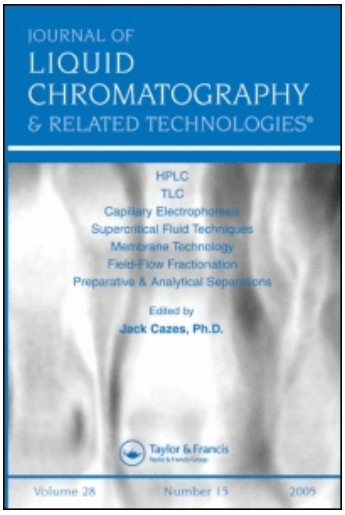

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Monoterpenoid Alkaloid Quantitation by *in situ* Densitometry-Thin Layer Chromatography

Elizabeta Hernández-Domínguez[a]; Felipe Vázquez-Flota[a]
[a] Unidad de Bioquímica y Biología Molecular de Plantas and Graduate Program in Plant Sciences and Biotechnology, Centro de Investigación Científica de Yucatán, Mérida Yucatán, México

To cite this Article Hernández-Domínguez, Elizabeta and Vázquez-Flota, Felipe(2006) 'Monoterpenoid Alkaloid Quantitation by *in situ* Densitometry-Thin Layer Chromatography', Journal of Liquid Chromatography & Related Technologies, 29: 4, 583 — 590
To link to this Article: DOI: 10.1080/10826070500479336
URL: http://dx.doi.org/10.1080/10826070500479336

Journal of Liquid Chromatography & Related Technologies®, 29: 583–590, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500479336

Monoterpenoid Alkaloid Quantitation by *in situ* Densitometry-Thin Layer Chromatography

Elizabeta Hernández-Domínguez and Felipe Vázquez-Flota
Unidad de Bioquímica y Biología Molecular de Plantas and Graduate Program in Plant Sciences and Biotechnology, Centro de Investigación Científica de Yucatán, Mérida Yucatán, México

Abstract: A simple and reproducible technique for the quantitation of the main monoterpenoid indole alkaloids from aerial tissues of *Catharanthus roseus*, based on *in situ* densitometry-thin layer chromatography, is described. The sensitivity of this method was enough to detect the amounts of ajmalicine, catharanthine, and vindoline present in 100 mg of dried leaf tissue and in different *in vitro* culture systems. The main advantage of this method is that it allows the proper separation of these alkaloids using a single solvent mixture, as well as the scanning of the corresponding spots at a single wavelength, reducing the number of chromatographic plates required and the time invested in the analysis.

Keywords: Ajmalicine, Catharanthine, *Catharanthus roseus*, Thin layer chromatography, Vindoline

INTRODUCTION

Thin layer chromatography (TLC) is a simple, rapid, and affordable technique for the analysis of natural products present in extracts from different sources. Although it is mainly used for qualitative purposes, combined with other analytical procedures, such as UV/visible spectrophotometry, it can become a reliable and sensitive quantitative method. This is particularly true for

Address correspondence to Felipe Vázquez-Flota, Unidad de Bioquímica y Biología Molecular de Plantas and Graduate Program in Plant Sciences and Biotechnology, Centro de Investigación Científica de Yucatán, Calle 43 # 130, Chuburna, 97200, Mérida Yucatán, México. E-mail: felipe@cicy.mx

compounds with well known chromatographic properties, which are expected to be present in the material under analysis. Monoterpenoid indole alkaloids (MIA) are commonly found in members of the Apocynacea family, such as *Catharanthus roseus*. This plant produces several alkaloids which, based on the molecular arrangement of the terpenoid moiety, can be grouped into eight different classes. Nevertheless, there are three main classes, defined as Aspidosperma, Corynanthea, and Iboga, with vindoline, ajmalicine, and catharanthine being the most representative members of each of the mentioned types.[1] Since some of the *Catharanthus* alkaloids have pharmacological applications, over the years, this plant has been under extensive research, resulting in the development of chromatographic methods for precise alkaloid measurement, such as those described by Tinkhomiroff and Jolicouer and references therein.[2] Most of these methods are based on high performance liquid chromatography (HPLC), which may represent a limiting condition for small budget laboratories.

Most reports on the chromatographic properties of MIA's on TLC plates were developed to provide quick information on the qualitative profile of the material under analysis.[3] Although some TLC quantitative methods are available,[4,5] they frequently require the use of more than one mobile phases in order to adequately separate the different alkaloids present in one extract, as well as the staining of the plates with chromogenic reagents, such as Dragendoff's. Those procedures can be time-consuming. We have developed a method that allows separating three of the most representative *Catharanthus* alkaloids: ajmalicine, catharanthine and vindoline, with just one single mobile phase. The resolution of the spots corresponding to these alkaloids was good enough to be scanned using an automatic densitometer, without signal overlapping. Furthermore, no staining of the plates was necessary. By using this method, we have been able to reduce by half the number of TLC plates required to analyze MIA's in different tissues, as well as the amount of time invested in their determination. This report describes the procedures involved in this improved separation and quantitation of *C. roseus* alkaloids.

EXPERIMENTAL

Biological Material

Tissues used for alkaloid extraction came from different *Catharanthus roseus in vitro* cultures,[6] seven day old seedlings or from nursery grown plants.

Alkaloid Extraction

Tissues were harvested or collected, immediately frozen with liquid nitrogen, and kept at -80°C until analysis. Tissues were freeze-dried and submitted to an

acid-base extraction as described previously.[4] Briefly, 100 mg of freeze-dried tissue was incubated in methanol for 2 h at 55°C and, after debris filtration, the methanol extract was evaporated to dryness. The residue was dissolved in 2.5% sulfuric acid and extracted thrice with ethyl acetate, keeping the aqueous phase each time. The pH of this phase was adjusted to above 9 with ammonium hydroxide. Alkaloids were then extracted with ethyl acetate, and the organic phase was reduced to dryness at reduced pressure, redissolving the residue in methanol. Total alkaloids were quantified spectrophotometrically at 280 nm.[4]

Chromatographic Separation

Plates were heat activated at 55°C for 30 min prior to use. Extract concentrations were adjusted with methanol in such a way that 5 μL represented 0.05 mg dry weight; this volume was loaded onto 20 cm high silica gel 60 TLC plates with fluorescence indicator (E. Merck Art, 105554, Darmstadt, Germany). Samples were manually applied 1.0 cm above the edge of the plate using a micropipette (Digital Finnpipette 0.5–10 μL, Finland) with ultramicrotips (Daigger TX20573, Mexico). Sample volume was delivered in a single, uniform hit and a one cm gap was left between adjacent samples. Diameter of the application spot was less than 4 mm in all cases. After loading, application spots were blown with hot air from a hair drier for 30 sec. Different mobile phases (Table 1) were prepared just before use and added to the chromatography tank between 10 and 15 min before plate development, so the chamber atmosphere was fully saturated. All solvents were chromatographic grade (Merk, J. T. Baker and Sigma-Aldrich Co).

Alkaloid Identification

After separation, plates were air dried and visualized by fluorescence quenching under 254 nm UV light. To identify the resolved alkaloids, the R_f values of chromatographic spots from the extracts were compared to those of authentic alkaloid standards. To assign identity, the chromatographic behavior of alkaloids using different solvent mixtures was considered, along with their colored reaction when sprayed with CAS reagent (ceric ammonium sulfate 1% in 85% phosphoric acid).[3] Plate staining was solely performed to confirm alkaloid identity and was not utilized for the quantitative purposes. In order to confirm that the analyzed chromatographic bands corresponded to single alkaloids, such spots were individually eluted from the plates and subjected to double dimension TLC (2D-TLC). To this end, stains corresponding to each alkaloid were individually scrapped from plates and the silica gel flakes (around 20 mg) were vigorously shaken with 500 μL of methanol. After centrifugation, the supernatant was collected and the flakes were re-extracted, pooling together the two eluates. Eluates were

Table 1. Mobile phases employed for the separation of alkaloids present in extracts from *C. roseus* tissues

Key	Mobile phase	Observations
I	MeOH	Alkaloids remained at the origin.
II	CHL : MeOH 6 : 4	No separation of AJM (0.78), CAT (0.74) and VIN (0.76).
III	CHL : MEOH 9 : 1	No separation of AJM (0.79), CAT (0.80) and VIN (0.80).
IV	CHL : ETOAC 1 : 1	VIN (0.42) separated from AJM (0.54) and CAT (0.52), which remained unresolved.
V	CHL : MEOH 1 : 1	No separation of AJM (0.76), CAT (0.74) and VIN (0.76).
VI	CHL : BEN 3 : 1	Separation of CAT (0.85) from VIN (0.70) whereas AJM remained at the origin.
VII	CHL : ACE 8 : 2	Separation of CAT (0.39) from VIN (0.56) whereas AJM remained at the origin.
VIII	CHL	Alkaloids remained at the origin.
IX	ETOAC : ETOH 1 : 1	Separation of AJM (0.71) from CAT (0.65) and VIN (0.64) which remained unresolved. Spots were diffused.
X	ETOAC : NH_4OH (110 ml : 3 drops)	Separation of AJM (0.56) from CAT (0.37) and VIN (0.25). Sharp and well defined bands.
XI	ETOAC : MEOH 75 : 5	Separation of AJM (0.68) from CAT (0.80) and VIN (0.81), which remained unresolved.
XII	HEX : ACE 6 : 4	Separation of CAT (0.38) from AJM (0.41) and VIN (0.40), which remained unresolved.

Alkaloids: AJM ajmalicine; CAT catharanthine; VIN vindoline. **Solvents:** ACE acetone; BEN benzene; CHL chloroform; ETOAC ethyl acetate; ETOH absolute ethanol; HEX hexane; MEOH methanol. Figures between brackets represent the R_f values for each alkaloid in the solvent mixture employed.

then evaporated to dryness and the residue dissolved in 10 μL methanol and subjected to 2-D TLC, in 10 × 10 cm plates using the mobile phases described under the Results section. Alkaloid standards came from our collection, which has been formed with kind gifts from Prof. V. De Luca (Brock University, Ontario, Canada) and Eli Lilly Company (Indianapolis, IN). Their purity was higher than 96% and all of them have been used before.[4,6,7,8]

Alkaloid Quantitation

Ajmalicine, catharanthine, and vindoline were quantified by *in situ* densitometry using a Shimadzu CS-930 dual wavelength chromato scanner (Kyoto, Japan), equipped with a DR 2 data collector, set in absorbance mode at

280 nm with a slit size of 1.2 × 1.2 mm in all cases. Peak area was compared to that of known amounts of the true standards. Linearity range for calibration curves was between 0.2 and 10 μg for the three analyzed alkaloids.

HPLC Analysis

Extracts from seedlings, obtained as described above, were subjected to HPLC analysis to validate the TLC method. Briefly, extracts were filtered and chromatographed on a Hypersil 5 μm ODS column (200 × 2.1 mm; Hewlett-Packard, Palo Alto, CA) using an HPLC system equipped with a 600E controlled system and a 991 photodiode array detector (Waters, Milford, MA). The eluent system consisted of 42% acetonitrile in water (0.1% triethylamine) with, after 10 min, a linear rise over 20 min in the percentage of acetonitrile to 65%.[8]

RESULTS AND DISCUSSION

Catharanthus shoot cultures presented an alkaloid profile similar to that of leaves and light grown seedlings, with vindoline as the main alkaloid and low levels of catharanthine and ajmalicine.[6] On the other hand, callus cell suspension and root cultures did not accumulate vindoline.[7] Our analysis concentrated on these three alkaloids since they are the most representative of the different MIA families.[1] Alkaloid identity was established by comparing the chromatographic behavior of true standards with those of the samples, using different mobile phases, as well as for the chromogenic reaction to CAS reagent. On the plate, ajmalicine acquired a pale yellow coloration immediately after CAS spraying, remaining without changes over a 24-h period. Catharanthine developed as a yellow centered spot, surrounded by a light blue area, whereas vindoline reacted as a dark pink stain, which developed a yellow center after one hour, slowly turning into a solid blue spot after 24 h.[3] The availability of a single solvent mixture allowing the proper separation of these alkaloids reduced significantly the time invested in their analysis. A total of 12 solvent mixtures were assayed (Table 1). In some of those, two of the three analyzed alkaloids were adequately separated, whereas in others, alkaloids remained at the origin. In other mixtures, even when alkaloids did separate, no well defined bands were obtained (Table 1). Mixtures IX and X (Table 1) could resolve the three alkaloids; however, only mixture X produced sharp, well defined spots, with a 55 mm separation among them (Fig. 1A). Furthermore, no other compounds were resolved between the alkaloids of interest, resulting in the detection of a single, non-overlapping, signal for each alkaloid in the densitometer (Fig. 1B). To confirm that the analyzed chromatographic bands corresponded to single alkaloids, such spots were eluted from the plates and subjected to 2D-TLC using mixture X (Table 1) for the first dimension for

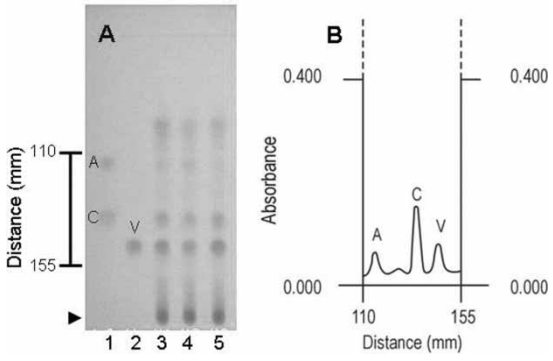

Figure 1. Chromatographic separation of monoterpenoid indole alkaloids from extracts of different tissues of *Catharanthus roseus*. Mobil phase: ethyl acetate with NH_4OH (see Table 1 for details). Panel **A** shows the chromatographic plate visualized under UV light at 254 nm loaded with standard ajmalicine (**A**) plus catharanthine (**C**) (lane 1) and vindoline (**V**), and extracts from seven day old seedlings (lane 3); third pair of leaves (lane 4); and 24 day old *in vitro* shoot cultures (lane 5). The arrowhead indicates the loading spot. Panel **B** displays the densitometry chromatogram of lane 5 (extract from a 24 day-old *in vitro* shoot culture) scanned at 280 nm.

the three alkaloids. As mobile phase for the second dimension, mixture VII was used for both catharanthine and vindoline, whereas mixture IX was chosen for ajmalicine (Table 1). Single spots were observed for the three alkaloids analyzed, even after spraying with Dragendorff's reagent.

Based on its resolution, mobile phase X was chosen for the analysis of the samples. After chromatography, the area where the three alkaloids resolved in each individual lane was scanned at 280 nm (Fig. 1). We chose this single wavelength since the reported maximal wavelengths for ajmalicine, catharanthine and vindoline on *in situ*-densitometry TLC were 284, 280 and 310 nm, respectively.[4,5] These values corresponded to our observations (data not shown). No significant differences in the readings were detected for ajmalicine at either 280 or 284 nm. *In situ* spectra of vindoline showed that readings at 280 nm were reduced up to 15% in comparison to those taken at 310 nm. Nevertheless, since calibration curves were obtained also at 280 nm, this difference did not interfere with the results obtained when the vindoline amount in the analyzed sample was at least 1 μg (see below).

Extracts from *in vitro* cultures,[9] including hairy roots, cell suspensions, and shoot cultures, were analyzed along with extracts from seven day old seedlings and mature plant leaves (Table 2). Content of the different MIA's was established by comparing the corresponding peak area of the samples with those of know amounts of standards. To validate the methodology, alkaloids from a previously HPLC analyzed extract from *Catharanthus* seedlings[8] were also quantified by *in situ* densitometry. Although values obtained using the densitometer were slightly lower than those obtained using HPLC, no statistically significant

Table 2. Alkaloid content in different *C. roseus* tissues. Average of three different analysis of the same extract, with the standard deviation in each case

	Alkaloid (mg/g DW)		
Tissue	Ajmalicine	Catharanthine	Vindoline
Hairy roots (14 day-old)	3.4 ± 0.34	4.2 ± 0.51	nd
Cell suspension (14 day-old)	1.2 ± 0.21	1.6 ± 0.31	nd
Shoots (24 day-old)	0.2 ± 0.01	0.4 ± 0.10	1.9 ± 0.30
Leaves (3rd pair)	0.9 ± 0.17	0.7 ± 0.18	4.6 ± 0.72
Seedlings (7 day-old)	1.4 ± 0.40	2.0 ± 0.41	3.9 ± 0.61

nd: Not detected.

differences could be assessed (1.8 ± 0.3 *vs* 1.4 ± 0.5; 2.4 ± 0.2 *vs* 2.0 ± 0.4 and 4.4 ± 0.3 *vs* $3.9 \pm 0.6\,\mu g/g$ DW for ajmalicine, catharanthine, and vindoline, respectively).

Even though other *in situ* densitometry methods have been described previously,[3,4] the main advantage of the one presented here is that it allows the separation of three of the main *Catharanthus* alkaloids: ajmalicine, catharanthine, and vindoline; using a single solvent system, as well as their quantitation in a single wavelength scan, reducing the amount of time and TLC plates required to perform a complete analysis. The sensitivity of this method was below 1 μg for the three analyzed alkaloids. For all the studied tissues, such values are usually present in less than 100 mg of dry matter.[9]

Although HPLC's sensitivity may be higher, this should not constitute a limiting condition for the application of this method when enough starting material is available. Besides, the sensitivity for the determination of vindoline may be increased by scanning the corresponding spots at 310 nm. However, in our experience, this is rarely necessary when the amount of plant material is not limiting. We found that reproducibility was not compromised if proper care was taken during loading of the samples. Additionally, the method takes only a relatively short time to complete, since up to 20 samples could be analyzed in *ca*. 4 hours, from the extraction process to the final scanning. The identity of the alkaloids can be easily established, based on the well known chromatographic behavior of the *Catharanthus* alkaloids.

In summary we have developed a simple, reproducible, and affordable technique for the quantitation of the main alkaloids in *C. roseus* aerial tissues.

ACKNOWLEDGMENTS

This work was supported by National Council for Research and Technology (CONACyT, Mexico) grant 31608B and with funds from PIFOP (National

Program for Graduate Studies, CONACyT). EH-D was recipient of a CONACyT scholarship for doctoral studies.

The authors wish to thank M. Sc. Miriam Monforte-González and Mildred Carrillo-Pech for their skillful technical assistance in TLC development and to Dr. Maria de Lourdes Miranda-Ham for her critical review of the manuscript.

REFERENCES

1. Loyola-Vargas, V.M.; Sánchez-Iturbe, P.; Canto-Canché, B.; Gutiérrez-Pacheco, L.C.; Galaz-Ávalos, R.M.; Moreno-Valenzuela, O. Biosíntesis de los alcaloides indólicos. Una revisión crítica. Rev. Soc. Quím. Méx. **2004**, *48*, 67–94.
2. Tinkhomiroff, C.; Jolicouer, M. Screnning of *Catharanthus roseus* secondary metabolites by high performance liquid chromatography. J. Chromatogr. A **2002**, *955*, 87–93.
3. Farnsworth, N.R.; Blomster, R.N.; Damratoski, D.; Meer, W.A.; Cammarato, V. Studies on *Catharanthus* alkaloids. VI. Evaluation by means of thin-layer chromatography and ceric ammonium sulfate spray reagent. Lloydia **1964**, *27*, 302–314.
4. Monforte-González, M.; Ayora-Talavera, T.; Maldonado-Mendoza, I.E.; Loyola-Vargas, V.M. Quantitative analysis of serpentine and ajmalicine in plant tissues of *Catharanthus roseus* and hyoscyamine and scopolamine in root tissues of *Datura stramonium* by thin layer chromatography-densitometry. Phytochem. Anal. **1992**, *3*, 117–121.
5. Tam, M.N.; Nikolova-Damyanova, B.; Pyuskyulev, B. Quantitative thin layer chromatography of indole alkaloids. II. Catharanthine and vindoline. J. Liq. Chromatogr. **1995**, *18*, 849–858.
6. Hernández-Domínguez, E.; Campos-Tamayo, F.; Vázquez-Flota, F. Vindoline synthesis in *in vitro* shoot cultures of *Catharanthus* roseus. Biotechnol. Lett. **2004**, *26*, 671–674.
7. Vázquez-Flota, F.; De Luca, V.; Carrillo-Pech, M.; Canto-Flick, A.; Miranda-Ham, ML. Vindoline biosynthesis is transcriptionally blocked in *Catharanthus roseus* cell suspension cultures. Mol. Biotechnol. **2002**, *22*, 1–8.
8. Vázquez-Flota, F.; Carrillo-Pech, M.; Minero-García, Y.; Miranda-Ham, M.L. Alkaloid metabolism in wounded *Catharanthus roseus* seedlings. Plant Physiol. Biochem. **2004**, *42*, 623–628.
9. Loyola-Vargas, V.M.; Vázquez-Flota, F. *In vitro* plant cell culture as the basis for the development of a research institute in Mexico: Centro de Investigación Científica de Yucatán. *In vitro* Cell Dev. Biol.-Plant **2003**, *39*, 250–258.

Received May 1, 2005
Accepted July 6, 2005
Manuscript 6647