation conditions as in Fig. 2 except mobile phase: isocratic, 35% 0.1 *N* acetic acid in methanol.

(Fig. 2). As with the Spherisorb ODS column, rather steep gradients had to be used to achieve separations in a reasonable time. Shorter column lengths would also be suitable. Although 4-Cl-IAA is expected to be more polar than IAA it was retained longer than IAA. On the other hand 5-OH-IAA was retained only for a short time.

The analytical RP-8 column can be used in an isocratic mode if the main purpose is to recover IAA from its substituted compounds (Fig. 3). This is especially important if IAA is to be quantified by the indolo-α-pyrone method[8]. 5-OH-IAA and 4-Cl-IAA, which both interfere with that method, can easily be excluded, since 5-OH-IAA is eluted and the methyl ester of 4-Cl-IAA is retained on the column.

REFERENCES

1 R. C. Durley, T. Kannangara and G. M. Simpson, *Can. J. Bot.*, 56 (1978) 157.
2 M. L. Brenner, *Proc. Amer. Chem. Soc.*, 111 (1979) 215.
3 P. B. Sweetser and D. G. Swartzfager, *Plant Physiol.*, 61 (1978) 254.
4 O. Beck, G. Palmskog and E. Hultman, *Clin. Chim. Acta*, 79 (1977) 149.
5 K. C. Engvild, H. Egsgaard and E. Larsen, *Physiol. Plant.*, 42 (1978) 365.
6 M. Hofinger and M. Böttger, *Phytochemistry*, 18 (1979) 653.
7 G. B. West, *J. Pharm. Pharmacol., Suppl.*, 11 (1959) 275.
8 M. Böttger, K. C. Engvild and P. Kaiser, *Physiol. Plant.*, 43 (1978) 62.
9 E. Knegt and J. Bruinsma, *Phytochemistry*, 12 (1973) 753.
10 L. Eliason, L.-H. Strömquist and L. Tillberg, *Physiol. Plant.*, 36 (1976) 16.