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## Molecular composition and chemosystematic aspects of Cretaceous amber from the Amazonas, Araripe and Recôncavo basins, Brazil

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### ABSTRACT

Amber occurrences in Brazil are rare. In this regard, the molecular composition of three such fossil resin samples from Brazilian Cretaceous sedimentary basins has been analyzed to determine the botanical origin of the resins. The samples were collected from the Amazonas (Alter do Chão Formation), Araripe (Santana Formation, Crato Member) and Recôncavo (Maracangalha Formation, Caruaçu Member) basins. The mono-, sesqui- and diterpenoids in the extracts were used as chemosystematic markers when compared with terpenoids in extant conifers. The compounds were mainly diterpenoids and their degradation products from the labdane, podocarpene, pimarane and isopimarane classes, in addition to paraffins, methoxyphenols and carboxylic acids. Tetracyclic diterpenoids such as phyllocladane, kauranol and kauranol were also present. The biomarker compositions are certainly typical for conifers and, given the absence of triterpenoids and diterpenoids such as ozic acid, angiosperms can be excluded as a botanical source. The absence of phenolic diterpenoids (ferruginol, totarol) and their derivatives excludes podocarpaceous affinities for the ambers from the Amazonas and Araripe basins. The fossil records of the embedding sediments (e.g. *Araucariaceae* pollen and leaves) support the proposal of an *Araucariaceae* origin for these ambers, but *Cupressaceae* and *Cheirolepidiaceae* cannot be excluded. On the other hand, the presence of phyllocladane and kaurane derivatives is evidence for *Araucariaceae* or *Podocarpaceae* origins for the amber from the Recôncavo basin, but *Cupressaceae* cannot be excluded.

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### 1. Introduction

Amber is the polymerized and fossilized remains of tree resins. Its chemical composition provides an opportunity for the observation of evidence for biochemical evolution within higher plant populations and for increasing current understanding of geochemical pathways for terpenoid transformations during diagenesis (Grimalt et al., 1988). In this sense, knowledge about amber and fossil resins in organic geochemistry is important, although such knowledge lags behind that available for other geological materials such as sediments, crude oils or coals. Furthermore, knowledge about the chemical composition of amber is necessary for chemosystematics. Chemosystematics or chemotaxonomy is a common tool for the evaluation of the systematics and phylogeny of extant plants (Otto et al., 2002, 2003).

Solid state spectroscopic techniques have been applied to the structural elucidation of amber samples. Infrared absorption spectroscopy and solid state Fourier transform infrared have been widely used to study samples from several localities and ages (Beck et al., 1964; Langenheim and Beck, 1968; Edwards and Farwell,

1996; Brody et al., 2001; Trevisani et al., 2005; Guiliano et al., 2006, 2007; Peñalver et al., 2007; Edwards et al., 2007). Solid state <sup>13</sup>C nuclear magnetic resonance (<sup>13</sup>C NMR) studies of fossilized resins from Mexico, The Dominican Republic and Poland (Baltic) have also been reported (Cunningham et al., 1983; Lambert et al., 1985, 1988; Martinez-Richa et al., 2000). Lambert et al. (1995) proposed criteria for distinguishing between fossilized and recent resin on the basis of <sup>13</sup>C NMR spectroscopy. Later, Lambert et al. (1996) analyzed several amber samples of Cretaceous age from Alaska, Canada, United States, Lebanon, Jordan and Israel using <sup>13</sup>C NMR. Synchronous luminescence analysis was also performed in order to identify aromatic compounds in solvent extracts of Baltic amber (Matuszewska and Czaja, 2002).

Unfortunately, most ambers and fossilized resins exhibit rather similar patterns when examined using spectroscopic methods, which obviously represents a major limitation for classification purposes. Moreover, these bulk techniques usually do not allow recognition of individual components, so comparison with the major terpenoid derivatives produced from their higher plant precursors is very limited (Grimalt et al., 1988). Gas chromatography–mass spectrometry (GC–MS) allows, on the other hand, individual components of amber extracts to be separated and provides mass spectra for structural elucidation. Thus,

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knowledge of characteristic natural products entrapped in the polymerized amber matrix may yield important information for the systematic assignment of unknown fossil species, for the reconstruction of fossil flora in sediments without anatomically preserved structures and the reconstruction of phylogenetic relationships (Thomas, 1986; Eglinton and Logan, 1991).

Terpenoids are common constituents of resins and gums and higher land plants are the major sources of these compounds. The diterpenoids originate mainly from conifers (gymnosperms), while triterpenoids (e.g. of the oleanane, ursane and lupane series) derive from angiosperms (Simoneit et al., 1986; Otto and Wilde, 2001; Otto et al., 2005; Yamamoto et al., 2006).

In a review article, Otto and Wilde (2001) reported on characteristic distribution patterns of sesqui-, di- and triterpenoids in conifer families. Anderson and coworkers have published extensive pyrolysis (Py)-GC-MS analyses of fossil resins, including Cretaceous amber samples (Anderson, 1994, 1995, 2006; Anderson and Botto, 1993; Anderson and LePage, 1995; Anderson et al., 1992; Bray and Anderson, 2008). In addition, GC-MS analysis of amber extracts has been well described (e.g. Grantham and Douglas, 1980; Mills et al., 1984; Mosini and Samperi, 1985; Simoneit et al., 1986; Grimalt et al., 1988; Czechowski et al., 1996; Otto et al., 2002; Chalor and Grimalt, 2005). Furthermore, GC-MS analysis of biomarkers in fossil plants, coals and the surrounding sediments (Simoneit et al., 1986, 2003; Otto et al.; Otto and Simoneit, 2001, 2002; Otto et al., 1997, 2002, 2003, 2005, 2007; Simoneit et al.; Marynowski et al., 2007) has provided valuable information on the specific plant sources of geoterpenoids related to the gymnosperm and angiosperm families (e.g. *Podocarpaceae*, *Cupressaceae*, *Pinaceae*, *Araucariaceae* and *Leguminosae*).

The earliest known fossilized resins are from Upper Carboniferous pteridosperms of England (van Bergen et al., 1995), but amber did not become abundant in the fossil record until the Early Cretaceous, with the rise of the coniferous *Araucariaceae*, particularly in tropical and subtropical forests (Martinez-Delclòs et al., 2004). The *Araucariaceae* family contains three genera, namely *Araucaria*, *Agathis* and *Wollemia*. Of these, *Agathis* produces great quantities of resin, and it, or a relative, appears to have been a very important amber producer in many deposits from the Early Cretaceous to the Pliocene. Moreover, all families in the order Coniferales synthesize terpenoid resins, but only members of the *Pinaceae* and *Araucariaceae* families produce appreciable quantities nowadays (Langenheim, 1969, 1990, 1995).

Considering that available information about Brazilian amber is scarce, a brief review on the existing data is warranted. Amber occurrences in Brazil are rare, samples having been found mainly in Upper Aptian-Lower Albian deposits. In the Araripe basin, it occurs as small pieces in siltstones at the top of the Crato Member and in lacustrine sandstones from the Recôncavo basin, Maracangalha Formation (Pereira et al., 2007 and references therein). Brazilian fossil resins from the Recôncavo (Maracangalha Formation) and the Parnaíba (Itapecuru Formation) basins have been analyzed using GC-MS and their chemical composition led to the suggestion that their botanical origin was related to the *Araucariaceae* family (Carvalho et al., 2000). According to the published data, no macroscopic inclusions are known from the latter fragments. Martill et al. (2005) reported amber from the Crato Member, associated with resin-filled cones, foliage and palynomorphs attributed to the *Araucariaceae* family. However, Langenheim and Beck (1968) found a single piece of amber collected from the Pirabas Formation near Capanema (Pará, Brazil). This formation is considered to be early Miocene in age and is accompanied by numerous dicotyledoneous leaves. The infrared spectrum of this amber sample closely resembled that of resins from the extant *Hymenaea courbaril*.

In the present study, the extracts of three Cretaceous amber samples from Brazil were analyzed using GC-MS and their organic composition characterized in order to assess their possible botanical origin. The work provides, to the best of our knowledge, the first study of the molecular composition and possible biological source(s) of ambers from Brazil. Because of the limited information on Brazilian amber, it also enhances our knowledge of such resins, which have not been studied in systematic detail to date.

## 2. Samples and methods

### 2.1. Geological setting, sampling and sample characteristics

The samples were collected from three Early Cretaceous basins in Brazil. The representative sample from the Amazonas basin (Alter do Chão Formation) was found in fluvial sediments of Albian age (Dino et al., 1999), while those from the Araripe (Santana Formation, Crato Member) and the Recôncavo basins (Maracangalha Formation, Caruaçu Member) were present in lacustrine sediments of Aptian and Berriasian–Barremian ages (Assine, 2007; Silva et al., 2007), respectively. The samples are from the paleobotanical collection of the Geology Department of the Federal University of Rio de Janeiro [register numbers 759Pb (Amazonas amber), 932Pb (Araripe amber) and 762Pb (Recôncavo amber)].

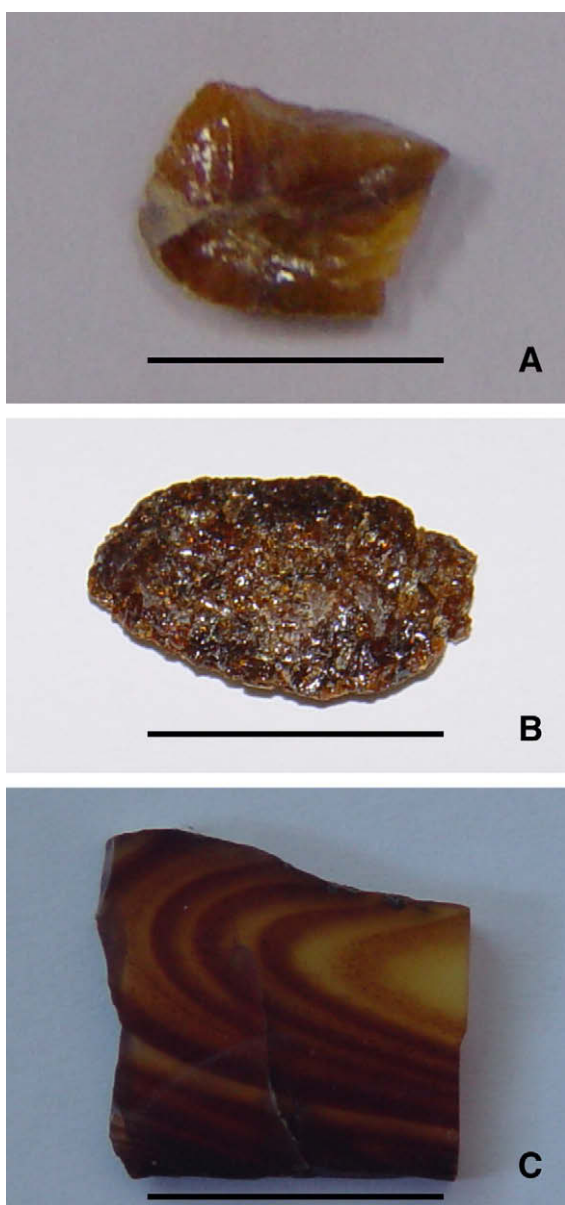
The samples occur as subspherical to angular clasts or as irregular masses, between 1.0 and 2.0 cm in diameter, as illustrated in Fig. 1. The color varies from yellow to dark brown and the samples are brittle and contain fractures. The Araripe sample was found in a laminated limestone of the Crato Member, Santana Formation. Carbonate was removed with HCl (10%) by carefully dropping the acid solution over the piece until it disaggregated, so that the amber could be separated from the rock (Fig. 1B). The amber samples from the Amazonas and Recôncavo basins (Fig. 1A and C) were analyzed after washing the exterior with solvent, powdering and extraction.

Occasionally, alternating clear and dark bands, representing successive resin flows are preserved in amber. This characteristic was found in the sample from the Recôncavo Basin (Fig. 1C). Free radicals, induced by solar radiation and exposure to air, contribute to the polymerization of resins. During higher daytime temperatures, resin is fluidized and exuded by the tree, while during the night, the temperature decreases, causing an increase in viscosity and a reduction in exudation. In this way, the darker layers, usually thinner than the clear ones, are the result of rapid drying by sunlight and wind (Martinez-Delclòs et al., 2004).

These Brazilian amber samples do not have fossil inclusions such as arthropods and insects. Although no macroscopic inclusions were observed in the pieces examined, micro-inclusions of fungal spores have been reported in the Amazon amber (Pereira et al., 2007).

### 2.2. Extraction and derivatization

The powdered samples (ca. 100 mg each) were sonicated ( $3 \times 5$  min) with dichloromethane (DCM):CH<sub>3</sub>OH; 1:1; v:v). The combined extracts were evaporated with a rotary evaporator, transferred prior to drying to a vial and the residual solvent was removed under N<sub>2</sub>. Aliquots of the weighed total extract were derivatized using two methods: (i) reaction with *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) for 30 min at 60 °C to convert polar functional groups to trimethylsilyl derivatives; (ii) treatment with diazomethane (CH<sub>2</sub>N<sub>2</sub>) to methylate carboxylic acids and, just before GC-MS, conversion of residual OH groups to trimethylsilyl ethers by reaction with BSTFA for 30 min at 60 °C.



**Fig. 1.** Cretaceous amber from Brazil. Scale bars represent 1 cm: (A) amber from the Alter do Chão Formation (Lower Cretaceous, Albian) of the Amazonas Basin; (B) amber clast isolated from the laminated limestone of the Crato Member, Santana Formation (Lower Cretaceous, Aptian) of the Araripe Basin; and (C) amber from the Caruaçu Member, Maracangalha Formation (Lower Cretaceous, Berriasian–Barremian) of the Recôncavo Basin, showing alternating bands representing successive resin flows preserved.

### 2.3. GC–MS

GC–MS analysis of the underivatized, derivatized with BSTFA, and derivatized with  $\text{CH}_2\text{N}_2/\text{BSTFA}$  extracts of the three samples were performed with an Agilent Technologies HP5890 gas chromatograph coupled to a HP5972 mass selective detector. Separation was achieved with a DB5 fused silica column (30 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness). The GC oven operating conditions were: 60–300  $^\circ\text{C}$  (held 10 min) at 6  $^\circ\text{C min}^{-1}$ . He was used as carrier gas. Samples were injected in splitless mode with the injector temperature at 290  $^\circ\text{C}$ . The mass spectrometer was operated in the full scan mode from  $m/z$  50–500 and with electron impact ionization (70 eV). The mass range is adequate

because no late eluting, high molecular weight compounds were detected.

Individual compounds were identified by comparison of mass spectra with those of standards, and with literature and library data (e.g. Gallegos, 1973; Bendoraitis, 1974; Grantham and Douglas, 1980; Simoneit and Mazurek, 1982; Mills et al., 1984; Philp, 1985; Summons and Powell, 1987; Adams, 1995; Czechowski et al., 1996; Otto et al., 2002; Otto and Simoneit, 2002; Cox et al., 2007; Bray and Anderson, 2008; Wiley275 and NIST MS Libraries). Relative retention times and interpretation of MS fragmentation patterns were used.

### 3. Results and discussion

The total extracts contain terpenoids, paraffins, alkyl benzenes, alkyl naphthalenes, alkyl hydronaphthalenes, phenols and carboxylic acids. The compounds found in the derivatized (with  $\text{CH}_2\text{N}_2/\text{BSTFA}$  or BSTFA) and underivatized total extracts are given in Table 1, along with their occurrence in the samples. Total ion chromatograms (TICs) for underivatized extracts are exposed in Fig. 2. The most significant aspect of the data is the large predominance of several diterpenoids in all the samples. Sesquiterpenoids and monoterpenoids are secondary components in some cases; alkyl benzenes, alkyl naphthalenes, alkyl tetralins (or alkyl hydronaphthalenes), phenols and carboxylic acids are present in all the samples; paraffins occur only in the Araripe sample. Some peaks correspond to unknown compounds (Fig. 2); however, their fragmentation patterns and molecular weights are indicative of diterpenoids.

#### 3.1. Monoterpenoids and sesquiterpenoids

Fenchone (**1**) [numbers refer to Table 1, Fig. 2 and Appendix A] and camphor (**2**) are the only monoterpenes. Camphor is present in small proportions in the Amazonas and Araripe samples, while fenchone was detected only in the Amazonas sample. Only calamenene (**3**), a sesquiterpenoid of the cadinane class, was detected in the Araripe and Recôncavo samples.

#### 3.2. Diterpenoids

Diterpenoids of the labdane, abietane and podocarpane types are important groups in our fossil resin extracts. The three samples contain demethylated aromatic and/or ketonic norabietanes and podocarpanes, as for instance 16,17,19-trisnorabiet-8-en-7-one (**4**), 16,17,18-trisnorabieta-8,11,13-triene (**7**), 7-oxo-15-norpodocarpane (**9**), 7-oxo-16,17,19-trisnorabieta-8,11,13-triene (**11**), 7-oxo-16,17,18-trisnorabieta-8,11,13-triene (**13**), 16,17-bisnorcalitric acid (**16**) and 16,17-bisnordehydroabietic acid (**17**). Dehydroabietane (**14**) was the only aromatized abietane in the Amazonas and Recôncavo samples. Important labdane skeleton components in the samples from the Amazonas and Araripe basins were norlabdenoic acids (**18**, **19**, **20**), noragathic acids (**21**, **22**), 14,15-bisnorlabda-8(20),11-dien-13-on-18-ol (**23**) and communic acid (**24**). Norlabdenoic acids (**18**, **19**) occur in the amber from Recôncavo as minor compounds.

The diterpenoids with the pimarane and isopimarane skeletons in the extracts of the Brazilian amber samples comprised minor amounts of isopimar-8,15-dien-18-oic acid (**25**) only in the Araripe sample, isopimar-8-en-18-oic acid (**26**) in the Araripe and Recôncavo samples and pimar-8-en-18-oic acid (**27**) in the Amazonas and Araripe samples. Diterpenoids of the kaurane and phyllocladane types are present only in the extracts of the amber sample from Recôncavo basin, such as phyllocladane (**28**), kaur-16-en-19-ol (**29**) and kauran-13-ol (**30**).

**Table 1**  
Compounds in derivatized and underivatized extracts of Brazilian ambers.

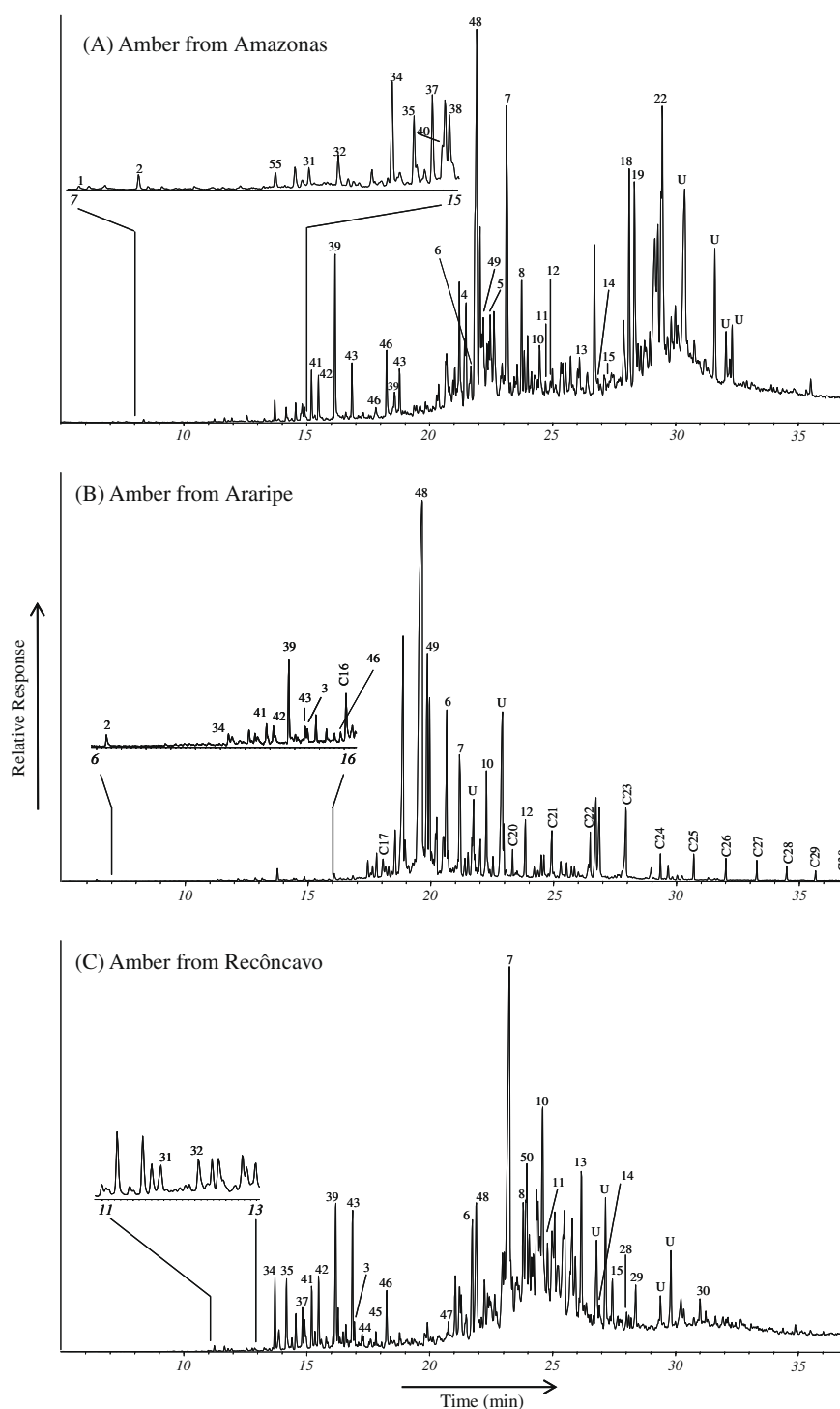
No	Compound	Composition	MW	Occurrence in sample <sup>a</sup>			Reference <sup>b</sup>
				Amazonas	Araripe	Recôncavo	
<b>Terpenoids</b>							
<i>Monoterpenoids</i>							
1	Fenchone	C <sub>10</sub> H <sub>16</sub> O	152	P	ND	ND	S, 1, 5, 8, 9
2	Camphor	C <sub>10</sub> H <sub>16</sub> O	152	P	P	ND	S, 1, 5, 9
<i>Sesquiterpenoids</i>							
3	Calamenene	C <sub>15</sub> H <sub>22</sub>	202	ND	P	P	S, 4, 11, 18
<b>Diterpenoids</b>							
<i>Abietanes and Podocarpanes</i>							
4	16,17,19-Trisnorabiet-8-en-7-one	C <sub>17</sub> H <sub>26</sub> O	246	P	P	P	I
5	16,17,18-Trisnorabiet-8-en-7-one	C <sub>17</sub> H <sub>26</sub> O	246	P	P	P	I
6	16,17,19-Trisnorabieta-8,11,13-triene	C <sub>17</sub> H <sub>24</sub>	228	P	ND	P	11
7	16,17,18-Trisnorabieta-8,11,13-triene	C <sub>17</sub> H <sub>24</sub>	228	P	P	P	11
8	16,17,18-Trisnorabietan-14-one	C <sub>17</sub> H <sub>28</sub> O	248	P	ND	ND	I
9	7-Oxo-15-norpodocarpane	C <sub>16</sub> H <sub>20</sub> O	228	P	P	P	S, 11
10	16,17-Bisnordehydroabietane (13-methylpodocarpane)	C <sub>18</sub> H <sub>26</sub>	242	ND	P	P	10, 11
11	7-Oxo-16,17,19-trisnorabieta-8,11,13-triene	C <sub>17</sub> H <sub>22</sub> O	242	P	P	P	S, 11
12	Bisnorsimonellite	C <sub>17</sub> H <sub>20</sub>	224	P	ND	ND	S, 6
13	7-Oxo-16,17,18-trisnorabieta-8,11,13-triene	C <sub>17</sub> H <sub>22</sub> O	242	P	P	P	S, 11
14	Dehydroabietane	C <sub>20</sub> H <sub>30</sub>	270	P	ND	P	S, 1, 2, 8
15	14-Methyl-16,17-bisnordehydroabietane	C <sub>19</sub> H <sub>28</sub>	256	P	ND	P	I
16	16,17-Bisnorcallitrisic acid <sup>c,d</sup>	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub>	272	P	P	P	12, I
17	16,17-Bisnordehydroabietic acid <sup>c</sup>	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub>	272	P	P	T	S, 11
<i>Labdanes</i>							
18	Z-14,15-Bisnorlabda-8(20),12-dien-18-oic acid <sup>c</sup>	C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>	276	P	P	T	10, I
19	E-14,15-Bisnorlabda-8(20),12-dien-18-oic acid <sup>c</sup>	C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>	276	P	P	T	10, I
20	Z-19-Norlabda-8(20),11-dien-15-oic acid <sup>d</sup>	C <sub>19</sub> H <sub>30</sub> O <sub>2</sub>	290	P	P	ND	10, I
21	Z-19-Noragathic acid <sup>d</sup>	C <sub>19</sub> H <sub>30</sub> O <sub>2</sub>	290	P	P	ND	10, I
22	E-19-Noragathic acid <sup>c,d</sup>	C <sub>19</sub> H <sub>30</sub> O <sub>2</sub>	290	P	P	ND	10, I
23	14,15-Bisnorlabda-8(20),11-dien-13-on-18-ol <sup>c</sup>	C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>	276	P	P	ND	I
24	Communic acid <sup>c</sup>	C <sub>20</sub> H <sub>30</sub> O <sub>2</sub>	302	P	P	ND	S, 2
<i>Pimaranes and Isopimaranes</i>							
25	Isopimara-8,15-dien-18-oic acid <sup>c</sup>	C <sub>20</sub> H <sub>30</sub> O <sub>2</sub>	302	ND	T	ND	11
26	Isopimar-8-en-18-oic acid <sup>c</sup>	C <sub>20</sub> H <sub>32</sub> O <sub>2</sub>	304	ND	P	P	11
27	Pimar-8-en-18-oic acid <sup>d</sup>	C <sub>20</sub> H <sub>32</sub> O <sub>2</sub>	304	P	P	ND	12
<i>Kauranes and Phyllocladanes</i>							
28	α-Phyllocladane	C <sub>20</sub> H <sub>34</sub>	274	ND	ND	P	S, 6
29	Kaur-16-en-19-ol <sup>c</sup>	C <sub>20</sub> H <sub>32</sub> O	288	ND	ND	P	1, 2
30	Kauran-13-ol <sup>c</sup>	C <sub>20</sub> H <sub>34</sub> O	290	ND	ND	P	1, 2
<i>Alkyl benzenes, alkyl naphthalenes, alkyl tetralins</i>							
31	Butyltrimethylbenzene	C <sub>13</sub> H <sub>20</sub>	176	P	ND	P	1
32	1,1,3-Trimethylindane	C <sub>12</sub> H <sub>16</sub>	160	P	ND	P	1
33	Trimethylisopentylbenzene	C <sub>14</sub> H <sub>22</sub>	190	ND	P	ND	7
34	1,1,5,6-Tetramethyl-5,6-dehydrodecalin	C <sub>14</sub> H <sub>24</sub>	192	P	ND	P	13
35	1,4a,5,6-Tetramethyldecalin	C <sub>14</sub> H <sub>26</sub>	194	P	ND	P	5, I
36	1,1,4a-Trimethyl-6-oxo-7,8-dehydrodecalin	C <sub>13</sub> H <sub>20</sub> O	192	P	ND	P	I
37	1,1,5,6-Tetramethyl-2,3-dehydrodecalin	C <sub>14</sub> H <sub>24</sub>	192	P	ND	P	13, I
38	1,1,5,6-Tetramethyl-8,8a-dehydrodecalin	C <sub>14</sub> H <sub>24</sub>	192	P	ND	P	13, I
39	Ionene	C <sub>13</sub> H <sub>18</sub>	174	P	P	P	5, 6, 9
40	Dimethylnaphthalene	C <sub>12</sub> H <sub>12</sub>	156	P	ND	P	6
41	Dimethylhexylbenzene	C <sub>14</sub> H <sub>22</sub>	190	P	P	P	I
42	Tetramethylbutylbenzene	C <sub>14</sub> H <sub>22</sub>	190	P	P	P	11
43	Methylionene	C <sub>14</sub> H <sub>20</sub>	188	P	P	P	11
44	Dimethylheptylbenzene	C <sub>15</sub> H <sub>24</sub>	204	ND	ND	P	3
45	Trimethylhexylbenzene	C <sub>15</sub> H <sub>24</sub>	204	ND	ND	P	3
46	Trimethylnaphthalene	C <sub>13</sub> H <sub>14</sub>	170	P	P	P	6
47	2,5,6-(or 2,5,8-) Trimethyl-1-propyltetralin	C <sub>16</sub> H <sub>24</sub>	216	P	ND	P	3, 6, I
48	2,5,6-Trimethyl-1-butyltetralin	C <sub>17</sub> H <sub>26</sub>	230	P	P	P	3, 6, I
49	Dimethyldecylbenzene	C <sub>18</sub> H <sub>30</sub>	246	P	P	P	3, 6, I
50	2,5,6-(or 2,5,8-) Trimethyl-1-(3'-methylbutyl)tetralin	C <sub>18</sub> H <sub>28</sub>	244	P	P	P	3, 6, I
51	2,5,6,8-Tetramethyl-1-butyltetralin	C <sub>18</sub> H <sub>28</sub>	244	ND	P	ND	3, 6, I
<i>Carboxylic acids, phenols<sup>c</sup></i>							
52	Benzoic acid	C <sub>7</sub> H <sub>6</sub> O <sub>2</sub>	122	P	P	ND	S, 1
53	Vanillin	C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>	152	P	ND	ND	S, 1, 8
54	E-Isoeugenol	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>	164	P	P	P	S, 1, 8
55	Guaiacyl acetone	C <sub>10</sub> H <sub>12</sub> O <sub>3</sub>	180	P	ND	P	S, 1
56	Vanillic acid	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	168	P	ND	ND	S, 1
57	n-Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	P	ND	P	S, 1
58	n-Octadecanoic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284	P	ND	P	S, 1

<sup>a</sup> Compound occurrence in sample: P, present; T, trace; ND, not detected. Compound numbers refer to Fig. 2 and Appendix A.

<sup>b</sup> Comparison of mass spectra with standard (S), electronic libraries (1) Wiley 275; (2) Nist, and literature data: (3) Gallegos (1973); (4) Simoneit and Mazurek (1982); (5) Mills et al. (1984); (6) Philp (1985); (7) Summons and Powell (1987); (8) Adams (1995); (9) Czechowski et al. (1996); (10) Otto and Simoneit (2002); (11) Otto et al. (2002); (12) Cox et al. (2007); (13) Bray and Anderson (2008), and interpretation (I) of mass spectrometric fragmentation patterns.

<sup>c</sup> Analyzed as TMS derivatives.

<sup>d</sup> Analyzed as methylated derivatives.



**Fig. 2.** GC-MS TIC traces of underivatized extracts from amber samples from: (A) Amazonas Basin; (B) Araripe Basin; (C) Recôncavo Basin. Peak numbers refer to Table 1 and Appendix A (U, unidentified components).

### 3.3. Other compounds (of minor systematic value)

Numerous alkyl benzenes, alkyl naphthalenes and alkyl tetralins (**31–51**) were present in all the extracts. Benzoic acid (**52**), vanillin (**53**), E-isoeugenol (**54**), guaiacyl acetone (**55**) and vanillic acid (**56**) were identified mainly in the extract of the Amazonas amber. E-isoeugenol, the only isomer, was present in all three samples. The samples from the Amazonas and Recôncavo basins contained C<sub>16</sub> (hexadecanoic acid, **57**) and C<sub>18</sub> (octadecanoic acid, **58**) *n*-alkanoic acids. A homologous series of paraffins was present only in the

Araripe amber. The alkanes range from C<sub>15</sub> to C<sub>35</sub>, with C<sub>max</sub> (dominant peak) at C<sub>23</sub> and a carbon preference index (CPI) of about unity. The possibility that these compounds represent contaminants from solvents was excluded on the basis of blank analysis.

### 3.4. Chemosystematic implications

Mosini et al. (1980) have shown that monoterpenes like fenchyl alcohol (**1**, Roman numerals refer to compounds derived from citations, but not detected in the samples; their structures are shown

in Appendix B), borneol (II) and other oxygenated terpenoids can be formed from pinenes by an “aging” process via heating pine resins in water at 110 °C for 30–60 days. Thus, they inferred that the presence of these monoterpenoids in amber is consistent with an origin from *Pinus*. However, Mills et al. (1984) reported that the results are equally consistent with many other coniferous sources, because the occurrence of pinenes is widespread. These authors detected camphor and fenchone in fossil kauri resins derived from *Agathis* (*Araucariaceae* family). The same monoterpenes were also reported to occur in amber derived from the *Araucariaceae* and *Podocarpaceae* families (Grimalt et al., 1988). Although monoterpenes predominate in the volatile fraction of the resins of conifers, the volatile fraction of resins in the family *Burseraceae*, for example, contains large proportions of mono- and sesquiterpenes (Langenheim, 2003). So, monoterpenes are not definitive chemotaxonomic indicators and have been preserved unchanged (at least partly) in the Brazilian fossil resins.

Bicyclic sesquiterpenoids have been found in Recent and fossil sediments, coal and amber (Mills et al., 1984; Simoneit et al., 1986; Alonso et al., 2000; Otto and Simoneit, 2001; Otto et al., 2002, 2003, 2007; Chaler and Grimalt, 2005; Yamamoto et al., 2006). The cadalane derivatives calamenene, 5,6,7,8-tetrahydrocadalene (III) and cadalene (IV) are among the most abundant sesquiterpenes in the geosphere (Simoneit et al., 1986). According to Sukh Dev (1989), the main sources are *Compositae*, *Dipterocarpaceae* and *Myrtaceae*, all belonging to the angiosperm plant family and, additionally, *Cupressaceae* belonging to the gymnosperm plant family. In this way, precursors of the cadalane derivatives (cadinols, bisabolol) are widely distributed among higher plants and are therefore not source specific (Otto and Simoneit, 2001).

Nevertheless, some successful approaches in the chemotaxonomy of fossil conifers have been reported by using sesquiterpenoids. Derivatives of cedranes and cuparanes [ $\alpha$ -cedrene (V), cuparene (VI), cuparenic acid (VII)] are observed only in modern species of the *Cupressaceae* and may be used as characteristic markers of fossil resins from this conifer family (Grantham and Douglas, 1980). However, they were not observed in our samples.

The major diterpenoid skeletal structure types found in biota are labdanes, abietanes, pimaranes, kauranes, phyllocladanes and podocarpanes, with minor amounts of other skeletal types. The abietane, pimarane, kaurane and podocarpane skeletons are produced primarily by vascular plants (Simoneit et al., 1986). The distribution of diterpenoids in conifers must be interpreted with great care. Nevertheless, some trends in the distributions have been reported and can be used as general chemical characteristics of distinct conifer families, because certain diterpenoids were hitherto observed only in individual or clusters of conifer families.

The formal demethylation of the abietane skeleton leads to podocarpanes, which are mostly represented in conifers from the *Podocarpaceae* family by, for example, podocarpic acid (VIII) (Otto and Wilde, 2001; Cox et al., 2007). Otto and Wilde (2001) pointed out that acids are predominant among the abietane class, together with alcohols, phenolic derivatives and hydrocarbons. The reports of abietane derivatives reveal two groups with different distributions: “regular” abietanes and phenolic abietanes. Regular abietanes, such as acids, some alcohols and hydrocarbons, are widely distributed in the conifer families. Resins from *Pinaceae*, on the other hand, usually have predominant abietanoic acids and lack phenolic abietanes such as ferruginol (IX), totarol (X), sugiol (XI) and their derivatives. The phenolic abietanes have been described in other conifer families, especially *Cupressaceae* and *Podocarpaceae* (Otto and Wilde, 2001).

Labdane-type compounds are the most common diterpenoids in conifers and have been described in all families except *Cephalotaxaceae*. Otto and Wilde (2001) reported that the major part of the labdanes are acids, alcohols and other oxygenated compounds.

Labdane and abietane type diterpenoids are common constituents of the resins of many extant conifer families, but typically occur in conjunction with a variety of other types of diterpenoids (Bray and Anderson, 2008). In modern resins, the exclusive presence of labdane and abietane diterpenoids is an indicator of the *Pinaceae* family (Otto and Wilde, 2001; Otto and Simoneit, 2002; Otto et al., 2002; Hautevelle et al., 2006; Bray and Anderson, 2008).

On the other hand, callitrisic acid (XII) was first identified as a unique natural product isolated from the oleoresin of *Callitris columellaris*, *Cupressaceae* family (Carman and Deeth, 1967). Afterwards, Anderson (2006) pointed out that the occurrence of callitrisates in modern conifer resins is limited to the *Cupressaceae*.

The most abundant pimaranes in conifer resins are pimaradiene (XIII) and pimarinol (XIV), while sandaracopimaric acid (XV) is a common constituent with the isopimarane skeleton (Sukh Dev, 1989). Pimaranes and isopimaranes are common and widely distributed among extant conifers (Otto and Wilde, 2001). They are also found in the *Araucariaceae*, *Podocarpaceae*, *Cupressaceae* and *Pinaceae* (Grimalt et al., 1988; Otto and Simoneit, 2001; Otto et al., 2003; Cox et al., 2007).

$\alpha$ - and  $\beta$ -Phyllocladane have been identified in many sediments, coals, fossil plants and ambers (Grimalt et al., 1988; Otto and Simoneit, 2002; Otto et al., 1997, 2003, 2005). They have been proposed as biomarkers for *Araucariaceae* and *Podocarpaceae*, because high amounts of phyllocladane precursors have been detected in recent species of these conifer families (Noble et al., 1985). Among the recent Coniferales families, phyllocladane- and kaurane-type diterpenoids are widespread in species of *Podocarpaceae*, *Araucariaceae* and *Cupressaceae*, but not in the *Pinaceae* (Otto and Wilde, 2001).

The interpretation of the significance biomarkers in the Brazilian ambers is complex, because many non-specific conifer diterpenoids were found. The overall biomarker composition is typical for conifers, but clear assignment of the ambers to an individual conifer family is not possible on the sole basis of biomarker composition. However, some possible paleobotanical origins may be proposed by consideration of the absence or presence of specific diterpenoid compounds as follows:

- (i) The absence of phenolic diterpenoids such as ferruginol, totarol and their derivatives points to a conifer family other than *Podocarpaceae* for the amber from the Amazonas and Araripe basins (according to Otto and Wilde (2001) and Otto et al. (2002)).
- (ii) The presence of phyllocladanes and kauranes in the amber from Recôncavo is evidence for an *Araucariaceae*, *Cupressaceae* or *Podocarpaceae* source, but excludes *Pinaceae* (according to Otto and Wilde (2001) and Otto et al. (2002)).
- (iii) The detection of 16,17-bisnorcallitrisic acid in the three samples is indicative of a *Cupressaceae* botanical origin (according to Anderson (2006)).
- (iv) The exclusive presence of labdane and abietane diterpenoids was not observed in any of the samples. So, this aspect allows us not to consider the *Pinaceae* family (according to Otto and Wilde (2001) and Bray and Anderson (2008)).
- (v) No triterpenoid compounds were found, so most angiosperms are not to be considered as the source (Yamamoto et al., 2006). Furthermore, the compositions of the extracts are very different from the abundant diterpenoid resins derived from the tropical angiosperm family Leguminosae, such as those from *Hymenaea*, a source of large deposits of Tertiary amber from the Dominican Republic and Mexico (Grimalt et al., 1988). Diterpenoids of labdatriene-type acids, such as ozic acid (XVI), are important components of leguminous resins (Anderson et al., 1992; Langenheim, 2003) and were not found in the extracts.

In summary, the biomarker composition of the extracts points to the following families: *Cupressaceae* or *Araucariaceae* for the amber from the Amazonas and Araripe Basins; *Cupressaceae*, *Araucariaceae* or *Podocarpaceae* for the amber from the Recôncavo Basin.

The minor benzoic acid, vanillin, E-isoeugenol, guaiacyl acetone and vanillic acid were interpreted as being lignin degradation products. Lignin and the phenolic derivatives are widely distributed in higher plants and are thus of limited chemosystematic value (Otto and Simoneit, 2001). There is no other literature report of the presence of isoeugenol in amber samples.

Numerous studies have proposed diagenetic pathways for the degradation of diterpenoids. Simoneit and Mazurek (1982) analyzed diterpenoids in aerosols and inferred diagenetic pathways for diterpenoids of abietane and pimarane skeletons. Diterpenoids undergo aromatization (oxidation), reduction, disproportionation and polymerization reactions, leading progressively to aromatized and hydrogenated derivatives.

Microbial degradation and non-biological alteration of diterpenoids have been discussed as major diagenetic processes. The disproportionation of terpenoids to saturated and aromatic derivatives has been described (Skrigan, 1964; Frenkel and Heller-Kallai, 1977). Investigations of recent sediments and buried wood have indicated that aromatic diterpenoids (e.g. simonellite, retene) may form under aerobic conditions, whereas saturated diterpenoids (e.g. fichtelite) are generated in anaerobic environments (Laflamme and Hites, 1978; Wakeham et al., 1980; Simoneit, 1986; Simoneit et al., 1986; Otto and Simoneit, 2002). Both aerobic and anaerobic environmental conditions may simultaneously generate these diterpenoids (Barnes and Barnes, 1983). Laboratory tests of bacterial alteration of diterpenoids have been reported, but produced diterpenoid ketones and ultimately diterpenoid dicarboxylic acids without any aromatic or saturated derivatives (Biellmann et al., 1973a,b; Tavendale et al., 1997a,b). Diterpenoid degradation by fungi has not been examined. Thus, the formation of altered saturated and aromatic diterpenoids may be governed by non-biological or fungal processes, especially in massive organic detritus such as plant resins, the precursors of amber.

Diterpenoids were heated with Pd/C in dehydrogenation experiments performed by Ellis et al. (1996), producing isohexylalkyl benzenes, alkyl naphthalenes and alkyl phenanthrenes. These authors proposed a scheme for the formation of isohexylalkyl benzenes and alkyl phenanthrenes from phyllocladane-, labdane- and pimarane-type precursors. Martin et al. (1999), in a review article, summarized the proposed biochemical pathways for abietane degradation by microbial activity. Recently, Armstroff et al. (2006) presented a scheme suggesting that communic and agathic (XVII) acids and manool (XVIII) may be possible biological precursors of trimethylnaphthalenes. Otto and coworkers (Otto et al., 2002, 2003; Otto and Simoneit, 2002; Stefanova et al., 2002) proposed diagenetic pathways for the degradation of abietic acid, regular and phenolic abietanes, labdanes, isopimaranes and pimaranes.

Based in part on the reports cited above and on the structures identified in our extracts, we present a scheme showing possible diagenetic modifications for the biomarkers in the Brazilian amber samples (Fig. 3). The decalin and tetralin compounds could be oxidative degradation products from labdane diterpenoid precursors such as, for example, the norlabdenoic acids described above. In the same way, norabietane compounds could come from isopimarane diterpenoid precursors such as isopimaric acids. The alkyl benzenes, alkyl naphthalenes and alkyl tetralins are highly degraded diagenetic products of various sesqui- and diterpenoid precursors. Thus, not all of these compounds can be assigned to specific terpenoid classes, because the basic structure of the parent molecules has been extensively altered by oxidation during diagenesis (Otto et al., 2002).

The homologous series of paraffins, without any carbon number predominance, in the extract of the Araripe amber is unusual. It is known that such a petroleum-like *n*-alkane distribution is inconsistent with the presence of acids (notably unsaturated ones) such as compounds 17–23 and 57–58. However, in Baltic ambers both paraffins and functionalized compounds were also detected (Czechowski et al., 1996). Homologous *n*-alkanes, ranging from C<sub>22</sub> to C<sub>33</sub> with C<sub>max</sub> at C<sub>26</sub> or C<sub>27</sub>, abietane and pimarane carboxylic acids, and alkanolic acids (mainly C<sub>16</sub> and C<sub>18</sub>) were reported by Czechowski et al. (1996). The classification of source species based on these aliphatic compounds and fatty acids is difficult, because extensive variability in the lipids within the same families, genera or even species has been observed (Herbin and Robbins, 1969; Otto et al., 2005).

### 3.5. Plants and palynomorphs related to amber-bearing deposits and possible botanical origins

Correlations between macro- and micro-fossil evidence and chemosystematics of co-deposited amber need to be used with care. Anderson (2006) reported that (i) amber in any deposit will reflect the occurrence of resin producing species and copious resin producers may be disproportionately represented; (ii) non-resin producers or species with minor resin production will not be represented in the resin record, although these may dominate other palynological or paleontological indicators.

On the other hand, the most direct evidence for the botanical source of amber is fossilized wood or other plant organs containing resin in the tissue, where it was synthesized (Langenheim, 2003). The Brazilian amber samples under study are dissociated from the parent trees; therefore, plant and pollen fossils in the amber-bearing strata provide a circumstantial indication for the source of the resins. So, the paleobotanical significance of the strata (their micro- and macro-fossil remains) adds further evidence in the identification of the possible botanical sources of the Brazilian ambers.

The Crato Member has a fossil record which is relatively well documented. Amber associated with resin-filled cones, foliage and palynomorphs attributed to the *Araucariaceae* family was reported in this geological formation (Martill et al., 2005). Araucarian leaves have also been well described by Duarte (1993), while Arai and Machado Coelho (2001) and Neumann et al. (2002) reported pollen from *Araucariaceae*, *Cupressaceae* and *Cheirolepidiaceae*. Nevertheless, Mohr et al. (2006) describe pollen from *Cupressaceae* and *Pinaceae* and Coimbra et al. (2002) reported pollen from *Araucariaceae* and *Cheirolepidiaceae*. Thus, the reported fossil records of gymnosperms in the Crato Member have been associated with several conifer families. In addition, the chemical composition of the amber from the Araripe Basin excludes *Podocarpaceae* and *Pinaceae*. As a result, the data are indicative of the *Araucariaceae*, *Cupressaceae* or *Cheirolepidiaceae* families being the possible botanical sources for the Araripe amber.

In the Alter do Chão Formation, Amazonas Basin, Dino et al. (1999) reported pollen from *Araucariaceae*, *Cupressaceae* and *Cheirolepidiaceae* in the same core from which this amber was collected. The occurrence of such pollen associated with the amber from Amazonas is strong evidence for an association with one of these three families. Thus, the *Araucariaceae*, *Cupressaceae* or *Cheirolepidiaceae* families are also suggested as the botanical sources for this amber.

Only one possibly *Cheirolepidiaceae* amber has been reported (Schmidt et al., 2006) and no rigorous geochemical analysis of amber unambiguously associated with the *Cheirolepidiaceae* has been described (Alonso et al., 2000). Bray and Anderson (2008) reported Early Cretaceous resins where the terpene distribution is dominated by abietane and labdane diterpenes, having similar distributions to those found in some species of extant *Pinaceae* family members. These authors reported a fossil record including *Pinaceae* and *Cheirolepidiaceae* in the sediments where the ambers were



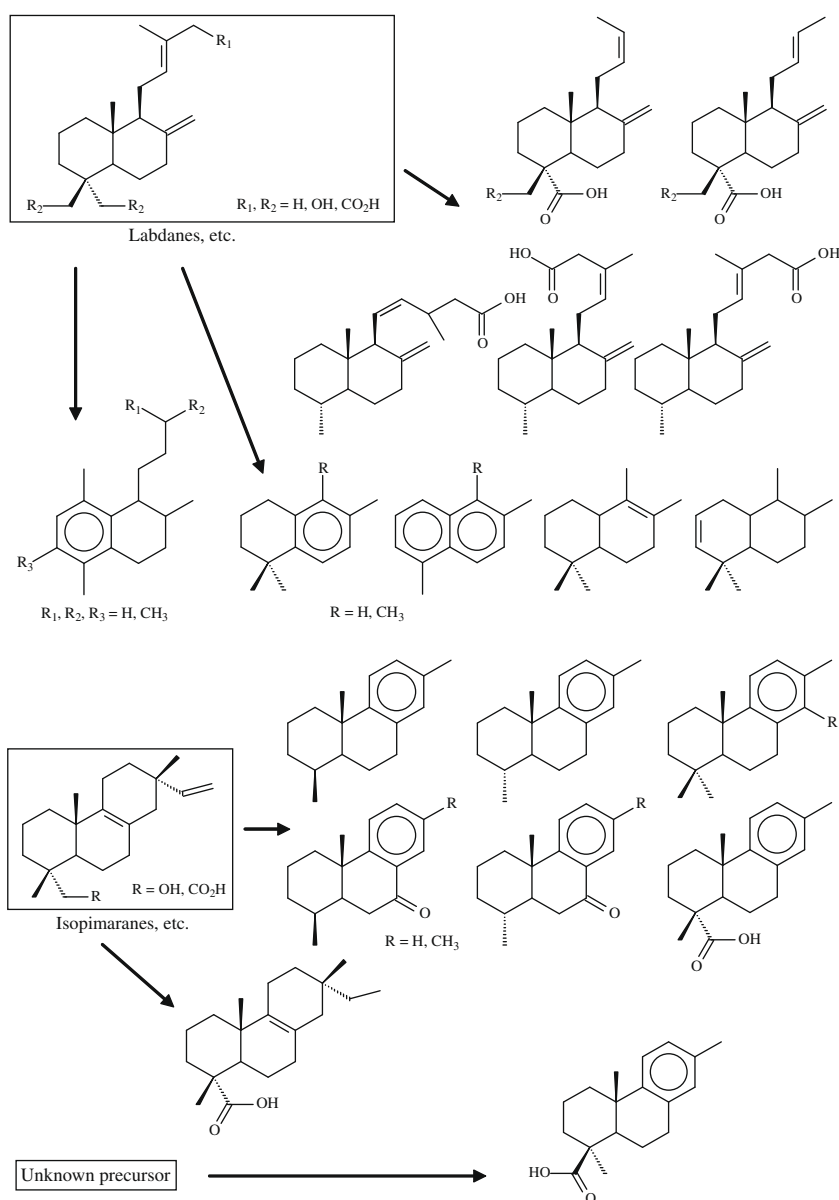


Fig. 3. Inferred product–precursor relationships for the diterpenoids found in the Brazilian amber samples.

found and included the *Cheirolepidiaceae* family as a possible botanical source for the fossil resins analyzed. So, given the absence of evidence concerning the chemotaxonomy of *Cheirolepidiaceae*, this family cannot be excluded as a possible source for the amber mentioned. Hence, the lack of geochemical data, as well as the presence of the *Cheirolepidiaceae* within the Araripe and Amazonas deposits, precludes the a priori exclusion of this family as a potential source of these samples. Therefore, in the absence of additional data, *Cheirolepidiaceae* must be included as a possible source for the Araripe and Recôncavo amber samples characterized in the present study.

In the Caruaçu Member, Recôncavo Basin, the record of plant fossils and pollen is not well documented, making it difficult to determine a possible botanical origin for the amber from this geological formation. Furthermore, Regali (1989) reported pollen from angiosperms (*Tucanopollis*) of Aptian age in the Recôncavo Basin, but the data are not useful in determining the origin of the Recôncavo amber. However, the detection of phyllocladane and kaurane related compounds in this sample supports an origin from *Podocarpaceae*, *Araucariaceae* or *Cupressaceae*.

#### 4. Conclusions

The chemical composition of extracts from three samples of Cretaceous amber from the Amazonas, Araripe and Recôncavo Basins, Brazil were analyzed using gas chromatography–mass spectrometry to determine their botanical origin. The terpenoid compounds found were from numerous classes: labdane, podocarpane, abietane, isopimarane, pimarane, phyllocladane and kaurane. Important compounds were  $\alpha$ -phyllocladane, kaur-16-en-19-ol, kauran-13-ol, isopimar-8,15-dien-18-oic acid, isopimar-8-en-18-oic acid, pimar-8-en-18-oic acid, 16,17,19-trisnorabiet-8-en-7-one, 16,17,18-trisnorabiet-8-en-7-one, 16,17,18-trisnorabieta-8,11,13-triene, 7-oxo-15-norpodocarpane, 7-oxo-16,17,18-trisnorabieta-8,11,13-triene, 16,17-bisnorcallitrisic acid, 16,17-bisnordehydroabietic acid, bisnorsimonellite and dehydroabietane. Phenolic diterpenoids (e.g. ferruginol), cedranes and cuparanes were not detected. Angiosperms were eliminated by way of the absence of triterpenes as well as diterpenes that characterize the Leguminosae. The terpenoid distributions are consistent with many coniferous sources. Lastly, *Araucariaceae*, *Cupressaceae* or *Cheirolepidiaceae* families are

suggested as the sources for the samples from the Amazonas and Araripe Basins on the basis of their biomarker compositions and previous reports of fossil occurrences (mainly pollen and leaves) from these conifers in the same strata. The biomarkers in the amber from the Recôncavo Basin indicate an origin from *Araucariaceae*, *Cupressaceae* or *Podocarpaceae* families. Thus, the amber samples in this work have coniferous sources, most probably related to *Araucariaceae*, *Podocarpaceae*, *Cupressaceae* or *Cheirolepidiaceae*.

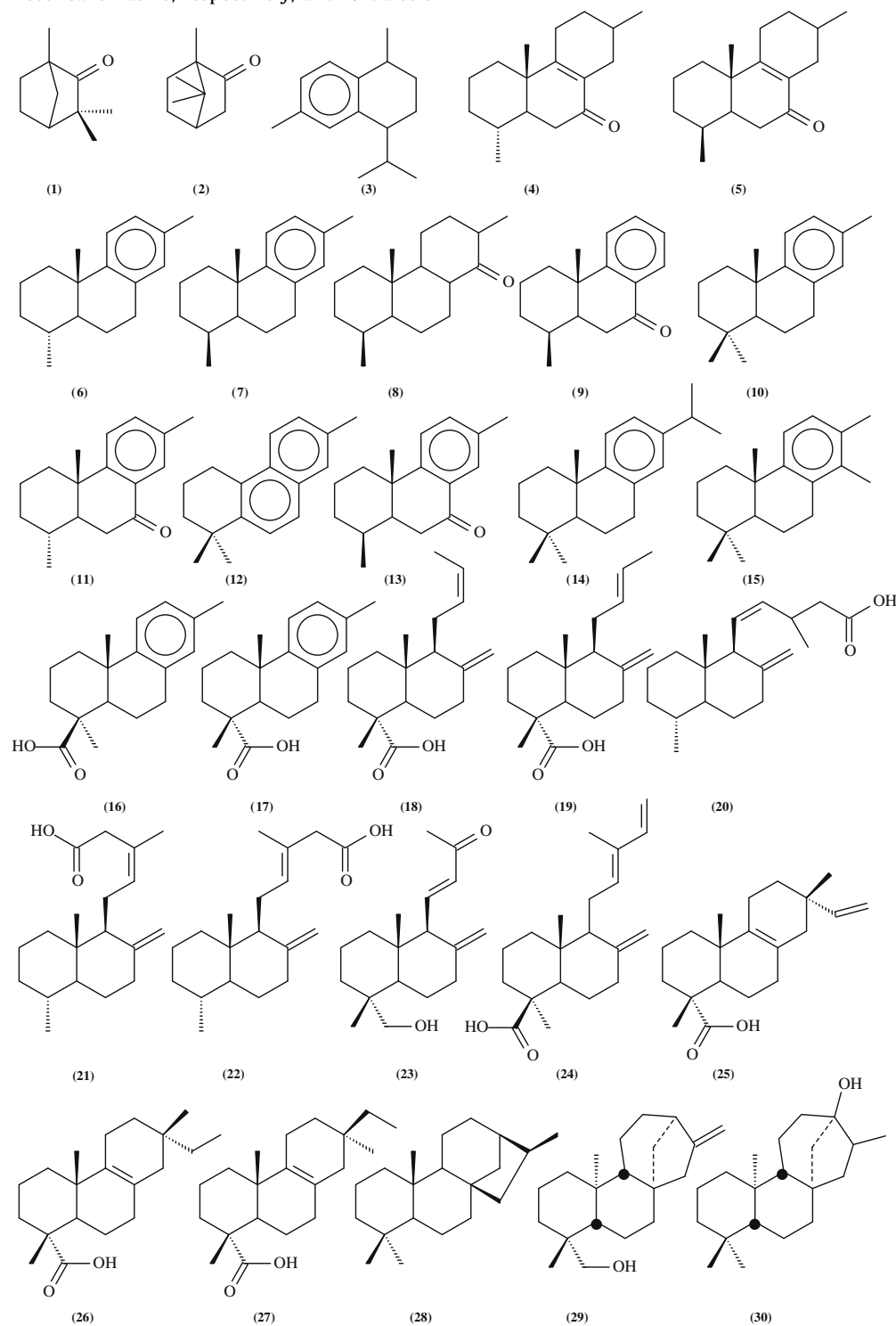
### Acknowledgements

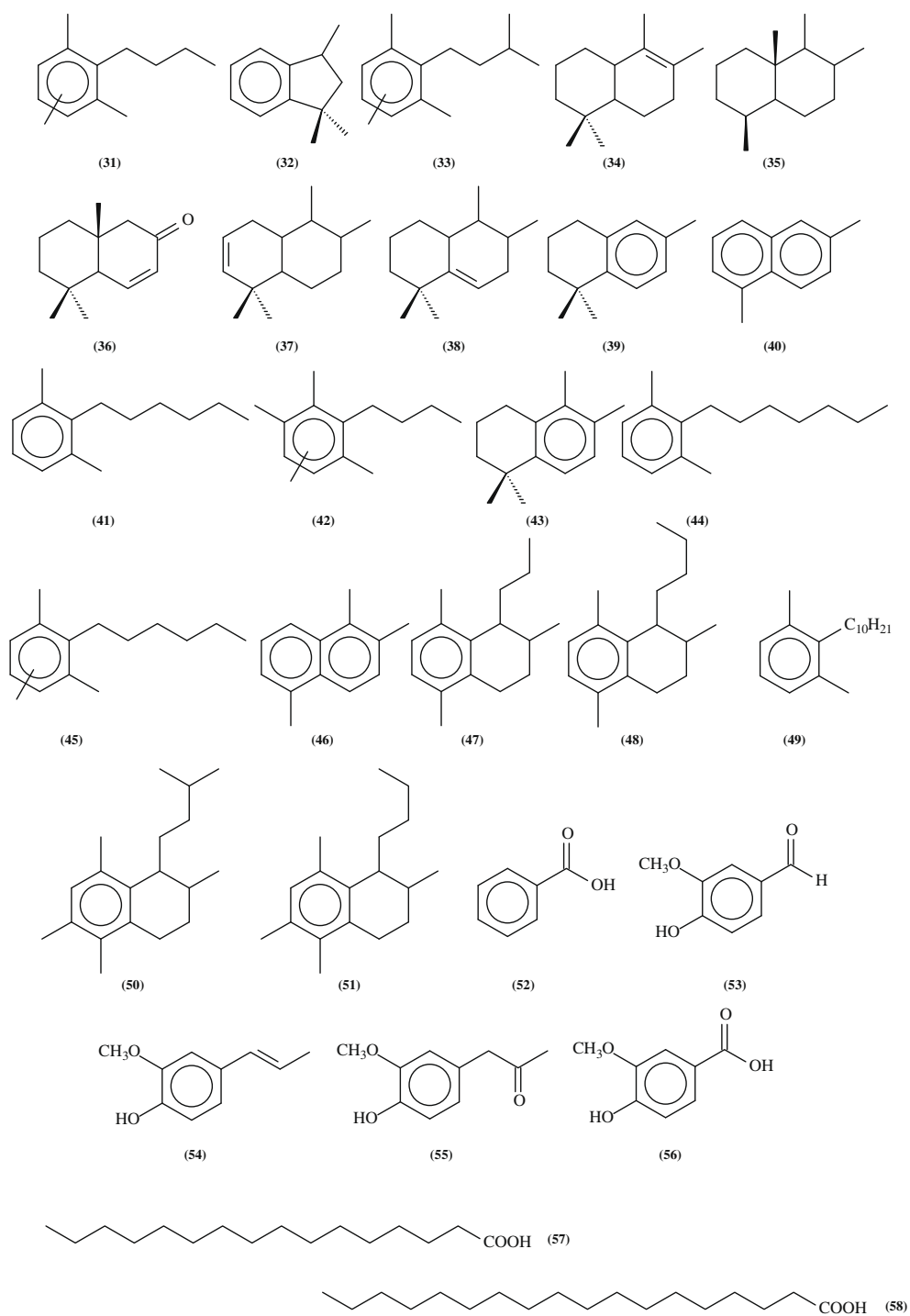
The authors thank R. Dino and L. Borghi for the samples from the Amazonas and Recôncavo Basins, respectively, and F. Idalécio

de Freitas for the sample from the Araripe Basin. We thank J. Graciano Mendonça Filho, M. de Araújo Carvalho, M. Aguiar de Barros, R. P. Philp and J. H. Langenheim for contributions and suggestions. We thank Thanh T. Nguyen Tu and Maya D. Stefanova for and review comments which improved the manuscript. Financial support from CAPES, CNPq, FAPERJ and FUJB is gratefully acknowledged.

### Appendix A

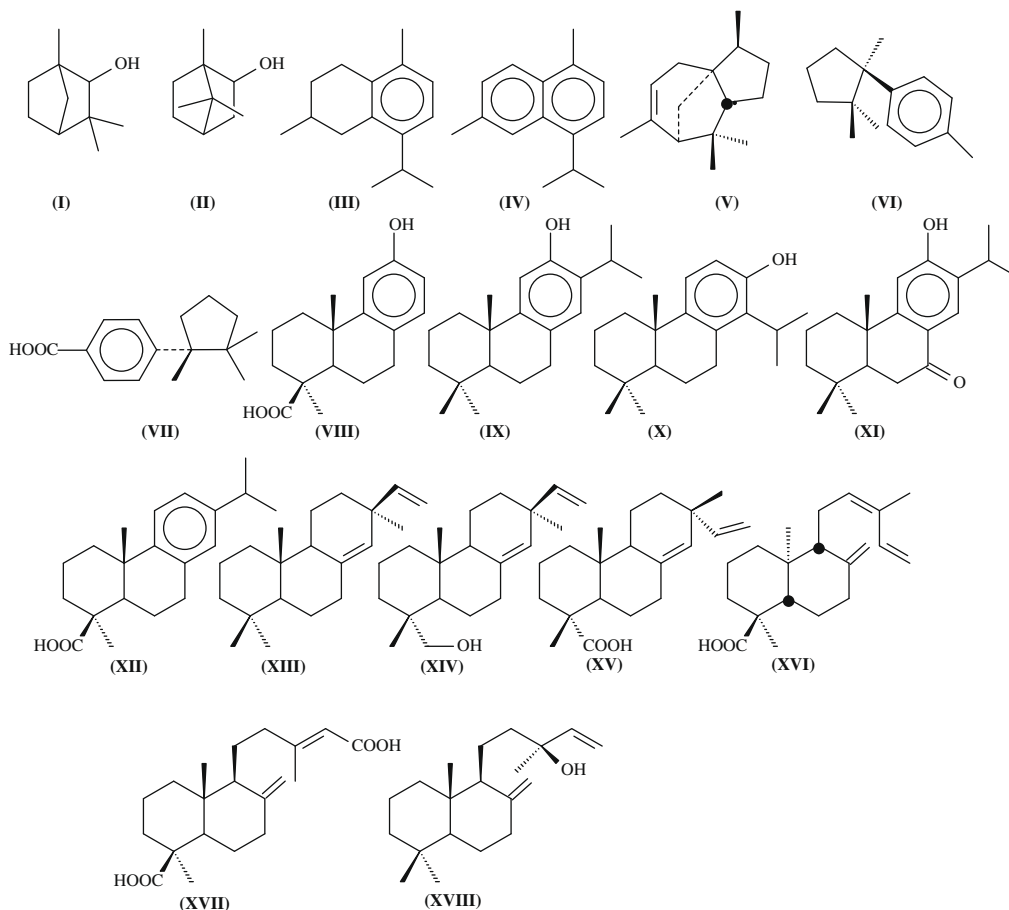
Structures cited in the text and detected in the samples.





## Appendix B

Chemical structures cited in this text, but not detected in the samples.



Associate Editor—P. Schaeffer

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