Phytochemistry, 1980, Vol. 19, pp. 219–221. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0201-0219 $02.00/0

A METHOD FOR THE QUANTITATION OF INDOLE AUXINS IN THE PICOGRAM RANGE BY HIGH PERFORMANCE GAS CHROMATOGRAPHY OF THEIR *N*-HEPTAFLUOROBUTYRYL METHYL ESTERS

MICHEL HOFINGER

Laboratory of Plant Biology, University of Liège, Quai Van Beneden, 22 B-4020 Liège, Belgium

(*Revised received* 23 *May* 1979)

Key Word Index—Indolic compounds; plant hormones; HPGC–ECD; derivatization; *N*-methyl-*bis*-(heptafluorobutyramide).

Abstract—A method is described for the extraction and HPGC–ECD quantitation of indolic acids in the picogram range. It includes an improved GLC technique and a new derivatization reagent; it is applied to the estimation of IAA in *Bryonia* leaves.

INTRODUCTION

A good sensitive method for the determination of trace amounts of indolic compounds that is applicable to extracts of small plant samples is becoming more than ever an essential tool for the understanding of physiological processes. Several sensitive techniques are available and some give satisfactory results with pure synthetic compounds or simple mixtures. Difficulties arise when most of them are applied to complex biological extracts. The impurities of the extracts often cause interferences preventing accurate determinations.

Other methods are not sensitive enough. Paper and thin-layer chromatographic methods associated with bioassays are not suitable due to their lack of specificity and to the highly probable interaction of impurities. The indolo-α-pyrone spectrofluorimetric method [1–3] seems very attractive but it has several limitations. Its use is restricted to 3-indolylacetic acids but it cannot distinguish between them (for example: IAA, 4-chloro IAA, 5-hydroxy IAA) [4]. Furthermore, this method does not allow the determination of other indolic compounds. Some more difficulties arise due to insoluble and coloured compounds in the extract [5]. Methods using HPLC have recently been developed [6, 7] but are rather time-consuming because they require purification by column chromatography (on Sephadex) before HPLC.

GLC methods have been used for a long time. The earliest methods used flame ionisation detection of the methyl esters [8–15] or silylated derivatives [10, 14, 16, 17] of indolic compounds. This detection technique is not selective so that impurities often present in the extract at relatively high concentrations can obscure the determination of indole compounds. Furthermore this detector is not very sensitive. The use of an electron capture detector (ECD) considerably increases the sensitivity of the GLC method. It is also selective because it requires the presence of highly electronegative groups in the molecule for high sensitivity. Halogen atoms have the highest electron affinity and therefore are the best groups from this point of view.

Some authors have derivatized indolic acids by introducing halogen atoms into the molecule. Bittner and Even-Chen [18] esterified IAA with trichloroethanol, so decreasing the threshold of sensitivity 500-fold. However this method increases the sensitivity towards all the acidic impurities at the same time and, according to the authors themselves, poses a big problem when applied to plant extracts.

A better method was proposed by several authors. It consists of the methylation of the carboxyl group followed by the derivatization of the *N*-indolic using trifluoroacetic (TFAA) [19] or heptafluorobutyric (HFBA) [20] anhydride, this latter reagent more effectively increasing the sensitivity. This method gives a selective increase of sensitivity and is based on a very sound principle. A mass spectrometer has been used as a detector, and especially the selected ion monitoring. Rivier and Pilet [21] applied this method (multiple ion detection) to the HFB derivative of IAA; Little *et al.* [22] used single ion detection of its trimethylsilyl derivative. This is certainly the best technique but it is very expensive and hence cannot be easily used in most laboratories.

This paper describes a new high performance gas chromatography technique for the estimation of indolic compounds. A new heptafluorobutyrylation reagent is compared with others, namely: *N*-methyl-*bis*(heptafluorobutyramide) (MBHFBA).

RESULTS AND DISCUSSION

Comparison of HFBA *and* MBHFBA

HFBA and MBHFBA were compared with regard to their chromatographic properties. As shown in Fig. 1, MBHFBA is eluted much more rapidly than HFBA

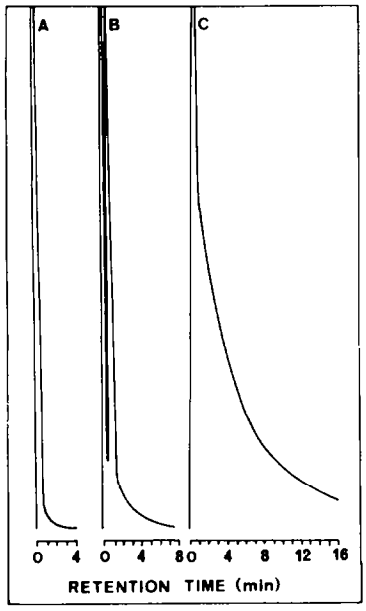

Fig. 1. GLC of MBHFBA (A), MBHFBA+DMAP (5 mg/ml) (B) and HFBA (C); 0.5 μl each, OV-101, 200°, 0.8 bar, direct injection, FID 64×10^{-11} A/mV, 10^{-3} V full scale.

which shows an important tailing. DMAP used with MBHFBA as catalyst is eluted rapidly and when ECD is used the peak of DMAP is very small and does not present any drawback.

Both reagents were compared for their acylation properties. HFBA is more powerful than MBHFBA, the reaction being more rapid at lower temperature than with MBHFBA. However the acid released during the reaction can damage some acid-sensitive indolic compounds, while heptafluorobutyramide, the by-product of MBHFBA, is neutral. HFBA could be used in the presence of triethylamine, an aprotic base absorbing the released acid, but in this case the triethylamine heptafluorobutyrate cannot be removed by evaporation. When using HFBA, and also heptafluorobutyrylimidazole (that has not been used in this study) partitioning should be made between an aqueous and a nonpolar solvent such as toluene or *n*-hexane [21]. MBHFBA is itself an excellent solvent and can therefore be used on its own.

Conditions of derivatization with MBHFBA

At 120°, most of the tested compounds were completely derivatized in 2 hr. Some compounds are derivatized more rapidly (1 hr for IAcrA, 30 min for IPyA) but can be maintained at 120° for a long time without damage. For ILA the formation of the di-HFB derivative takes longer. There is still 10% mono-HFB after 2 hr (7% after 3 hr).

Chromatography

Figure 2 shows the separation of some pure indolic acids by the HPGC system.

Quantitation of IAA *in the leaves of* Bryonia dioica

Figure 3 shows the chromatography of *Bryonia* leaves extract. The IAA content is estimated at 62 ng/g fresh leaves.

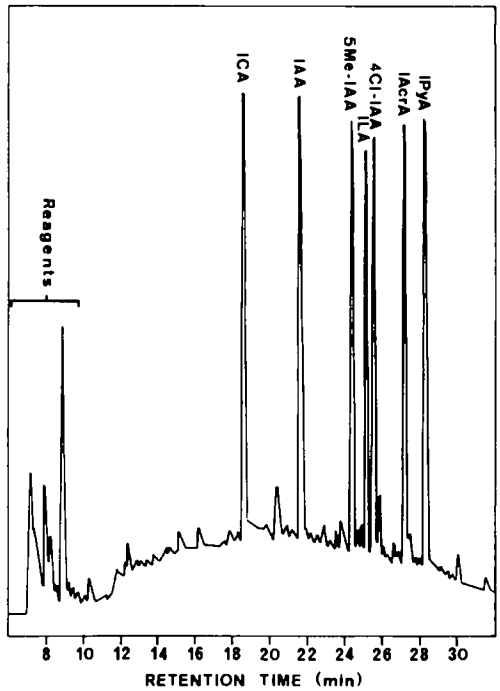

Fig. 2. HPGC–ECD of some synthetic *N*-HFB-indolic acid methyl esters: indolyl-carboxylic (ICA), -acetic (IAA), -lactic (ILA), -acrylic (IAcrA), -pyruvic (IPyA), 5-methylindolyl-acetic (5 Me–IAA) and 4-chloro-indolylacetic (4 Cl–IAA) acids (*ca* 5 ng each). 1.2 bar, program temperature 130–235°, 4°/min, attenuation: 2^8. For other conditions see Experimental.

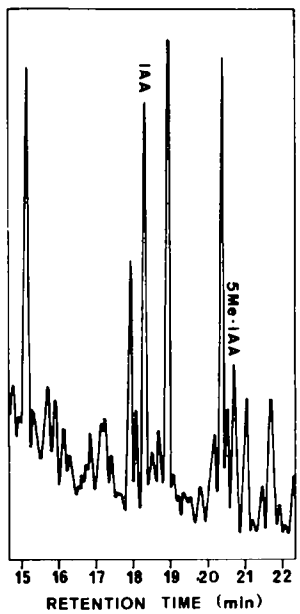

Fig. 3. HPGC–ECD of *Bryonia dioica* leaves extract, derivatized as described in Experimental. 1 bar, program temperature 160–235°, 4°/min, attenuation: 2^8. For other conditions see Experimental. 5 Me–IAA is the internal standard.

Confirmation by GC–MS of the presence of IAA *in* Bryonia *leaves*

The single ion detection method records peaks for ions at *m/e* 385 and 326, characteristic for N-HFB-IAA methyl ester [21], only at the retention time of the synthetic compound.

EXPERIMENTAL

Reagents and solvents. HFBA, MSHFBA (*N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide), DMCS are from Macherey-Nagel & Co. 4-Dimethylaminopyridine (DMAP) from Aldrich. Indolic compounds from Fluka, except 4-Cl-IAA which is a generous gift from K. C. Engvild, Denmark. Acetonitrile silyl grade from Pierce. All other solvents are of the grade 'for the analysis of pesticide residue' from Carlo Erba.

Extraction procedure. All procedures were carried out in N_2. Plant material (0.1–1 g) was homogenized in a refrigerated Potter homogenizer (0°) with 2 ml cold degassed Na_2CO_3 (100 mM, pH 9.0) containing 5 mg/ml Na ascorbate and saturated with NaCl. 5 Me-IAA [21] (20 ng; 10 μl of a MeOH solution 2 mg/dm^3) was added as internal standard. After homogenization, the mixture was transferred to a glass centrifuge tube provided with a screw cap and PTPE-faced disc; 5 ml cold EtOAc were added, the tube was capped, agitated 1 min and centrifuged (−2°, 2000 g, 5 min). The EtOAc layer was removed by suction and discraded. The aq. phase was adjusted to pH 3 by addition of a previously determined vol. of H_3PO_4 0.5 N; 5 ml cold EtOAc were added. After agitation and separation, the EtOAc layer was transferred to another tube containing 2 ml Na_2CO_3 buffer. After agitation, the EtOAc layer was sucked out and the aq. phase adjusted to pH 3.0. Cold EtOAc (1 ml) was added. After agitation and centrifugation, as much EtOAc as possible was rapidly transferred to a 1 ml cone-shaped vial (silylated by a 5% DMCS solution in toluene, rinsed with MeOH, then with Me_2CO).

Methylation. The vial was screwed into an original device allowing simultaneous methylation of up to six samples by CH_2N_2 by a procedure adapted from Fales *et al.* [23]. After methylation (30 min at 0°), the samples were evapd. in a N_2 stream. Acetonitrile (20 μl) was added and similarly evapd. The vial was filled with dry N_2 and closed with a 'mininert valve' (Pierce).

Heptafluorobutyrylation. MBHFBA (5–20 μl) containing 5 μg/μl DMAP as acylation catalyst was added to each sample. The vial was heated at 120° for 2 hr.

Synthesis of MBHFBA. The procedure described by Donike [24] for MBTFA was followed. An equimolecular mixture of MSHFBA and HFBA (with a small excess of HFBA) was prepared and distilled under atmos. press. in dry conditions. HFBA boiled at 107–110°, the heptafluorobutyrate trimethylsilyl ester at 115–117° and MBHFBA at 155–157° (yield higher than 90%). MBHFBA was stored under dry N_2; it is liquid at room temp. but crystallizes at 4°.

Chromatography. We used an Intersmat IGC 120 fitted with a moving needle solid injector and an ATC-140 Sc^3H electron capture detector coupled with a linearized electrometer. A 50 m × 0.25 mm WCOT glass column (SE-30, 0.2 μm) was used. The injection and detection temps. were 260 and 290°, respectively. Make-up 100 ml/min. The column temp. and carrier (Ar/CH_4, 9:1) velocity must be adapted to each plant material. A linear program was often necessary. Injection: 0.5–1 μl. The IAA was quantified by the IAA: 5-Me IAA peak height ratio. Ancillary experiments were carried out with a FID detector and a 1.8 m × 3 mm glass column filled with 2% OV-101 on Gaschrom Q 60–80 mesh, direct injection.

GC–MS. A Varian 1400 equipped with a 50 m × 0.5 mm glass capillary column containing SE-30 was interfaced to a Varian Mat 112, fitted with a single ion detector.

Plant material. Seeds of *Bryonia dioica* sown in vermiculite imbibed with a standard mineral mixture solution were allowed to grow at 25° under fluorescent light (16 hr light). Leaves were collected from 25-day-old plants.

Acknowledgement—This work was supported by a Belgian FRFC grant (No. 2.9009).

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