Rapid Screening of Natural Products by High-Resolution High-Temperature Gas Chromatography

Silvia Albero Carbonell, Francisco Radler de Aquino Neto, Jari Nobrega Cardoso, and Alberto dos Santos Pereira*
LADETEC, Instituto de Química, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Cidade Universitaria, CT, Bloco A, Sala 607, Rio de Janeiro, RJ - Brazil 21949-900

Ana Claudia F. Amaral
Lab. de Química de Produtos Naturais (LQPN), Far-Manguinhos, RJ - Brazil 21041-250

Roderick A. Barnes
Núcleo de Pesquisa de Produtos Naturais, Centro de Ciências da Saúde, Bloco H, Universidade Federal do Rio de Janeiro, RJ - Brazil 21941-590

Abstract

The crude ethanol extracts from the leaves of three Croton hemiarigyrus hemiarigyreus plants are fractionated by thin-layer chromatography, yielding five fractions. The fractions and the crude extract are analyzed by high-temperature high-resolution gas chromatography coupled with mass spectrometry (HT-HRGC–MS). Several natural products, including thermolabile components, can be characterized directly in the samples, such as alkaloids, terpenes, flavonoids, acids, alcohols, etc. The cold on-column technique proves to be appropriate for the injection of these thermolabile compounds. HT-HRGC–MS is shown to be a valuable tool and an alternative technique to classical phytochemical procedures for the simple and fast routine analysis of natural products in crude extracts.

Introduction

The genus Croton (Euphorbiaceae) is widely distributed in Brazil. In the state of Rio de Janeiro alone, 39 species have been identified (1). The isolation of morphinandienone, aporphine, and proaporphine alkaloids has been described for this genus (2,3). Some species have been used in folk medicine in a large number of applications, including anticancer drugs or agents (4).

The study of metabolic pathways and the role of secondary metabolites in defensive or attractive functions within a plant increased the interest in assessing the distributions and structures of the hundreds of minor compounds that, until now, have been largely overlooked (5). Important classes of secondary metabolites of plants are the flavonoids; some were shown to inhibit the replication of picornaviruses such as poliomyelitis and rhinoviruses. Compounds such as 4′-ethoxy-2′-hydroxy-4,6′-dimethoxychalcone interact directly with specific sites on the viral capsid proteins, producing an uncoating of the virus with consequent liberation of viral RNA (6).

The usual systematic methodology in natural product analyses involves a step of isolation by column chromatography, thin-layer chromatography (TLC), or high-performance liquid chromatography (HPLC) before identification by the usual spectroscopic methods (ultraviolet, infrared, and nuclear magnetic resonance) (5,7). As a result, identification of multiple components by classical phytochemistry is extremely slow.

It is now becoming common knowledge in the gas chromatographic (GC) community that high-resolution GC (HRGC) can be used to analyze many compounds traditionally considered intractable as a result of being polar or “thermolabile”. This knowledge, however, has not been completely disseminated to the domains of environmental, medicinal, clinical, pharmaceutical, and fine chemical analyses. Old limitations of GC for the analysis of polar compounds (e.g., low resolution and sensitivity) were extended to HRGC without further investigation, and researchers in these varied fields preferred to elect HPLC as their technique of choice. Yet, in many cases where the nature of the samples is adequate, HRGC has distinct advantages in comparison with HPLC (offers higher resolution, faster separation, ease of coupling with a wide selection of detectors, no use of solvent, etc.) (8). Today, HRGC coupled with mass spectrometry (HRGC–MS) is an important and consolidated method for the systematic analysis of natural products (5,7). In the study of medicinal plants, however, HRGC–MS is generally restricted to the analysis of low-polarity compounds of low molecular weight (7).

The on-column sample injection is advantageous, or even essential, particularly for high molecular weight (HMW) compounds. Since the sample is deposited directly into the column, cold on-column injection gives the highest reproducibility, lowest discrimination, and lowest sample decomposition (9,10).
However, it can be deficient to inject samples directly into narrow bore capillary columns (11).

Today, the apolar and medium polar high-temperature capillary columns for high-temperature HRGC (HT-HRGC) can be conveniently operated at temperatures up to 420°C. At first, the extension of the working range from 320°C to above 400°C may appear to be of little significance. However, in practice, it is highly relevant. Expressed in mass units of the compounds, which can be analyzed, the working range can be extended by more than 400 daltons (12). The use of HT-HRGC and HT-HRGC–MS in the analysis of crude extracts and fractions of natural products permitted the direct characterization of several compounds without derivatization or, in some cases, even clean-up procedures. These compounds include flavonoids, rotenoids, fatty esters of terpenols, and long-chain alcohols (13–15). The possibility of directly analyzing crude extracts can be extremely useful for the systematic study of medicinal plants and other sources of biologically active compounds as a quick screening method, which could guide subsequent phytochemical or pharmacological work.

**Experimental**

**Samples**

**Material**

The leaves and stems of *Croton hemiargyrus* Muell. Arg. var. hemiargyreus were collected near Nova Friburgo (Rio de Janeiro, Brazil). The species was identified by Prof. Arline Souza de Oliveira (National Museum, Federal University of Rio de Janeiro). The voucher sample has been deposited in the herbarium of the Federal University of Rio de Janeiro with the specimen number R182775.

**Extraction**

Leaves and stems were ground and extracted with 75% ethanol by percolation. The ethanol extract was concentrated first on a rotary evaporator and then using a stream of nitrogen gas.

**Fractionation of extract**

Approximately 20 mg of crude ethanolic extract of powdered leaves and stems as separated by preparative TLC using 20- × 20-cm plates prepared in our laboratory with Merck (Darmstadt, Germany) silica gel 60 Å with a fluorescent indicator (254 nm) and hexane–ethyl ether (95:5) after removal of the material eluting with the solvent front. Four more fractions were obtained after elution in sequence of the same preparative thin-layer plates with hexane–ethyl acetate (95:5). The TLC elution regions were scraped off, eluted with CH$_2$Cl$_2$, concentrated by rotatory evaporation followed by nitrogen blowdown, and transferred to 2-mL vials.

The fractions were weighed after solvent removal and drying in vacuum desiccators with P$_2$O$_5$ and gave values of 0.0029 g (F1), 0.0007 g (F2), 0.0012 g (F3), 0.0006 g (F4), and 0.0069 g (F5). All extracts and fractions were kept in a refrigerator until analyzed.

**GC**

**Columns**

GC was performed on borosilicate capillary columns (25 m × 0.25-mm i.d.; Duran-50, Vidrolex, Sao Paulo, Brazil) coated with 0.2 µm of PS-086 (5% phenyl–95% methylpolysiloxane, Petrarch Systems, Bristol, PA). The columns were prepared in our laboratory according to a procedure in the literature (16).

Prior to analysis by HRGC–MS, the capillary column was connected using a fused glass-to-silica connection as described by Grob (17) to a 2-m piece of an empty capillary tube (interface GC–MS) prepared from 0.25-mm-i.d. high-temperature fused-silica (HTFS, J&W Scientific, Folsom, CA). The HTFS was purged with hydrogen at 180°C for 15 min and deactivated by flushing with hexamethyldisilazane (HMDS)–1,3-diphenyl-1,1,3,3-tetramethyldisilazane (DPTMDS) 1:1 (Petrarch Systems), sealing the capillary, and heating at 400°C for 12 h. The tubing was then rinsed with hexane, methanol, and diethyl ether.

Column performance was checked by the Grob test (18,19) prior to use.

**GC conditions**

An on-column injector (part number 4269608, Carlo Erba, Rodano, Italy) was mounted on a Hewlett-Packard (Palo Alto, CA) model 5890-II GC. The column temperature was maintained at 40°C during injection, then programmed to 390°C at 10°C/min and held for 10 min. The flame ionization detector (FID) and the on-column injector were operated at 400°C and room temperature, respectively.
Hydrogen was used as a carrier gas at a linear velocity of 50 cm/s, and the sample volume injected was 0.5 µL. GC data were acquired and processed with an HP 3396-II integrator (Hewlett-Packard).

**MS conditions**

HT-HRGC–MS analyses were carried out on an HP 5972 MS (Hewlett-Packard) under electron impact ionization (70 eV). The GC operating conditions were the same as previously described. The on-column injector and the transfer line temperatures were set at 40 and 390ºC, respectively, and the ion source temperature was 300ºC (MS scan range, 40–700 amu).

**Compound identification**

The compounds were characterized by mass spectral interpretation and comparison with reference spectra (library search). Library searches were of relatively limited help in the case of the HMW compounds, because many of these compounds had not been previously characterized.

### Table I. Compounds Characterized in the Crude Ethanolic Extract of Powdered Leaves and Stems of Croton hemiargyrous

<table>
<thead>
<tr>
<th>Number</th>
<th>t&lt;sub&gt;1&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>Characteristic ions and relative abundances (%)</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.45</td>
<td>Δ-Cadinene</td>
<td>204 (46), 189 (14), 161 (100), 147 (8), 134 (51), 119 (65), 105 (61), 91 (40)</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>10.81</td>
<td>Veridiflorol</td>
<td>222 (1), 204 (14), 189 (11), 161 (43), 147 (14), 135 (13), 109 (40), 43 (100)</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>11.17</td>
<td>Humulene epoxide II</td>
<td>161 (3), 149 (26), 138 (91), 123 (48), 109 (100), 96 (100), 67 (100), 43 (61)</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>11.28</td>
<td>Calarene</td>
<td>204 (31), 189 (26), 161 (72), 147 (17), 121 (46), 105 (58), 93 (45), 43 (45)</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>11.48</td>
<td>α-Muurolol</td>
<td>204 (31), 189 (14), 161 (100), 147 (12), 119 (38), 105 (58), 81 (65), 43 (66)</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>11.72</td>
<td>τ-Muurolol</td>
<td>204 (35), 189 (6), 161 (35), 121 (72), 109 (31), 105 (25), 95 (100), 43 (66)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

F2

<table>
<thead>
<tr>
<th>Number</th>
<th>t&lt;sub&gt;1&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>Characteristic ions and relative abundances (%)</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>9.83</td>
<td>Guaiol</td>
<td>222 (3), 204 (18), 189 (20), 161 (100), 149 (20), 119 (30), 107 (55), 59 (54)</td>
<td>0.3</td>
</tr>
<tr>
<td>18</td>
<td>11.71</td>
<td>7-acetyl-2-hydroxy-2-methyl-5-isopropyl-bicyclo[4,3,0]nonane</td>
<td>238 (3), 220 (3), 153 (46), 135 (34), 109 (8), 95 (8), 81 (11), 71 (17), 43 (100)</td>
<td>0.3</td>
</tr>
<tr>
<td>19</td>
<td>21.15</td>
<td>Farnesyl acetate</td>
<td>189 (52), 161 (3), 136 (12), 121 (15), 107 (9), 95 (28), 81 (78), 69 (100), 41 (25)</td>
<td>0.2</td>
</tr>
<tr>
<td>20</td>
<td>23.41</td>
<td>Vitamin E</td>
<td>430 (52), 364 (2), 205 (10), 165 (100), 121 (5), 95 (2), 69 (10)</td>
<td>0.5</td>
</tr>
<tr>
<td>21</td>
<td>24.48</td>
<td>α-Amyrin</td>
<td>426 (5), 411 (4), 218 (100), 203 (58), 189 (18), 147 (8), 135 (12), 95 (71)</td>
<td>0.2</td>
</tr>
<tr>
<td>22</td>
<td>24.75</td>
<td>α-Amyrin</td>
<td>426 (6), 411 (5), 218 (100), 203 (32), 189 (40), 147 (20), 135 (32), 69 (15)</td>
<td>0.4</td>
</tr>
<tr>
<td>23</td>
<td>24.88</td>
<td>Stigmaster-4-en-3-one</td>
<td>412 (12), 397 (6), 370 (7), 289 (14), 259 (12), 229 (40), 124 (100), 69 (31)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

F3

<table>
<thead>
<tr>
<th>Number</th>
<th>t&lt;sub&gt;1&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>Characteristic ions and relative abundances (%)</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>25.47</td>
<td>Ergost-5-en-3-ol</td>
<td>400 (6), 382 (16), 340 (6), 289 (6), 261 (9), 213 (15), 145 (49), 107 (36), 44 (100)</td>
<td>0.3</td>
</tr>
<tr>
<td>25</td>
<td>25.60</td>
<td>Stigmaster-5,22-dien-3-ol</td>
<td>412 (2), 394 (6), 379 (5), 255 (13), 213 (10), 145 (24), 69 (46), 43 (100)</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>26</td>
<td>26.03</td>
<td>β-Stigmaster-5-en-ol</td>
<td>414 (30), 396 (60), 381 (38), 329 (26), 303 (26), 255 (41), 213 (47), 145 (81)</td>
<td>5.7</td>
</tr>
</tbody>
</table>

F4

<table>
<thead>
<tr>
<th>Number</th>
<th>t&lt;sub&gt;1&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>Characteristic ions and relative abundances (%)</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>12.00</td>
<td>β-Eudesmol</td>
<td>222 (3), 204 (21), 189 (40), 161 (35), 149 (80), 123 (29), 90 (41), 59 (100)</td>
<td>0.3</td>
</tr>
<tr>
<td>27</td>
<td>25.48</td>
<td>Œpis-πsi-taraxastanonol</td>
<td>424 (12), 205 (60), 189 (35), 149 (37), 121 (52), 95 (83), 81 (48), 67 (54), 44 (100)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

F5

<table>
<thead>
<tr>
<th>Number</th>
<th>t&lt;sub&gt;1&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>Characteristic ions and relative abundances (%)</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>20.66</td>
<td>Glaucine</td>
<td>355 (94), 354 (100), 340 (50), 324 (31), 281 (26)</td>
<td>7.7</td>
</tr>
<tr>
<td>29</td>
<td>21.45</td>
<td>Cordyline&lt;sup&gt;3&lt;/sup&gt;</td>
<td>341 (62), 340 (100), 326 (17), 310 (17), 294 (14), 267 (8)</td>
<td>1.6</td>
</tr>
<tr>
<td>30</td>
<td>22.05</td>
<td>Dimethoxy-Chrysin</td>
<td>314 (100), 299 (12), 283 (6), 271 (15), 242 (8), 135 (9), 77 (5)</td>
<td>0.3</td>
</tr>
<tr>
<td>31</td>
<td>25.49</td>
<td>Dimethoxy-quececin</td>
<td>330 (100), 315 (41), 301 (6), 287 (9), 297 (10), 259 (9), 151 (29), 135 (8), 79 (3)</td>
<td>0.3</td>
</tr>
<tr>
<td>32</td>
<td>26.48</td>
<td>Dehydroglaucine</td>
<td>353 (100), 338 (72), 322 (5), 307 (10), 280 (31), 265 (3), 209 (7), 176 (9)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Peak numbering as shown in Figure 1.
† Estimate of concentration using a response factor for the FID equal to 1.
‡ Corydine or Isocorydine.
been analyzed previously by GC–MS. In this regard, comparison with authentic standards (when available) was essential to elucidate the final structures.

Results and Discussion

HRGC analyses of the fractionated and crude ethanolic extracts of powdered leaves and stems of the *Croton hemiargyrus* hemiargyreus separated previously by TLC are presented in Figure 1. Figure 1B (fraction F1) shows the efficiency of the fractionation of the crude ethanolic extract by TLC with the increase of relative concentration of HMW compounds. This preconcentration by TLC permitted the characterization of more than 50 compounds (Table I) of different classes of natural products (*n*-alkanes, alcohols, terpenes, alkaloids, flavonoids, triterpenyl fatty acid esters, etc.).

**Compound characterization and quantification**

Various classes of compounds were detected in this survey, but the full characterization of their structures was frequently not possible due to the unavailability of published reference mass spectra. For some classes of natural products such as flavonoids, the inclusive characterization by mass spectral analysis alone is rather difficult because of the number of isomers and, in many cases, only minor differences between their mass spectra. Usually only probable structures can be suggested using the mass spectral data together with biogenetic considerations. The presence of a 4'-methoxyphenyl moiety in the flavonoids, for example, was inferred based on the biogenetic formation of the flavonoids from shikimic acid (20). Discussions of the electron impact mass spectral fragmentation characteristics of flavonoids were made by Porter (21) and Takayama et al. (22). Their characteristics could be used to interpret the MS data more thoroughly.

The characterized compounds are shown in Table I with their respective retention times. It is obvious that precise quantitation is very difficult because of the complexity of unfractionated natural product samples. This difficulty results from the very high variation in the detector response for the different classes of natural products. An estimate of the concentrations using a response factor of 1 for the flame ionization detector (FID) is also shown in Table I.

**Hydrocarbons**

The usual distribution of *n*-alkanes in higher plant extracts with 16 (retention time $t_R = 9.45$ min) up to 33 ($t_R = 21.85$ min) carbons with odd carbon number predominance and maxima at $C_{29}$ was characterized. Hexadecene ($t_R = 9.80$ min), octadecene ($t_R = 12.12$ min), hexacosene ($t_R = 16.19$ min), and squalene ($t_R = 21.57$ min) were also detected. These compounds were characterized in the solvent front fraction in TLC (Figure 1B).

**Alcohols**

The *n*-alkanol distribution in the ethanol extract was $C_{18}$ ($t_R = 14.29$ min), $C_{25}$ ($t_R = 20.42$ min), $C_{27}$ ($t_R = 21.90$ min), and $C_{29}$ ($t_R = 23.04$ min). Phytol ($t_R = 21.57$ min) was also detected. All these compounds were characterized in intermediate fractions of TLC (Figure 1C, fraction F2). Inositol ($t_R = 18.54$ min) was characterized in the origin of the TLC (Figure 1F, fraction F5) in an estimated concentration of 6.8 mg/g.

**Terpenes**

The terpenoids constitute the largest class of natural products found in abundance in higher plants (23). Compounds such as triterpenes can
have an elaborate structure that requires detailed analysis for elucidation. However, even such structures could be rapidly detected through the monitoring of characteristic fragments and molecular ions. The $\Delta^{12}$-oleananes and $\Delta^{12}$-ursenes are the most common naturally occurring pentacyclic triterpenes. Also known as the $\alpha$ and $\beta$ amyrins series, they have a characteristic double bond at C12–C13. This feature proved to be readily recognizable by MS, because the molecular ion undergoes the equivalent of a Retro-Diels-Alder fragmentation to generate a very characteristic peak ($m/z$ 218). Several other terpenoids were characterized in the TLC fractions (Table I).

Triterpenyl alkanoates

The mass spectra of triterpenyl fatty acid esters (TFAE), characterized for the first time in Croton hemiargyrus hemiargyreus, are fairly simple considering their relatively complex structures. Essentially, the fragmentation pattern consists of the molecular ion ($M^+$) and ($M^+ - \text{CH}_3$, $M^+$ – fatty acid), as well as fragments characteristic of the esterified triterpenol; the fragmentation of TFAE has been reported previously by Elias et al. (24). The dominant fragmentation is cleavage of the ester bond directly or by H-transfer via a McLafferty-type rearrangement to yield the triterpenyl ions ($m/z$ 408 and 409, respectively). Figure 2 shows example mass spectra of the triterpenols esterified to tetradecanoic acid representative of the TFAE series. The low abundance of the ions with mass-to-charge ratios higher than 500 did not preclude the characterization of the TFAE series. Figure 3 shows the characteristic ions (base peaks) of the amyril and taraxasteryl compounds ($m/z$ 218 and 189) and the $M^+$ ions ($m/z$ 608, 636, and 664) of the TFAE of dodecanoid, tetradecanoic, and hexadecanoic acids, respectively.

Alkaloids

The isolation of morphinandienone, aphoriphine, and proaphorphine alkaloids has been described for this genus (2,3,25). Previous results in the literature (26) showed that unsaturation in the heterocyclic ring increases the GC retention time with regards to the saturated analog because of the enhanced planarity of the fused rings due to $sp^2$ carbons and resonance with the nitrogen lone electron pair. These findings are in accordance with the results observed for compounds glaucine and dehydroglaucine (Figure 4): the difference of 3.8 min (38°C) in retention time illustrates the increase in the distribution constant ($K$) in the apolar phase for dehydroglaucine.

In the mass spectra characteristic of the alkaloids (aporphine group), the base peak is the molecular ion or the ion formed by the loss of a hydrogen atom. Other important ions are ($M – 15$) and ($M – 31$), formed by the loss of methyl and methoxyl groups from one of the aromatic rings (27). The general fragmentation patterns of aporphinic alkaloids have been reported previously (26). Compared with the mass spectra obtained by direct introduction (27,28), the data generated in this HT-HRGC–MS study showed the same typical fragmentation pattern and only slight differences in relative peak intensities, allowing direct spectral recognition by computerized routines (e.g., a library search). The MS data of all analyzed alkaloids are shown in Table I.

HMW compounds in higher plants

The analysis of fractions of higher plants by HT-HRGC and HT-HRGC–MS may permit the charac-
terization of HMW compounds not previously reported because of their poor elution on capillary columns (HRGC and HRGC–MS) coated with phases that have rather low temperature limits (approximately 300°C), such as (5%-phenyl)-methylpolysiloxane.

However, compounds such as the triterpenyl fatty acid esters characterized in this paper for the first time in *Croton hemiarigyurus* hemiarigyeus are common in higher plants. An extended TFAE series with acyl carbon chain lengths (for example, from 5 to 20 carbons) was previously characterized in the smoke extract collected from burning the Castanha-do Pará plant (Brazil nut, *Bertholletia excelsa*) (13).

The nondetection of higher molecular weight compounds in extracts or fractions of higher plants is of serious concern, especially for the quantitative analysis of medicinal plants. In this regard, HT-HRGC may overcome the usual limitations and prove to be a powerful tool for the detection of new HMW organic compounds in higher plants.

**Conclusion**

Old limitations of GC analyses have been largely overcome, as illustrated by the characterization of high molecular weight triterpenyl fatty acid esters, reported for the first time in *Croton hemiarigyurus* hemiarigyeus. These compounds have not been reported as such in HRGC and HRGC–MS analyses. HT-HRGC and HT-HRGC–MS have proven to be powerful tools for the characterization of these higher molecular weight compounds without the need for isolation or extensive clean-up.

These techniques allow the rapid qualitative analysis (e.g., profiling) of alkaloids and other secondary metabolites, offering an alternative and advantage in comparison with the classical phytochemical methodology, especially where the number of samples is very large. The HT-HRGC analyses can be used notably for the analysis of trace compounds in fractions (e.g., TLC fractions). Several natural products of different structural classes (e.g., alkanes, alcohols, terpenes, alkaloids, flavonoids, triterpenyl fatty acid esters, etc.) were characterized and could be simultaneously detected in crude plant extracts.

This methodology opens new possibilities for the analysis of crude extracts and fractions and can be extremely useful for the systematic study of medicinal plants and other sources of biologically active compounds. Analyses of natural products by HT-HRGC provide a quick screening method that could guide subsequent natural product chemistry or pharmacological work.

**Acknowledgments**

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

**References**


Manuscript accepted April 26, 2000.