ELSEVIER

Journal of Chromatography A, 693 (1995) 281–287

JOURNAL OF CHROMATOGRAPHY A

HPLC separation and determination of naphtho[2,3-b]furan-4,9-diones and related compounds in extracts of *Tabebuia avellanedae* (Bignoniaceae)

Jürgen Steinert*, Hosni Khalaf, Manfred Rimpler

Institut für Medizinische Chemie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 30625 Hannover, Germany

First received 17 February 1994; revised manuscript received 17 October 1994

Abstract

A HPLC method for the separation of some naturally occurring naphtho[2,3-b]furan-4,9-diones and related compounds on a RP column is reported. These compounds were determined in aqueous and various non-aqueous extracts of the inner bark of *Tabebuia avellanedae* (Bignoniaceae). 8-Hydroxy-2-hydroxyethyl-naphtho[2,3-b]furan-4,9-dione, 2-hydroxyethyl-naphtho[2,3-b]furan-4,9-dione and 2,3-dihydro- 2-(1-methylethenyl-)naphtho[2,3-b] furan-4,9-dione were predominating in all extracts investigated. Lapachol could not be detected in aqueous extracts known as "lapacho tea".

1. Introduction

Tabebuia avellanedae (Bignoniaceae) is a tree growing in South America. Preparations from its inner bark are known as "Lapacho", "Ipe roxo" or "Pau d'arco". They are used as adjuvants in cancer therapy in South America and the USA [1–3].

For about ten years it has been known that there are some naphtho[2,3-b]furan-4,9-diones with interesting pharmacological properties occurring in the inner bark of *T. avellanedae*. For example, 2-(1-hydroxy-ethyl-)-naphtho[2,3-b] furan-4,9-dione is active against leucemia cells in mice [4,5]. This compound can also influence the immune reaction of human granulocytes and lymphocytes [6].

Lapachol, a constituent of *T. avellanedae* discovered in the last century, has already been tested in clinical studies [7,8]. It is active against the intramuscular Walker carcinosarcoma [9] and shows antiviral [10], antischistosomal [11,12], and anti-inflammatory [13] activities.

So far all publications about the constituents of *T. avellanedae* are dealing with their isolation from stem wood or inner bark by means of various preparative chromatographic techniques (CC, TLC, HPLC) followed by ^{1}H- or ^{13}C-NMR and MS for identification of the pure compounds [4,5,14–16]. It has not been tried to quantify the various quinones in preparations of the inner bark in one analytical run.

So we developed a HPLC method for the separation of some naphtho[2,3-b]furan-4,9-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)01128-1

diones and related compounds and examined aqueous and non-aqueous extracts of the inner bark of *T. avellanedae*.

We prepared aqueous extracts because they are drunken as a tea ("lapacho tea") and on the other hand non-aqueous extracts, because up to now isolation and identification of the constituents has been done from extracts prepared with organic solvents. Furthermore we wanted to find out which organic solvent gave the highest amount of extractables and which one is best suited for a following HPLC analysis.

Our investigations included nine compounds. The formulas are given in Table 1.

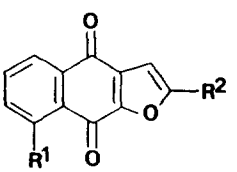
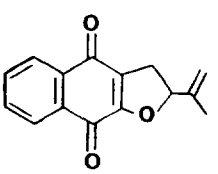
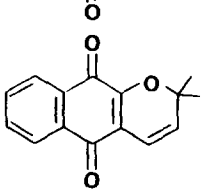
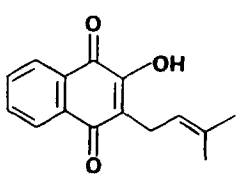
Wagner et al. [15] could identify compounds **1–4** and **7–9** in a $CHCl_3$ extract of the inner bark from *T. avellanedae*, **6** is a minor constituent of various Bignoniaceae species and **5** was used as a further standard, although it has not yet been found occurring naturally. Up to the present time a separation of the compounds **1–9** has not yet been described in literature.

2. Experimental

2.1. Apparatus

A Model 2249 liquid chromatograph (LKB Pharmacia, Bromma, Sweden) was used, fitted with a LKB 2141 UV–VIS detector and a Rheodyne injection valve (20 μl loop), connected

Table 1
Structural formulas of the investigated compounds

Compound	Formula	Name
		Naphtho[2,3-b]furan-4,9-diones
		R^1 R^2
1		H $COCH_3$
2		H $CH(OH)CH_3$
3		OH $COCH_3$
4		OH $CH(OH)CH_3$
5		H CH_2CH_3
6		H $CH(CH_3)_2$
7		2,3-Dihydro-2-(1-methyl-ethenyl)-naphtho[2,3-b]furan-4,9-dione
8		Dehydro-α-lapachon
9		Lapachol

with a LKB 2221 integrator with a two-channel module.

2.2. Chromatography

A Spherisorb (Grom, Herrenberg, Germany) ODS-2 column (250 mm × 4 mm I.D.; 5 μm particle size) was used. The mobile phases consisted of water–methanol–acetonitrile mixtures in various ratios. The water was always acidified with phosphoric acid and the flow rate was 1.3 ml/min. The detector was set at 254 nm, and at 280 nm on the second channel.

2.3. Chemicals and materials

Methanol (MeOH), acetonitrile (ACN), water (all purchased from Merck, Darmstadt, Germany), and tetrahydrofuran (THF, from Fluka, Neu-Ulm, Germany) were of HPLC grade. Petroleum ether (50–70°C, PE), diethylether (Et_2O), chloroform ($CHCl_3$), methylene chloride (CH_2Cl_2) ethyl acetate (EtOAc), acetone and phosphoric acid (H_3PO_4, 85%) were all purchased from Merck and were of analytical grade.

Silica gel 60 (deactivated with 15% (w/w) H_2O), citric acid monohydrate and sodium sulfate were purchased from Merck and of analytical grade.

A buffer solution pH 9 (boric acid–potassium chloride–sodium hydroxide) was purchased from Merck and diluted tenfold prior to use.

Membrane filters (type 0-45/15, 0.45 μm) were purchased from Macherey-Nagel (Dueren, Germany).

Lapachol (**9**) was purchased from Fluka. The other compounds were prepared according to partly modified published procedures: **5**, **1**, and **2** were prepared from 2-hydroxy-1,4-naphthoquinone (2-OH-NQ) and butanal [17–20], **6** from 2-OH-NQ and 3-methyl-butanal [17,18], and **8** from 2-OH-NQ and 3-methyl-2-butenal [21]. 1,5-dihydroxynaphthalene was transformed in three steps into 2,8-dihydroxy-1,4-naphthoquinone [22–26], which in five and six steps, respectively, gave **3** and **4** [17–20]. **7** was obtained from **1** in two steps [27].

Stock standard solutions were prepared by dissolving 5–10 mg of compounds **1–9** in methanol in a 10-ml volumetric flask, except **1** which was dissolved in MeOH–THF (4:1, v/v).

Working standard solutions were obtained by dilution with methanol.

They were used as external standards to determine the content of **1–9** in the T. *avellanedae* inner bark extracts.

2.4. Aqueous extracts

10.0 g of the finely ground inner bark were brought into 500 ml of boiling water or buffer pH 9 (in a 1-l beaker). After covering the beaker with a watch glass the water was kept boiling for 5 min. Then the heater was removed and after 10 min the solution was filtered into a separation funnel. The beaker was washed two times with 25 ml H_2O. After cooling to room temperature the filtrate was acidified with 5 g of citric acid monohydrate and then extracted three times with 100 ml Et_2O. The combined organic layers were washed with 25 ml of H_2O, dried over anhydrous sodium sulfate, filtered, evaporated, and purified (see purification of the extracts).

2.5. Non-aqueous extracts

In each case 10 g of the finely ground inner bark were extracted in a Soxhlet apparatus with 180 ml of PE, Et_2O, $CHCl_3$, CH_2Cl_2, EtOAc, acetone, ACN or MeOH each for 16 h, and then purified.

2.6. Purification of the extracts

The solvent was evaporated under reduced pressure. The residue was taken up in MeOH–THF (4:1, v/v), filtered into a 10-ml graduated flask and filled up. An aliquot was evaporated to dryness, taken up in 0.5 ml of CH_2Cl_2–MeOH (4:1, v/v), brought on a column (20 cm × 1 cm ID; filled with 5.5 g of silica gel) and eluted with a total of 15 ml CH_2Cl_2–MeOH (9:1, v/v). The first 3 ml were discarded; the remaining effluent was evaporated under reduced pressure. Last residuals of solvent were blown off under a

stream of nitrogen. The residue was taken up in 1.0 ml of MeOH–THF (4:1, v/v) and after membrane filtration used for HPLC.

3. Results and discussion

3.1. Separation of pure compounds

Trials to separate compounds **1–9** with some simple MeOH–H_2O–H_3PO_4 or ACN–H_2O–H_3PO_4 mixtures as eluents on a RP material as stationary phase led to the coelution of some compounds. Compounds **1** and **4** could not be separated with the methanolic eluent, whereas with acetonitrile **7** and **8** coeluted. Separation was successful with a mixture of MeOH–ACN–H_2O–H_3PO_4 (25:35:40:0.1, v/v/v/v) in less than 15 min (Fig. 1). As can be seen from Fig. 1 retention depends on the polarity of the side chain of the furan ring: **2** and **4** are bearing a hydroxyethyl group and elute before **1** and **3** which possess a less polar acetyl group. The compounds with alkyl side chains (**5**, **6**, and **9**) have the longest retention times.

The compounds were also separated by means of gradient elution. It is evident from Fig. 1, that the higher quantity of water in the eluent in the beginning of the chromatographic development results in longer retention times of the analytes. This should be helpful for the investigation of plant extracts, so that co-extracted more polar substances elute earlier and do not interfere with the analytes.

The eluents used were acidified with phosphoric acid to repress the dissociation of phenolic OH groups of compounds **3**, **4**, and **9**, which would lead to a tailing of the corresponding peaks.

The detector signal was linear in a wide range of concentration (0.2–50 mg/l), which was proved by injection of standard solutions with various concentrations. The correlation coefficients calculated were greater than 0.999 for all compounds with the exception of **2**, which had a correlation coefficient of 0.996.

When investigating plant extracts the identity of peaks should be verified. One possibility is the

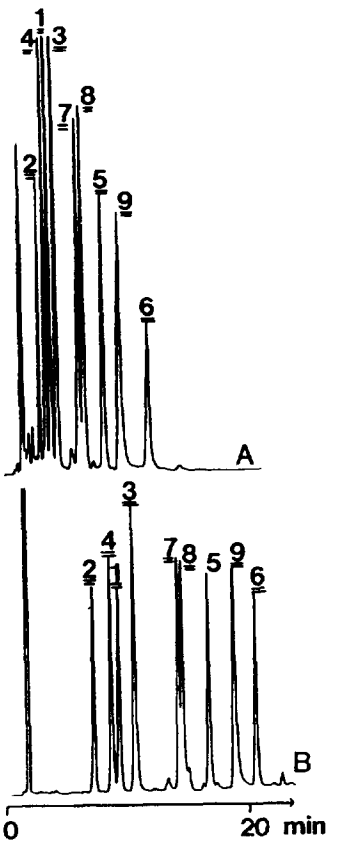


Fig. 1. HPLC separation of *1–9*. Flow rate 1.3 ml/min; detection UV at 254 nm; 0.10 AUFS. (A) eluent: MeOH–ACN–H_2O–H_3PO_4 (25:35:40:0.1, v/v/v/v); (B) gradient elution starting with MeOH–ACN–0.1 vol% H_3PO_4 (25:20:55, v/v/v) for 5 min, then in 20 min with linear increase to MeOH–ACN–0.1 vol% H_3PO_4 (25:45:20, v/v/v).

simultaneous detection at two different wavelengths. So the detector was set on 254 nm and 280 nm and the ratios of peak areas were calculated. The ratios of peak areas (280 nm/254 nm) differed over a wide range: from 0.11 (**5** and **6**), 0.44 (**7**) to 1.00 (**8**).

3.2. Choice of extraction solvents and sample clean-up

Extracts were prepared from the air-dried and finely ground inner bark of *T. avellanedae* with various organic solvents because the qualitative

and quantitative composition of extracts from plant material depends on the structure of extractable compounds and on the nature of the solvent used [28]. The selected low-boiling solvents belonged to different chemical groups and included a wide range of polarity: PE, Et_2O, $CHCl_3$, CH_2Cl_2, EtOAc, acetone, ACN and MeOH.

The extracts obtained varied widely in colour and content. There is a relationship to the polarity of the solvent: with increasing polarity of the solvent, the colour of the extracts changed from slightly yellowish (PE) to almost brown (MeOH) and the amount of extractables increased from 0.76 g/100 g (PE) to 15.27 g/100 g (MeOH).

Before the extracts were subjected to HPLC they had to be cleaned up by column chromatography. TLC investigations of the extracts (on silica gel plates and CH_2Cl_2–MeOH (9:1, v/v) as mobile phase) showed that the analytes wandered near to the solvent front, while the other solutes almost remained on the starting spot. By using this solvent system for column chromatography the effluents of the extracts were clear yellowish solutions, which were ready to use for HPLC. For recovery assay of the analytes, standard mixtures were treated like the samples. The recovery ranged from 67.4 to 98.0%.

In Fig. 2 typical chromatograms of various extracts are shown.

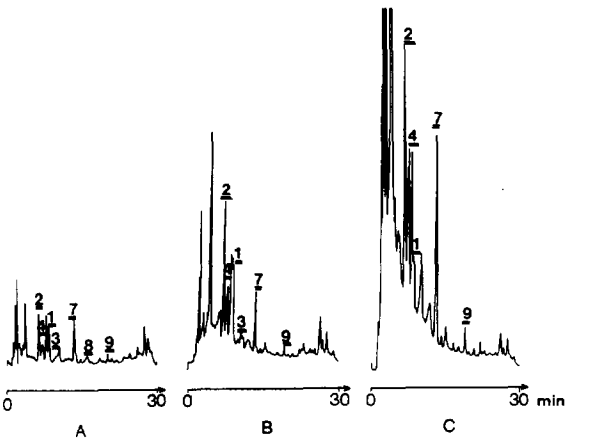


Fig. 2. Chromatograms of various extracts. (A) PE (1.0 g/ml; 0.05 AUFS), (B) Et_2O (0.5 g/ml; 0.05 AUFS) and (C) MeOH (0.2 g/ml; 0.10 AUFS); HPLC conditions as in Fig. 1 with gradient elution.

3.3. Results from HPLC investigations of non-aqueous extracts

The quantitative results are graphically shown in Fig. 3.

Clearly the lowest quinone content is in the PE extract and the highest in the MeOH extract. **4** is the main quinone in all extracts: up to 180 mg/kg are extractable (MeOH, CH_2Cl_2). Then there are **7** and **2** with 128 mg/kg and 91 mg/kg, respectively (MeOH extract). **3** is best extractable with acetone (26.4 mg/kg) or EtOAc (22.8 mg/kg) while **1** is especially extractable with ACN (72.7 mg/kg).

Only small amounts of **9** (lapachol) were found in all extracts (less than 10 mg/kg). That means that **9** is a minor constituent of the inner bark whereas it is one of the major constituents

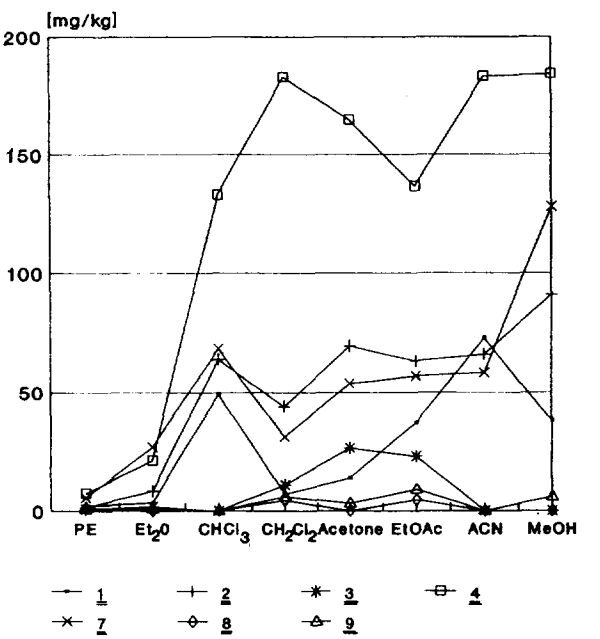


Fig. 3. Quantification of quinonoid constituents from the inner bark of *T. avellanedae* after extraction with different organic solvents; (results in mg/kg air-dried material).

of the heart wood of *T. avellanedae* (for example 2–7% according to [29] or 4% according to [14]).

It was also found that some compounds are not detectable in all extracts. **8**, for example, could only be measured in PE-, CH_2Cl_2- and EtOAc extracts. There are several explanations: first of all some compounds are almost insoluble in the chosen solvents. On the other hand the investigated material was only mechanically ground, so that cell walls largely stay intact. So the cell content is not completely accessible for the following extraction. The cell walls could be corroded with strong acids, which would also cleave the bindings between the quinonoid compounds and carbohydrates or proteins. But this treatment was not chosen because under acidic catalysis changes like isomerizations and cyclizations could occur.

6 and **5** are not detectable in any of the extracts. This is in agreement with literature where **6** has not yet been identified as naturally occurring and **5** has only been known from the wood of another Tabebuia species.

Furthermore there are distinct differences in the quinone specimen. That means the ratios of the quinones among themselves vary from solvent to solvent.

When comparing the chromatograms with regard to the appearance of other compounds than the analytes or to overlapping peaks and quantitation, respectively, than PE or Et_2O are the extraction solvents of choice (Fig. 2). On the other hand when extracts with high concentrations of quinones (especially **1**, **2**, **4**, **7**) have to be prepared a very polar solvent should be used (Fig. 2). That means that PE and Et_2O are the best suited extraction solvents for a following analysis whereas for preparative use ACN and MeOH are to be preferred.

All given values correspond to the investigation of one charge of the inner bark of *T. avellanedae*. So the absolute amounts of extractable quinonoid compounds may vary when analysing another charge due to the fact that the content of plant constituents depends on climate, location of the plant, time of harvest, conditions of storage etc.

3.4. Results from HPLC investigations of aqueous extracts

HPLC determination of the quinones in aqueous extracts was possible after re-extraction with an appropriate organic solvent. We compared Et_2O, EtOAc and CH_2Cl_2 as extraction solvents and found that Et_2O was best suited because the compounds had higher partition coefficients between Et_2O and H_2O compared to CH_2Cl_2–H_2O and EtOAc–H_2O. Furthermore re-extraction with EtOAc often led to emulsions.

The quantitative results are graphically shown in Fig. 4.

In aqueous extracts, which correspond to the "lapacho tea", only five of the investigated quinonoid compounds (**1**, **2**, **3**, **4**, and **7**) could be detected. The dominating quinone was **4** (up to 7.1 mg/l), followed by **2** and **7** (1.5 and 1.2 mg/l). The concentrations of **1** and **3** were about 0.4 mg/l each. **9** (lapachol) and **8** (an oxidation product of **9**) were not detectable (limit of detection: 0.04 mg/l).

There is little influence of boiling time (10 min instead of 5 min) and extraction time (30 min instead of 10 min).

Interestingly there is a lower quinone content in a weakly alkaline extract (buffer pH 9). One reason could be chemical changes which decreased the amount of the quinones. The so

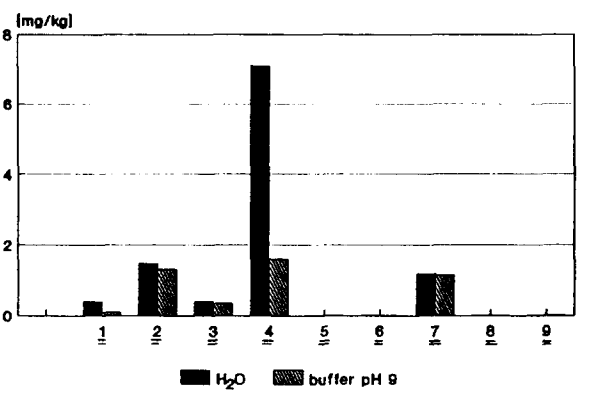


Fig. 4. Quantification of the quinonoid constituents in aqueous extracts (results in mg/l tea; tea preparation: 10 g of material/500 ml H_2O or buffer pH 9).

formed products could not be identified under the conditions chosen. Furthermore the buffer salts could have influenced the extractability of the plant material.

3.5. Conclusions

A method is presented which allows analysis of some quinonoid constituents of the inner bark of *T. avellanedae* or products from it, known as "lapacho", "pau d'arco", "ipe roxo" or "taheebo", by extraction with PE or Et_2O, clean-up, and separation by HPLC.

Lapachol, earlier said to be the active principle of preparations from *T. avellanedae* is only a minor constituent of the inner bark and not detectable in aqueous extracts.

References

[1] V.E. Tyler, *The new honest herbal*, Philadelphia, PA, 1988, pp. 176–177.
[2] F.W. Freise, *Bol. Agric. Secr. Agric. (Sao Paulo)*, 34 (1933) 252–496.
[3] J.L. Hartwell, *Lloydia*, 31 (1968) 71–170.
[4] D.G.I. Kingston and M.M. Rao, *Planta Med.*, 39 (1980) 230–231.
[5] M.M. Rao and D.G.I. Kingston, *Planta Med.*, 45 (1982) 600–604.
[6] B. Kreher, H. Lotter, G.A. Cordell and H. Wagner, *Planta Med.*, 54 (1988) 562–563.
[7] J.B. Block, A.A. Serpick, P. Wiernik, P.K. Nayak and D. Mollins, *Fed. Proc.*, 29 (1970) 682.
[8] J.B. Block, A.A. Serpick, W. Miller and P. Wiernik, *Cancer Chemother. Rep. Part 2*, 4 (1974) 27–28.
[9] K.V. Rao, T.J. McBride and J.J. Oleson, *Proc. Am. Assoc. Cancer Res.*, 8 (1967) 55.
[10] M.H.D.C. Lagrota, M.D. Wigg, L.O.B. Pereira, M.E.F. Fonseca, N.A. Pereira and J.C. Guimares, *Rev. Microbiol.*, 14 (1983) 21–26.
[11] B. Gilbert, J.P. DeSouza, M. Fascio, M. Kitagawa, S.S.C. Nascimento, C.C. Fortes and J. Pellegrino, *An. Acad. Bras. Cienc.*, 42 (Supl.) (1970) 397–400.
[12] A.V. Pinto, M.D.C.R. Pinto and B. Gilbert, *Trans. Roy. Soc. Trop. Med. Hyg.*, 71 (1975) 133–135.
[13] E.R. de Almeida, A.A. da Silva Filho, E.R. dos Santos and C.A.C. Lopez, *J. Ethnopharmacol.*, 29 (1990) 239–241.
[14] A.R. Burnett and R.H. Thomson, *J. Chem. Soc. C*, (1967) 2100–2104.
[15] H. Wagner, B. Kreher and H. Lotter, *Helv. Chim. Acta*, 72 (1989) 659–667.
[16] M. Girard, D. Kindack, B.A. Dawson, J.C. Ethier and D.V.C. Awang, *J. Nat. Prod.*, 51 (1988) 1023–1024.
[17] S.C. Hooker, *J. Am. Chem. Soc.*, 58 (1936) 1163–1167.
[18] V.F. Ferreira, A.V. Pinto, M.C.F.R. Pinto, M.C. da Cruz and A. Clarino, *Synth. Comm.*, 19 (1989) 1061–1069.
[19] K.H. Dudley and H.W. Miller, *J. Org. Chem.*, 32 (1967) 2341–2344.
[20] C.C. Lopes, R.S.C. Lopes, A.V. Pinto and P.R.R. Costa, *J. Heterocyclic Chem.*, 21 (1984) 621–622.
[21] A.V. Pinto, V.F. Ferreira and L.C.M. Coutada, *An. Acad. Bras. Cienc.*, 52 (1980) 477–479.
[22] C. Grundmann, *Synthesis*, (1977) 644–645.
[23] N.K. Kapoor, R.B. Gupta and R.N. Khanna, *Tetrahedron Lett.*, 21 (1980) 5083–5084.
[24] N.K. Kapoor, R.B. Gupta and R.N. Khanna, *Acta Chim. Hung.*, 112 (1983) 163–166.
[25] R.H. Thomson, *J. Org. Chem.*, 13 (1948) 870–878.
[26] A.S. Wheeler and B. Naiman, *J. Am. Chem. Soc.*, 44 (1922) 2331–2334.
[27] A.V. Pinto, M.D.C.R. Pinto, M.A. Aguiar and R.S. Capella, *An. Acad. Bras. Cienc.*, 54 (1982) 115–120.
[28] D. Fengel and G. Wegener, *Wood: Chemistry, Ultrastructure and Reactions*, W. de Gruyter, Berlin and New York, 1984, pp. 240–267.
[29] H.A. Hoppe, *Drogenkunde*, Cram, De Gruyter and Co, Hamburg, 1958, p. 889.