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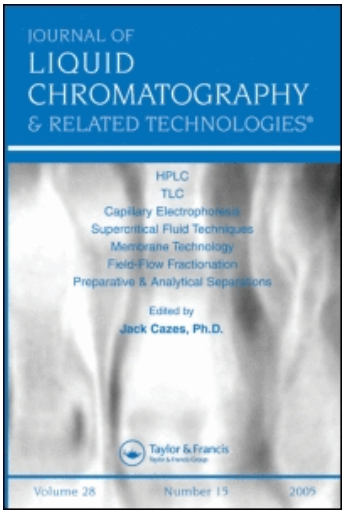

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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THE DEVELOPMENT AND EVALUATION OF AN HPLC-DAD METHOD FOR THE ANALYSIS OF THE PHENOLIC FRACTIONS FROM *IN VITRO* AND *IN VITRO* BIOMASS OF *HYPERICUM* SPECIES

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Online publication date: 28 January 1999

To cite this Article Dias, A. C. P. , Seabra, R. M. , Andrade, P. B. and Fernandes-Ferreira, M.(1999) 'THE DEVELOPMENT AND EVALUATION OF AN HPLC-DAD METHOD FOR THE ANALYSIS OF THE PHENOLIC FRACTIONS FROM *IN VITRO* AND *IN VITRO* BIOMASS OF *HYPERICUM* SPECIES', Journal of Liquid Chromatography & Related Technologies, 22: 2, 215 — 227
To link to this Article: DOI: 10.1081/JLC-100101655
URL: http://dx.doi.org/10.1081/JLC-100101655

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J. LIQ. CHROM. & REL. TECHNOL., 22(2), 215–227 (1999)

THE DEVELOPMENT AND EVALUATION OF AN HPLC-DAD METHOD FOR THE ANALYSIS OF THE PHENOLIC FRACTIONS FROM *IN VIVO* AND *IN VITRO* BIOMASS OF *HYPERICUM* SPECIES

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ABSTRACT

A new HPLC-DAD method for separation and identification of the main phenolics present in *in vivo* and *in vitro* biomass of *Hypericum perforatum* and *Hypericum androsaemum* has been developed. This method accomplished the direct identification of 22 compounds including flavonoids, hypericins, phloroglucinols, and phenolic acids. The HPLC profiles obtained in the course of this work clearly evidenced a distinct phenolic production between *in vivo* and *in vitro* biomass. For example *calli* and suspended cells produced mainly xanthones while in *in vivo* plants of both species these compounds were not detected.

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Some luteolin flavone type compounds were identified in *calli* and suspended cells of *H. perforatum* while quercetin was found in suspended cells of *H. androsaemum*. Flavonoids, namely those related to quercetin, were the major metabolites in methanolic extracts from *in vivo* plants. Hypericins were detected in *in vivo* plants and in *in vitro* shoots of *H. perforatum* but not in *calli* or suspended cells of the same species. Aurones, produced by *in vitro* cultures were not detected in *in vivo* plants of *H. perforatum*.

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INTRODUCTION

Hypericum perforatum L. (St. John's wort) and *Hypericum androsaemum* L. (tutsan) are plant species widely used in folk medicine. *H. perforatum* plants are well known as anti-inflammatory and healing agents as well as by their anti-depressive and anti-viral applications.[1] *H. androsaemum* plants have been largely used in Portugal by their hepatic protective properties.

Some type of phenolics, namelly flavonoid compounds, are considered responsible for some of the therapeutic effects attributed to those plant species. Hypericin, a bianthraquinone found in *H. perforatum* has been considered effective against several viral infections including AIDS.[2] Several methods of identification of hypericins, namely by TLC-densitometry,[3] visible spectrophotometry,[4,5] HPLC,[6] and HPLC-MS[7] have been reported.

Recently HPLC analysis of flavonoids, hypericins, and phloroglucinols from *in vivo*[8,9] and *in vitro*[10] *H. perforatum* biomass have been described. Quercetin related flavonoids were identified by TLC in *in vivo* plants of *H. androsaemum*.[11] However those methods proved inadequate for the analysis of similar phenolic fractions produced by *in vitro* cultures of *H. androsaemum* and *H. perforatum.*

In our opinion those methods failed because phenolic fractions produced by *in vitro* cultures of these species include compounds more apolar than those produced by *in vivo* plants.

This paper describes an adequate, simple, reproducible, and accurate HPLC-DAD method for simultaneous analysis of phenolic acids, flavonoids, xanthones, aurones, and phloroglucinols, from *in vivo* and *in vitro* biomass of *H. perforatum* and *H. androsaemum*. To our knowledge, so far, no reports on HPLC analysis of methanolic extracts of *in vivo H. androsaemum* plants have been published.

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EXPERIMENTAL

Chemicals

HPLC grade solvents from Merck (Darmstadt, Germany) and ultra-pure water were filtered through a 0.2 µm nylon filter (Lida, WI, USA) prior to HPLC utilization. Phenolic standards from Extrasynthese (Geney, France) were used to confirm identification.

Plant Materials

In vivo H. androsaemum and *H. perforatum* plants were collected at National Park of Peneda-Gerês in the north of Portugal. *In vitro* shoots, *calli* and cells suspension cultures of *Hypericum sp.* were obtained in solid or in liquid MS medium supplemented with several phytohormones. Extracts were prepared from freeze-dried biomass (0.1-0.5 g) by maceration at room temperature with 5 mL of a methanol-water solution (80:20). Solutions were filtered through a 0.2-µm filter (Lida, WI, USA) and were analysed by HPLC-DAD using injection sample amounts of 20 µL.

HPLC-DAD Analysis

The HPLC analysis were carried out in a Gilson system (Gilson Medical Electronics, Villiers le Bel, France) equipped with a type 305 pump, a type 302 pump, and a type 7125 injection valve with a 20 µL loop (Rheodyne, Cotati, CA, USA). Separations were performed on a LichroCart RP18 end-capped supersphere column (150x4 mm I.D.; 4 µm) from Merck (Darmstadt, Germany), equipped with a pre-column of the same material. For the mobile phase, water-formic acid (95:5) was used as eluent A and methanol as eluent B. The final elution gradient selected for sample analysis consisted of a six-step gradient which is described in Table 1.

Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range 200-600 nm, and chromatograms were recorded at 260, 280, 350, and 590 nm. The data were processed on an UniPoint® system software (Gilson Medical Elecronics, Villiers le Bel, France). The phenolics were identified by comparing their UV-Vis spectra in the 200-600 nm range and their Rts with those of a library previously compiled by the authors or with those of authentic markers.

Table 1

Elution Gradient Selected for the Analysis of the Methanolic Extracts from *In Vivo* and *In Vitro* Biomass of *Hypericum* Species*

Time (min.)	%A	%B
0	90	10
2	70	30
8	70	30
13	65	35
20	50	50
22	50	50
30	30	70
35	30	70
45	20	80
50	20	80
55	5	95
62	5	95
65	90	10

* Flow velocity = 1 mL min^{-1}.

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RESULTS AND DISCUSSION

Development and Validation of the Analysis Method

The analysis of phenolics from *in vivo* and *in vitro* biomass of *H. perforatum* by HPLC was already reported.[8-10] However, the application of the same methods, namely that already reported for *in vitro* cell cultures,[10] to phenolic analysis of our samples proved inadequate due to the poor resolution of several highly apolar aglycones, present in the methanolic extracts of *in vitro* cultures. To overcome that problem we established a chromatographic elution gradient by using a standard mixture of compounds which included some phenolics, usually found in *in vivo Hypericum sp.* plants and phenolics related to those found in *calli* and cell cultures. This mixture comprised a range of thirteen compounds with different elution characteristics including phenolic acids (ferulic acid, cinnamic acid, and 5-caffeoylquinic acid), flavonoids (the aglicones quercetin, kampferol, apigenin, and amentoflavone, and the glycosides rutin, hyperoside, and isoquercitrin), xanthone, a quinone (emodin) and a bianthraquinone (hypericin).

Table 2

Composition of the Standard Mixture and Retention Times of the Respective Compounds Analysed in the Conditions Described in Experimental

Compound	Retention Time
5-Caffeoylquinic acid (Chlorogenic acid)	4.25
Ferulic acid	7.95
Rutin	11.74
Hyperoside	12.39
Isoquercitrin	13.15
Cinnamic Acid	19.18
Quercetin	20.85
Kampferol	24.68
Apigenin	25.70
Xanthone	29.86
Amentoflavone	31.21
Emodin	39.36
Hypericin	60.18

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Several tests were performed starting with a gradient usually used in our lab for flavonoid analysis and employing this synthetic matrix. The gradient was finally tuned with samples of the methanolic extracts from *calli* and suspended cells of *H. perforatum* and *H. androsaemum*. The gradient selected consisted of six stages completed in a period of 65 min (Table 1). With this elution program the compounds of the mixture were well separated, as it is shown in Table 2. The same elution program afforded a linear relationship between the absorbance at 260, 280, and 350 nm and phenolic concentration, in the range of 5-200$\mu g.mL^{-1}$. The same was true for hypericin at 590 nm. Calibration curves for phenolics were obtained by triplicate determinations of each level of the calibration standards. Peak area values (arbitrary units) were plotted versus concentration ($\mu g.mL^{-1}$). The correlation coefficient for each standard curve invariably exceeded 0.99 for all phenolics. The relative percentage deviations of triplicates were less than 0.5% in all cases (results not shown).

To assure the repeatability of the method in such complex extracts we chose, arbitrarily, samples of *in vivo* and *in vitro H. perforatum* biomass and five injections of each sample were performed. The retention times and quantification, by areas of peaks, of the identified compounds and the major xanthones from *in vitro* biomass were submitted to statistical analysis (results

Table 3

Compounds Identified in the Methanolic Extracts of *H. Perforatum* and *H. Androsaemum* Biomass with their Respective Retention Times (RT)[a]

Peak	Compound	RT
1	3-Caffeioylquinic acid	3.26
2	5-Caffeioylquinic acid (chlorogenic acid)	4.26
3	Caffeic acid	5.10
4	Ferulic acid	7.95
5	Quercetin 3-sulphate	8.71
6	Luteolin 5-glucoside	10.21
7	Hyperoside (quercetin 3-glucoside)	12.39
8	Isoquercitrin (quercetin 3-glucoside)	13.15
9	Quercetin 3-xyloside	14.80
10	Quercitrin (quercetin 3-rhamnoside)	16.48
11	Luteolin 3'-glucoside	18.98
12	Quercetin	20.85
13	Luteolin	22.50
14	Kampferol	24.68
15	Apigenin	25.70
16	Luteolin 5,3'-dimethylether	26.34
17	I3, II8-biapigenin	29.27
18	Amentoflavone (I3', II8-biapigenin)	31.21
19	6-C-prenylluteolin	35.30
20	Pseudohypericin	48.91
21	Hiperforin	58.23
22	Hypericin	60.18

[a] Separation conditions with the elution gradient program described in Table 1.

not shown). Data showed that standard deviation in the retention times of all the compounds studied was less then 0.1 and that the relative standard deviation of each compound was less than 2 %. These results confirmed the high repeatability of the method devised.

Analysis

In spite of the complex and diverse phenolic composition of the methanolic extracts from *in vivo* and *in vitro* biomass of *H. perforatum* and *H. androsaemum*, well-resolved chromatograms were obtained with the HPLC

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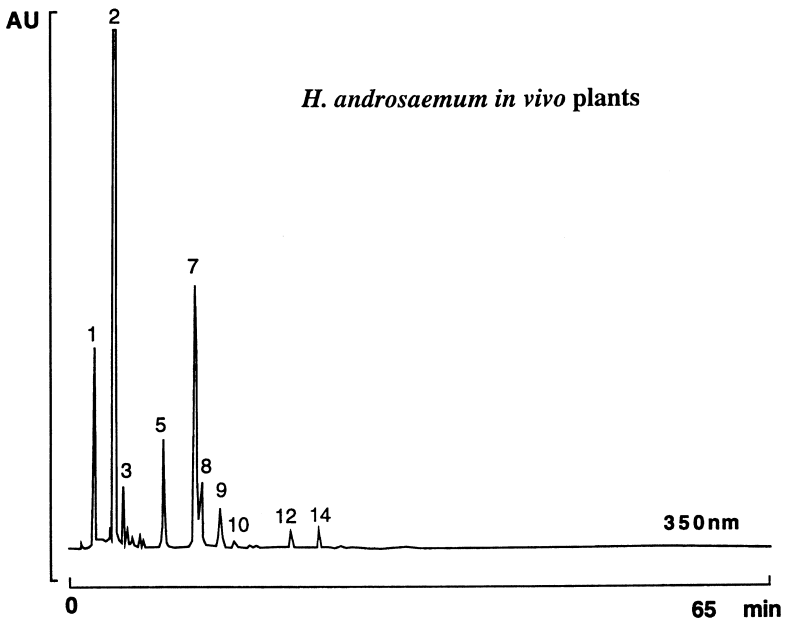

Figure 1. Chromatogram of a methanolic extract of *in vivo H. androsaemum* plants: peak numbers correspond to compounds shown in Table 3.

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gradient above described (Figures 1-5). The utilization of a longer column (250 mm) did not significantly improve the resolution. In such conditions, an undesirable very high pressure developed and the necessary time for column stabilization increased. The examination of the chromatograms at several wavelengths and the use of a photodiode-array detector clearly evidenced a different phenolic composition between the methanolic extracts of *in vivo* plants (Figures 1-2) and those of *in vitro* cultures (Figures 3-5). Table 3 lists the identified compounds with the respective retention times.

The phenolic profile of *in vivo H. androsaemum* plants is shown in Figure 1. Additionally, to those compounds already reported elsewhere,[11] kampferol and quercetin 3-xyloside were also identified.

Most of the flavonoids identified in methanolic extracts from *in vivo* plants (Figure 2) and from *in vitro* shoots (Figure 3) of *H. perforatum* were quercetin derivatives, such as it was already reported by other authors.[8-10] Several diferences however, can be pointed out when we compare the composition of our extracts with those of the above authors as well as the methanolic extracts of *calli* and suspended cells here reported (Figure 4).

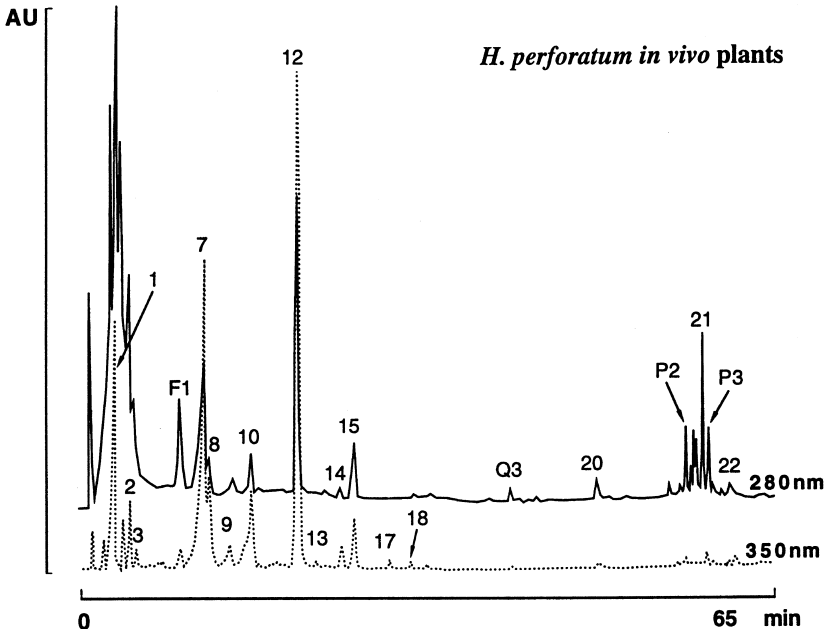

Figure 2. Chromatogram of a methanolic extract of *in vivo H. perforatum* plants: peak numbers correspond to compounds shown in Table 3.

Rutin was not detected either on HPLC nor on silica TLC (results not shown). An unidentified flavanone (peak F1 from Figures 2 and 3) was found both in *in vivo* plants and in *in vitro* shoots of *H. perforatum*. Two other unidentified highly apolar flavanones (peaks F2 and F3 from Figure 3) were found in *in vitro* shoots but were absent in *in vivo H. perforatum* plants. Additionally, to the reported chlorogenic acid (5-caffeoylquinic acid),[12] 3-caffeoylquinic acid and caffeic acid (peaks 2 and 3, respectively) were clearly identified both in *in vivo* plants and in *in vitro* shoots of *H. perforatum*. The identification of 3-caffeoylquinic acid was confirmed by analysing the extracts in a more appropriate HPLC eluent for phenolic acids and by comparisons with a mixture of monocaffeoylquinic acids prepared from 5-caffeoylquinic acid according to that reported elsewhere.[13] The acylphloroglucinol hyperforin (peak 21 in the chromatograms), identified by comparison with an authentic sample isolated from the flowers of *H. perforatum*, as well as several compounds (peaks P1-3, Figure 2 and 3) with retention times and UV spectra (max. between 270-278) similar to those of hyperforin were also found, namely in *in vitro H. perforatum* shoots. Three unidentified quinones (peaks Q1-3 in the chromatograms) were also found in *in vitro* shoots of *H. perforatum*. From these three quinones, only Q3 was detected in *in vivo* plants.

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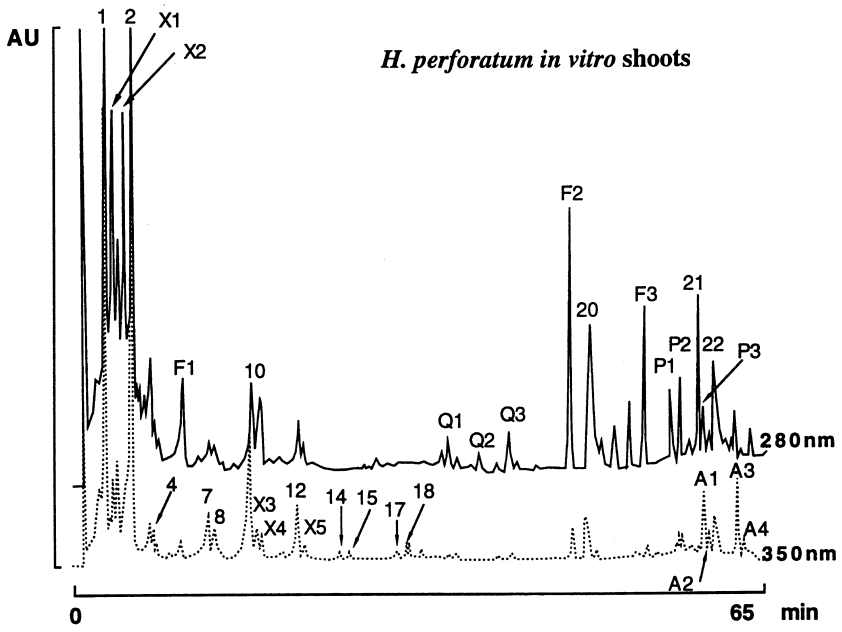

Figure 3. Chromatogram of a methanolic extract of *in vitro H. perforatum* shoots: peak numbers correspond to compounds shown in Table 3.

Pseudohypericin and Hypericin (peaks 20 and 22, respectively) both identified in *in vivo* plants and in *in vitro* shoots were not detected in cells nor in *calli* of *H. perforatum*, as clearly evidenced by the chromatograms recorded at 590 nm. However, the production of these compounds by cell cultures of *H. perforatum* was already reported.[10] This discrepancy could be attributed to different growth conditions (utilization of BAP and continuous light) or to differences in the cell lines. The peaks A1-4 present in *calli* and suspended cells of *H. perforatum* were clearly attributed to aurones due to their characteristic UV spectra. These compounds were also present in *in vitro* shoots but not detectable in *in vivo* plants of the same species. The UV spectra of the peaks X1-X4 present in *in vitro* shoots of *H. perforatum* were of the same type. On the basis of their UV characteristics they were classified as xanthone aglycones.

Xanthones correspond to the major peaks recorded in the UV traces of the different methanolic extracts from *calli* and suspended cells of *H. perforatum* (Figure 4) and *H. androsaemum* (Figure 5). However, they were not detected in the methanolic extracts from *in vivo* plants of the same species. The presence of xanthones in *H. perforatum* plants was already reported.[1] Several xanthones

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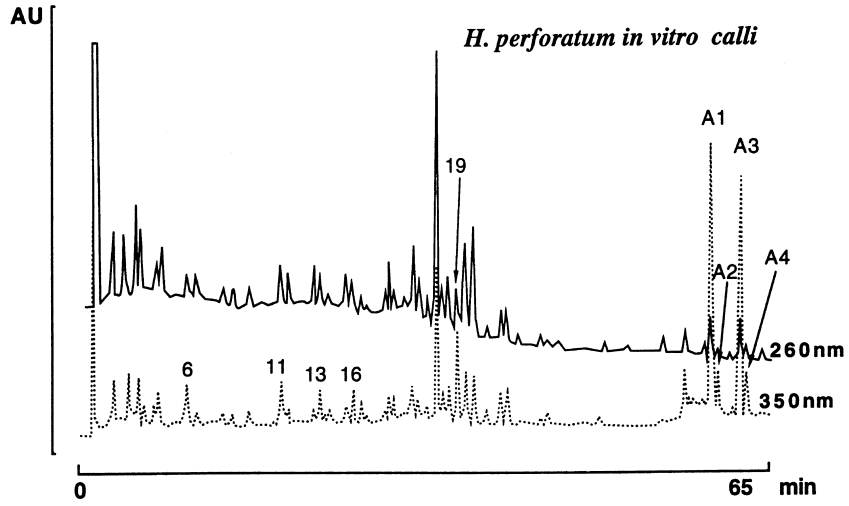

Figure 4. Chromatogram of a methanolic extract of *calli* of *H. perforatum*: peak numbers correspond to compounds shown in Table 3; peaks not assigned correspond to unidentified xanthones. Similar chromatogram profiles were obtained with methanolic extracts of suspended cells of *H. perforatum.*

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were isolated from the roots of *H. androsaemum*[14] and mangiferin was also identified in the aerial parts of *H. androsaemum.*[15] The accumulation of unidentified C-glucosylated and/or prenylated 1,3,6,7-tetraoxygenated xanthones in *H. androsaemum* cell cultures were already reported.[16] Several xanthones were also identified in cell cultures from other *Hypericum* species.[16-18] It seems, therefore, that the production of xanthones is a characteristic of *in vitro* cultures of *Hypericum sp.*

Flavonoids from *in vivo* plants of *H. perforatum* (Figure 2) and *H. androsaemum* (Figure 1) were almost absent in the respective *calli* and suspended cells (Figure 4-5). Nevertheless, quercetin was identified in the methanolic extract of *H. androsaemum calli* and cells, as it was confirmed by comparison with a standard (Figure 5). Flavones were also found in the extracts of *calli* and suspended cells of *H. perforatum* (Figure 4) as they were identified after isolation from calli biomass of *H. perforatum.*[19] These flavones were of luteolin type and, with the exception of luteolin itself, none of them were detected in *in vivo* plants of *H. perforatum.*

To conclude, by means of direct injection in a HPLC coupled to a photodiode array detector, a method of analysis of phenolics from methanolic extracts of *in vivo* and *in vitro* biomass of *Hypericum sp.* was confirmed as

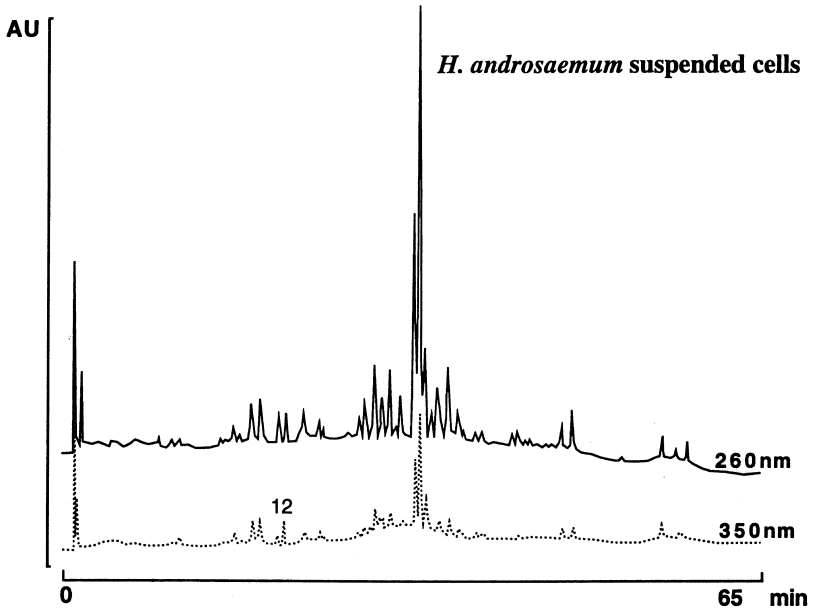

Figure 5. Chromatogram of a methanolic extract of suspended cells of *H. androsaemum*: peak numbers correspond to compounds shown in Table 3; peaks not assigned correspond to unidentified xanthones. Similar chromatogram profiles were obtained with methanolic extracts of *calli* of *H. androsaemum.*

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valid. This method allows an accurate separation, identification, and quantification of the main phenolics present in these type of extracts, in spite of their complexity and chemical diversity. The examination of the chromatograms clearly indicates distinct phenolic production between *in vivo* plants and *in vitro* cultures.

The utilization of this method will allow us to study the production of phenolics in *in vitro* biomass at several media conditions, promoting a better knowledge of the phenolic pathways in these species.

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Received April 1, 1998
Accepted April 28, 1998
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