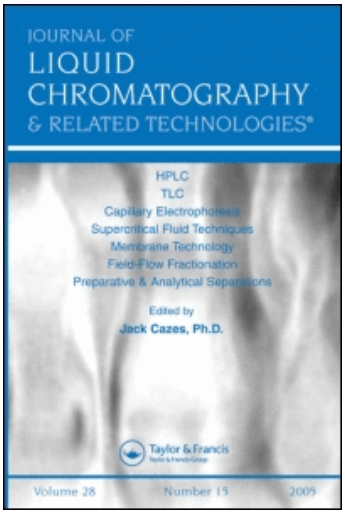

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Abstract: A method for measuring the content of total capsaicinoids by *in situ* densitometry on thin layer chromatography (TLC) plates is described. This method is easy to perform, reproducible, and can detect up to 0.5 μg of capsaicinoids in unpurified organic extracts. Identity of the compounds was assigned by comparing the chromatographic behavior of the putative capsaicinoids with that of standards. Under the assayed conditions, capsaicin could not be separated from dihydrocapsaicin by TLC; however, the amounts detected in different tissues was always equal to the sum of the individual compounds, as quantified by gas chromatography, suggesting that the method is reliable.

Keywords: Amides, Capsaicinoids, Chili peppers, *In situ* TLC densitometry

INTRODUCTION

The typical hot flavour of chili peppers is due to the presence of capsaicinoids, a group of around nine different compounds formed by a phenolic moiety

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condensed with a fatty acid chain. The phenol moiety, vanillylamine, is derived from phenylalanine through the phenylpropanoid pathway, whereas the acyl chain is formed from valine.[1,2] Differences among capsaicinoids are associated with differences in the length of the fatty acid chain, the number of double bonds, and their position on this chain. Capsaicin and dihydrocapsaicin are the two major capsaicinoids, representing together over 90% of the total content in ripe fruits. Capsaicin and its derivatives are synthesized in the epidermal cells of the placental tissue, where they are also accumulated.[3,4] Since hotness is an important agronomical trait for peppers, a number of techniques to estimate it have been developed. Some of them are based on organoleptic tests, whereas others rely on measuring the actual amount of capsaicinoids in the pods. Most analytical methods devised for caspsaicinoid analysis are based on the chromatographic separation of organic extracts by high performance liquid chromatography (HPLC) or gas chromatography (GC). Although, these methods are highly sensitive, they require expensive instrumentation, which could make them unaffordable to small budget laboratories. Thin layer chromatography (TLC) is a relatively simple technique for the fractionation of components present in complex mixtures, such as extracts from plant tissues. Combined with the proper detection methods, TLC can turn into a reliable quantitative technique, particularly for compounds with well characterized chromatographic properties.[5] Such is the case of capsaicinoids. We have developed a simple, affordable, and sensitive method for capsaicinoid analysis by TLC-*in situ* densitometry (TLC-D). This method has sufficient resolution to separate a mixture of capsaicinoids from other compounds extracted from *Capsicum* tissues and allows the detection up to 0.5 μg of the compound.

EXPERIMENTAL

Materials

Reagents and solvents were either analytical or chromatographic grade and were used without further purification. Standards of capsaicin, dihydrocapsaicin, and vanillin were purchased from Sigma Chemical Co (USA) and dissolved in absolute ethanol. Precoated, aluminum backed silica-gel$_{60}$ chromatographic sheets (20 × 20 cm), with a fluorescence indicator (F_{254}) were used (Merck, art. 105554, Darmstadt Germany).

Instrumental

Densitometric measurements were carried out on a Shimadzu CS-930 dual wavelength equipped with a DR-2 data recorder (Tokyo Japan). Gas chromatography was performed in a Hewlett Packard GC 5890, series II

gas chromatographer equipped with a flame ionization detector (FID), using an ULTRA 2 high resolution column (0.32 mm × 25 m; Agilent Technologies).

Extraction of Capsaicinoids

Pods from local cultivars of Habanero pepper (*Capsicum chinense* Jacq.) were collected, and placental tissue was separated. After weighing, tissues were freeze dried, ground to a fine powder, and stored in a sealed plastic bag in a glass desiccator at room temperature until analysis. For the extraction, 100 to 200 mg were mixed with 10 mL acetone and incubated for 5 h at room temperature in the dark with orbital shaking (50 RPM). Debris was separated by centrifugation and the organic phase was evaporated to dryness at reduced pressure. The residue was dissolved in one mL of acetone, dried, and then concentrated in 50 μL of absolute ethanol.

Chromatography Development

Samples, up to 2 μL of the crude extract (representing between 4–8 mg of dry tissue) were manually applied on the plates 10 mm above the lower edge, using a micropipette (Digital Finnpippette 0.5–10 μL, Finland) with ultramicrotips (Daigger TX20573, Mexico). Sample volume was delivered in a single, uniform hit, and a 5 mm gap was used as separation between adjacent samples. The diameter of the application spot was less than 2 mm in all cases. After loading, application spots were dried with hot air for 30 seconds. Separation was performed using either solvent mixture A (ciclohexane:chloroform:acetic acid, 70:20:10), mixture B (ciclohexane:acetone, 40:50) or mixture C (chloroform:methanol:acetic acid, 95:1:5).[6,7] Plates in mixture A were developed twice in order to obtain a better resolution. Solvent mixtures were prepared just before use and added to the chromatography tank 15 min before development, so the chamber atmosphere was fully saturated.

Capsaicinoid Identification

After separation, plates were air dried and visualized under UV light (254 nm). To identify capsaicinoids, the Rf's values of the chromatographic spots from the extracts were compared to those of commercial standards, using different solvent mixtures. Capsaicin and dihydrocapsaicin identities were further confirmed by gas chromatography, comparing their retention times with that

of the standards. Separation was performed using helium as the carrier gas and a temperature gradient from 180 to 290°C.

RESULTS AND DISCUSSION

Even when solvent system A produced a low polarity mixture, it allowed the proper separation of capsaicin (Rf = 0.16) and some biosynthetic intermediaries, such as vanillin (Rf = 0.38), ferulic acid (Rf = 0.22), and coumaric acid (Rf = 0.10) (Figure 1A). These compounds are not uncommon in peppers' placentas. Resolution was improved by running the same plate twice in the same mobile phase (Figure 1). Sufficient separation of these compounds occurred to allow detection as single peaks on the chromatograms (Figure 1B). Capsaicin and vanillin identities were confirmed by comparing the chromatographic behavior of placenta extracts with those of the standards, using different mobile phases. In this way, the putative capsaicin and vanillin chromatographic spots displayed Rf values of 0.66 and 0.59,

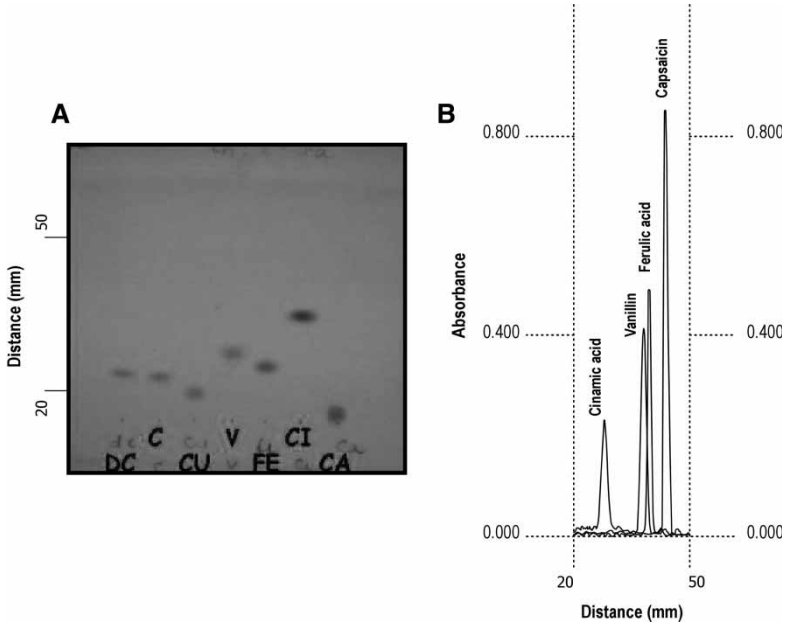

Figure 1. Chomatographic separation of capsaicinoids from their biosynthetic intermediares. (A) Chromatogram visualized under UV light (254 nm). DC, dihydrocapsacin; C, capsaicin; CU, coumaric acid; V, vanillin, FE, ferulic acid, CA, cinnamic acid. Mobile phase: cyclohexane:chloroform:acetic acid (70:20:10). (B) Densitometric estimation of the chromatographic spots.

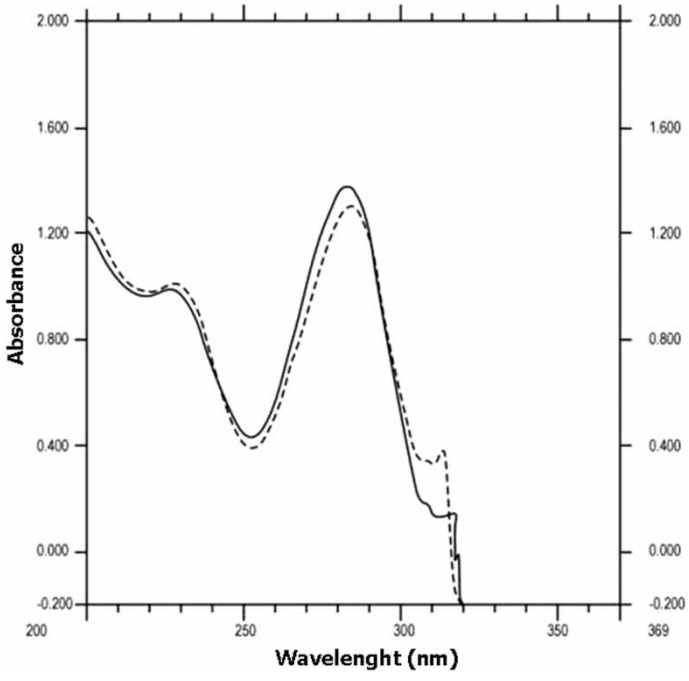

Figure 2. Comparison of an *in situ* absorbance spectrum of capsaicin after TLC separation. Pepper's placenta extract (dashed line); a 99% pure commercial standard (solid line). Chromatographic separation was performed as described in Figure 1.

respectively, using the mixture B as mobile phase and 0.70 and 0.77 using mixture C (data not shown). In addition, putative capsaicin from the pepper's placenta displayed an identical UV spectrum (λ_{max} 281 nm) to that of the standard, when analyzed by *in situ* densitometry (Figure 2). However, capsaicin and dihydrocapsaicin displayed very similar chromatographic behaviors and remained unseparated from each other, despite the use of different mobile phases. In this way, this method allowed the separation of total capsaicinoids from compounds of different character, but not between them. To assure the nature of the compounds, extracts from the pepper's placenta were separated by gas chromatography. Along the peak corresponding to capsaicin ($R_t = 9.753$), another minor peak, closely associated to the first one ($R_t = 9.890$), was also observed (Figure 3). It was identified as dihydrocapsaicin and represented about 33% of the one corresponding to capsaicin. In this way, the TLC method apparently was not able to separate this mixture. However, it is commonly reported that the mixture of capsaicin and its dihydro derivative account for more than 95% of the total capsaicinoid content in peppers.[8,9]

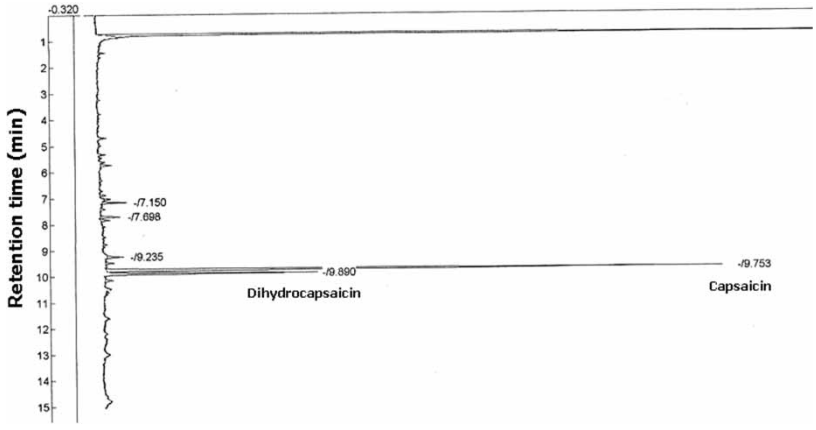

Figure 3. Gas chromatogram of a pepper's placenta extract. Capsaicin ($R_t = 9.753$ min) and dihydrocapsaicin ($R_t = 9.890$ min) were identified comparing their retention times with those of standards. Conditions as described under Experimental. Peak at 1.00 min corresponds to the internal standard (vanillin).

Since solvent mixture A allowed the proper separation of capsaicinoids from vanillin and other compounds, it was employed in conjunction to *in situ* densitometry in order to develop a quantitative method. Thus, after chromatography, plates were kept in the dark for 15 min at room temperature, to allow solvent evaporation, and then scanned in a Shimatzu CS-930 densitometer, using a zigzag absorbance mode set at 281 nm, with a slit size of 1.2 mm × 1.2 mm. A linear response was found in the range of 0.5–5 μg for capsaicin and 0.1–0.5 μg for vanillin (data not shown). To improve resolution, plates were chromatographed twice in the same solvent mixture.

The same extracts were also measured by GC to compare the accuracy of the method (Table 1). Gas chromatography demonstrated that capsaicinoids present in the placental tissues corresponded almost exclusively to capsaicin and its dihydro derivate (Figure 3), which were separated with sufficient resolution to be independently quantified (Table 1). Since the TLC separation was not able to resolve capsaicin from its dihydro derivative, it only can estimate the total capsaicinoid content (Table 1). However, it is noteworthy that the sum of them, as measured by GC, is practically the same as that detected by *in situ* densitometry, even when extracts from different cultivars were analyzed (Table 1).

Summarizing, we have developed an easy, affordable, and reproducible method to quantify capsaicinoids from chili pepper tissues. We have observed that minor training is needed to obtain reproducible results, and up to 20 samples can be processed within a 2 hour period (not considering the incubation time required for extraction). Though it cannot quantify

Table 1. Comparison of total capsaicinoid content (TCS) in placentas of habanero pepper (*C. chinense*) using gas chromatography (GC) or thin layer *in situ* densitometry (TLC-D). CP, capsaicin; DHCP, dihydrocapsaicin; SHU, Scoville Heat Units

	Method[a]					
	TLC-D		GC			
Cultivar	TCS (mg/g DW)	SHU[b]	CP (mg/g DW)	DHCP (mg/g DW)	TCS (mg/g DW)	SHU[b]
Refers to the color of ripe pods						
Red (ripe)	41	615 000	21	17	38	570 000
Yellow (ripe)	56	840 000	24	24	48	720 000
Orange (ripe)	50	750 000	36	12	48	720 000
Orange (unripe 1)[c]	47	705 000	34	10	44	660 000
Orange (unripe 2)[c]	32	480 000	24	7	31	465 000

[a]The same extract was analyzed either by TLC or GC. Averages or three different measurements of the same extract. Standard deviation was always lower than 5% of the average in each case.

[b]One SHU equals 0.015 mg/g DW of TCS (0.0015% on DW basis). DW, Dry weight.

[c]Peppers at different developmental stages.

capsaicin and dihydrocapsacin independently, for agronomical and food technology purposes an actual estimation of hotness or pungency, which is a reflection of total capsaicinoid content, is more often required than the precise composition of the capsaicinoid mixture. For this reason, hotness is frequently reported in Scoville Heat Units (SHU), which can be calculated from the total capsaicinoid content.[10] Interestingly, SHU values were very similar in the tissues analyzed, either by GC or by the method here developed (Table 1).

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