An improved high performance liquid chromatography method for separation of lipophilic triterpenes from *wunderlichia crulsiana* followed by gas chromatography analysis

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Dedicated to Professor Otto Gottlieb on his 85th birthday

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Abstract

A reverse-phase high performance liquid chromatographic (RP-HPLC) method was developed for the separation of lipophilic triterpenes found in *Wunderlichia crulsiana*. Three triterpenic fractions were obtained when stems extract was submitted to chromatography on silica gel; (1) triterpenes esterified with fatty acids, (2) triterpenes esterified with acetic acid and (3) free triterpenols. Fraction 2 was separated by RP-HPLC, using either methanol or acetonitrile as the mobile phase, yielding 13 identified triterpenes. Fraction 3 and 1, after hydrolysis, were acetylated, and compared by gas chromatography with the triterpenes isolated from fraction 2, allowing their identification.

Keywords: HPLC separation, lipophilic triterpenes GC analysis, *Wunderlichia crulsiana*, Asteraceae

Introduction

The genus *Wunderlichia* Riedel ex Benth. belongs to the tribe Mutisieae, of the Asteraceae family, which is endemic to the Northwest of Brazil.[1] This genus contains six species described, of which just *W. mirabilis* has been studied chemically; sesquiterpene lactones, triterpenes and polyacetylene were found.[2] In this paper we deal with *W. crulsiana* Taulb. which grows in rocky places of Bahia, Goiás, São Paulo and Rio de Janeiro States. Only a chemical study has been done and flavonoids were isolated.[3]

Triterpenes are natural products widespread in nature, found from bacteria to humans. They play an important role as membrane stabilizers in plants[4] and can act as anti-inflammatory,[5] skin cancer chemo preventive,[6] antiulcer[7] and anti-HIV[8] agents in humans.

Triterpene mixtures are very difficult to separate and isolate due to similarities in chemical structure. Pentacyclic triterpenes, for example, can differ only in double bond positions and/or methyl groups.[9] It is possible to achieve a fraction that contains solely triterpenes with classic chromatographic columns. However, only high resolution chromatography procedures, such as HPLC, can separate these compounds. Few HPLC methods have been developed for this purpose.[10]

This paper describes a reverse-phase (RP-HPLC) method for the isolation of acetate triterpenes and 3-oxo triterpenes followed by gas chromatography (GC) analysis of these compounds from *W. crulsiana*.

Results and Discussion

A preliminary chemical investigation of stems of *W. crulsiana* showed that the dichloromethane extract is rich in triterpene mixture, mainly triterpenes esterified with acetate and fatty acids and sesquiterpene lactones, which were described elsewhere.[11]

Figure 1 shows the fractionation of the dichloromethane extract of stems of *W. crulsiana*. The first three fractions obtained from the chromatographic column were identified by ^{1}H NMR as triterpenes esterified with fatty acids (fraction 1), triterpenes esterified with acetic acid (fraction 2) and hydroxy triterpenes (fraction 3). Fraction 2 and 3 were analyzed by GC and fraction 2 showed the highest complexity, which was separated by HPLC. Since the λ_{max} of this fraction was at 201 nm we decided to use RP-HPLC because the solvents employed did not interfere with the detection method. Various C18-HPLC analytical columns and conditions were tested and Luna (Phenomenex®) showed the best chromatogram resolution (Fig. 2), with an isocratic mobile phase. The chromatogram showed at least nine well separated peaks, which were collected using a semi-preparative column (Luna, Phenomenex®). Mixtures of triterpenes were identified using the methodology described by Gallegos and Roque[12] which allowed to analyze the ^{13}C-NMR data of each triterpene. The identification of pure triterpenes was carried out by comparison with the ^{13}C-NMR data reported in the literature.[9]

Table 1 shows the identified triterpenes, their concentration and their GC retention times. Figure 3 shows the chemical structure of the triterpenes identified.

Fractions F2.6 and F2.8 containing the two minor compounds not identified were mixed and separated by HPLC (mobile phase: ACN) (Fig. 1). This procedure led to the identification of fern-8-en-3-one and β-amirinyl acetate.

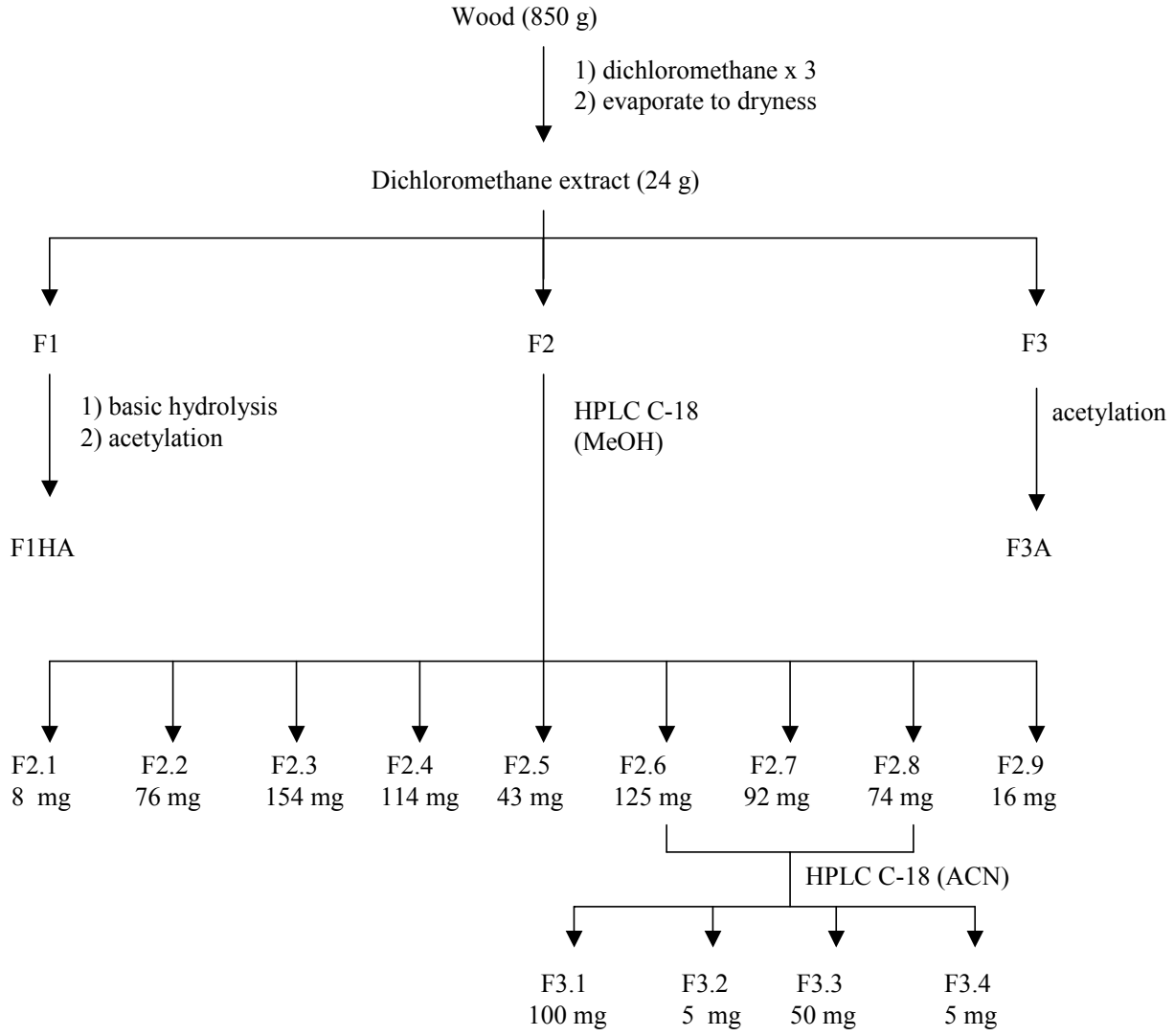


Figure 1. Dichloromethane extract fractionation of *Wunderlichia crulsiana* stems.

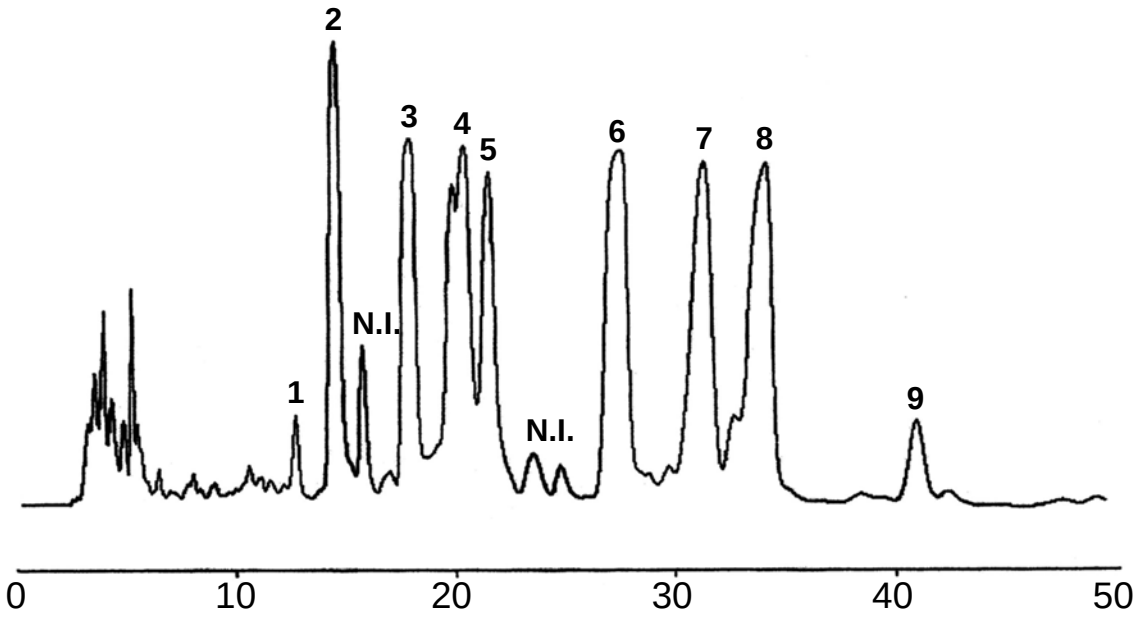
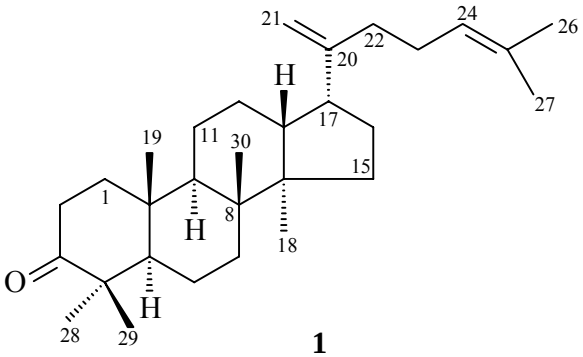


Figure 2. HPLC chromatogram (UV detection at 201 nm) of fraction 2. (For chromatographic protocol see Experimental section).

Table 1. Fractions obtained by HPLC, showing weight, purity and GC retention time of identified triterpenes from *Wunderlichia crulsiana*

Fraction	Mass (mg)	RT (min)	k′	SD (n=4)	%	Identified substance
F2.1	8	9.79	4.82	0.29	100	dammara-20,24-dien-3-one 1
F2.2	76	11.09	5.60	0.07	100	lupen-3-one 2
F2.3	154	10.40	5.33	0.23	3	not identified
		11.08	5.56	0.10	97	lupen-3-one 2
F2.4	114	10.28	5.01	0.13	49	β-amirin-3-one 3
		10.40	5.14	0.07	24	germanic-3-one 4
		12.78	6.21	0.12	18	pseudotaraxaster-3-one 5
		13.03	6.33	0.10	8	taraxaster-3-one 6
F2.5	43	11.05	5.59	0.11	6	lupen-3-one 2
		12.05	6.11	0.16	3	not identified
		12.77	6.34	0.07	91	pseudotaraxaster-3-one 5
F2.6	125	11.94	5.83	0.19	7	fern-8-en-3-one 11
		13.37	6.50	0.18	93	lupenyl acetate 10
F2.7	92	12.41	6.23	0.15	72	β-amirin acetate 7
		15.80	7.75	0.12	22	taraxasteryl acetate 8
F2.8	74	13.34	6.63	0.15	9	α-amirin acetate 13
		15.51	7.71	0.09	91	pseudotaraxasteryl acetate 12
F2.9	16	14.46	7.12	0.16	100	fern-8-en-3β-yl acetate 9
F3.1	100	11.92	6.05	0.06	3	fern-8-en-3-one 11
		13.38	6.78	0.04	97	lupenyl acetate 10
F3.2	5	12.00	6.05	0.03	55	fern-8-en-3-one 11
		13.42	6.65	0.08	45	lupenyl acetate 10
F3.3	50	12.39	6.10	0.10	6	β-amirin acetate 7
		15.49	7.67	0.15	94	pseudotaraxasteryl acetate 12
F3.4	5	13.33	6.51	0.24	100	α-amirin acetate 13
F1HA	1300	7.520	3.63	0.08	7	not identified
		12.582	6.08	0.08	20	β-amirin acetate 7
		12.716	6.17	0.10	11	pseudotaraxaster-3-one 5
		13.666	6.58	0.20	42	lupenyl acetate 10
		14.593	7.04	0.21	4	fern-8-en-3β-yl acetate 9
		15.654	7.59	0.18	9	pseudotaraxasteryl acetate 12
		15.890	7.71	0.12	2	taraxasteryl acetate 8
F3A	1000	12.415	6.22	0.01	55	β-amirin acetate 7
		13.598	6.80	0.02	30	lupenyl acetate 10
		15.524	7.77	0.01	15	pseudotaraxasteryl acetate 12



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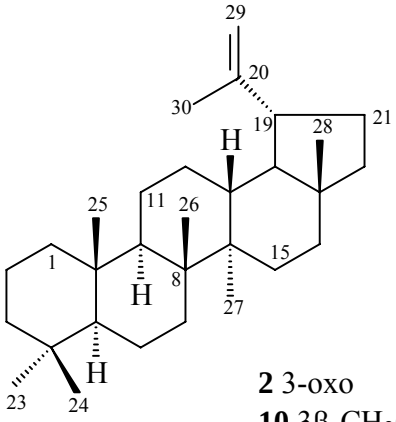
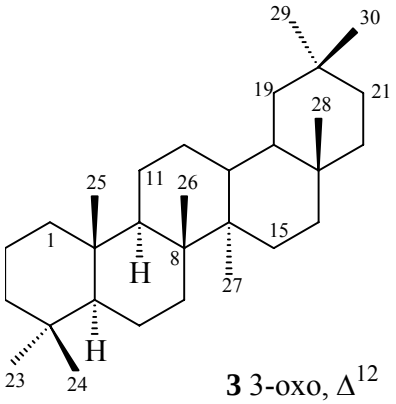
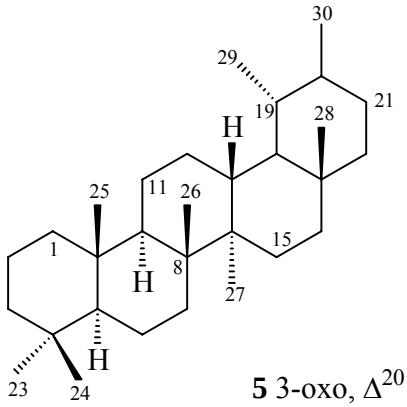


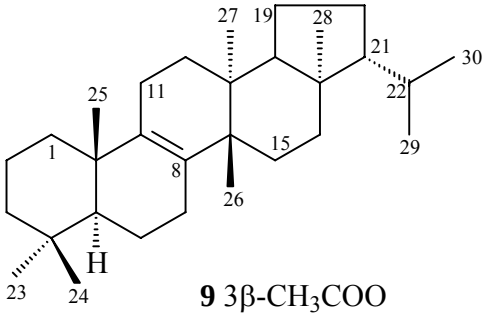
2 3-oxo
10 3β-CH_3COO



3 3-oxo, Δ^{12}
4 3-oxo, Δ^{18}
7 3β-CH_3COO, Δ^{12}



5 3-oxo, Δ^{20}
6 3-oxo, $\Delta^{20(30)}$
8 3β-CH_3COO, $\Delta^{20(30)}$
12 3β-CH_3COO, Δ^{20}



9 3β-CH_3COO
11 3-oxo

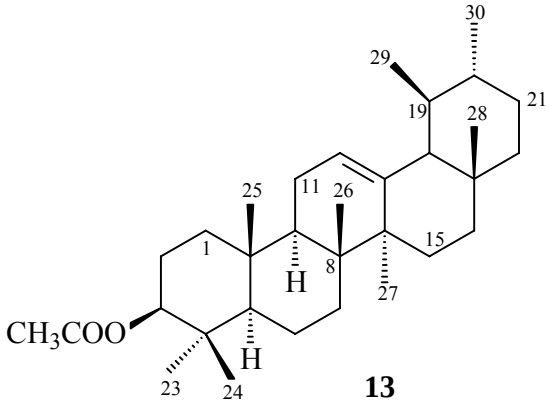


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Figure 3. Identified triterpenes from *Wunderlichia crulsiana*.

Conclusions

This methodology with isocratic HPLC condition is useful to separate lipophilic triterpenes and can be a sensitive tool to follow triterpenes biosynthesis or quantify these compounds in other plant extracts. Also, with the isolated standards, it is possible to identify triterpenes from other sources by GC analysis.

To know the kind of triterpenes present in the fraction 1 and 3, the fraction 1 was hydrolyzed and then acetylated and fraction 3 was acetylated. Then, both fractions were analyzed by GC, using the triterpenes isolated or in mixture well known as standard.

Experimental Section

Material. All solvents used for chromatographic purposes were HPLC grade. Other solvents were ACS grade or equivalent.

Plant material. The plant material was collected in Morro do Pai Inácio (S 12^{o} 27′ 326″ and W 41^{o} 28′ 509‴), Chapada Diamantina, Bahia State, Brazil, in September 19, 1998. The identification of plant material was made by Prof. Dr. M. L. Guedes (Instituto de Botânica – Universidade Federal da Bahia) and by Prof. Dr. J. Pirani (Instituto de Botânica – Universidade de São Paulo). Voucher specimens are deposited in SPF Herbarium of Instituto de Botânica da Universidade de São Paulo under collector′s number: Nunez C.V.-004.

Extraction of triterpenes. Dried powdered stems were extracted with dichloromethane. The dichloromethane extract (24 g) was submitted to column chromatography on silica gel 60 (Merck, Darmstadt, Germany) using n-hexane/dichloromethane 1:1 (fractions 1 and 2), n-hexane/dichloromethane 2:8 (fraction 3) as mobile phase.

Thin layer chromatography. The TLC system employed in this study was silica GF_{254} plates (Aldrich, Wisconsin, USA) with dichloromethane as mobile phase and ceric sulfate: sulfuric acid (1:1,2 w/w) as visualizing agent.

High performance liquid chromatography. HPLC was performed using a Shimadzu system consisting of a SPD-10AV UV detector, SPD-M10AV diode array detector and CBM 10A – Communications Bus Module. HPLC columns were Luna C-18 Phenomenex (250 x 4.6 mm i.d. and 250 x 10.0 mm, both with 5 μm particle diameter). In order to protect the integrity of the analytical and semi-preparative column, all analyses were performed with a coupled C-18 guard column (4 x 3 mm, 5 μm particle diameter). The first fractionation was made using methanol (MeOH) as the mobile phase at a flow rate of 4.7 mL/min and the samples were monitored at 201 nm. The second fractionation was made using acetonitrile as the mobile phase at the same flow rate and monitored at 210 nm. To dissolve the samples a mixture of *iso*-propanol:MeOH 1:1 was used.

Gas chromatography. The triterpenes isolated by HPLC were analyzed by GC. The GC analyses were performed using a Hewlett Packard 5890 series II GC, HP 7673 automatic injector

and HP 3396A integrator, a 30 m capillary column (5% phenyl in 95% methyl-silicon), Helium as carrier gas and an FID detector. The oven temperature was 290 ^{0}C, injector temperature 280 ^{0}C and detector temperature 310 ^{0}C.

NMR spectroscopy. NMR spectra were recorded at 125 MHz for ^{13}C and 500 MHz for ^{1}H (Bruker DRX-500) using $CDCl_3$ (Aldrich) as solvent and internal standard.

Reaction conditions. The fraction containing triterpenes esterified with fatty acids (fraction 1) was hydrolyzed using KOH and MeOH, in the proportion of 1 g of sample, 1 g of KOH and 125 mL of MeOH. The reaction time was 1 hour under reflux. The solution was then concentrated to 100 mL under vacuum and the final volume was adjusted with distilled H_2O. The aqueous MeOH was partitioned against diethyl ether (5 x 200 mL). The diethyl ether solution was concentrated under vacuum to 100 mL and partitioned against 0.1 M KOH (1 x 100 mL) and distillated H_2O (2 x 100 mL). The diethyl ether phase was dried with Na_2SO_4, filtered and concentrated.

Since the standards were triterpenes esterified with acetic acid, the fraction hydrolyzed above and the fraction which contained the free triterpenols (fraction 3) were acetylated using acetic anhydride and pyridine, the proportion being 1 mL of acetic anhydride and 1 mL of pyridine for 20 mg of sample. The solution was heated for 24 hours at 60 ^{o}C. The solution was rinsed with distilled H_2O, and diethyl ether was added to extract the acetylated compounds.

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