Seasonal Variation of the Chemical Constituents from *Croton* Species[§]

Alberto dos Santos Pereira[a,*], Ana Claudia Fernandes do Amaral[b], Margareth de Araujo Silva[a] and Francisco Radler de Aquino Neto[a]

[a] LADETEC, Instituto de Qímica, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Cidade Universitaria, CT, Bloco A, Sala 607, Rio de Janeiro, RJ – Brazil 21949–900. E-mail: ladetec@iq.ufrj.br

[b] Lab. de Qímica de Produtos Naturais (LQPN), Far-Manguinhos, RJ – Brazil 21041–250

*Author for correspondence and reprint requests

Z. Naturforsch. **56c,** 357–362 (2001); received October 9, 2000/February 20, 2001

Croton, Chemical Constituents, Seasonal Variation

The terpenes, sterols, alkaloid (glaucine) and α-tocopherol show seasonal variation for *Croton hemiargyreus hemiargyreus* and *Croton* echinocarpus. The amounts of triterpenes are higher during the tropical summer and in most samples the major sesquiterpene was characterized as caryophyllene. The seasonal variation of glaucine showed a maximum between June and October for *C. hemiargyreus*, and was present only in January and June in *C. echinocarpus*.

Introduction

The genus *Croton* (Euphorbiaceae) is widely distributed in Brazil: in the state of Rio de Janeiro alone, 39 species have been identified (Amaral, 1996). Some species have been used in folk medicine in a large number of applications, including as anticancer drugs (Hartwell, 1969; Farnsworth *et al.*, 1969). The isolation of morphinandienone, aporphine and proaporphine alkaloids has been described in this genus (Barnes, 1964; Bertolo and Scarpati, 1979). Ecophysiological factors (physical and environmental factors) that affect the general metabolism can modify qualitatively and quantitatively the occurrence of therapeutically interesting constituents (Vanhaelen *et al.*, 1991). Biotic (e.g. fungal, bacterial and viral infections; presence of insects or deposition of eggs) and genetic factors can also affect plant metabolism (Vanhaelen *et al.*, 1991). Previous work in our laboratories showed the easy characterization of different natural product families of substances, including alkaloids and high molecular weight compounds, in leaves and stems of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus* (Pereira *et al.*, 1999; Carbonell *et al.*, 2000), this fact is due to the use of high temperature high resolution gas chromatography (HT-HRGC) capillary columns and cold on-column injection (Pereira and Aquino Neto, 1999).

This paper reports the seasonal variation in the composition of the constituents, including isoquinoline alkaloids, of two croton species (*Croton hemiargyreus hemiargyreus* and *Croton echinocarpus*), as well as among individuals of about the same age growing in the same site.

Experimental

Capillary columns

Gas chromatography was performed on 20 m lengths of 0.30 mm i.d. Borosilicate capillary column (Duran-50, Vidrolex, Brazil) coated with a film (0.1 μm) of OV-1701-OH (88% methyl, 7% cyanopropyl, 5% phenylpolysiloxane, Ohio Valley Speciality Chem. Co., Marietta, Ohio, USA). The capillary column was prepared according to the literature (Blum, 1985).

The column used in HRGC-MS, was interfaced to the MS ion source through a 2 m length of 0.25 mm i.d. High Temperature Fused Silica (HTFS, J&W, USA) through a "press-fit" type connection. The HTFS was purged with hydrogen at 180 °C for 15 min and deactivated by flushing with HMDS/DPTMDS 1:1 v/v, sealing the capillary, and heating at 400 °C for 12 h. The tubing was then rinsed with hexane, methanol and diethyl

[§] In memoriam to Professor Roderick A. Barnes, who has contributed so much to Brazilian phytochemistry.

 © 2001 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com ·

ether. Column performance was checked by the Grob test (Grob *et al.*, 1978; Grob Jr. *et al.*, 1981).

High temperature high resolution gaschromatography

An on-column injector (Carlo Erba, Rodano, Italy) was mounted on a Hewlett Packard (HP) model 5890-II gas chromatograph. The column temperature was maintained at 40 °C for 0.5 min then programmed to 370 °C at 10 °C/min and held isothermal for 10 min. The flame ionization detector (FID) and on-column injector were operated at 400 °C and room temperature, respectively. Hydrogen was used as carrier gas at a flow rate of 2.5 ml/min and the sample volume injected was 0.5 µl. GC data were acquired with a HP 3396-II integrator.

High temperature high resolution gas chromatography coupled to mass spectrometry

HT-HRGC-MS analyses were carried out on a HP 5972 MSD (Hewlett Packard, Palo Alto, USA). The GC operating conditions were the same as described above. The on-column injector and the transfer line temperatures were set at 40 °C and 350 °C, respectively. The mass spectrometer was operated in the electron impact (EI, 70 eV) mode and MS scan range was 40 to 700 daltons.

Plant material

The leaves and stems of *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus* (R182775) and *C. echinocarpus* Muell. Arg. (R185345) were collected near Nova Friburgo (Rio de Janeiro, Brazil). These species were identified by Prof. Arline Souza de Oliveira (Museu Nacional, Universidade Federal do Rio de Janeiro) and voucher samples have been deposited in the herbarium of the Universidade Federal do Rio de Janeiro with the specimen numbers indicated above.

Extraction

5 g of the powdered plant (leaves and stems) were extracted with 50 ml of ethanol 75% by percolation. The hydroalcoholic extract was first concentrated on a rotary evaporator and then by a stream of nitrogen gas. The extract was weighed after solvent removal under vacuum and dried in a vacuum desiccator with P_2O_5, yielding about 1.0 to 1.2 g of an olive green solid residue. Samples were prepared for HT-HRGC-MS analysis by dissolution of the residue with methanol to a 10,000 ppm solution.

Results and Discussion

Seasonal variation

The leaves and stems of three *Croton hemiargyreus* hemiargyreus and three *Croton echinocarpus* individuals, at the same geographical localization (see experimental), were simultaneously collected for the evaluation of seasonal composition. Sampling was realized between June of 1998 and March of 1999. Weather parameters (by data of the Brazilian Meteorology Institute) in the months at the area of the samples collection are shown in Table I. The characterization of the components was based in mass spectra interpretation, retention time and comparison with the mass spectra library Wiley 275.l.

It is obvious that a precise quantitation is very difficult due to the complexity of unfractionated natural product samples. This difficulty results from substantial variation in detector response for the different classes of natural products. An estimate of the concentrations was therefore performed using a response factor of 1 (one) for the flame ionization detector (FID) for all compounds; that is, the peak

Table I. Regional weather parameters of the sample collection period.

	Jun/98	Jul/98	Aug/98	Sep/98	Nov/98	Jan/99	Mar/99
Average temp. (°C)	20	n.d.*	23	24	23	28	27
Rainfall (mm)	15	n.d.	75	10	100	12	205
Sunstroke (H)	148	n.d.	170	160	98	180	200

* Not determined.

areas integrated from the chromatograms were used directly without a correction factor.

Tables II and III show the relative concentrations of several constituents of both *Croton* sp. hydroalcoholic extracts analyzed (based in percentage areas of the chromatogram). The values shown represent averaged values for the three individuals within each *Croton* sp. studied.

It is interesting to note that among *Croton hemiargyreus* hemiargyreus individuals, CH–3 showed all measured constituents in quite distinct relative amounts from CH–1 and CH–2 (see Fig. 1), thus

Table II. Seasonal variation of different lipid classes from the hydroalcoholic extract of the leaves and stems of three *Croton hemiargyreus* hemiargyreus (CH) and three *Croton echinocarpus* (CE) individuals.

	Jun/98	Jul/98	Aug/98	Sep/98	Nov/98	Jan/99	Mar/99
				Alkanes			
CH	3.4±1.6[a]	3.8±2.1	2.4±0.1	2.5±0.8	9.4±10.8	1.7±2.6	4.0±3.9
CE	3.0±2.1	9.3±6.4	5.8±4.0	5.4±3.1	2.5±2.7	4.5±5.3	1.1±1.2
				Alcohols			
CH	7.3±5.4	11.9±14.3	4.2±3.1	4.8±4.5	7.7±3.8	6.6±4.6	3.3±3.7
CE	3.2±4.2	3.2±4.4	2.4±2.0	11.8±7.1	2.8±4.1	3.1±3.9	8.4±8.4
				Sesquiterpenes			
CH	1.7±1.6	2.3±1.7	4.0±4.9	2.5±1.9	2.3±2.0	0.3±0.3	0.4±0.7
CE	0.6±1.0	0.3±0.2	0.7±0.4	1.6±0.9	0.2±0.1	Trace	0.2±0.3
				Triterpenes			
CH	8.2±8.3	7.9±5.4	6.4±3.4	6.5±2.4	6.7±4.5	21.2±8.7	9.4±8.9
CE	9.0±1.1	5.4±4.3	5.7±2.3	13.5±3.9	9.3±8.2	6.0±3.4	8.1±3.9
				Sterols			
CH	7.3±10.3	7.0±8.1	1.0±0.1	5.0±3.3	28.3±13.0	8.7±4.3	1.3±2.2
CE	8.2±3.8	1.5±1.3	4.2± 2.0	7.6±1.7	4.9±8.4	9.4±7.6	9.5±5.8

[a] Mean relative concentration of the different natural product classes for the three individuals of the same *Croton* species and their standard deviation.

Table III. Seasonal variation of tocopherols and glaucine from the hydroalcoholic extract of the leaves and stems of three *Croton hemiargyreus* hemiargyreus (CH) and three *Croton echinocarpus* (CE) individuals.

	Jun/98	Jul/98	Aug/98	Sep/98	Nov/98	Jan/99	Mar/99
				α-Tocopherol			
CH	0.4±0.3[a]	0.6±0.3	0.8±0.7	1.7±1.0	0.1±0.2	0.2±0.3	1.0±1.7
CE	10.1±11.1	11.1±8.8	12.1±10.3	5.2±0.8	0.9±0.8	Trace	0.7±1.2
				β-Tocopherol			
CH	Trace	Trace	0.2±0.3	Trace	Trace	Trace	Trace
CE	0.4±0.7	1.3±1.6	1.5± 1.2	0.9±0.9	0.3±0.6	Trace	Trace
				γ-Tocopherol			
CH	0.2±0.2	0.5±0.2	0.5± 0.4	0.7±0.9	Trace	0.5±0.4	Trace
CE	1.2±0.1	1.8±0.9	1.4± 0.7	1.4±1.6	0.5±0.5	Trace	0.2±0.3
				δ-Tocopherol			
CH	0.5±0.4	0.1± 0.2	0.1±0.2	0.3±0.5	0.2±0.4	Trace	Trace
CE	0.7±0.2	1.2±1.0	0.8±0.6	0.9±0.9	1.0±1.7	Trace	0.1±0.2
				Glaucine			
CH	3.5±1.5	2.8±4.7	5.9±8.3	4.9±2.5	0.8±1.4	1.7±1.0	0.7±1.2
CE	5.6±5.8	Trace	Trace	Trace	Trace	Trace	9.2±13.8

[a] Mean relative concentration of the different natural product classes for the three individuals of the same *Croton* species and their standard deviation.

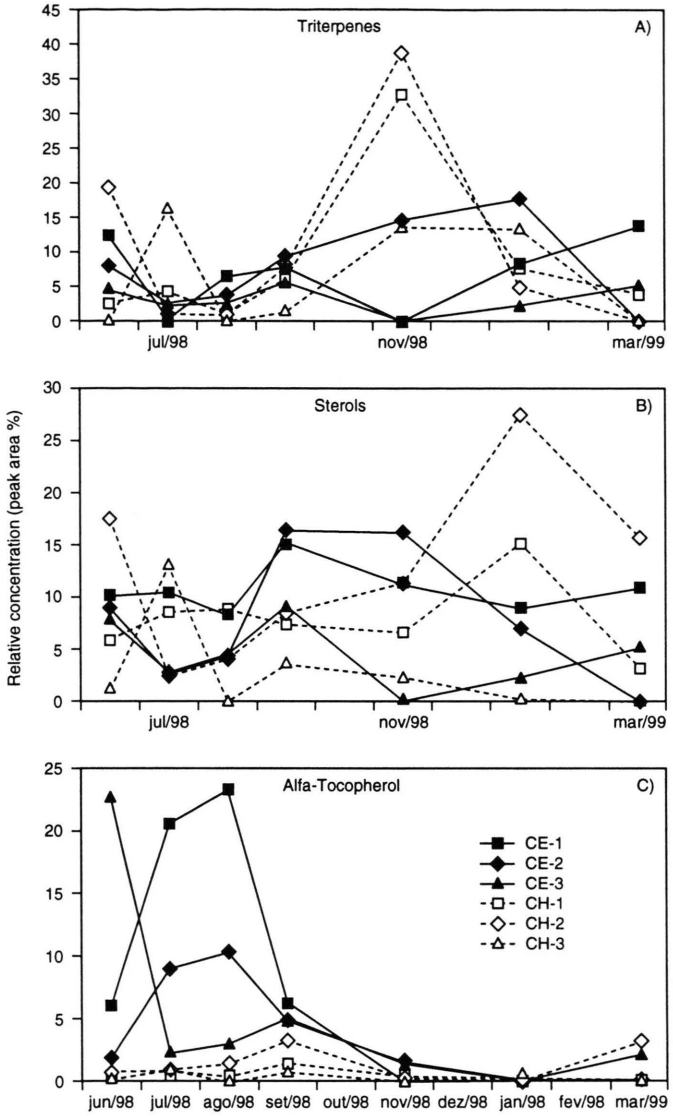

Fig. 1. Monthly variation of A) triterpenes; B) sterols and C) α-tocopherol, in *Croton hemiargyreus* Muell. Arg. var. *hemiargyreus* (three individuals; CH–1, CH–2 and CH–3) and *C. echinocarpus* Muell *Arg.* (three individuals; CE–1, CE–2 and CE–3), between June 1998 and March 1999.

emphasizing the high standard deviations observed for that particular sample.

Alkanes and alcohols

The variation observed in the amounts of alkanes and alcohols (Table II) illustrates that high a dispersion of values can be obtained from different *Croton* individuals. The more abundant constituents of these classes of natural products were heptacosane and 1-nonacosanol.

Terpenes

The terpenes were divided in sesquiterpenes and triterpenes and both classes were higher in *C. hemiargyreus hemiargyreus* in relation to *C. echinocarpus.*

Terpenoids are synthesized in various cellular organelles and are then stored in specialized secretory structures, thus protecting the plant from their toxic effects (Langenheim, 1994). In most samples the major sesquiterpene was characterized as caryophyllene. High levels of caryophyllene in *Hymenae* sp. resin were correlated with ef-

fects on mortality or low levels of insect herbivors (Langenheim, 1994). However there is a wealth of evidence regarding variation in total amount of terpenoids due to abiotic environmental factors. Generally, it is considered that enhanced accumulation occurs with increased light intensity, and depletion occurs with N, P or K fertilization (Langenheim, 1994).

In *C. hemiargyreus hemiargyreus* the amounts of triterpenes is higher (Table I) between January and March, during the tropical summer, when light intensity, and the rainfall, is at its peak (Table I). Previous results show that fungal infections (e.g.: *Pythium* and *Phytophthora* species) also increase the accumulation of terpenes due to alteration of the biogenetic route of glycoalkaloides (D'Mello, 1997). The main triterpenes in all samples were α- and β-amyrine.

All samples also showed α- and β-amyrin alkanoates (between dodecanoate to hexadecanoate) in low concentration, previous results showed that the amounts of amyrin esters were similar both for emerging and mature leaves of the *Rubus idaues* L. (Shepherd *et al.*, 1999).

An interesting fact was the decrease of the sesquiterpene amount when triterpenes increased. In *C. echinocarpus* sesquiterpenes as well as triterpenes did not show a significant variation.

Sterols

The composition of sterols in the plant kingdom does not appear quite so simple at the present time. The majority of angiosperm species examined to date are those containing sitosterol, stigmasterol and campesterol, but a substantial number of angiosperms have a composition dominated by spinasterol and 7-stigmasterol (Patterson, 1994). The sterol concentration is reduced in wax from younger leaves and their amount increases with maturation (Avato *et al.*, 1987). In *C. hemiargyreus* the higher amount of sterols occurred between September and December, and the main constituent was stigmasterol. In *C. echinocarpus* the variation of sterol amount was random (Table II).

Tocopherols

Tocopherols have been found in plant cuticular waxes, and were less abundant in wax from emerging leaves than in mature leaves (Shepherd *et al.*, 1999). However, in our results, three members of the tocopherol family were characterized in all samples, α-, β- and γ- tocopherol (Table III). In *C. echinocarpus* the amount of tocopherols was higher than that in *C. hemiargyreus*, mainly during June and September (Fig. 1C), and α-tocopherol was the most abundant in all samples, the other tocopherols were found in low concentration.

Alkaloids

Alkaloids are the largest group of secondary metabolites present in higher plants and are structurally highly diverse. Four aporphine alkaloids were characterized: glaucine and dehydroglaucine in *C. hemiargyreus* and glaucine, laurelliptine and dehydrolaurelliptine in *C. echinocarpus*. The seasonal variation of glaucine showed a maximum between June and October for *C. hemiargyreus*, and was present only in January and June in *C. echinocarpus* (Table III). Other alkaloids were characterized in low concentration.

Acknowledgements

The authors wish to thank, FINEP, FAPERJ, CNPq, and FUJB for financial support and/or fellowships.

Amaral A. C. F. (1996), Contribuição á qímica do gênero *Croton* (Euphorbiaceae). Componentes de cinco entidades, DSc. Thesis, Universidade Federal do Rio de Janeiro, Brazil.

Avato P., Bianchi G. and Pogna N. (1990), Chemosystematics of surface lipids from maize and some related species. Phytochemistry **29**, 1571–1576.

Barnes R. A. (1964), The structure of salutaridine. An. Acad. Bras. Cien. **36**, 238–239.

Bertolo R. M. and Scarpati M. L. (1979), Alkaloids of *Croton draconoides*. Phytochemistry **18**, 520–521.

Blum W. (1985), Preparation of inert and high-temperature stable apolar and medium polar glass-capillary columns using OH-terminated polysiloxane stationary phases. J. High Resol. Chromatogr. **8**, 718–726.

Carbonell S. A., Aquino Neto F. R., Cardoso J. N., Pereira A. S., Amaral A. C. F. and Barnes R. A. (2000), Rapid screening of natural products by high-products by high-resolution high-temperature gas chromatography. J. Chromatogr. Sci. **38**, 234–240.

D'Mello F. P. J. (1997), Handbook of Plant and Fungal Toxicants, p. 23, New York, CRC Press.

Fansworth N. R., Biomster R. N., Messmer W. N., King J. C., Persinos G. J. and Wilkes J. D. (1969), A phytochemical and biological review of the genus *Croton*. Lloydia **32**, 1–28.

Grob K., Grob G. and Grob Jr. K. (1981), Comprehensive standardised quality test for glass capillary columns. J. Chromatogr. **219**, 13–20.

Grob Jr. K., Grob G. and Grob K. (1978), Testing capillary gas chromatographic columns. J. Chromatogr. **156**, 1–20.

Hartwell H. (1969), Plants used against cancer. Lloydia **32**, 153–205.

Langenheim J. H. (1994), Higher plant terpenoids: A phytocentric overview of their ecological roles. J. Chem. Ecol. **20**, 1223–1280.

Patterson G. W. (1994), Phylogenetic distribution of sterols. In: Nes W. D., Isopentenoids and Other Natural Products: Evolution and Function, p. 255, Washington, DC, ACS series 562.

Pereira A. S. and Aquino Neto F. R. (1999), High-temperature high-resolution gas chromatography: breaching the barrier to the analysis of polar and high molecular weight compounds. Trends Anal. Chem. **18**, 126–136.

Pereira A. S., Amaral A. C. F., Barnes R. A., Cardoso J. N. and Aquino Neto F. R. (1999), Identification of isoquinoline alkaloids in crude extracts by high temperature gas chromatography-mass spectrometry. Phytochem. Anal. **10**, 254–258.

Shepherd T., Robertson G. W., Griffiths D. W. and Birch A. N. E. (1999), Epicuticular wax ester and triacylglycerol composition in relation to aphid infestation and resistance in red raspberry (*Rubus idaeus* L.) Phytochemistry **52**, 1255–1267.

Vanhaelen M., Lejoly J., Hanocq M. and Molle L. (1991), Climatic and geographic aspects of medicinal plant constituents. In: The Medicinal Plant Industry (Wijesekera R. O. B., ed.). Boca Raton, Florida: CRC Press, p. 269.