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Rapid Quantitative Analysis of a Gibberellin-Sterol Inhibitor Using High-Performance Liquid Chromatographic Cartridge Columns

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Abstract. A rapid, sensitive, and economical chemical analysis of the triazole, gibberellin-inhibitor, paclobutrazol (PP333, [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4 triazol-1-yl) pentan-3-ol]) was sought, featuring high-performance liquid chromatography (HPLC) as the final quantitation step. Three C_{18}-reverse phase columns (conventional, 250 × 4.6 mm; cartridge type, 125 × 4.6 mm; and minicolumn, 33 × 4.6 mm) were evaluated for their performance in HPLC separation and quantitation of PP333 applied to soil and plant foliage. The 125-mm Whatman Partisil 5 ODS-3 cartridge column was superior to the standard 250-mm DuPont Zorbax ODS unit, and provided enhanced resolution and reduced solvent consumption, analysis time, and cost. A Perkin-Elmer Pecosphere 3 × 3C-C_{18} cartridge system was also superior to the 125-mm column with respect to these parameters. Although this minicolumn necessitated an additional purification step prior to HPLC analysis, its exceptionally fast analysis time and recovery period coupled with a high degree of sensitivity rendered it the most superior unit. This HPLC technology provided an efficient means of assaying for PP333 in large-scale experiments dealing with the chemical's absorption, translocation, and physiological response.

Substituted triazoles, such as PP333 ([(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4 triazol-1-yl) pentan-3-ol]); commercial trade names, Cultar, Bonzi, Clipper, have been shown to be potent gibberellin biosynthesis inhibitors, with a growth-retarding characteristic applicable to agriculture (Steffens 1988; Sterrett 1985). Studies pertaining to the physiology and efficacy of the compound (Mauk et al. 1986) have necessitated advances in the technology for PP333 extraction, purification, and determination in plant and soil samples. Several methodologies that assayed for PP333 featured gas chromatography (GC) as the final quantitation step (Reed 1988; Stahly and Buchanan 1986; Wang et al. 1986). Stahly and Buchanan (1986) utilized high-performance liquid chromatography (HPLC) as a preparative or purification step prior to GC analysis. Previously reported PP333 resolution and sensitivity limits for conventional C_{18}-reverse phase HPLC columns were in the range of 200–300 ng, 10- to 30-fold less than GC (Early and Martin 1988; Fujimoto et al. 1988; Hseuh et al. 1986). Although longer columns (Mauk et al. 1988) and semi-preparative units (Early and Martin 1988) afforded greater sensitivity with larger sample volumes, analysis time was increased, in addition to solvent consumption and analysis cost. To accommodate large-scale sample numbers, while maintaining a reasonable budget for analysis, improved means of separation and determination of PP333 were needed. The use of C_{18}-reverse phase, cartridge minicolumns featuring reduced pore size and increased sensitivity (Dong and Gant 1984), was explored as an alternative to existing methods for PP333 analysis.

Materials and Methods

PP333 Extraction and Purification

PP333 recovery from soil and apple (*Malus domestica* Borkh.)

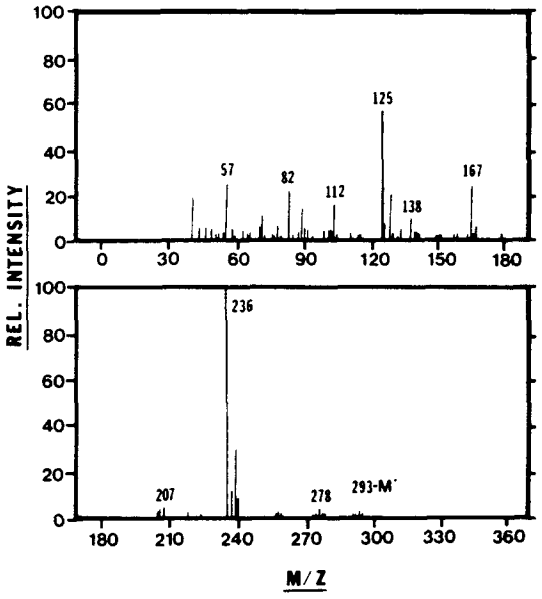

Fig. 1. Complete mass spectrum for PP333 (MW 293, designated M*), depicting significant mass fragments (refer to Table 1 for individual chemical structures). Mass spectrum operating conditions are covered in Materials and Methods.

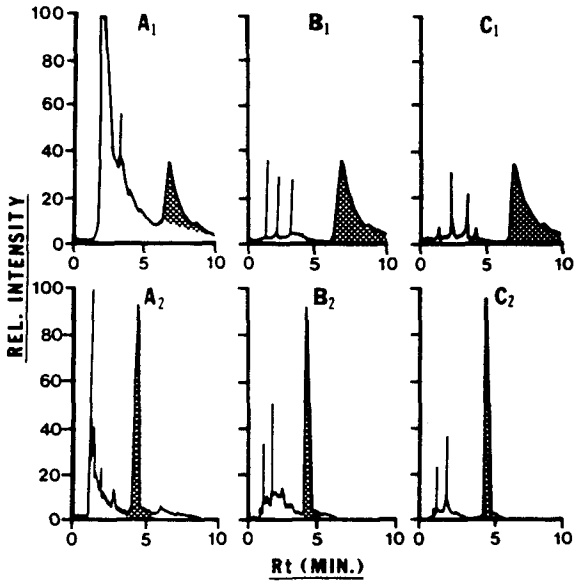

Fig. 2. HPLC separation of PP333 extracted from soil samples following a 1.0 g C_{18}-preparatory extraction column (A) and elution through 1.0 and 2.0 g of Florisil (B and C, respectively). Chromatograms A_1–C_1 were run at 60% CH_3CN on a Zorbax-ODS analytical column (250 × 4.6 mm) with PP333 eluting at a R_t = 6.2 min. Chromatograms A_2–C_2 were run at 50% CH_3CN on a Partisil 5 ODS-3 cartridge column (125 × 4.6 mm) with PP333 eluting at an R_t = 4.4 min. PP333 peaks in all chromatograms are equivalent to 400 ng of purified standard. HPLC operating conditions are covered in further detail in Table 1.

foliage has been described previously (Mauk et al. 1988); however, certain modifications and improvements were made. Plant samples were maintained at 1 g dry wt; whereas, the soil sample size was increased to 4.0 g dry wt/aliquot, while maintaining a ratio of 1.0 g:10 ml 80% MeOH for extraction. Samples were dried at 35°C under a gentle stream of nitrogen to decrease solvent volume reduction time and reduce PP333 degradation. Both C_{18} and Florisil preparatory extraction columns were conditioned three times, instead of once with their respective solvents which noticeably enhanced subsequent PP333 separation, purification, and recovery from both plant and soil samples. PP333 quantitation from samples spiked with either purified PP333 or diclobutrazol (PP296) consistently yielded 67–71% recovery for either soil or foliage sources.

PP333 for standard usage was purified and recrystallized from technical grade material (ICI Americas, Inc., Goldsboro, NC, USA) utilizing the method of extraction and purification above. Approximately 5–10 mg of the recrystallized source material were inserted by direct probe into a Hewlett-Packard Model HP-5985-B mass spectrometer operated in an electron-impact mode at 70 eV. The compound was scanned from 40–350 amu under a temperature gradient of 50–300°C at a rate of 20°C/min.

Quantitative PP333 HPLC

HPLC analyses were conducted with a ternary system (Isco Inc., Lincoln, NE, USA) at a fixed wavelength of 220 nm (maximum absorbance for PP333) with an isocratic solvent system varying from 50–70% CH_3CN (in water) depending on the column used for analysis. A sensitivity of 0.02% AUFS was chosen to achieve a compromise between PP333 detection sensitivity and baseline noise.

Initial HPLC quantitation of PP333 was performed with a standard 250 × 4.6 mm, C_{18}-reverse phase Zorbax ODS column (DuPont, Wilmington, DE, USA) under specified operating conditions (see Table 1). The second system featured a 125 × 4.6 mm Partisil 5 ODS-3 column (Whatman Inc., Clifton, NJ, USA) with a replaceable column-packing cartridge. The column hardware in this case was a one-time purchase. A guard column used with this system featured a replaceable C_{18}-cartridge. The third column, a Pecosphere 3 × 3C-C_{18} (Perkin-Elmer Corp., Norwalk, CN, USA) featured a replaceable cartridge with packing material of a smaller pore size (3 vs. 5 μM) and a reduced column length of 33 mm.

Results and Discussion

The identity and authenticity of the purified PP333 derived from the technical grade source was confirmed by comparison of the mass spectrum in Fig. 1 with information previously reported (Wang et al. 1986). The chemical structure of all the significant fragmented ions in the mass spectrum of PP333 were accounted for. Ion fragments M*-15 (m/z 278), M*-57 (m/z 236), and M*-86 (m/z 207) denoted the parent molecule of PP333 M* (m/z 293), with functional groups deleted and later reflected in the lower portion of the mass spectrum (m/z 57, 82; Fig. 1).

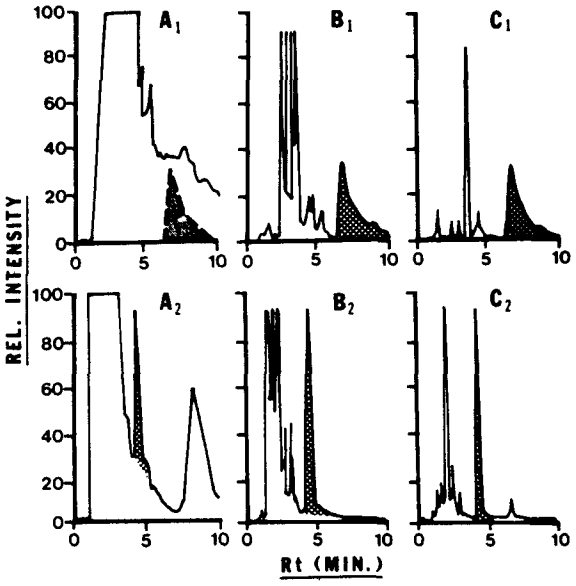

Fig. 3. HPLC separation of PP333 extracted from foliar samples following a 1.0 g C_{18}-preparatory extraction column (A) and elution through 1.0 and 2.0 g Florisil (B and C, respectively). Chromatogram characteristics and HPLC operating conditions are the same as in Fig. 2.

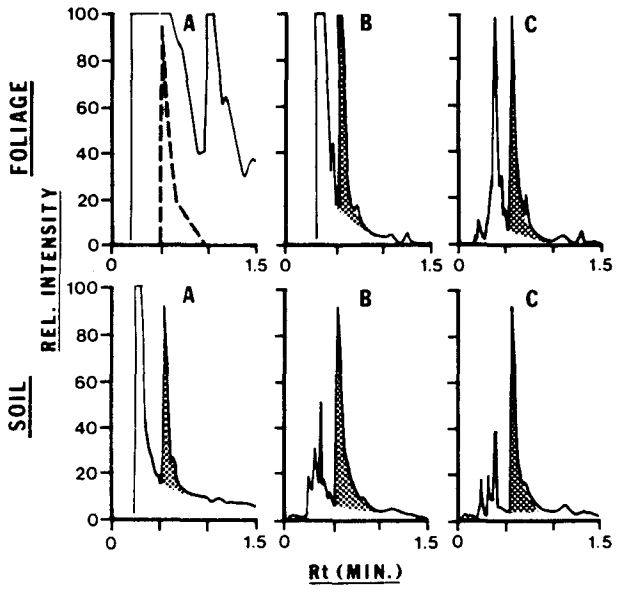

Fig. 4. HPLC separation of PP333 extracted from foliar and soil samples following 1.0 g C_{18}-preparatory extraction column (A) and elution through 1.0 and 2.0 g of Florisil (B and C, respectively). Samples were run at 70% CH_3CN on a Pecosphere 3 × 3C-C_{18} cartridge column (33 × 4.6 mm). PP333 eluted at an R_t = 0.6 min, and the peak was equivalent to 250 ng of purified standard.

The conclusion of the mass spectral evaluation was that the resultant purified material was indeed PP333, and it was of sufficient purity to warrant its use as a quantitative and qualitative chemical standard in HPLC analyses.

Purified PP333 was utilized in evaluating the performance of selected columns in the separation and quantitation of PP333 extracted from PP333-treated soil and plant tissue (Figs. 2–4). PP333 analysis of PP333-treated soil was simplified due to the lack of interfering contaminants following a standardized extraction and purification procedure (Mauk et al. 1988). The procedure was condensed by the omission of the Florisil step, since the C_{18}-preparatory column yielded sufficient clean-up for HPLC analysis (B and C, Fig. 2).

There were significant differences in PP333 HPLC analysis when comparing a conventional column (250 × 4.6 mm) with a reduced-length (125 mm) cartridge system (Fig. 2; A_1–C_1 vs. A_2–C_2, respectively). The shorter cartridge column was superior in all characteristics to the longer unit (Table 1). Most noticeable were enhanced resolution, a 10- to 20-fold increase in PP333 detection limits, and a 50% reduction in run time (resulting in 50% less solvent consumed). The R_t of PP333 could have been reduced even further by increasing to 60% CH_3CN and using the Florisil purification step (2 g) to remove contaminants that would potentially co-elute with PP333. Cost expenditure was another consideration where the cartridge system represented a 20% savings over the conventional longer column for the initial system and a 42% reduction in cost each time replacement was warranted (Table 1). The detection limits of the cartridge column system (10–20 ng) approach those reported for PP333 GC analysis (Reed 1988; Stahly and Buchanan 1986) and certainly are superior to previously documented HPLC analyses (Fujimoto et al. 1988; Hseuh et al. 1986).

A further comparison was made between the 250- and 125-mm columns with respect to HPLC analysis of foliar-derived PP333 (Fig. 3). The PP333 peak was not resolved after cleanup with the C_{18}-preparatory column and subsequent analysis on the Zorbax-ODS column (Fig. 3, A_1) and was only partially resolved under corresponding conditions for the Partisil 5 ODS-3 unit (Fig. 3, A_2). The Florisil extraction was a necessary purification step for foliar-derived PP333 prior to HPLC analysis with both columns (Fig. 3). A similar degree of purification was noted for foliar-extracted PP333 prior to GC quantitation (Reed 1988). As with soil-derived PP333, the superior attributes of the Whatman cartridge system still applied compared to the conventional longer DuPont column (Table 1).

Table 1. Comparison of HPLC column features, operating conditions, and efficiency of PP333 separation, resolution, and detection.

Column[a]	Size (L × o.d., mm)	Particle pore size (μm)	Column flow rate (ml/min)	% CH_3CN[b]	PP333 R+ (min)	Detection limit (ng)	Total run time (min)	Solvent consumed (ml)	Column cost[c] ($)
Dupont Zorbax ODS	250 × 4.6	5	1.5	60	6.2	200	12–22	18–33	250.00; 250.00
Whatman Partisil 5 ODS-3[d]	125 × 4.6	5	1.5	50	4.4	10–20	6– 8	9–12	200.00; 145.00
Perkin-Elmer Percosphere 3 × 3C-C_{18}	33 × 4.6	3	1.5	70	0.6	5–10	2	3	180.00; 75.00

[a] All column packings were C_{18}-reverse phase, octadecylsilyl derivatives.
[b] Elutions were done with an isocratic gradient that ranged from 50–70% CH_3CN depending on the column used.
[c] Column cost is separated into an initial figure for all the hardware and a second one to replace spent column-packing material.
[d] These are cartridge columns that feature a replaceable column-packing module.

The third alternative for PP333 analysis was the use of a minicolumn cartridge, with a reduced length (33 mm) and column packing (3 μM) (Table 1). HPLC analysis of both soil and foliar-extracted PP333 using the Pecosphere 3 × 3C-C_{18} column necessitated both C_{18} and Florisil (2 g) purification steps (Fig. 4). Although the entire purification procedure of Mauk et al. (1988) was necessary, the benefits of the minicolumn far outweighed the disadvantages of utilizing the entire purification procedure. Solvent consumption (3 ml) and total HPLC run time (2 min) were sharply cut by a drastically reduced R_t of only 36 s (Table 1). Excellent resolution was achieved by running at 70% CH_3CN as the mobile phase and decreasing the sample load volume to 5 μl. The column resolution for PP333 was two to four times that of the Partisil 5 ODS-3 unit and the cost of a replacement cartridge represented a 50% reduction.

Summary

Advances in HPLC technology now afford the means to analyze growth retardants and gibberellin inhibitors at levels approaching GC. Thus, HPLC can be utilized as a quantitation alternative to GC analysis of PP333 and as a preparative tool. The use of replaceable, inexpensive cartridge minicolumns represents a substantial savings in solvent consumption and analysis run time. Their high degree of resolution and brief analysis time per sample can facilitate large-scale experiments that are necessary to fully understand the uptake, translocation, and fate of exogenous growth regulators.

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