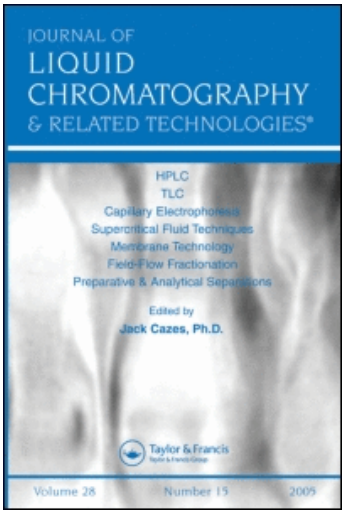

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Quantitative Determination of Cucurbitacins by High Performance Liquid Chromatography and High Performance Thin Layer Chromatography

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QUANTITATIVE DETERMINATION OF CUCURBITACINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

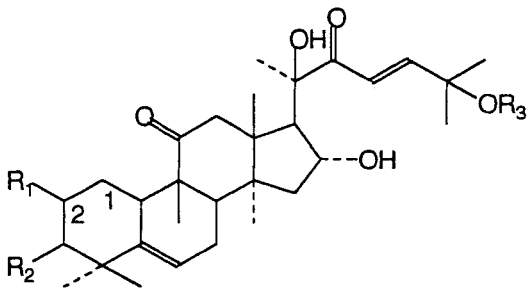
A simple and precise method using High-Performance liquid [HPLC] and High-Performance Thin-Layer Chromatography [HPTLC] was developed for simultaneous determination of the frequently encountered cucurbitacins B **(1)**, D **(2)**, 3-epi-iso-cucurbitacin D **(3)**, E **(4)** and I **(5)** as well as cucurbitacin glycosides E **(6)** and I **(7)**. A reverse phase column [C-18] was used and elution achieved with acetonitrile-water [2:8, solvent A and 45:55, solvent B] with gradient elution analysis. Reverse-phase HPTLC plates were developed with methanol:water (7:3) and normal-phase plates were developed with toluene-ethylacetate (25:75). Developed plates were scanned with Thin-Layer Scanning Instrumentation. A clean-up procedure was perfected before sample loading to both the HPLC and HPTLC.

INTRODUCTION

The cucurbitacins are a group of highly oxygenated tetracylic triterpenes that are produced mainly by Cucurbitaceae and a few plants of other families[1,2]. This group of compounds exhibits a great diversity of

biological and chemical effects, such as acute mammalian toxicity and anti-neoplastic activity[3-5]. Furthermore, the extreme bitterness of cucurbitacins and the fact that they can be induced to higher concentrations by insect feeding damage[6] suggests that these compounds are involved in plant protection against herbivores[7,8]. In spite of their repellency toward most herbivores, cucurbitacins act as arrestants and phagostimulants for diabroticite chrysomelid beetles[7]; even nanogram quantities of these compounds trigger compulsive feeding in diabroticites with no perceivable harmful effects. The phagostimulatory properties of cucurbitacins are so powerful that they currently form the basis of a diabroticite control program using toxic baits impregnated with cucurbitacins. Thus, cucurbitacins also have considerable importance to agriculture since several diabroticites are serious pests of corn, cucurbits and sugar beets world-wide[10] .

Though scientific interest in cucurbitacins has increased dramatically in the past decade, the lack of accessibility to accurate, and sensitive quantification procedures for these compounds has slowed progress in many laboratories. All work published to date in this regard involves HPLC methodology, is typically limited to only one cucurbitacin aglycone[11] , or has focused on the qualitative and quantitative analysis of crude chloroform extracts using either internal or external standards[12]. Furthermore, analysis of crude extracts necessarily limits sample number; without prior clean-up, HPLC column life is drastically reduced. Here we present a simplified approach for identifying and quantifying the common cucurbitacins B, D, E, I and cucurbitacin E and I glucosides using both HPLC and TLC technology.



		R1	R2	R3	$\Delta^{1,2}$
1	cu B	OH	=O	COCH3	-
2	cu D	OH	=O	H	-
3	cu iso-D	=O	OH	H	-
4	cu E	OH	=O	COCH3	+
5	cu I	OH	=O	H	+
6	cu E-gl.	glucose	=O	COCH3	+
7	cu I-gl.	glucose	=O	H	+

Scheme 1: Cucurbitacins used in the assay

EXPERIMENTAL

Plant Materials and Reference Compounds:

Fresh samples of *Cucurbita andreana*, *C. texana* and *C. okeechobeensis* were obtained from the greenhouse of the University of Delaware, Agriculture Experimental Station, Newark, DE.. The original source of these species was the USDA seed bank. Pure seed stock was maintained by hand pollination every flowering season. Cucurbitacins B, D, 3-epi-iso-cucurbitacin D, E and I were isolated and their structures were confirmed by spectroscopic methods [UV, IR, ^{1}-NMR, ^{13}-C-NMR and MS]. Cucurbitacin E and I glucosides were also isolated from *C. andreana* fruits and were structurally elucidated spectroscopically.

Apparatus

High-performance liquid chromatography analysis was performed with a Perkin-Elmer S100 and a Hewlett Packard reverse column [Rp-18, 10 μm, 200x4.6 mm] fitted with a Supelco guard column LC-18. A Shimadzu C9000U Dual Wavelength Flying Spot Scanner was used for TLC scanning.

HPLC Conditions

An acetonitrile-water mixture was used in two proportions as follows; solvent A, acetonitrile-water 2:8; solvent B, acetonitrile -water 45:55 [IV]. Elution was accomplished via a gradient analysis starting with 0% solvent B which gradually increased to 100% at 35 minutes. Flow rate was adjusted to 2 ml/min. and the column was equilibrated for 10 min. with solvent A before each run. Detection was achieved by a UV detector [Beckmen 160] at wavelength 229 nm and chromatgram data were processed with a Perkin-Elmer LCI-100.

TLC Conditions:

HPTLC-NP plates [HPTLC-GHLF,Inorganic binder, 10x20 cm, w/UV 254, Analtech] and NPTLC [Whatman, 20x20 , 0.250 mm, GF_{254}] were developed with ethylacetate-toluene (75:25) [I]. For HPTLC-RP plates [HPTLC-RP 18F, 10x20 cm, w/UV 254, Analtech], methanol-water (7:3) [III] was used as the solvent system and the plates were conditioned for 30 minutes with the solvent before development.

Assay Procedure:

Sample Preparation:

1- 0.2 gm of freshly obtained or frozen plant materials were always transferred in triplicate to test tubes containing 5 ml of chloroform;

2- The samples were then homogenized for three minutes with a biohomogenizer and the latter was washed with 2 ml chloroform which was then added to the homogenate;
3- The homogenate was vigorously shaken by vortex for one minute and filtered through Whatman filter paper [9 cm]. The vacant test tube was subsequently washed with 5 ml chloroform, shaken, and also transferred to the filter paper;
4- The chloroform extract was evaporated under nitrogen in a Pierce Reacti-Therm III Heating Module at 45 C till dryness;

Sample Purification:
1- A solid phase extraction column packed with 0.2 gm of C-18 reverse phase silica gel [10 μm, 14% carbon load, Analtech] was first activated by washing with 3 ml methanol and was dried under vacuum in an Extra-Sep extraction unit.
2- The sample residue was then dissolved in 60 μl chloroform, vortexed for 10 seconds until it dissolved, and transferred to the column bed. At this point the chloroform was evaporated with an air-dryer for 30 seconds;
3- The columns were then eluted with 3 ml of methanol-water [8:2], allowed to filter slowly until the solvent completely drained, and finally were placed under vacuum for 2 minutes for complete solvent elution;
4- The hydro-alcohol extract was evaporated to dryness under vacuum at 45 °C and samples were refrigerated until HPLC or TLC analysis;
5- For HPLC analysis , samples were dissolved in 200 μl methanol and filtered through a 0.45 μm nylon filter directly into autosampler vials;
6- For TLC analysis, samples were dissolved in 60 μl chloroform and half of this volume was applied to the

plates using micro-pipettes and a Camag Nanomat III spotter guide [half of the chloroform volume here was 25 µl as it was noticed that the volume decreased after shaking with the vortex].

Recovery experiments:

Plant tissue samples were spiked with a known concentration of cucurbitacin D before homogenization in order to measure the efficacy of the extraction at this step. The homogenate was then centrifuged at 10,000 rpm for 10 min. and the contents of the supernatant were estimated by HPTLC. We also measured recovery rates following filtration and sample purification. Recovery rates from the solid extraction column prepared with Analtech packing was compared with those from extraction columns purchased from Baxter [B&J solid phase system column (C-18)] and Thomason Instruments [Spe-ed cartridge].

RESULTS AND DISCUSION

Extraction conditions:

When the efficiency with which chloroform, methanol, and chloroform-methanol [1:1, v/v] extracted cucurbitacins from plant tissues was compared, we found the most efficient solvent to be chloroform in terms of total extractable materials [84.5%] and sample purification. While methanol extraction efficiency was higher [93%], samples extracted with it had a very sticky residue after evaporation, which resisted total solubility in methanol. This was especially true for fruit samples and made it difficult to redissolve the samples for further analysis. The optimum homogenization time for efficient extraction was three minutes at high speed. Also, samples shaken for one minute by vortex before transfer to the filter paper provided constant and steady recovery values.

TABLE 1
Recovery data of cucurbitacins throughout the assay.

Process	Recovery %	S.E of mean
Homogenization	84.50	1.15
Filteration	76.25	0.90
Clean-up	63.85	1.25

Recovery rates and clean-up efficiency from the Baxter and Thomason Instruments extraction column were 25% less than those from the column we prepared with Analtech packing. We attributed these differences to lower carbon loads (8%) of the C-18 phase used in the former two columns as compared to the 14% carbon load of the Analtech packing material. Analtech higher carbon load also helped produce a stable baseline on resulting chromatograms. Total recovery evaluations from all steps in the assay (from extraction through filtration and sample purification) consistently averaged 63.85% [Table 1].

HPLC Conditions:

Elution parameters such as the organic contents of the mobile phase and gradient system combinations were varied to establish the optimum elution parameters on both normal and reverse phase columns. Using both normal phase and reverse phase columns, it was not possible to separate the isomeric cucurbitacin D from 3-epi-iso-cucurbitacin D, with isochratic elution. Similarly, cucurbitacin B could not be separated from interfering material, most probably iso-B (solvent used was

TABLE 2

Retention Data of Cucurbitacins in the Assay.

Compound	R_f I	R_f II	R_f III	R_t IV	R_f V
cu B	0.72	0.85	0.40	28.95	-
cu D	0.41	0.70	0.51	18.80	-
cu iso-D	0.47	0.72	0.52	19.78	-
cu E	0.81	0.88	0.36	33.92	-
cu I	0.55	0.74	0.47	21.87	-
cu E-glu.	-	0.28	0.56	20.70	0.34
cu I-glu.	-	0.19	0.67	10.40	0.24

I-V are solvent systems.

methanol:water 7:3, 75:25 8:2). To alleviate this problem we employed acetonitrile-water mixtures in two combinations: acetonitrile-water, 2:8 (solvent A), and 45:55 (solvent B) [IV] as a mobile phase in the gradient elution. This solvent is very sensitive and minor changes in either its proportions or its flow rate create significant changes in R_t values and in resolution. Thus, it is a suitable solvent for analysing extracts from other species that may require further changes in these parameters.

TLC Conditions:

Normal phase TLC and HPTLC showed excellent resolution with standards [Table 2], but when tested with assay preparations, a non-stable base line was obtained whenever any of chlorophyll remained. Consequently, a clean extract was essential. While reverse phase plates showed a reasonable resolution [Table 2] and a steady

base line, any remaining chlorophyll was retained on the spotting line. Conditioning the plates for 30 minutes with the mobile phase before development improved the stability of base line. We also noticed that washing the TLC plates with methanol, followed by drying them, improved the sensitivity of detection about 40%, especially at lower cucurbitacin concentrations [0.25-1 μg]. The separation of glycosides from aglycones on TLC was not accomplished using one solvent system such as chloroform-methanol of different polarities (from 19:1 - 9:1). Glycosides remained very close to the base line when developed with solvent less polar than 19:1, while increasing the polarity of the chloroform-methanol mixture above 94:6 made aglycone resolution unsatisfactory [Table 2]. Glycoside resolution was excellent, however, with chloroform:methanol at 9:1 [V]. Obviously TLC estimation of both the aglycones and glycosides using one solvent system was not posssible, but they could be assayed very well using two different solvent systems. Such was the rational for saving half of the purified extract for the aglycone assay and half for glycoside analysis using systems I and V respectively (Table 2).

Calibration Graphs, Precision, and Detection Limits:

A serial dilution of the five standard cucurbitacins containing between 0.01 and 0.32 mg/ml was prepared in methanol. These were used for HPLC injection, giving a load of 0.5, 1, 2, 4, 6, 8, and 16 ug [a 50 μl loop was used]. The data obtained were used for preparing calibration graphs (Tables 3 and 4). A similar set of solutions from cucurbitacin glucosides was prepared for the same reason. In the case of TLC, a set of dilutions from the aglycones and the glycosides was prepared with the loaded concentrations ranging from 0.25 μg to 16 μg.

TABLE 3

Calibration Graphs of Cucurbitacins on TLC.

Compound	Reg. equation	r	Linear range
I-RP-HP:			
cu B	y=470.7+15481x	0.999	0.25-12 μg
cu D+	y=211.1+15886X	0.999	0.25-12 μg
cu E	y=13042+22465x	0.995	0.25-16 μg
cu I	y=15356+19904x	0.993	0.25-16 μg
cu E-gl.	y=23927+16208x	0.996	0.25-16 μg
cu I-gl.	y=34048+15056x	0.989	0.25-16 μg
II-NP*			
cu B	y=22481+14680x	0.991	1-16 μg
cu D	y=15551+17417x	0.997	1-16 μg
cu E	y=81438+19847X	0.999	1-16 μg
cu I	y=-1147+23002x	1.000	1-16 μg
cu E-gl.	y=479680+28051x	0.990	1-12 μg
cu I-gl.	y=27137+23272X	0.990	1-12 μg

+ cu D and 3-epi-iso-cu D.

* both HP and NP TLC.

TABLE 4

Calibration Graphs of Cucurbitacins in HPLC:

Compound	Reg. equation	r	Linear range
cu B	y=-237950+34332x	0.960	2-12 μg
cu D*	y=-958558+360035x	0.985	2-12 μg
cu E	y=-2389390+626813x	0.995	2-12 μg
cu I	y=-1018193+372590X	0.993	2-12 μg
cu E glu.	y=-963702+334629x	0.998	2-12 μg
cu I glu.	y=-396978+221205x	0.996	2-12 μg

* both cu D and 3-epi-iso-D.

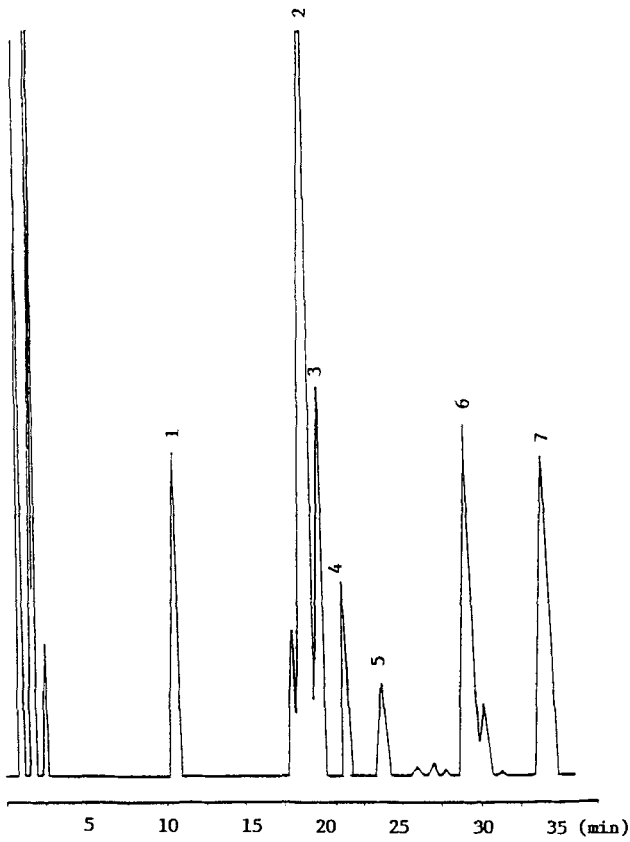


FIGURE 1. HPLC chromatogram of *C. texana* chloroform extract, developed with solvent system IV. 1= cu I-gl., 2= cu D, 3= iso-D, 4= cu E-gl., 5= cu I, 6= cu B and 7= cu E.

Analytical Results:

Figure 1 illustrates an example of an HPLC chromatogram of a chloroform extract from *C. texana* fruit. Figure 2 provides TLC chromatograms developed with solvents I (A) and III (B), also obtained from a chloroform extract of *C. texana* friut. Comparison of HPLC and TlC chromatograms show that HPLC provides the most

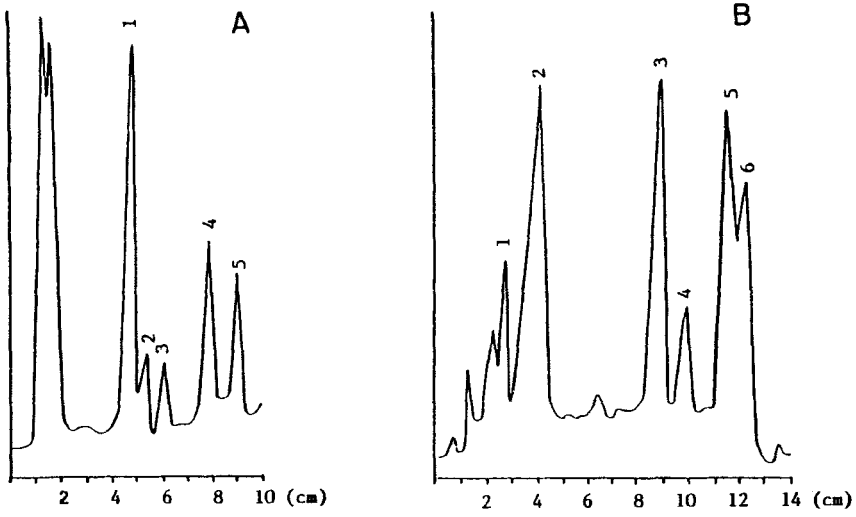


FIGURE 2 A). NP-TLC chromatogram of *C. texana* chloroform extract, developed with solvent I, 1= cu D, 2= cu iso-D, 3= cu B, 4=cu I and 5=cu E.
2 B). NP-TLC chromatogram of *C. texana* chloroform extract, developed with solvent II, 1=cu I-gl., 2= cu E-gl., 3= cu D, 4= cu I, 5= cu B and 6= cu E.

TABLE 5

Total Cucurbitacin Contents* of Fruits from some *Cucurbita* spp.

Spp.	RP-TLC[1]	NP-TLC[2]	HPLC3[3]
C. andreana	1.05(0.06)	0.95(0.06)	0.96(0.08)
C. okeecheebensis	0.54(0.03)	0.50(0.04)	0.58(0.02)
C. texana	0.82(0.03)	0.73(0.02)	0.78(0.02)

* mg/g fresh weight (+ S.E., N=3).
1,2 and 3 are solvents III, I & V and IV used for development repectively.

comprehensive resolution and achieves analysis in only one step, while TLC provides resonable resolution, but analysis requires a two steps as discussed above. Using one solvent system for TLC analysis of total cucurbitacin [Figure 2 B] yields unsatisfactory resolution of the glycosides and the isomeric compounds. In terms of analysis time and costs, however, TLC is incomparable.

Table 5 displays the total cucurbitacin content of fruit samples from *C. andreana*, *C. texana* and *C. okeecheebesis* analyzed by different chromatographic techniques. The results were remarkably consistent and suggest that either of the techniques can be used depending on the available instrumentation. We have also employed our assay in studies measuring cucurbitacin induction following mechanical and/or insect damage to foliage of *C. okeecheebensis* and *C. maxima* (Authors, in preparation). Again the assay has proven to be a reproducible and effecient means of analyzing 100's of samples.

CONCLUSION

Both reverse phase HPLC [gradient analysis] and TLC methods were developed for the rapid and repetitive analysis of the cucurbitacin content of various plant tissues. Several advances have been accomplished, particularly in reducing interference from chlorophyll by more than 95%. For this reason alone, this assay is an appropriate approach for other natural product purifications. The sensitivity, speed, and accuracy of this approach makes it ideal for ecological, pharmacological and biosynthetic studies of cucurbitacins that require analysis of 100's or even 1000's of replicate samples.

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