ELSEVIER

Journal of Chromatography A, 835 (1999) 59–65

JOURNAL OF CHROMATOGRAPHY A

Quantitative reversed-phase high-performance liquid chromatography of procyanidins in *Crataegus* leaves and flowers

Gabriela E. Rohr[a], Beat Meier[b], Otto Sticher[a,*]

[a]*Department of Pharmacy, Swiss Federal Institute of Technology (ETH) Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland*
[b]*Zeller AG, CH-8590 Romanshorn, Switzerland*

Received 4 August 1998; received in revised form 8 December 1998; accepted 4 January 1999

Abstract

A quantitative reversed-phase high-performance liquid chromatographic method using UV diode array detection has been developed for the determination of (−)-epicatechin, procyanidins B2 and B5 in *Crataegus* leaves and flowers. Validation experiments revealed that if evaluations are based on height counts procyanidins B4 also yields good and procyanidin C1 acceptable recovery rates. This uncommon behavior of procyanidins B4 and C1 is postulated to be due to matrix effects. A sample preparation procedure including a filtration step over C_{18} cartridges is proposed for quantitative determinations.

Keywords: *Crataegus*; Procyanidins; Epicatechin; Polyphenols; Flavan-3-ols

1. Introduction

Crataegus (hawthorn) was used by the Romans and is considered as one of the oldest medicinal plants of the western world [1]. Nowadays preparations of *Crataegus* are given in cases of declining cardiac performance equivalent to stages I and II of the NYHA (New York Heart Association) classification [2]. The pharmacological effects are ascribed to the flavonoid fraction (hyperoside, vitexin, vitexin-2′-*O*-rhamnoside) as well as to the procyanidin fraction [(−)-epicatechin, procyanidins B2, B5, C1 and oligomers] [3,4]. Procyanidins occurring in *Crataegus* are primarily composed of (−)-epicatechin units while the diastereoisomeric (+)-catechin is only represented as a minor component. Further structural diversity is based on the different types of interflavanoid bonds (C4→C8 or C4→C6) and on the different degrees of polymerization.

The quantitative analysis of the flavonoids in *Crataegus* is well established [5]. For the analysis of oligomeric procyandins however there still does not exist a validated analytical method. For quantitative analysis of total procyanidin contents colorimetric determination of cyanidin after acid hydrolysis has been widely used [6–9]. Hiermann et al. [10] colorimetrically analyzed an oligomeric and a polymeric fraction obtained from chromatography over Sephadex G25. This method has also been applied in studies on seasonal changes of procyanidin contents [11,12] as well as in a study on cell cultures of *Crataegus* [13]. The major drawbacks of colorimetric assays are that the reactions are not stoichiometric and that they often lack reproducibility [14]. For the determination of individual procyanidins a variety of

*Corresponding author. Tel.: +41-1-635-6050; fax: +41-1-635-6882; e-mail: sticher@pharma.ethz.ch

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00043-6

different qualitative HPLC (high-performance liquid chromatography) methods have been described [4,7,15,16], but resolution is generally too bad for a quantitative determination. Two densitometric protocols have been published. The method of Vanhaelen and Vanhaelen-Fastre [17] is based on thin layer chromatography and detection with vanillin reagent after sample clean-up over Sephadex LH-20. The quantitation is hampered by considerable background absorbance. Kappenberg [18] derivatized sample solutions of inflorescenses of *Crataegus* with dansylchloride prior to thin-layer chromatography on silica. Drawbacks of this method are that it is sensitive to light and that so far, no studies on reaction kinetics of procyanidins have been performed.

The analysis of procyanidins is not only hampered by their structural diversity, but also by the instability of this class of polyphenolic compounds. Procyanidins are subject to enzymatic and spontaneous oxidation reactions [19,20], they are thermally labile [21,22] and may undergo molecular rearrangements (i.e., epimerization) [23–25]. With numerous compounds like proteins and carbohydrates they can form reversible as well as irreversible complexes [26].

The scope of the present work was to develop a validated HPLC protocol using UV diode array detection for the determination of the major low-molecular-mass procyanidins in *Crataegus* leaves and flowers.

2. Experimental

2.1. Materials

Crataegus leaves and flowers (Crataegi folium cum flore Ph.Helv.VII / DAB10) were obtained from Dixa (St. Gallen, Switzerland). (−)-Epicatechin (E; HPLC grade) was purchased from Extrasynthese (Genay, France). Chlorogenic acid was of CHR grade and was obtained from Roth (Reinach, Switzerland). Procyanidin dimers B2 [(−)-epicatechin-(4β→8)-(−)-epicatechin], B5 [(−)-epicatechin-(4β→6)-(−)-epicatechin], B4 [(+)-catechin-(4α→8)-(−)-epicatechin] and trimer C1 [(−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin] were isolated from *Crataegus* leaves and flowers as described elsewhere [27]. Methanol, acetonitrile, tetrahydrofuran and acetone were of HPLC grade (Romil, Shepshed, UK). *Ortho*-phosphoric acid (Ph.Helv.VI/Ph.Eur.1) was purchased from Hänseler (Herisau, Switzerland) and acetic acid (analytical-reagent grade) from Merck (Dietikon, Switzerland). Water was obtained using a NANOpure cartridge system (Skan, Basel, Switzerland). Celite 535 was purchased from Fluka (Buchs, Switzerland). Sep-Pak tC_{18} solid-phase extraction columns (3 ml, 500 mg) were bought from Waters (Milford, MA, USA). Nylon filters (Spartan 30/B, 0.45 μm) were obtained from Spartan (Dassel, Germany).

2.2. Instrumentation

All HPLC analyses were performed using a Hewlett-Packard instrument (Model 79994A analytical workstation, Model 1090 liquid chromatograph, Model 1040 diode array detector). A Knauer (Berlin, Germany) prepacked cartridge column (250×4 mm I.D., 5 μm) filled with LiChrosorb RP-18 (for validation experiments: batch L233233646, series MD 186) and a guard column (10×4 mm I.D.) of the same material was used for the final chromatographic separation. For lyophilization an alpha 1-4 Model of Christ (Osterode am Harz, Germany) was used. Sonification was performed with a Model TOC-300 (35 kHz) instrument of Merck (Dietikon, Switzerland).

2.3. Chromatographic conditions

The mobile phase of the optimized chromatographic method consisted of solvent A (methanol) and solvent B [0.5% (v/v) *ortho*-phosphoric acid in water]. The elution profile was: 0 min 18% A in B, 0–10 min 18% to 24% A in B, 10–20 min 24% A in B, 20–45 min 24% to 40% A in B, 45.01–50 min 100% A (wash-out), 50.01–60 min 18% A in B (re-conditioning). All gradients were linear. The flow-rate was set to 1.0 ml/min, the column temperature was fixed at 24.0°C and the injection volume was 5 μl. The detection wavelengths were set at 280 nm and 220 nm, spectra were recorded in the range 190 nm to 600 nm. Peak purity was checked using the software of the photodiode array

detector. Upslope, apex and downslope spectra of the peak under consideration were normalized and matches were calculated. Perfect matches are assigned values of 1000; values below 990 are considered to be indicative of co-eluting impurities.

2.4. Sample preparation

2.4.1. Sample preparation procedure

15.00 g of dried and pulverized (mesh 1 mm) plant material was macerated with 50 ml of aqueous acetone 70% (v/v) for approximately 15 min. The suspension was transferred to a column (dimensions: ∅ 250×20 mm) and was percolated at an approximate speed of 1 drop/s. Two hundred and fifty ml of extract was collected (requiring 2.5 to 3 h). 25.0 ml of the extract (=extract solution) was transferred to a tared flask. The extract solution was evaporated to approximately 5 ml applying temperatures below 30°C. The flask was again weighed and a sufficient volume of methanol was added to produce a 50% (v/w) aqueous solution. The suspension was poured over a sintered glass filter (3G1, dimensions: ∅ 40×40 mm) containing an approximately 5 mm layer of Celite 535. The flask and filter was washed in portions using another 30 ml of 50% (v/v) aqueous methanol. If necessary, the flask was held in an ultrasonic bath for a short time. The methanol was evaporated below 30°C and the aqueous extract solution was immediately frozen and lyophilized. The brown residue was dissolved in 50% (v/v) aqueous methanol and was transferred to a 5.0-ml volumetric flask (=sample solution). Sep-Pak tC_{18} cartridges were activated with 10 ml of methanol and pre-conditioned using 5 ml 50% (v/v) aqueous methanol. 2.0 ml of the sample solution was applied and the filtrate was collected in a 5-ml volumetric flask. The cartridge was further eluted with 2.3 ml of 50% (v/v) aqueous methanol using a 5.0 ml Pipetman of Gilson (Villiers-le-Bel, France). During the whole filtration the vacuum was adjusted to approximately −400 mbar which resulted in a speed of filtration of 1 drop/s. The volumetric flask was filled up to the mark with 50% (v/v) aqueous methanol and was directly used for HPLC analysis.

2.4.2. Validation experiments

Validation samples to determine recovery rates were prepared by adding weighed amounts of reference compounds to the extract solutions. The spiking levels were chosen to approximate 0.5- to 2-times the levels detected in unspiked samples. Six validation samples were analyzed for (−)-epicatechin, procyanidins B2 and B5. For procyanidins B4 and C1 three and five samples, respectively, were prepared. To each validation set three unspiked samples were worked up, the average of which were used to calculate the recovery rates. The results of the recovery experiments are given in Table 1.

Quantitation was carried out by the external standard method on the basis of area and height counts at 280 nm as well as 220 nm. All procyanidins were also quantified in terms of (−)-epicatechin equivalents using calibration curves of (−)-epicatechin. Calibration graphs were generated using six to eight calibration solutions. All graphs were linear. Correlation coefficients for graphs of procyanidins were better than 0.9993, while correlation coefficients for (−)-epicatechin graphs were better than 0.9969. Detection limits (amount of analyte that produces a signal-to-noise ratio of two to five) are given in Table 2 and were determined using

Table 1
Results of recovery experiments

Reference compound	Recovery (%) calculation based on			
	280 nm area counts	280 nm height counts	220 nm area counts	220 nm height counts
E	95	99	96	99
B2	88	88	86	89
B5	99	98	95	96
B4	79	95	82	97
C1	67	82	71	85

Table 2
Limits of detection (ng)

Reference compound	280 nm	220 nm
E	20.9	10.5
B2	5.5	2.8
B5	14.0	5.6
B4	8.2	3.3
C1	44.2	17.7

additional calibration solutions. The repeatability of the method was determined with 13 replicates. Results are given in Table 3.

3. Results and discussion

3.1. Optimization of the analytical method

The sample preparation of procyanidin containing extracts is not trivial. Liquid–liquid partitioning is not quantitative due to the amphiphilic nature of procyanidins while their complexing ability limits the use of solid supports in sample preparation procedures [14]. The method presented here makes use of the insolubility of chlorophylls and lipids in 50% aqueous methanol. It is necessary to weigh the remaining amount of aqueous extract after evaporation of the acetone, since an exceeding amount of methanol is capable of dissolving significant amounts of more lipophilic matrix. On the other hand, if the methanol content is too low, precipitates are extremely sticky, clinging to the glass wall of the flasks and tending to clog in the following filtration step.

Preliminary tests revealed that the collection of 250 ml of aqueous acetone extract is exhaustive. More than 99% of the total amounts of extractable (−)-epicatechin and procyanidins could be recovered. Attempts to develop a solid phase extraction procedure specific for low-molecular-mass procyanidins using commercially available solid-phase extraction cartridges were unsuccessful because of the limited loadability of the cartridges [27]. The procedure used in this study only represents a filtration step over C_{18} material. Higher molecular mass procyanidins are retained and analytical chromatography is therefore less affected by these compounds which tend to be spread over the whole chromatogram. The recovery rates of calibration solutions applied to the C_{18} cartridges were above 95%.

The chromatographic method was optimized with respect to stationary phase, eluent composition, temperature, flow-rate and injection volume. Using the final chromatographic method peak purities of all compounds were better than 990, which indicates the absence of impurities originating from non-procyanidins. The simultaneous resolution of procyanidin C1 and (−)-epicatechin on one hand and the separation of procyanidin B2 from matrix components on the other hand, remain critical. Care should be taken to keep chromatographic parameters constant, since already small changes (i.e., temperature) may affect the separation. An example of a typical chromatogram of an unspiked sample is given in Fig. 1.

Table 3
Determination of repeatability

Reference compound	Relative standard deviation (%) of 13 replicates[a], calculation based on			
	280 nm area counts	280 nm height counts	220 nm area counts	220 nm height counts
E	4.6	6.2	4.0	5.9
B2	2.6	3.1	3.8	4.6
B5	6.5	6.4	8.4	4.6
B4	3.6[b]	5.2	3.1[b]	5.7[b]
C1	6.1[b]	8.4	5.1	7.2

[a] Based on detected concentrations expressed in (−)-epicatechin equivalents.
[b] One value was considered as an outlier (values beyond ±2 s).

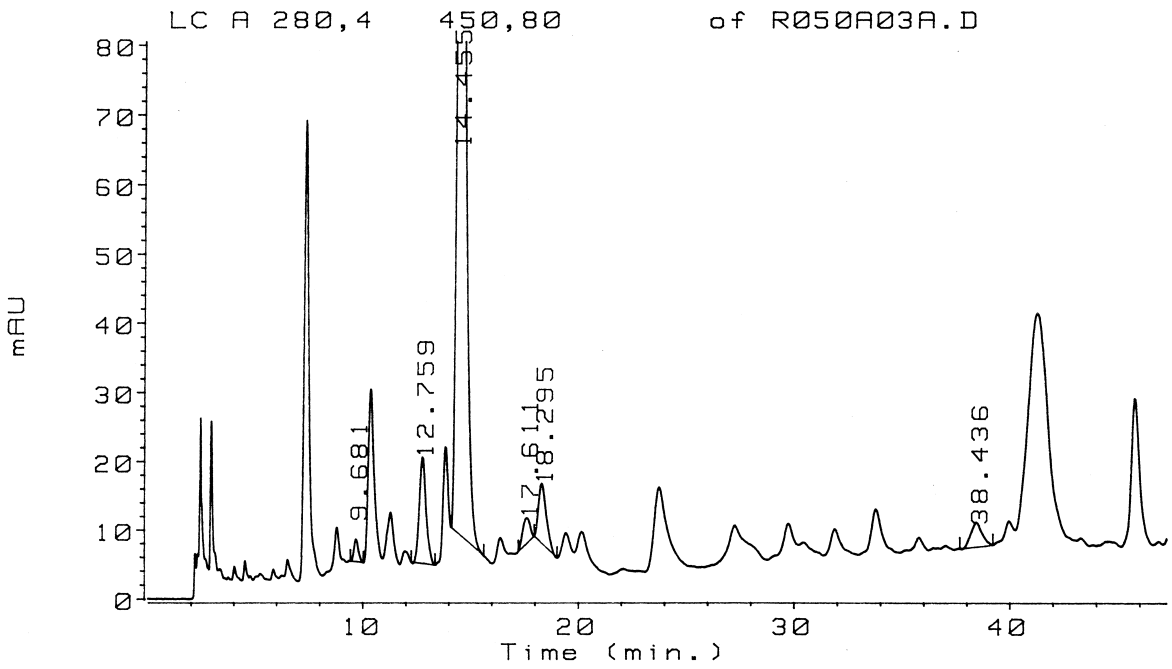

Fig. 1. Typical chromatogram of an unspiked sample recorded at 280 nm. Retention times (min): B4=9.7, B2=12.8, chlorogenic acid=14.5, C1=17.6, (−)-epicatechin=18.3 and B5=38.4. Different integration modes for the quantitation of procyanidin C1 and (−)-epicatechin were evaluated. Integration in terms of ''baseline-all-valleys'' yielded the best results. For conditions, see Section 2.3.

3.2. Validation

Although quantitation of procyanidins is usually carried out at a detection wavelength of 280 nm the validation results clearly demonstrate that quantitation can as well be performed at 220 nm (see Table 1). So far this approach has only been applied to the quantitative analysis of apple juice [28]. The major advantage of the detection at 220 nm is its increased sensitivity (see Table 2). Although all procyanidins possess the same chromophore, differences in the limits of detection arise primarily from different elution times. Furthermore, higher molecular mass procyanidins tend to elute as broader peaks, which can be seen from the relatively high limit of detection that was obtained for procyanidin C1. If sensitivity is not a problem, quantitation should however rather be based on a detection wavelength of 280 nm, because possible interferences are reduced at this higher wavelength.

The validation experiments revealed that recovery rates calculated on the basis of height counts are significantly higher than the ones based on area counts for procyanidins B4 and C1 (see Table 1). It is postulated that this effect is due to interferences from minor procyanidin oligomers. The presence of procyanidin oligomers of higher degree of polymerization in the vicinity of B4 and C1 could be demonstrated in qualitative liquid chromatography–mass spectrometry (LC–MS) analyses [27]. Therefore, if procyanidins B4 and C1 are to be quantified, the evaluation should be based on height counts at 280 or 220 nm. The lower stability of the trimeric procyanidin C1 is a possible explanation for its lower overall recovery compared to the other compounds [27]. The application of a nitrogen atmosphere during the filtration procedure over the C_{18} cartridges and the use of a cooled sample tray for the vials during the HPLC analysis are considered to be suitable measures to improve the recovery of procyanidin C1.

The recovery rates obtained for the validated method are comparable to the ones described in the literature for other matrices. In grape tissue re-

coveries of procyanidin B3 were 86 to 91% (five replicates) [29], in wine recoveries for procyanidins B1 and B3 were 88 to 96% [30], while validation experiments in apple cider and must yielded recoveries of 84 to 110% (2 replicates, nature of spiked procyanidins is not specified) [31]. Better recoveries were obtained with synthetic wine solutions (92 to 101% for dimers, trimers and tetramers) [32] and in another protocol for the analysis of wine (94 to 101% for dimers and trimers) [33].

Because procyanidins are not commercially available the quantitation of procyanidins in terms of (−)-epicatechin equivalents is very widespread, but has never been demonstrated to be valid. All recovery experiments were therefore also conducted using (−)-epicatechin calibration graphs. The recovery rates for the determinations based on 280 nm area counts corresponded very well with the respective values of the experiments run with individual procyanidin calibration graphs. Calculations based on height counts at 280 or 220 nm as well as calculations based on area counts at 220 nm generally led to significant overestimations and should therefore not be applied.

The following contents in *Crataegus* leaves and flowers were detected: (−)-epicatechin: 0.120%, B2: 0.106%, C1: 0.060%, B5: 0.052% and B4: 0.051%. Literature values for procyanidin contents in *Crataegus* leaves and flowers are between 1.9% and 4% [7,10,11,17,34]. However, all these results are based on colorimetric detection methods which are known to be not quantitative. Furthermore, all methods (exception: Ref. [17]) do not quantify individual procyanidins but the total of oligomeric and/or polymeric procyanidins.

4. Conclusions

The recovery rates using the validated protocol amount to: 99% for (−)-epicatechin, 88% for B2, 96% for B5, 95% for B4 and 82% for C1 (basis: 280 nm height counts). Recovery values calculated from area counts are much lower for procyanidins B4 and C1 which is postulated to be due to matrix effects. Quantitation in terms of (−)-epicatechin equivalents should be based on area counts at 280 nm.

The sample clean-up procedure is proposed as a general procedure for the analysis of procyanidins in chlorophyll containing plant material. It is a suitable alternative to the time consuming chromatography over polyamide or Sephadex LH-20.

References

[1] H.P.T. Ammon, R. Kaul, Dtsch. Apoth.-Ztg. 134 (1994) 2433.
[2] Commission E, monograph: Crataegi folium cum flore, Bundesanzeiger, No. 133, 7.19.1994.
[3] R. Kaul, Der Weißdorn. Botanik, Inhaltsstoffe, Qualitätskontrolle, Pharmakologie, Toxikologie und Klinik, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1998, Ch. 5, p. 77.
[4] A. Leukel-Lenz, Untersuchung zur pharmakologischen Wirkung von Crataegusfraktionen und deren analytische Charakterisierung, Thesis, Philipps-Universität, Marburg, 1988.
[5] A. Rehwald, B. Meier, O. Sticher, J. Chromatogr. A 677 (1994) 25.
[6] M. Oswiecimska, J. Sendra, A. Sibiga, K. Kmiec, A. Polak, Farm. Pol. 42 (1986) 116.
[7] G. Petri, Á. Kéry, U. Krawczyk, B. Herényi, Á. Vadász, Bull. Liaison Groupe Polyphenols 14 (1988) 115.
[8] Th. Bahorun, B. Gressier, F. Trotin, C. Brunet, Th. Dine, M. Luyckx, J. Vasseur, M. Cazin, J.-C. Cazin, M. Pinkas, Arzneim.-Forsch./Drug Res. 46 (1996) 1086.
[9] Bundesvereinigung Deutscher Apothekerverbände (Ed.), Deutscher Arzneimittel-Codex 1986, 4. Ergänzung 1992, Govi-Verlag, Frankfurt am Main, Deutscher Apotheker Verlag, Stuttgart, 1986, monograph: Weißdornbeeren.
[10] A. Hiermann, Th. Kartnig, S. Azzam, Sci. Pharm. 54 (1986) 331.
[11] Th. Kartnig, A. Hiermann, S. Azzam, Sci. Pharm. 55 (1987) 95.
[12] Th. Kartnig, A. Gruber, Sci. Pharm. 56 (1988) 271.
[13] Th. Kartnig, G. Kögl, B. Heydel, Planta Med. 59 (1993) 537.
[14] G.E. Rohr, B. Meier, O. Sticher, in: Atta-ur-Rahman (Ed.), Recent Advances in Analytical Techniques, Harwood Academic Press, The Netherlands, 1999.
[15] J. Oszmianski, M. Bourzeix, Pol. J. Food Nutr. Sci. 45 (1995) 89.
[16] U. Krawczyk, G. Petri, Á. Kéry, Arch. Pharm. 324 (1991) 97.
[17] M. Vanhaelen, R. Vanhaelen-Fastre, J. Pharm. Biomed. Anal. 7 (1989) 1871.
[18] F.-J. Kappenberg, Die quantitative DC in der Drogenstandardisierung am Beispiel procyanidinhaltiger Drogen sowie des Knoblauchs, Thesis, Westfälische Wilhelms-Universität, Münster, 1992.
[19] M.J. Amiot, M. Tacchini, S. Aubert, J. Nicolas, J. Food Sci. 57 (1992) 958.
[20] S. Guyot, J. Vercauteren, V. Cheynier, Phytochemistry 42 (1996) 1279.
[21] T. Hernandez, N. Ausín, B. Bartolomé, L. Bengoechea, I. Estrella, Z. Lebensm. Unters. Forsch. A 204 (1997) 151.

[22] C. Dallas, J.M. Ricardo da Silva, O. Laureano, Vitis 34 (1995) 51.
[23] L.J. Porter, in: P.M. Dey, J.B. Harborne (Eds.), Methods in Plant Biochemistry, Vol. 1 Plant Phenolics, Academic Press, London, San Diego, 1989, p. 389.
[24] C. Dallas, J.M. Ricardo da Silva, O. Laureano, J. Agric. Food Chem. 44 (1996) 2402.
[25] C. Dallas, J.M. Ricardo da Silva, O. Laureano, J. Sci. Food Agric. 70 (1996) 493.
[26] P.G. Waterman, S. Mole, in: J.H. Lawton, G.E. Likens (Eds.), Methods in Ecology. Analysis of Phenolic Plant Metabolites, Blackwell Scientific, London, Edinburgh, 1994, p. 104.
[27] G.E. Rohr, Analytical investigation on and isolation of procyanidins from Crataegus leaves and flowers, Thesis, Swiss Federal Institute of Technology, Zürich, in press.
[28] E. Delage, G. Bohuon, A. Baron, J.-F. Drilleau, J. Chromatogr. 555 (1991) 125.
[29] J. Oszmianski, C.Y. Lee, Am. J. Enol. Vitic. 41 (1990) 204.
[30] B. Sun, C. Leandro, J.M. Ricardo da Silva, I. Spranger, J. Agric. Food Chem. 46 (1998) 1390.
[31] B. Suárez, A. Picinelli, J.J. Mangas, J. Chromatogr. A 727 (1996) 203.
[32] E. Revilla, E. Alonso, M. Bourzeix, N. Heredia, in: G. Charalambous (Ed.), Flavors and Off-Flavors, Proceedings of the 6th International Flavor Conference, Rethymnon, Crete, Greece, July 1989, p. 53.
[33] J.M. Ricardo da Silva, J.-P. Rosec, M. Bourzeix, N. Heredia, J. Sci. Food Agric. 53 (1990) 85.
[34] J. Hölzl, A. Strauch, Planta Med. 32 (1977) 141.