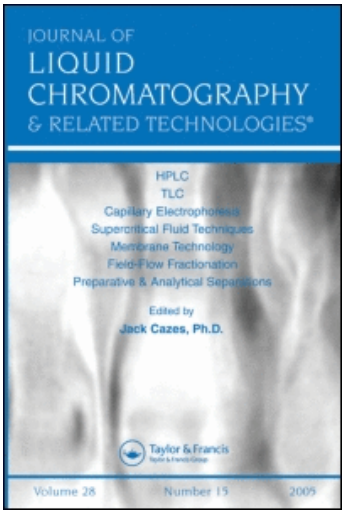

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Pepperman Jr., Armand B. and Wartelle, Lynda H.(1987) 'Quantitative HPLC Assay of Strigol and Epistrigol-Witchweed Germination Stimulants', Journal of Liquid Chromatography & Related Technologies, 10: 7, 1485 — 1495
To link to this Article: DOI: 10.1080/01483918708066781
URL: http://dx.doi.org/10.1080/01483918708066781

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(7), 1485-1495 (1987)

QUANTITATIVE HPLC ASSAY OF STRIGOL AND EPISTRIGOL–WITCHWEED GERMINATION STIMULANTS

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ABSTRACT

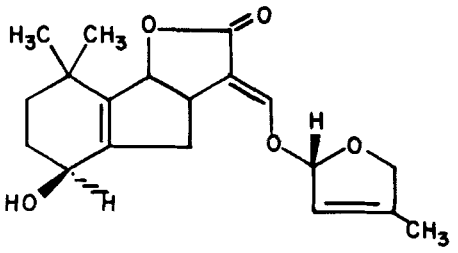
In the preparation of strigol, the last step of an 18-step synthesis produces strigol and its diastereomer, epistrigol. The two can be separated by column chromatography and recrystallization. It would be advantageous to be able to monitor the purity of each of these isomers in the presence of the other at each step of purification. Also, strigol is a much more potent witchweed seed germination stimulant than epistrigol and it is important to know the purity of samples submitted for bioassay. A satisfactory method was devised which utilized HPLC on a C-18 reverse phase column with a ternary solvent mixture of acetonitrile-methanol-water (10-50-40). The method gave good separation and reproducibility of analyses of mixtures of

epistrigol and strigol and was sensitive enough to detect 1-2% of either isomer in the presence of the other.

INTRODUCTION

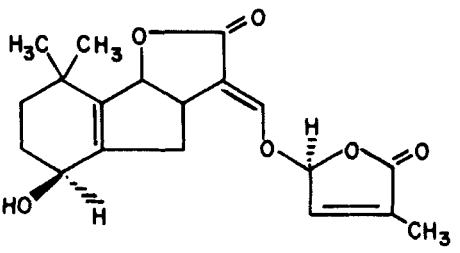
Witchweed (Striga asiatica (L.) Kuntze) is an obligate parasite of the Gramineae, including such economically important crops as corn, grain sorghum, and sugar cane. (1) Control of witchweed is complicated since the seeds will remain dormant until exposed to a chemical exuded from the roots of the host plant and some non-host plants. (2) A potent witchweed seed germination stimulant was isolated from root exudates of cotton (a non-host plant) by Cook, et al. (3) The compound was given the trivial name strigol and its first total synthesis was reported by Heather, et al. in 1974. (4) When witchweed seeds are induced to germinate in the absence of a host plant, the seedlings die of starvation; this process is known as "suicidal germination" (5), and effects some measure of control.

Two pairs of diastereomers are produced in the last step of the synthesis (6); d,l-strigol and d,l-4'-epistrigol (See Figure 1). These diastereomers are separated chromatographically and by fractional recrystallization. Since their activities as seed germination stimulants are vastly different, it is necessary to avoid contamination of one isomer with the other. A 10^{-10} M concentration of strigol or a 10^{-6} M concentration of epistrigol will induce 80% germination of witchweed (7). The activity of these compounds at hormonal levels indicates that they should be tested in other systems where the activities conceivably could be



STRIGOL

requires 10^{-10}m concentration to give 80% germination



EPISTRIGOL

requires 10^{-6}m concentration to give 80% germination

Figure 1. Structures of strigol and epistrigol

reversed. It is generally accepted that the presence of any impurities or other active molecules compromise the interpretation of the bioassay (8). Since the spectral properties of the diastereomers are very similar (6), it would be difficult to develop a quantitative method for analysis of mixtures of the two by spectroscopic methods. In this work, a method was developed based on HPLC techniques, which gives quantitative measurement of either isomer in the presence of the other.

MATERIALS AND METHODS

Analyses were performed using a Beckman Model 324 HPLC system (9) consisting of two Model 100A pumps, a Model 156 fixed wavelength ultraviolet detector (254 nm), a Model 421 controller, and a Kipp and Zonen BD 41 recorder. Peak area and retention time data were obtained using a Hewlett-Packard Laboratory Automation System (LAS) Model-3356.

The four columns used in the analyses were C-8 and C-18 columns from Alltech Associates and Dupont Instruments. All columns were 4.6 mm X 25 cm. long with 5μ packing. The guard column used in the analyses was also from Alltech and was 4.6 mm X 10 cm with 5μ C-18 packing.

Solvents used were acetonitrile - UV (Burdick and Jackson Laboratories), methanol - UV (Burdick and Jackson Laboratories), and deionized water purified in a Water I (Gelman Scientific) system. The various solvent mixtures used in this study were mixed, filtered, and degassed before use. Details on the mixtures are given in the Results and Discussion Section.

Strigol and epistrigol were synthesized according to the procedure developed by Heather, et al (4,6). Stock solutions of strigol and epistrigol were prepared by weighing out 0.050g. and diluting to 50ml. in acetonitrile. These solutions contained 0.1% w/v of the compound. The standard mixtures contained a total of 0.1% of the two compounds. Standard mixtures were prepared by combining the stock solutions in appropriate volumes from 1 ml to 0 ml to give solutions contaning only strigol to only epistrigol in 10% increments. These mixtures contained 1000 ppm of the two compounds combined and were injected twice (injection volume 80 μl) onto each of the four columns to check for quality of separation.

RESULTS AND DISCUSSION

Preliminary experiments were conducted using the Zorbax ODS (C-18) and Zorbax C-8 columns from DuPont on a Waters HPLC

system. In this work, it was shown that a reasonable separation of strigol and epistrigol could be achieved with a binary mobile phase of acetonitrile-water (CH_3CN-H_2O), with the most effective ratio determined to be 35-65 respectively. Smaller amounts of acetonitrile (30% and 25%) caused tailing of peaks and mechanical difficulties due to precipitation of solids on the column, whereas larger amounts of acetonitrile (40% and 45%) caused overlapping peaks.

Since strigol and epistrigol are essentially water insoluble, some precipitation of solids on the column was encountered (sample was injected from 100% CH_3CN) even with the 35-65 CH_3CN-H_2O mobile phase. These problems were evidenced by longer retention times after several injections, and the appearance of stray peaks caused by the precipitated strigol and epistrigol redissolving in the mobile phase. As these problems occurred, flushing the column with 100% CH_3CN for an hour or more would clear them up but the symptoms reappeared after a few injections. For this binary system satisfactory results could be obtained if regular flushing of the column with 100% CH_3CN was performed.

The use of a guard column proved helpful in maintaining consistent results through several runs, but caused increased pressure in the system. To alleviate the excessive pressure, it was necessary to reduce the amount of water in the mobile phase and to lower the flow rate to 0.8 ml/min. A ternary mobile phase of 20-40-40 CH_3CN- CH_3OH-H_2O was found to give baseline

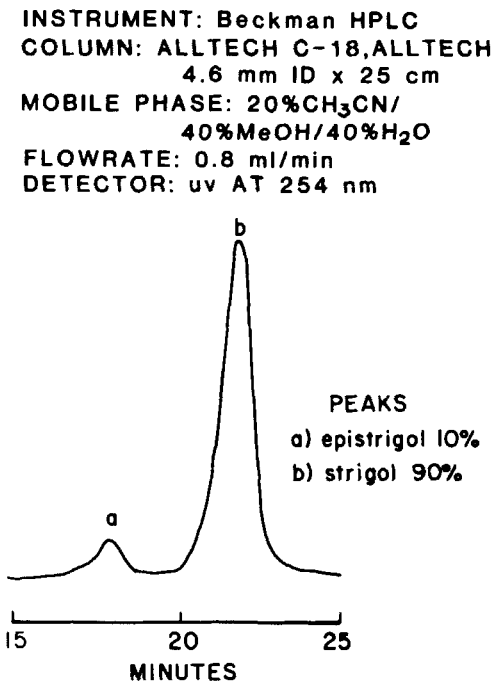


Figure 2. HPLC chromatogram of a 90-10 mixture of strigol-epistrigol

separation (See Figure 2) and good resolution under these conditions.

The results comparing all four columns using the 20-40-40 CH_3CN-CH_3OH-H_2O mobile phase are shown in Table 1. The chromatographic parameters are comparable for all four columns, with the resolution (R) being best for the Alltech C-18 column. Retention times are longer, but are still acceptable, due to the lower flow rates. The accuracy for all four columns was acceptable and no statistically significant differences (paired

TABLE 1.

Chromatographic Parameters for Four Columns. With Ternary Mobile Phase of CH_3CN-CH_3OH-H_2O (20-40-40)

Column	k'_1	k'_2	α	R	Retention Time (Min) Strigol	Epistrigol
Alltech C-8	2.03	2.56	1.28	1.96	22.7	19.1
Alltech C-18	2.17	2.91	1.34	2.61	23.4	19.0
Dupont C-8	2.16	2.77	1.28	1.86	21.0	17.6
Dupont C-18	2.35	3.18	1.36	2.17	22.4	18.0

AUFS UV_{254}= 0.64
Flowrate = 0.8 ml/min
Pressure = 2980 psi

t-test) were found between the theoretical percentages of isomers injected and the actual values obtained from the chromatogram. The Alltech C-18 column was chosen for further work primarily because it gave better resolution.

Varying the ratios in the ternary solvent mixture caused the pressure to increase dramatically, requiring the flow rate to be decreased further. The R values and retention times are shown in Table 2 for several of these mixtures. To maintain a pressure of 3000 psi or lower, flow rates were decreased to as low as 0.5 ml/min. Resolution was best with the lowest amount of CH_3CN and became generally poorer as the concentration CH_3CN increased.

However, retention times were in excess of thirty minutes, for both strigol and epistrigol, with only 5% acetonitrile in the mixture, since the flow rate had to be lowered to 0.5 ml/min. With the 20-40-40 and 10-50-40 mixtures at flow rates of 0.6

TABLE 2.

Comparison of Solvent Systems for Separation of Strigol and Epistrigol on Alltech C-18 Column

Solvent System	Resolution R	Retention Time (min) Strigol	Epistrigol	Flowrate ml/min	Pressure psi
CH_3CN-MeOH-H_2O(5-55-40)	3.80	40.7	30.1	0.5	3140
CH_3CN-MeOH-H_2O(10-50-40)	3.43	32.8	25.1	0.6	3430
CH_3CN-MeOH-H_2O(20-40-40)	2.91	30.2	24.6	0.6	2980
CH_3CN-MeOH-H_2O(30-40-30)	1.40	11.3	10.2	0.8	2860
CH_3CN-MeOH-H_2O(30-70)	2.04	18.6	15.5	0.5	3070
CH_3CN-MeOH(10-90)		no separation 5.50 min		0.8	2680

UV_{254} = 0.01 AUFS

ml/min, retention times were comparable, being only slightly longer for the solvent lower in acetonitrile. For the 30-40-30 solvent mixture, resolution and retention times dropped substantially, and the separation was no longer baseline. The binary systems of H_2O-MeOH and CH_3CN-MeOH also gave poor separation of the peaks.

A precision analysis was carried out using the two solvent systems which gave the best overall performance characteristics; the 20-40-40 and the 10-50-40 CH_3CN-MeOH-H_2O. The 20% CH_3CN solvent analyses were carried out at 0.8 ml/min flow rate while the 10% CH_3CN analyses were carried out at 0.6 ml/min flow rate. Two separately prepared stock solutions of strigol and epistrigol were used to prepare 98-2 and 2-98 percent mixtures, respectively (Experiments 1 and 2 in Table 3). The mean of five injections

TABLE 3.

Precision Analysis Data for Two Ternary Solvent Systems of CH_3CN-CH_3OH-H_2O

% Strigol Injected	% Epistrigol Injected	Injections per Experiment	Mean ± Confidence Interval (C.I) for Strigol /a Experiment 1	Experiment 2
		20%CH_3CN - 40% CH_3OH - 40% H_2O/b		
98	2	5	97.91±0.536	97.09±0.999
2	98	5	3.604±1.539	2.265±1.366
		10% CH_3CN - 50% CH_3OH - 40% H_2O/c		
98	2	5	97.91±0.865	97.99±0.772
2	98	5	3.31±1.89	3.13±0.745

/a There was no significant difference between data in Experiment 1 and Experiment 2 according to the Students t-Analysis

/b C.I. for this solvent system is at 98% level.

/c C.I. for this solvent system is at 99% level.

and the confidence interval is given for each experiment. Good agreement, between amount injected and amount analyzed for, occurred for the 98% strigol-2% epistrigol standards in both solvent systems. For the 2-98 standards, a high analysis for strigol results in both solvent systems. Still, the correlation coefficient for the least squares line of the combined data is 0.999. Since the ultraviolet spectra for strigol and epistrigol are identical, (6) the differences cannot be attributed to any differences in extinction coefficient. Rather, it appears that the area integration mode of the LAS system is weighted slightly in favor of the broader peak thus giving a higher value for the

compound eluting last (strigol). This effect is magnified when the broader peak is very small. The precision analysis indicates that good reproducibility is possible with reasonable precision and acceptable accuracy. The method is also quite sensitive as the 2% in the mixtures above corresponds to 20 ppm. This level was readily detectable and sensitivity was shown to extend to the 1-2 ppm range. With an 80 µl injection, the minimum detectable content is 8.0 ng.

Due to the low solubility of strigol and epistrigol, the preferred method uses a ternary solvent mixture and a guard column to give consistent and reproducible results. The method is sensitive (low ppm range for either analyte), accurate, and reproducible under these conditions.

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